Antimalarial Activity of Aqueous and 80% Methanol Crude Seed Extracts and Solvent Fractions of *Schinus molle* Linnaeus (*Anacardiaceae*) in *Plasmodium berghei* Infected Mice

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This is to certify that the thesis prepared by Getu Habte, entitled 'antimalarial activity of aqueous and 80% methanol crude seed extracts and solvent fractions of *Schinus molle* Linnaeus (*Anacardiaceae*) in *Plasmodium berghei* infected mice' and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Pharmacology complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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Abstract

Antimalarial activity of aqueous and 80% methanol crude seed extracts and solvent fractions of *Schinus molle* Linnaeus (*Anacardiaceae*) in *Plasmodium berghei* infected mice

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Malaria is one of the major global public health problems. Resistance to antimalarial drugs has been a major challenge in controlling the disease. This issue makes the development of novel antimalarial drugs a necessity. Medicinal plants had been one of the important sources in discovering antimalarial drugs. *Schinus molle* Linnaeus is among medicinal plants that are used for the treatment of malaria in Ethiopia and exhibited *in vitro* antimalarial activity. Nevertheless, it lacks further *in vivo* pharmacological investigations to substantiate its antimalarial activity. As a result, this study was aimed at investigating *in vivo* antimalarial activity of aqueous and 80% methanol crude seed extracts and solvent fractions of *Schinus molle* in *Plasmodium berghei* infected mice using peter's four day suppressive test. The crude extracts and fractions were administered at doses of 100, 200 and 400mg/kg. Parameters including body weight, packed cell volume, temperature, survival time and parasitemia were then determined. The crude extracts and solvent fractions exerted significant inhibition of parasitemia compared to the negative control. Chemosuppressive effects exerted by the crude extracts and solvent fractions were in a range of 27.18-66.91% and 15.64-55.60%, respectively. The highest inhibition of parasitemia was exhibited by the highest dose of 80% methanol crude extract. Among the fractions of the 80% methanol crude extract tested, chloroform fraction demonstrated the highest inhibition. Moreover, the crude extracts and solvent fractions prevented loss of weight, reduction in temperature and anemia compared to the negative control in a dose dependent manner. In addition, they significantly prolonged the survival time of infected mice except the aqueous fraction. In conclusion, the findings of the present study show that the seed of *Schinus molle* has *in vivo* antimalarial activity which supports the traditional claim and promising to be a source for developing more effective and safer antimalarial drugs, and alerts in depth investigations on the plant.

Key words: *Schinus molle*, crude extracts, fractions, *Plasmodium berghei*, antimalarial activity, *in vivo*.

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List of Abbreviations and Acronyms

ACT	Artemisinin Based Combination Therapy
ANOVA	Analysis of Variance
CD	Cluster of Differentiation
CDC	Centers for Disease Control and Prevention
CQ	Chloroquine
CQP	Chloroquine Phosphate
CSA	Chondroitin Sulfate A
EPHI	Ethiopian Public Health Institute
Fe(II)PPIX	Ferrousprotoporphyrin IX
Fe(III)PPIX	Ferricprotoporphyrin IX
FMoH	Federal Ministry of Health
G6PD	Glucose 6 Phosphate Dehydrogenase
GPI	Glycosylphosphatidylinositol
Hb	Haemoglobin
Hz	Haemozoin
ICAM-1	Intercellular Adhesion Molecule 1
IPT	Intermittent Preventive Treatment
IRS	Indoor Residual Spraying
ITNs	Insecticide Treated Nets
KAHRP	Knob Associated Histidine Rich Protein
LLINs	Long Lasting Insecticide Impregnated Nets
MSP	Merozoite Surface Protein
MST	Mean Survival Time
NCAM	Neural Cell Adhesion Molecule
OECD	Organization for Economic Cooperation and Development
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PfEMP	*Plasmodium falciparum* Erythrocyte Membrane Protein
PP	Percentage Parasitemia

PPS	Percentage Parasitemia Suppression
PRBC	Parasitized Red Blood Cell
PVM	Parasitophorous Vacuolar Membrane
RBC	Red Blood Cell
RDT	Rapid Diagnostic Test
rpm	Revolutions per Minute
SEM	Standard Error of the Mean
SPSS	Statistical Package for Social Science
TM	Traditional Medicine
WHO	World Health Organization

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1. Introduction

1.1. Definition and History of Malaria

Malaria is a protozoal disease caused by parasites of the genus *Plasmodium* and transmitted to human and other animals by certain species of female *Anopheline* mosquitoes [Cox, 2010; Kalra *et al*., 2006; Mojarrab *et al*., 2014]. The term malaria was derived from the Italian 'mala aria' meaning foul air or bad air. The condition was called so as the Italians considered the foul air in marshy areas to be the cause of the disease [Dutta, 2015].

Malaria is an ancient disease [Cunha and Cunha, 2008]. In 1880, Laveran discovered the causative agent of malaria [Bruce-Chwatt, 1981; Krettli and Miller, 2001]. In 1897, Ronald Ross demonstrated that *Anopheles* mosquito transmits malaria. The same year, William MacCallum discovered the sexual stages of malaria parasite. In 1948, Garnham and colleagues discovered the tissue and erythrocytic stages of malaria. Shortt and colleagues later described the complete life cycle of *Plasmodium falciparum* [Cox, 2010]. Malaria eradication program was launched in the 1950s and 1960s and dramatically decreased the percentage of the world population at risk of the disease. However, the number of people at risk has increased from 2.1 billion in 1975 to about 3 billion in 2002 [Breman *et al*., 2004; Moreno-Madriñán and Turell, 2018]. The malaria eradication program had little success in many parts of sub-Saharan Africa. The number of people at risk of malaria in this Region grew to over 74% at the end of the 20th century [Hay *et al*., 2004]. Ethiopia, like other sub-Saharan African countries, shares the intolerable burden of malaria, which has become a leading public health problem in the country [Alelign and Dejene, 2016; Deressa *et al*., 2006].

1.2. Etiology and Transmission of Malaria

The protozoan parasites which belong to the genus *Plasmodium* and phylum *Apicomplexa* are responsible for malaria. Five of the *Plasmodium* species, *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* have been well known to cause human malaria [Dondorp, 2005; Sabbatani *et al*., 2010; Trung *et al*., 2004; William and Menon, 2014]. *P. falciparum* is the most prevalent on the African continent and responsible for most deaths from malaria [WHO, 2017]. *P. vivax*, on the other hand, is the parasite that poses the greatest challenge from malaria outside the African continent [Liu *et al*., 2014]. *P. malariae* causes sporadic infections in Africa, parts of Asia and South America, whereas *P. ovale* is restricted to tropical Africa, New Guinea, and the Philippines. *P. knowlesi* is

found in South East Asian countries [Collins and Jeffery, 2007; Greenwood *et al*., 2005; Igbeneghu and Odaibo, 2012; Walker *et al*., 2010; WHO, 2016]. *P. falciparum* and *P. vivax* are the most dominant malaria parasites in Ethiopia, the former causing greater burden from malaria [FMoH, 2014].

Malaria is mainly transmitted through female *Anopheles* mosquitoes [CDC, 2018a]. There are about 465 species of mosquitoes in the *Anopheles* genus, 70 of these species have the capacity to transmit human malaria parasites and 41 are considered to be dominant vector species, capable of transmitting malaria at a level of major concern to public health. In Africa, *A. gambiae*, *A. arabiensis* and *A. funestus* are co-dominant across much of the continent, whereas in the Asian- Pacific region there is a highly complex situation with multi-species coexistence and variable species dominance. *A. arabiensis* and *A. pharoensis* are the main malaria vectors in Ethiopia. The effectiveness of these vectors to transmit the disease differs from species to species. Uncommon modes of malaria transmission are congenital, blood transfusions, organ transplantation and contaminated needles [Alelign and Dejene, 2016; CDC, 2015; Owusu-Ofori *et al*., 2010; Sinka *et al*., 2012; WHO, 2014].

1.3. Epidemiology of Malaria

Globally, an estimated 3.2 billion people are at risk of being infected with malaria [CDC, 2018b]. The incidence rate of malaria is estimated to have decreased by 18% globally between 2010 and 2016 though, between 2014 and 2016, substantial increases in case incidence occurred. The burden is heaviest in the African region, where an estimated 90% of all malaria deaths occur [WHO, 2017]. Children aged less than 5 years account for 78% of all deaths [WHO, 2014]. The risk of malaria in Ethiopia is also high. Only about 25% of the population of Ethiopia live in areas that are free of malaria [Alelign and Dejene, 2016; Ayele *et al*., 2013a; FMoH, 2014]. The risk is highest in the western lowlands of Oromia, Amhara, Tigray and almost the entire areas of Gambella and Benishangul Gumuz regions [Alelign and Dejene, 2016].

Besides public health risks, malaria poses socioeconomic burdens too [CDC, 2018b; WHO, 2012]. The economic impact includes costs of health care, working days lost due to sickness, days lost in education, decreased productivity due to brain damage from cerebral malaria, loss of investment, tourism and trade. To fight malaria, over 19 billion US dollar was invested by governments of malaria endemic countries and international partners since 2010 [CDC, 2018b; WHO, 2017].

1.4. Factors Associated with the Development of Malaria

Factors attributed to the environment, the parasites, the mosquito vectors and the human host affect the occurrence of malaria [Byakika-Kibwika *et al*., 2009; CDC, 2018a; Ferreira *et al*., 2012; Luzzatto, 1974]. Marshy areas that are convenient for the development of the mosquito larva pose great risks in getting the disease [Tadesse *et al*., 2018; Tuyishimire *et al*., 2016]. Moreover, temperature, altitude and humidity affect the survival and reproduction of the vectors and the parasites that cause malaria. Malaria parasite which multiplies in short period of time poses a great risk than those that grow slowly. Moreover, the parasites that adhere to deep tissues cause more risk than others. The length of time the vectors can survive also matters in getting malaria. The higher the life span of the mosquitoes, the higher the probability for sexual stage of the parasite to occur in the mosquitoes, the higher the transmission of the disease [CDC, 2016; Minakawa *et al*., 2002; Sweeney *et al*., 2006].

Pregnant women and children are more vulnerable to malaria than adults as the immunity in these population groups is low [CDC, 2018b; Fana *et al*., 2015; Stauffer and Fischer, 2003; Ukaga *et al.*, 2007; Uneke, 2011]. On the contrary, neonates less than six weeks old are less affected by malaria compared to other population groups. Foetal hemoglobin and scarcity of Paraaminobezoic acid from the mother's breast is among the reasons hypothesized for the protection of neonates from the disease [D'Alessandro *et al*., 2012]. The genetic makeup of the human population is also found to play a role to acquire malaria [Dolo *et al*., 2005; Wassmer and Carlton, 2016]. People with glucose 6 phosphate dehydrogenase (G6PD) deficiency, negative for Duffy antigen on their red blood cells (RBCs), sickle cell diseases, thalassemia, blood group O and ovalocytosis show protection against malaria as opposed to others [De Mendonc *et al*., 2012; Durand and Coetzer, 2008; Nasr *et al*., 2012; Rowea *et al*., 2009].

1.5. Life Cycle of the Malaria Parasite

Plasmodium has a complex life cycle which involves two hosts [Fujiokaa and Aikawab, 2002]. Part of the cycle takes place inside a vertebrate host (asexual phase), in our case, human, and part of it takes place inside invertebrate host (sexual phase), a mosquito vector as shown in Figure 1. The human stage begins when a malaria infected *Anopheles* mosquito inoculates sporozoites into the human host during a blood meal. Then, the motile parasites are taken to the liver through the circulation [CDC, 2016; Ceusters and Smith, 2010; Greenwood *et al*., 2008]. Sporozoites in the

hepatocytes develop into exoerythrocytic schizonts. *P. vivax* and *P. ovale* have a dormant stage, named hypnozoite that may remain in the liver for weeks to many years before the development of pre-erythrocytic schizogony which results in relapses of the disease. A pre-erythrocytic schizont contains merozoites, which are released into the blood circulation and invade the RBCs [Bannister and Sherman, 2009; CDC, 2016].

In circulation, the merozoite develops within the erythrocyte through ring, trophozoite and schizont stages. The erythrocyte containing the segmented schizonts eventually ruptures and releases the newly formed merozoites that invade new erythrocytes [Bannister and Sherman, 2009]. Blood stage parasites are responsible for the clinical manifestations of the disease. Concomitantly, a small portion of the parasites differentiate from newly formed merozoites into sexual erythrocytic forms, the macrogametocyte (female) and microgametocyte (male) [Greenwood *et al*., 2008; Grüring *et al*., 2011]. Then, the sexual stage in the mosquito, mentioned earlier, the sporogonic cycle begins when mature macrogametocytes are taken into the midgut of the *Anopheles mosquito* escape from the erythrocyte to form macrogametes. Microgametocytes exflagellate, forming motile microgametes in the mosquito midgut [Bannister and Sherman, 2009]. The microgamete moves quickly to fertilize a macrogamete and forms a zygote. Then, the non-motile zygotes transform into motile ookinetes. After traversing the midgut epithelium, the ookinete transforms into an oocyst. Then, the motile sporozoites invade the salivary gland epithelium. When this infected mosquito bites a susceptible vertebrate host, the *Plasmodium* life cycle begins again [CDC, 2017; Ceusters and Smith, 2010; Grüring *et al*., 2011].

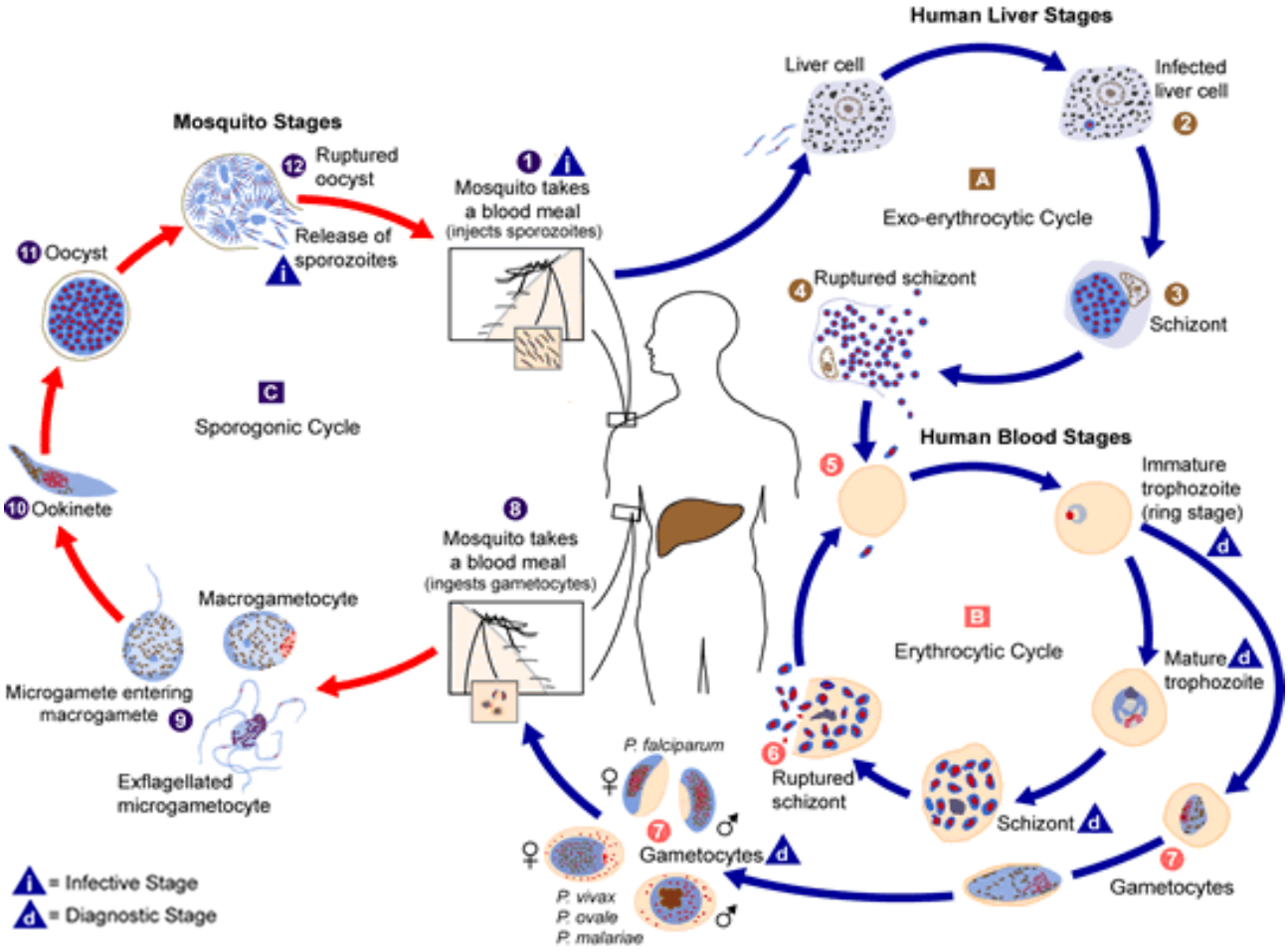

Figure 1: The life cycle of malaria parasites [CDC, 2017]. The malaria parasite life cycle involves two hosts. During a blood meal, a malaria-infected female *Anopheles mosquito* inoculates sporozoites into the human host ❶. Sporozoites infect liver cells ❷ and mature into schizonts ❸ which rupture and release merozoites ❹. Of note, in *P. vivax* and *P. ovale* a dormant stage (hypnozoites) can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later. 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 ❺. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites ❻. Some parasites differentiate into sexual erythrocytic stages (gametocytes) ❼. Blood stage parasites are responsible for the clinical manifestations of the disease. The gametocytes, male (microgametocytes, ♂) and female (macrogametocytes ♀), are ingested by an *Anopheles mosquito* during a blood meal ❽. The parasites' multiplication in the mosquito is known as the sporogonic cycle C. In the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes ❾. The zygotes in turn become motile and elongated (ookinetes) ❿ which invade the midgut wall of the mosquito where they develop into oocysts ⓫. The oocysts grow, rupture, and release sporozoites ⓬, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites ❶ into a new human host perpetuates the malaria life cycle.

1.6. Pathophysiology of Malaria

The manifestations of malaria illness are caused by the infection of the RBCs by the asexual forms of the parasite. Up on arrival to the circulation, the merozoites from the liver attach and enter RBCs via a ligand-receptor interaction. Both the parasite and erythrocyte components regulate parasite entry and intracellular growth by extensively remodeling host membranes. These remodeling events include: the invagination of the host cell membrane during parasite entry that results in the creation and maintenance of a parasitophorous vacuolar membrane (PVM) that surrounds the intracellular organism [Dondorp, 2005; Goel *et al*., 2003; Trampuz *et al*., 2003]. More specifically, merozoite surface protein-1 (MSP-1) associated with the parasite membrane via a glycosylphosphatidylinositol (GPI) anchor binds to the RBC surface proteins [Aikawa *et al*., 1978; Baldwin *et al*., 2015]. Then, a ring-like tight junction is formed between the parasite and host cell [Mayer *et al*., 2009; Mitchell *et al*., 2004]. PVM is then formed in the junction area and the parasite move via this annulus as it enters the expanding PVM. Eventually, the PVM and host cell membrane are closed [Baldwin *et al*., 2015; Dondorp, 2005; Maier *et al*., 2003; Trampuz *et al*., 2003].

Up on entry to RBC, the parasite begins degrading haemoglobin (Hb) to obtain building blocks for protein synthesis. In addition, four molecules of haem, the ferrousprotoporphyrin IX (Fe(II)PPIX), are released from each molecule of Hb. In the presence of molecular oxygen in the parasite food vacuole, Fe(II) PPIX is oxidized to form ferricprotoporphyrin IX, Fe(III)PPIX. Free Fe(III)PPIX is highly toxic [Dondorp, 2005; Huy *et al*., 2013; Kumar *et al*., 2007; Trampuz *et al*., 2003; Weatherall *et al*., 2002]. As a result, a cascade of events begins to prevent haem toxicity to the parasite. The parasite converts free Fe(III)PPIX into a crystalline form termed as haemozoin (Hz) which is safe to the parasite. The acidic environment within the food vacuole of the parasite is important for the formation of Hz. On the other hand, Hz is found to induce different immunological cascades after release in to the blood stream when the RBCs rapture [Coronado *et al*., 2014; Dondorp, 2005; Huy *et al*., 2013; Maier *et al*., 2003; Trampuz *et al*., 2003].

At the completion of the schizogony within the red cells, newly developed merozoites are released by the lysis of infected RBCs, and along with them waste substances such as red cell membrane products, Hz, *plasmodial* DNA and GPI are released into the blood. These products activate macrophages and endothelial cells to secrete cytokines and inflammatory mediators such as tumor necrosis factor, interferon-γ, interleukins and lymphotoxin [Angulo and Fresno, 2002; Weatherall *et al*., 2002]. The *plasmodial* DNA is presented by Hz to interact intracellularly with the Toll-like

receptor-9, leading to the release of proinflammatory cytokines that in turn induce cyclooxygenase up regulating prostaglandins leading to the induction of fever [Patel *et al*., 2003].

The intact parasitized red blood cells (PRBCs) can also be responsible for the pathogenesis of malaria. PRBCs modify the host by binding to endothelial cells of the vasculature (cytoadherence), platelets, tissues of the vital organs (sequestration) and non-infected RBCs (rosetting) resulting in the blockage of blood flow and induction of different immunological cascades. Parasites adhesion to endothelium allows them to escape clearance by the spleen and to hide from the immune system [Chakravorty *et al*., 2008; Rasti *et al*., 2004]. Cytoadherence, rosetting and sequestration are facilitated through the formation of Knobs on the PRBCs membrane. Knob-associated histidine rich protein (KAHRP) and *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) are two of several proteins which induce knob formation. PfEMP-1 has been anchored to the knobs by KAHRP and is exposed on the host RBC surface and function as a ligand. PfEMP-1 is encoded by var genes; out of a family of 60 var genes in a parasite, only one is expressed at a time, the others being silenced at a transcriptional level. This results in expression of an antigenically distinct PfEMP-1 protein [Rasti *et al*., 2004].

The binding between the PRBCs and the target involves ligand-receptor interaction. The adhesion receptors include cluster of differentiation 36 (CD36), intercellular adhesion molecule 1(ICAM-1), vascular cell adhesion molecule 1, neural cell adhesion molecule (NCAM), hyaluronan-binding protein 1 and chondroitin sulfate A (CSA) that are located on the endothelial cells and other tissues [Adams *et al*., 2014; Craig and Scherf, 2001; Njuguna and Newton, 2004; Ryan, 2001; Weatherall *et al*., 2002]. The variation in different PfEMP-1 proteins determines the ability of parasite isolates to adhere differentially to the wide array of host receptors. At specific vascular sites or tissues, unique arrays of adhesion receptors are expressed, resulting in preferential sequestration of parasites expressing different PfEMP-1 variants, potentially contributing to differential pathology. For instance, variant of PfEMP-1 specific to placenta binds with CSA and lead to sequestration of PRBCs to placenta. Furthermore, variant of PfEMP-1 specific to brain endothelium binds to ICAM-1 and/or NCAM and results in cerebral malaria. CD36 receptors are abundantly distributed on vasculature, and platelet and can bind many variant of PfEMP-1 [Adams *et al*., 2014; Craig and Scherf, 2001; Patel *et al*., 2003; Rasti *et al*., 2004].

1.7. Clinical Manifestations of Malaria

The signs and symptoms of malaria can be non-specific and resembles other diseases like viral infections and enteric fever or other acute febrile infections [Bartoloni and Zammarchi, 2012; Singh *et al*., 2013; WHO, 2015]. The classical cyclic fever, chills and shivering are the cardinal signs of the disease. The blood stage parasites are responsible for the signs and symptoms of malaria. Splenomegaly and hepatomegaly can also be manifested in patients having malaria [Hermansyah *et al*., 2017; Idro *et al*., 2005; WHO, 2015]. Severe anaemia, metabolic acidosis, hypoglycemia, acute renal failure, acute pulmonary oedema, cerebral malaria, coma and death are usually the manifestations of severe malaria [Bartoloni and Zammarchi, 2012; Hermansyah *et al*., 2017; Schofield and Grau, 2005]. Fatal outcome due to severe malaria is generally attributed to cytoadherence, rosetting and sequestration [Idro *et al*., 2005; Rasti *et al*., 2004; Trampuz *et al*., 2003; Weatherall *et al*., 2002].

1.8. Diagnosis of Malaria

The diagnosis of malaria could be categorized as clinical and parasitological diagnosis. The clinical diagnosis of malaria is based on the signs and symptoms obtained from physical examination [Ryan, 2001; Wongsrichanalai *et al*., 2007]. As mentioned in section 1.7, the signs and symptoms of malaria mimic that of other acute febrile infections. As a consequence, it is very important to consider those diseases as differential diagnosis. WHO recommends that in settings where the risk of malaria is low, clinical diagnosis of uncomplicated malaria should be suspected based on the possibility of exposure to malaria and a history of fever in the previous three days with no features of other severe diseases. In settings where the risk of malaria is high, clinical diagnosis should be based on a history of fever in the previous 24h and/or the presence of anemia, for which pallor of the palms appears to be the most reliable sign in young children [WHO, 2015].

The parasitological diagnosis of malaria involves detection of the malaria parasites in the blood and important for confirming the disease. Moreover, definitive diagnosis of malaria and differentiation among *Plasmodium* species requires laboratory testing to direct the treatment based on susceptibility of the parasite to a particular drug [Bronzan *et al*., 2008]. According to WHO, in all settings, clinical suspicion of malaria should be confirmed with a parasitological diagnosis. Nonetheless, in settings where parasitological diagnosis is not possible, the decision to provide antimalarial treatment must be

based on the prior probability of the illness being malaria [Bronzan *et al*., 2008; WHO, 2015; Wongsrichanalai *et al*., 2007].

There are different malaria parasite detection techniques including the light microscopy, rapid diagnostic test (RDT) and Polymerase chain reaction (PCR). The first two methods are most commonly used for the diagnosis of malaria in clinical settings. RDT is the method which is used to detect the malaria antigen and needs less expertise and skill and commonly applied in field areas and in remote areas of developing countries where malaria is endemic [Bronzan *et al*., 2008; Wongsrichanalai *et al*., 2007]. Despite this, it is not specific in differentiating the malaria parasites and predicting the load of the parasites and false positive may also occur when the same victim is tested again after certain time. Microscopy is the gold standard in clinical diagnosis of malaria. Furthermore, it shows the level of parasitemia and type of parasite so that improve in directing the treatment choice. The need for light source, laboratory experts and false negative where the parasites are low are the shortcomings of microscopy. PCR is not routinely used for the clinical confirmation of malaria, instead it could be employed for epidemiological identification of the type of malaria parasites [CDC, 2016; Snounou *et al.,* 1993; WHO, 2015].

1.9. Prevention of Malaria

1.9.1. Vector Control

Careful observance of favourable conditions for the malaria transmitting mosquitoes and targeting those conditions has been of great importance to the effective management of the disease. There are many of such tools available. Many of these techniques are targeted at inhibiting the bite of mosquitoes [Karunamoorthi, 2011; Naseem *et al*., 2016]. The role of personal and environmental measures is among the important preventive mechanisms indicated. Moreover, appropriately covering the whole body with clothes between dusk and dawn when mosquitoes biting is high, the use of insect repellents, appropriately closing the doors and windows, and avoiding accumulation of water in the environment to tackle the reproduction of mosquitoes are among such measures [Karunamoorthi, 2011; WHO, 2017].

Insecticide treated nets (ITNs), long lasting insecticide impregnated nets (LLINs), larviciding and indoor residual spraying (IRS) are widely used by the governments of malaria endemic countries as components of malaria control and elimination tools. ITNs and LLINs exert both chemical and

physical barrier to mosquitoes while IRS and larviciding involve the use of chemicals to kill mosquitoes and their larva in that order [WHO, 2017].

1.9.2. Chemoprophylaxis

For travelers, migrant labourers and militants exposed to highly malaria endemic countries, effective chemoprophylaxis is recommended. The use of the personal protection techniques discussed above along with the chemoprophylactic drugs is indicated for better protection from the disease [WHO, 2015]. The choice of the prophylactic drug is dependent on the type of parasite prevalent, resistance profile, tolerability to the drugs and the user's interest among others. Of great note is that prophylactic drugs are not 100% protecting against the disease, and after returning back to their home, if symptoms indicative of malaria such as fever is observed, informing the health professional of recent travel is mandatory [CDC, 2016].

Pregnant women and children under five years of age are required to get chemoprevention as these population groups are highly vulnerable to malaria specially in high transmission areas. The name given to chemoprevention in pregnancy and children living in high malaria transmission area is called Intermittent Preventive Treatment (IPT). IPT aims to reduce the possible complications during pregnancy such as severe anaemia and placental infections which can threaten the life of the mother and the fetus as well as to reduce under five years mortality [Gosling *et al*., 2010]. WHO recommends pregnant women and children under five years of age to take sulfadoxine-pyrimethamine as IPT to protect these special population groups from malaria as immunity in this population group is reduced. Ivermectin mass drug administration is also under investigation for the prophylaxis against malaria to prevent transmission where ivermectin kills the mosquitoes after taking it with blood meal [WHO, 2O15].

1.9.3. Malaria Vaccine

The current malaria tools are not sufficient to effectively control and eradicate the disease [WHO, 2017]. Moreover, the emergence and spread of drug and insecticide resistance has been the major obstacles to control the disease. As a result, safe and effective malaria vaccine is needed to aid the eradication of malaria from the globe. The malaria life cycle is very complex involving multiple stages that involve many antigenic molecules that could be a target for malaria vaccine [Fujiokaa and Aikawab, 2002; Verma *et al*., 2013]. Accordingly, vaccines targeting the different stages of the

malaria life has been extensively investigated including pre-erythrocytic vaccine, erythrocytic vaccine and transmission blocking vaccine [Lennartz *et al*., 2017].

The pre-erythrocytic vaccine targets the infective sporozoite proteins. One such vaccine is the RTS, S vaccine that is in phase III clinical trial and it is the most promising vaccine [WHO, 2015; WHO, 2017]. The blood stage vaccines that target MSP were also investigated, where MSP-l and MSP-2 has been found to be promising [Jepsen *et al*., 2013]. VAR2CSA, variant of pfEMP1 that target the receptors on placenta has been studied and shown promising protection against malaria complications occurring during pregnancy [Rogerson *et al*., 2007]. Furthermore, transmission blocking vaccines that target antigens on gametocytes and zygote to prevent parasite development in mosquito midgut by specific antibodies has also been investigated, pfs25 and pfs28 among others [*Arakawa et al*., 2005]. In general, different parasitic antigenic molecules are involved in the pathogenesis of malaria which made challenge to the development of effective malaria vaccine to be licensed [Lennartz *et al*., 2017; Ouattara and Laurens, 2015].

1.10. Treatment of Malaria

1.10.1. Chemotherapy of Malaria

There are different classes of drugs currently in use for the treatment of malaria. These drugs are used to tackle malaria, by targeting the different stages in the life cycle of the parasite outlined under section 1.5. Antimalarial drugs are categorized in to different classes based on their structure and mechanism of antimalarial activity: quinoline based antimalarial drugs that include the 4-aminoquinolines (chloroquine, amodiaquine, piperaquine), and 8-aminoquinolines (primaquine), arylaminoalcohols (quinine, mefloquine, halofantrine, lumefantrine), antimetabolites (pyrimethamine, dapson, proguanil, sulfadoxine), artemisinins (arteether, arthemeter, artesunate, dihydroarthemisinin, artemisone), hydroxoynapthoquinone (atovaquone) and antimalarial antibiotics (tetracycles, clindamycin) [Peter W, 1973; Saifi *et al.*, 2013]. The quinolone antimalarials work by inhibiting polymerization of haem to Hz. The antimetabolites inhibit the synthesis of folic acid. The antibiotic antimalarials inhibit protein synthesis while artemisinins are supposed to work by interfering with the parasite sarco-endoplasmic calcium ATPase and production of free radicals. These drugs can target the asymptomatic liver asexual stages to prevent relapse, asexual symptomatic blood stage or gametocytes of the blood stage and sporozoite formation in mosquitoes to prevent transmission. Most of the today's drugs act on asexual blood stage of the parasite. Artemisinins target both the asexual

blood stages and inhibit the development of early gametocytes, primaquine targets both the pre-erythrocytic stages and mature gametocytes of *plasmodium falciparum*, yet atovaquone and antifolates inhibit sporozoite formation [Saifi *et al.*, 2013; WHO, 2015].

According to WHO, artemisinin based combination therapies (ACTs) are recommended for the treatment of uncomplicated malaria caused by *P. falciparum* or other species resistant to chloroquine (CQ). Quinine plus clindamycin is recommended for the treatment of uncomplicated malaria in the first trimester of pregnancy as safety of ACTs is not clearly investigated in the early pregnancy. However, ACTs is the drug of choice in the second and third trimesters [CDC, 2013]. The reason behind is that artemisinin and its derivatives when combined with long acting antimalarials kill parasites more rapidly and are given for short duration of time enhancing adherence and also more safer than others. Injectable artesunate is recommended in the case of severe malaria followed by standard regimens of ACTs as indicated for uncomplicated malaria at least after 24h of treatment with artesunate. In the absence of artemisinins, quinine or quinidine plus doxycycline or clindamycin or tetracycline for seven days is recommended based on the patient conditions, costs, availability and safety profile of the drugs [CDC, 2013; WHO, 2015].

1.10.2. Traditional Medicine

WHO defines traditional medicine (TM) as practices, knowledge, and belief which use minerals, plants and animal based remedies, spiritual therapies and exercises to prevent, treat and maintain wellbeing. According to WHO, about 80% of the population of the world relies on TM, mostly herbal remedies, for their primary health care needs. In Africa, the use of plants for medicinal purpose trace back to the early period in history [WHO, 2013]. In some African countries up to 90% of the population depends on medicinal plants [Jamshidi-Kia *et al*., 2018; Kazembe *et al*., 2012]. Furthermore, in Ethiopia about 80% the population relies on plant derived traditional remedies [Asmare and Kesara, 2015; Kassaye *et al*., 2006]. Medicinal plants have been of great importance in the search for new antimalarial drugs especially these days where resistance to the mainstay antimalarial drugs is alarming. Many of the antimalarial drugs were derived from medicinal plants that had traditionally been used for the treatment of malaria [Jamshidi-Kia *et al*., 2018]. For instance, the antimalarial drug quinine was derived from cinchona bark. The same is true of artemisinin from *Artemisia annua.* Therefore, it is logical to have investigations on traditionally used medicinal plants on the way to discover new antimalarial drugs [Kassaye *et al*., 2006; Kazembe *et al*., 2012].

1.10.3. Investigational Antimalarial Agents

Different antimalarial molecules are in the pipeline at different preclinical and clinical trial stages. The molecules under development for malaria treatment like those previously developed and licensed ones can target the biochemical processes involved in the life cycle of the parasites perhaps in different or similar ways [Nwaka *et al*., 2004; Rowe *et al*., 2009]. While some tried the pre-existing compounds for malaria or other treatments, in single or combination, others tried absolutely new compounds [Nzila *et al*., 2011]. Accordingly, OZ439, acting the same way to artemisinins is under clinical trial II [Opsenica and Šolaja, 2009]. Moreover, combinations of antibiotics fosmidomycin-clindmycin is under clinical trial III for uncomplicated *falciparum* malaria. A combination chlorproguanil-dapsone-artesunate is under clinical trial III as well. Transmission blocking agents KAF 156 and KAE609 [Kuhen *et al*., 2014], 8-amino quinolone tafenoquine under phase III clinical trial [Rajapakse *et al*., 2015] and trimethoprim-sulfamethoxazole under phase III clinical trial are also promising [Janet *et al*., 2016]. The antihelminths, levamisole and ivermectin are also under clinical trials [Smit *et al*., 2018; Rowe *et al*., 2009]. Despite the promises, none of them were licensed to the market by Food and Drug Administration [Nwaka *et al*., 2004; Smit *et al*., 2018; WHO, 2017].

1.11. Antimalarial Drug Resistance

Development and spread of antimalarial drug resistance has been the major challenge towards the control of the disease [Bhattacharjee and Shivaprakash, 2016; WHO, 2015]. Moreover, it has emerged as one of the greatest challenges facing malaria control and also implicated in the spread of the disease to new areas and re-emergence of malaria in areas where the disease had been eradicated. Resistance to CQ, the cheapest perhaps the most widely used antimalarial drug, posed the most serious challenge among others. Nevertheless, CQ resistant strains can be controlled by artemisinins, the most active regimens in the current antimalarial drugs [White, 2004]. Yet, an alarming data is emerging from the Cambodian-Thailand border as well as reduced sensitivity in parts of Africa with artemisinin-resistant *P. falciparum* [Alelign and Dejene, 2016; Krishnaa *et al*., 2004]. The emergence of antimalarial drug-resistant parasites will not only obscure the malaria control efforts achieved but also alarms to look for new treatment regimens to treat and control the disease [Antony and Parija, 2016].

The mechanism of antimalarial drug resistance is different for different classes of antimalarial drugs [Antony and Parija, 2016; WHO, 2015]. Antimalarial drug resistance to quinolones and arylaminoalcohols involves the mutations of the genes encoding vacuolar transmembrane proteins that regulate the transport of these drugs at the target. For instance, CQ resistance in *P. falciparum* is mainly because of single nucleotide polymorphisms in the genes encoding pfcrt which is CQ transporter while polymorphisms in the genes encoding for the *P. falciparum* multidrug resistance (*pfMDR1*) is involved in mefloquine resistance [Farooq and Mahajan, 2004; White, 2004; WHO, 2015]. Resistance to antifolate drugs involves stepwise development of point mutations in the genes, pfdhps and pfdhfr, that encode for the enzymes taking part in the folate synthesis, dihydropteroate synthetase and dihydrofolate reductase, respectively [Gregson and Plowe, 2005]. Mutations in the genes encoding for the sarco-endoplasmic reticulum calcium ATPase, and Kelch 13 are indicated for artemisinins resistance while point mutations of cytochrome b gene of *P. falciparum* is responsible for the resistant to atovaquone [Krishnaa *et al.*, 2004; Saifi *et al.*, 2013].

1.12. The Experimental Plant

Schinus molle Linnaeus (Figure 2) is ever green tree originally from South America but has been introduced to different parts of the world. The plant belongs to the family *Anacardiaceae*. It is widely planted on roadsides, in graveyards and often used in subtropical climates for landscaping. It is the largest of all *Schinus* species and potentially the longest lived. The fruit are round drupes with woody seeds that turn from green to red, pink or purplish, carried in dense clusters of hundreds of berries that can be present year round. The bark, leaves and berries are aromatic when crushed. It is vernacularly named differently in different languages: Amharic (qundo berbere); Afan Oromo (Mimxa-Gumuz); Arabic (felfelkazib, filfilrafie); English (pepper tree) [Belhamel *et al*., 2008; Feyera and Abdisa, 2016; Kenea and Tekie, 2015].

It is used as spice mixed with commercial pepper. Extracts of coat of fruits of *S. molle* have been used as a flavor in drinks and syrups [Biwer and VanDerwarker, 2015]. Moreover, different parts of *S. molle* are used as medicinal remedy [Devec *et al*., 2010; Kenea and Tekie, 2015]. Extracts obtained from the fruits and aerial parts of the leaf of *S. molle* have been found to show analgesic, anti-inflammatory, anti-tumorous and anti-depressant effect [Ferrero *et al*., 2006; Rajput *et al*., 2011]. It also possesses potent anthelmintic, antibacterial, antiviral, antifungal, insecticidal and insect repellent properties [Chopa *et al*., 2006; Devec *et al*., 2010; Feyera and Abdisa, 2016; Hayouni *et al*., 2008;

Huerta *et al*., 2010; Zenebe *et al*., 2017]. The seed of the plant has been traditionally used for treatment of malaria in different parts of Ethiopia [Alebie *et al*., 2017; Giday *et al*., 2006; Kenea and Tekie, 2015]. The methanolic crude extract of the leaves and fruits of *S. molle* has also been found to show *in vitro* antimalarial activity with IC50 of 4.47and 8.21µg/ml, respectively [Abdel-Sattar *et al*., 2010].

Figure 2: Picture of *Schinus molle* Linnaeus with its berries

1.13. Methods for Screening Antimalarial Compounds

There are various *in vitro* and *in vivo* screening methods being used for antimalarial drug development. The *in vitro* methods for screening antimalarial compounds is based on the ability to culture the malaria parasites in human erythrocytes. Hypoxanthine rich culture media is the most standardized method to determine the level of parasite growth inhibition in this model. Compounds that show promising antimalarial activity in this model can pass to the next investigations [Kalra *et al*., 2006].

Compounds effective in *in vitro* screening tests are taken up for in vivo evaluation. The *in vivo* malaria models have been established in a variety of laboratory animals. Each model system has its individual characteristics. *Plasmodium* species that cause human disease are essentially unable to infect non primate animal models. So, *in vivo* evaluation of antimalarial compounds begins with the use of rodent malaria parasite. *P. berghei, P. yoelii, P. chabaudi, P. vinckei* have been used extensively in drug discovery and early development [Adumanya *et al*., 2014; Kalra *et al*., 2006].

The protocols which are used for the *in vivo* screening of antimalarial compounds include four day suppression test, prophylactic test and curative test. In the four day suppressive test, the effect of compounds on the early stage of the parasite is evaluated while curative test involves determination of the effect of compounds on established infection. In prophylactic test, on the other hand, the compounds' ability to prevent the development of the disease if given prior to the infection is assessed. In all these models percentage parasitemia is the most reliable parameter monitored [Adumanya *et al*., 2014; Kalra *et al*., 2006]. In the present study, the four day suppressive test is utilized for antimalarial screening pertaining to the following reasons. The traditional healer strongly mentioned that it should be given in the early stage of symptoms for better response. The ethnobotanical survey indicated that the plant seed is not given for prevention like that of Allium sativum except the use of leaf of the plant for insect repellency [Giday *et al*., 2006; Kenea and Tekie, 2015].

1.14. Rationale of the Study

Malaria is a major global public health problem. The burden is heaviest in sub-Saharan Africa especially among children under the age of five and pregnant women. In addition to its public health burden, the disease has also negative impact on socioeconomic development. Resistance to antimalarial drugs has been among the major challenges in controlling the disease. Previously sensitive parasites are now resistant to many of the antimalarial drugs. More importantly, there are alarming reports on parasite resistance to currently existing first line drug regimen, ACTs, in parts of Cambodia and Thailand as well as reduced sensitivity in parts of Africa [Alelign and Dejene, 2016; Dippmann *et al*., 2008; Dondorp *et al*., 2009]. Furthermore, these resistant strains have the potential to spread to different parts of the world including Ethiopia and subsequently become a global threat.

Therefore, this issue necessarily triggers a search for new and effective antimalarial drugs [CDC, 2016; Dondorp *et al*., 2009; WHO, 2015].

Medicinal plants played a crucial role as a source for antimalarial drugs including artemesinin and quinine [Tesfaye and Alamneh, 2014; Willcox and Bodeker, 2004]. *S. molle* is used as folk medicine for the treatment of malaria in Ethiopia [Alebie *et al*., 2017; Giday *et al*., 2006; Kenea and Tekie, 2015] and found to show *in vitro* antimalarial activity elsewhere [Abdel-Sattar *et al*., 2010]. Nevertheless, it lacks further *in vivo* pharmacological investigations for its antimalarial activity. Consequently, the crude seed extracts and solvent fractions of *S. molle* were assessed for the antimalarial activity following the peter's 4 day suppressive test against *P. berghei* in mice model in an effort to confirm the traditional claim and contribute to the scientific community to further investigate on the plant to discover antimalarial drug(s) from it.

2. Objectives

2.1. General Objective

- To evaluate the antimalarial activity of aqueous and 80% methanol crude seed extracts and solvent fractions of *S. molle* in *P. berghei* infected mice.

2.2. Specific Objectives

- To determine the acute toxicity of aqueous and 80% methanol crude seed extracts and solvent fractions of *S. molle.*
- To evaluate the antimalarial activity of aqueous and 80% methanol crude seed extracts of *S. molle* using the four day suppressive test.
- To assess the antimalarial activity of solvent fractions of *S. molle* using the four day suppressive test.
- To assess phytochemical composition of the aqueous and 80% methanol crude seed extracts and solvent fractions of *S. molle.*

3. Materials and Methods

3.1. Chemicals and Reagents

In this study, the following chemicals and reagents were used: trisodium citrate (BDH Chemicals Ltd, England), methanol (Carlo Erba, France), oil immersion (Neolab, India), Geimsa stain (BDH Chemicals Ltd, England), chloroform (Carlo Erba, France), normal saline (Fresenius Kabi, India), chloroquine phosphate (Ethiopian Pharmaceutical Manufacturing, Ethiopia), Wagner and Mayer reagents (Fisher Scientific, England), dilute ammonia (Riedal de Haen, Germany), glacial acetic acid (Lobe Chemi, India), ferric chloride (Fisher Scientific, England), hydrochloric acid (Riedal de Haen, Germany), distilled water (Ethiopian Pharmaceutical Manufacturing, Ethiopia), tween 80 (Lobe Chemi, India), n-butanol (Fischer Scientific, England). All the chemicals and reagents used in this study were of analytical grade.

3.2. Experimental Animals

For the *in vivo* test, healthy Swiss albino mice, weight 25-31g, age 6-8 weeks, female for toxicity and male for antimalarial activity were used. The animals were bred and maintained in the animal house at School of Pharmacy, Addis Ababa University. They were acclimatized for one week to the experimental environment before the actual experiment. They were then maintained in groups of six in plastic cage at 12 hour 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 United States National Academy of Sciences guidelines for the care and use of laboratory animals which is internationally accepted [National Academy of Sciences, 2011].

3.3. Rodent Malaria Parasite

Chloroquine sensitive *P. berghei* ANKA strain was obtained from Ethiopian Public Health Institute (EPHI). The parasites were then maintained by serial passage of blood from infected mice to non-infected ones every week until 30-37% parasitemia level was attained [Bantie *et al*., 2014].

3.4. Collection and Authentication of Experimental Plant

The ripened fruit of *S. molle* was collected in December 2017 GC, from Sasiga District, East Wollega Zone, Oromia Region, West Ethiopia, which is about 350 km away from Addis Ababa where it has a traditional claim for the treatment of malaria. The fruit was carried in a plastic bag during

transportation. In addition, for authentication purpose, the branch with its berries was cut and wrapped with newspaper sheet to maintain its architecture during transportation. Identification and authentication of the plant was done by a taxonomist at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University, where a voucher specimen was coded and deposited for future reference with voucher number GH 01/2017.

3.5. Preparation of Crude Extracts

The ripened fruits of the plant were thoroughly washed with tap water to remove dirt and cleaned with gauze. Then, they were air-dried under shade, and the covering was removed by meshing with hand. After that, the resulting woody seeds were pulverized using mortar and pestle to get coarse powder used for extraction. Then, the powder was separated in to 2 parts; 300g for aqueous extraction and the other 750g of the powder for 80% methanol extraction.

Then after, for aqueous extraction, 150mg of pulverized seeds was weighed using sensitive digital balance and soaked in 1500ml of distilled water in an Erlenmeyer flask. After that, the flask was put on mechanical shaker (Bibby Scientific Limited Stone Staffo Reshire, UK) and the extraction process was facilitated with occasional stirring at 120 rpm (revolutions per minute) for 72 hours. The flask was then removed from the shaker and put on the table for 30 minutes. Then, the resulting distillate containing the aqueous crude extract was separated from the marc with gauze, and further filtered by Whatman filter paper number 1 (Whatman®, England) under suction filtration. The same procedure was repeated two times by adding another fresh solvent into the marc. Moreover, the other 150mg of the coarse powder was extracted with the same procedure detailed above. The filtrates were then combined together in a round bottom flask, frozen in deep freezer overnight and then freeze dried with a lyophilizer (Operan, Korea Vacuum Limited, Korea) to remove the solvent at -50^{o}C and vacuum pressure of 200 mBar. Finally, the concentrated extract was transferred into vials and kept at -20°C until use.

The other extraction was that of 80% methanol crude extract that was used for antimalarial test and fractionation. Accordingly, 150mg of pulverized seeds was weighed using sensitive digital balance and soaked in 1500ml of 80% methanol in an Erlenmeyer flask. After that, the flask was put on mechanical shaker (Bibby Scientific Limited Stone Staffo Reshire, UK) and the extraction process was facilitated with occasional stirring at 120 rpm for three consecutive days. After three days, the flask was removed from the shaker and put on the table for 30 minutes. Then, the resulting distillate

containing the 80% methanol crude extract was separated from the marc with gauze, and further filtered by Whatman filter paper number 1 (Whatman®, England) under suction filtration. The same procedure was repeated two times by adding another fresh solvent into the marc. Moreover, the other 600mg of the coarse powder was divided in to four and extracted in the same way. The filtrates were then combined together in a round bottom flask and the solvents were removed. First, methanol was removed from the combined filtrates by evaporation under reduced pressure using rotary evaporator (Buchi Rota vapor R-200, Switzerland) at 45 rpm with temperature not exceeding 40°C. Then, the resulting extract was frozen in deep freezer overnight and freeze dried with a lyophilizer (Operan, Korea Vacuum Limited, Korea) to remove water at -50°C and vacuum pressure of 200 mBar. Finally, the concentrated extract was transferred into vials and kept at -20°C until use.

3.6. Fractionation of 80% Methanol Crude Extract

Forty-two grams of the 80% methanol crude extract, an extract with better antimalarial effect, was subjected to fractionation using solvents of differing polarity that include chloroform, n-butanol and water**.** The aforementioned amount of the crude extract was suspended in 350 ml of distilled water in a separatory funnel. Then, the suspension was shaken with 100ml of chloroform. The chloroform layer was collected to a beaker by careful opening of the funnel at its valve located near to the bottom. Then, the valve was closed and the aqueous layer was further shaken twice with 100ml of chloroform and the chloroform fraction was collected and combined to the previous one. The combined chloroform layers were then dried in dry oven with temperature not exceeding 40^{o}C.

The remaining aqueous layer was again shaken with 100ml of n-butanol. Then, the aqueous layer was first collected into a beaker by carefully opening the separatory funnel at its valve. Then, the remaining n-butanol layer in the separatory funnel was collected to a separate beaker. The aqueous layer was then transferred to the separatory funnel and shaken twice with 100ml of n-butanol, and the n-butanol fraction was collected and combined to the previous one. Then, combined n-butanol layers were transferred to a round bottom flask and concentrated in rotary evaporator (Buchi Rota vapor R-200, Switzerland) at 45 rpm and temperature not exceeding 40^{o}C. The remaining aqueous residue in the separatory funnel was transferred to a round bottom flask, frozen in deep freezer overnight and then freeze dried with a lyophilizer (Operan, Korea Vacuum Limited, Korea) to remove water at -50^{o}C and vacuum pressure of 200 mBar to obtain the aqueous fraction. Finally, after calculating the

yield, the fractions in all cases were kept in amber glass vials and stored in a refrigerator at -20°C until used for the test.

3.7. Acute Toxicity Test

Oral acute toxicity test was performed on the crude extracts and solvent fractions of the seed of *S. molle* before they were evaluated for their antimalarial activity according to the Organization for Economic Co-operation and Development (OECD) 425 guideline for crude extracts [OECD, 2008]. Female Swiss albino mice, age 6-8 weeks and weight 25-31g, were used for the toxicity study. The mice were fasted for three hours before the test. Following the period of fasting, the animals were weighed and 2000mg/kg of the crude extracts and fractions were administered by oral gavage as a single dose. Food was then withheld for further 2 hours. First, 5 female mice, one for each crude extract and fraction, was given 2000mg/kg of each of the extracts and fractions as a single dose by oral gavage. Then, each mouse was observed over a period of 24 hours. Since no death was observed, another four female mice for each crude extract and fraction, were given the same dose and observed for gross behavioral changes such as loss of appetite, hair erection, lacrimation, tremors, convulsions, mortality and the like which are signs of toxicity over a period of 14 days.

3.8. *In Vivo* Antimalarial Screening

3.8.1. Grouping and Dosing of Animals

For evaluation of *in vivo* antimalarial activity of the aqueous and 80% methanol crude seed extracts and the three solvent fractions (chloroform, n-butanol and aqueous fraction) of *S. molle*, infected male Swiss albino mice for each crude extract and fraction were randomly assigned to five groups of 6 mice in different cages. Three of the groups for each crude extract and fraction received the extracts and fractions at doses of 100mg/kg, 200mg/kg and 400mg/kg daily for four consecutive days. The remaining two groups for each crude extract and fraction served as negative and positive controls. The negative control groups received solvents for reconstitution; 10ml/kg of distilled water for aqueous extract and aqueous fraction, 10ml/kg of 2% tween 80 in case of the other three tests for the same days. Positive control groups in all the five tests received chloroquine phosphate (CQP) containing 10mg/kg of CQ for four days. Dose selection was made based on the result of acute toxicity and preliminary study conducted on the extracts. Each dose of the extracts, fractions and standard drug after reconstitution by the respective solvents, and the solvents for reconstitution in negative control groups were administered via the oral route using gavage. Volume administered was

calculated based on individual mouse body weight. The maximum volume administered was 0.31ml while the minimum was 0.25ml.

3.8.2. Parasite Inoculation

For *in vivo* antimalarial test, Swiss albino mice infected with *P. berghei* were obtained from EPHI as mentioned in section 3.3 and were used as donors. *In vivo* antimalarial activity of the crude extracts and solvent fractions against early chloroquine sensitive *P. berghei* infection was then carried out according to the four day suppressive test described by Peter *et al*. [1975]. First, the parasitaemia level of the donor mice were determined from the blood collected by cutting a 0.5 to 1mm section from tail of the mice with scissor. The parasites were then maintained by serial passage of blood from infected mice to non-infected ones every week until 30-37% parasitemia level was attained. Then, to infect the test mice, the donor mouse with a rising parasitaemia of aforementioned level [Deressa *et al*., 2010] were euthanized with cervical dislocation and infected blood obtained by cardiac puncture was collected in a falcon tube containing 2% trisodium citrate (BDH Chemicals, England) as anticoagulant [Bantie *et al*., 2014; Nureye *et al*., 2018].

The collected blood from all donor mice were pooled together to avoid variability and then diluted in normal saline so that the final suspension would contain about $1x10^7$ infected RBCs in every 0.2ml suspension [Bantie *et al*., 2014]. The dilution was made based on parasitemia of the donor mice and RBC count of the normal mice in such a way that 1ml blood contains $5x10^7$ infected RBCs [Asnake *et al*., 2015]. Then, on day 0 (d0), each mouse used was infected intraperitoneally with 0.2 ml of blood containing about $1x10^7$ *P. berghei* infected RBCs. Then, treatment was started three hours after mice had been inoculated with the parasite at day 01 (d01) and then continued daily with three doses at day 1 (d1, 24h from d01), day 2 (d2, 48h from d01) and day 3 (d3, 72h from d01). On day 4 (d4, 96h from d01), parameters detailed below were determined and the mice were monitored daily for 30 days for survival time [Peter *et al*., 1975].

3.8.3. Determination of Parasitemia and Survival Time

On d4, blood was collected from the tail of each mouse using clean, non-greasy slides and thin films made and allowed to air dry. The films were then fixed with few drops of methanol, left for about 15 minutes to air dry and transferred to slide box. Then, the slides were stained with freshly prepared 10% Giemsa for 15 minutes. The stain was then washed off with distilled water and slides were left to air dry. Then, the slides were viewed under the light microscope using the oil immersion objective

and parasites were counted using the X100 objective. The percentage parasitemia (PP) was obtained by counting the number of PRBCs out of erythrocytes in random fields of the microscope. Two stained slides for each mouse were examined. Three fields [Bantie *et al*., 2014] with approximately 200-500 cells were counted for each slide and PP for each mouse was determined using the following formula [Adumanya *et al*., 2014; Fidock *et al*., 2004; Mengiste *et al*., 2012]:

$$PP = \frac{PRBC}{total\ number\ of\ RBCs\ counted} \times 100$$

The mean PP of the groups in each crude extract and fraction, and the respective negative controls was determined. Then, the mean percentage parasitemia suppression (PPS) was calculated using the formula described below [Adumanya *et al*., 2014; Fidock *et al*., 2004]:

$$PPS = \frac{(\text{mean } PP\ in\ negative\ control - \text{mean } PP\ in\ treatment\ group)}{mean\ PP\ in\ negative\ control} \times 100$$

To assess the extracts' and fractions' effect on survival time, 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 (30 days). Then, the mean survival time (MST) for each group was calculated as follows [Asnake *et al*., 2015]:

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

3.8.4. Determination of Weight, Temperature and Packed Cell Volume

Weight and rectal temperature of each mouse in each group were recorded just before treatment, and after treatment on the d4 using sensitive digital weighing balance (Mettler Toledo, Switzerland) and digital rectal thermometer (G.S.T Corporation, New Delhi, India). The mean percentage changes prior to treatment and after treatment on d4 were then calculated and analyzed for each group [Peter *et al*., 1975]. In the same way, packed cell volume (PCV) was measured just before treatment and at the end of d4 using microhematocrit centrifuge (Centurion Scientific, UK). Blood was collected from the tail of each mouse in heparinized microhaematocrit capillary tubes. The capillary tubes were filled to 3/4th of their height with blood and 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 minutes [Bantie *et al*., 2014]. Finally, the tubes

were taken out of the centrifuge and PCV was determined using the standard hematocrit reader (Hawksley and Sons, England). PCV is a measure of the proportion of RBCs to plasma in the whole blood and determined using the formula shown below [Asnake *et al*., 2015, Dikasso *et al*., 2006; Mengiste *et al*., 2012]:

$$PCV = \frac{volume\ of\ erythrocyte\ in\ a\ given\ volume\ of\ blood}{total\ blood\ volume\ examined}\ X\ 100$$

3.9. Phytochemical Screening

The aqueous and 80% methanol crude seed extracts and solvent fractions of *S. molle* were screened for the presence of major phytochemical constituents. Accordingly, tests for alkaloids, saponins, tannins, flavonoids, phenols, cardiac glycosides, steroids and terpenoids were performed following standard procedures as described below.

Test for Alkaloids

Five hundred milligram of each of the two crude extracts and all the 3 solvent fractions were separately treated in test tubes with 10ml of 1% HCl for 30 minutes in a water bath, heated gently with occasional stirring and then filtered through cotton in to a separate test tube for each. Then, from each test tube, small portion was transferred into two test tubes, and to one of the test tubes five drops of Mayer's reagent, and to the other test tube five drops of Wagner's reagent were added. After that, turbidity of the resulting precipitate, the formation of whitish opalescence (Mayer's reagent) or reddish brown (Wagner's reagent), was inspected for the presence of alkaloids [Tiwari *et al*., 2011].

Test for Saponins

Five hundred milligram of each of the two crude extracts and all the three solvent fractions were mixed with 10ml of distilled water in a test tube and was shaken vigorously. Then, the formation of stable foam that persists for 30 minutes was visually inspected as an indication for the presence of saponins [Ajayi *et al*., 2011].

Test for Tannins

First, about two milligram of each of the two crude extracts and all the three solvent fractions were stirred with 2ml of distilled water and few drops of ferric chloride solution were added. Then, the formation of blue or greenish precipitate/solution was visually inspected as an indication for the presence of tannins [Njoku and Obi, 2009].

Test for Flavonoids

The crude extracts and fractions were treated with few drops of 20% sodium hydroxide solution. Then, formation of intense yellow colour which becomes colourless on addition of dilute hydrochloric acid was visually inspected as an indication for the presence of flavonoids [Ugochukwu *et al*., 2013].

Test for Phenols

First, appropriate amount of sample of the crude extracts and solvent fractions were dissolved or suspended in distilled water. Then, to 2ml of filtered solutions of the samples, freshly prepared three drops of a mixture of 1ml of 1% FeCl3 and 1ml of 1% KFe(CN)6 were added and the appearance of a green or blue color was visually inspected for the presence of flavonoids [Njoku and Obi, 2009].

Test for Cardiac Glycosides

Five milligram of each the two crude extracts and all the three solvent fractions were treated with 2ml of glacial acetic acid in a test tube and a drop of ferric chloride solution was added. The mixture was then poured into a test tube containing 1ml of concentrated H_2SO4. The appearance of a brown ring at the interface was inspected visually for the presence of deoxysugar, characteristic of cardenolides. A violet ring may appear below the ring while in the acetic acid layer, a greenish ring may form [Ugochukwu *et al*., 2013].

Test for Steroids

One gram of each of the two crude extracts and all the three solvent fractions were weighed and placed in a test tube. This was dissolved in 2ml 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 inspected for the presence of steroids [Tiwari *et* al., 2011].

Test for Terpenoids

Five milligram of the crude extracts and fractions dissolved in the solvent was mixed in 2ml of chloroform. Then, 2ml of concentrated H2SO4 was carefully added and heated for about 2 minutes. The appearance of a reddish brown color at the interface was inspected visually for the presence of terpenoids [Mohammeda *et al*., 2016].

4. Data Analysis

The collected data were organized and entered into windows statistical package for social science (SPSS) version 22 and then analyzed. Moreover, results obtained from the study were presented as mean plus or minus standard error of the mean (M ± SEM). One way analysis of variance (ANOVA) followed by Tukey post Hoc test was used to compare the mean PPS, MST, changes in mean body weight, PCV and rectal temperature of the *P. berghei* infected mice between the test groups and the respective control, and among different test groups in each test. The analysis at 95% confidence interval and p-value less than 0.05 was considered to be statistically significant.

5. Results

5.1. Percentage Yield of the Crude Extracts and Fractions

The percentage yield of the aqueous and 80% methanol crude seed extracts and solvent fractions of the seed of *S. molle* is presented in Table 1. The 80% methanol crude seed extract exhibited higher percentage of yield (11.2%) as compared to the aqueous crude extract (8.00%). Among the fractions of 80% methanol crude seed extract, the highest percentage yield was obtained from the chloroform fraction (45.52%) while the lowest was obtained from the aqueous fraction (18.27 %).

Table 1: Percentage yield of the aqueous and 80% methanol crude seed extracts and solvent fractions of the seed of *S. molle*.

Extract/Fraction	%yield (w/w)
80% methanol crude extract	11.20
Aqueous crude extract	8.00
Chloroform fraction	45.52
Butanol fraction	35.21
Aqueous fraction	18.27

5.2. Acute Toxicity Test

According to the acute toxicity test conducted to determine the safety level of the crude extracts and solvent fractions of the seed of *S. molle*, the lethal dose for both crude extracts and all the fractions was found to be above 2000 mg/kg. Furthermore, the test substances that were administered orally in a single dose of 2000 mg/kg to the laboratory bred Swiss albino mice caused no mortality within the first 24 h and the next 14 days of the observation period. In addition, the gross behavioral and physical observation of the experimental mice revealed that the substances caused no visible signs of acute toxicity such as lacrimation, loss of appetite, hair erection, salivation, diarrhoea, reduction in motor and feeding activities.

5.3. Effect of Crude Extracts on Parasitemia and Survival Time

The findings of the effect of aqueous and 80% methanol crude seed extracts of *S. molle* at different dose levels on parasitemia and survival time of Swiss albino mice infected with CQ sensitive *P. berghei* are summarized in Table 2. Furthermore, the results are expressed as mean PPS and MST in days in reference to the respective negative control mice treated with the vehicle. The highest percentage parasitemia inhibition (66.91%) was exhibited by 80% methanol crude seed extract at 400 mg/kg/day while the lowest inhibition (27.18%) was exhibited by aqueous crude seed extract at 100 mg/kg/day. Nevertheless, the effect produced by both the crude extracts was less than that of the standard drug which cleared the parasite to undetectable level. Significant inhibition of parasitemia ($p<0.05$) was exhibited by both the aqueous and 80% methanol crude extracts compared to their respective negative controls. In the same way, different dose levels in each crude seed extract exhibited statistically significant ($p<0.05$) difference in reducing parasite load at all dose levels evaluated in the study. Moreover, the higher the dose of each crude extract given, the higher the percentage inhibition of parasitemia exhibited.

The mice treated with different doses of aqueous and 80% methanol crude seed extracts of *S. molle* survived for more days than the respective negative control groups (Table 2). The highest mean survival time was recorded in mice that received the highest dose of the crude extracts. The 80% methanol crude seed extract was the one that exhibited the longest mean survival time (13.83 days) at the highest dose given. However, the maximum effect exerted by the highest dose of the 80% methanol crude extract was lower than that of CQP where none of the mice showed death in the follow up period. The aqueous crude seed extract, on the other hand, significantly prolonged mean survival time (8.00 days) only at the highest dose given ($p<0.05$) when compared to the respective negative control mice.

Table 2: The effect of crude seed extracts of *S. molle* on parasitemia and survival time of *P. berghei* infected mice.

Group	% Parasitemia	%Suppression	Survival Time (day)
TW	51.35±1.66	0.00	6.33±0.49
80ME100	33.01±0.92	35.72^{a*,c*,d*,e*}	9.50±0.43^{a*,d*,e*}
80ME200	22.75±0.83	55.70^{a*,b*d*,e*}	10.1667±0.48$^{a*,\ d*,e*}$
80ME400	16.99±0.73	66.91^{a*,b*,c*,e*}	13.83±0.87^{a*,b*,c*,e*}
CQ10	0.00±0.00	100.00 ^{a*}	>30.00±0.00 ^{a*}
DW	52.20±2.20	0.00	6.33±0.21
AE100	38.00±1.32	27.18^{a*,c*,d*,e*}	6.50± 0.34^{d*,e*}
AE200	35.42±1.20	32.15^{a*,b*,d*,e*}	6.67±0.33 ^{d*,e*}
AE400	33.89±1.32	35.08^{a*,b*,c*,e*}	8.00±0.37^{a*,b*,c*,e*}
CQ10	0.00±0.00	100.00 ^{a*}	>30.00±0.00 ^{a*}

Data are expressed as mean ± SEM (n = 6); a, compared to either DW or TW; b, compared to 100 mg/kg; c, compared to 200 mg/kg; d, compared to 400 mg/kg; e, compared to CQ10; $^{*}p<0.05$. AE=aqueous crude extract, DW= negative control, received distilled water (10 ml/kg), TW= negative control, received 2% tween 80 (10 ml/kg), CQ=chloroquine base, 80ME = 80% methanol crude extract. Numbers after letters in the first column refer to dose in mg/kg.

5.4. Effect of Crude Extracts on Body Weight and Rectal Temperature

The findings of the effect of aqueous and 80% methanol crude seed extracts of *S. molle* at different dose levels on body weight and rectal temperature of Swiss albino mice infected with CQ sensitive *P. berghei* are summarized in Table 3. Moreover, the results are expressed as the change in mean body weight and rectal temperature between the pre-treatment value (d0) and the post- treatment value (d4) for each group in reference to the change in mean values of the respective negative control mice.

Significant increment of weight and rectal temperature was exhibited in mice treated with both the aqueous and 80% methanol crude extracts compared to their respective negative controls (Table 3). Moreover, the crude extracts improved body weight and rectal temperature increment to different

levels between the d0 and the d4 compared to the respective negative control mice treated with the vehicle. The highest increment in both cases was exhibited by 80% methanol crude seed extract at 400 mg/kg/day while the lowest increment was exhibited by aqueous crude seed extract at 100 mg/kg/day (Table 3). Nevertheless, the effect produced was less than that of the standard drug, CQP which showed the highest protection from parasite-induced body weight loss and reduction in rectal temperature. When compared among themselves, different dose levels in each crude seed extract, exhibited statistically significant ($p<0.05$) difference in protection from parasite induced body weight loss and reduction in rectal temperature evaluated in this study. Moreover, the higher the dose of each crude extract given, the better the protection from infection induced body weight loss and reduction in rectal temperature.

Table 3: The effect of crude seed extracts of *S. molle* on body weight and rectal temperature of *P. berghei* infected mice.

Group	Weight (g)			Temperature (°C)		
	d0	d4	Change	d0	d4	Change
TW	28.17±0.82	24.54±0.5	-3.63±1.46	36.85±0.22	34.47±0.27	-2.38±0.06
80ME100	28.50±0.63	26.47±0.68	-2.03±0.17a*c*d*e*	36.70±0.35	35.50±0.36	-1.20±0.02a*c*d*e*
80ME200	28.66±0.66	27.53±0.69	-1.13±0.13a*b*d*e*	37.05±0.32	36.52±0.32	-0.53±0.01a*b*d*e*
80ME400	28.63±0.66	28.42±0.66	-0.21±0.017a*b*c*e*	36.94±0.27	36.70±0.27	-0.24±0.01a*b*c*e*
CQ10	28.52±0.79	28.80±0.83	0.28±a*	37.20±0.22	38.00±0.21	0.80±0.03a*
DW	28.22±0.85	24.74±0.88	-3.48±0.10	37.05±0.42	34.69±0.41	-2.36±0.04
AE100	28.14±0.77	24.68±0.77	-3.46±0.64d*e*	37.15±0.44	34.78±0.43	-2.37±0.01c*d*e*
AE200	28.35±0.55	24.89±0.55	-3.45±0.01d*e*	36.88±0.22	34.54±0.24	-2.34±0.00a*b*d*e*
AE400	28.54±0.86	25.38±0.84	-3.16±0.04a*b*c*e*	36.97±0.30	35.12±0.29	-1.85±0.01a*b*c*e*
CQ10	28.23±0.50	28.45±0.46	0.22±0.05667a*	36.99±0.28	37.67±0.27	0.68±0.04 a*

Data are expressed as mean ± SEM (n = 6); a, compared to either DW or TW; b, compared to 100 mg/kg; c, compared to 200 mg/kg; d, compared to 400 mg/kg; e, compared to CQ10; $^{*}p<0.05$. d0 = pre-treatment value on day 0, d4 = post-treatment value on day four. AE=aqueous crude extract, DW= negative control, received distilled water (10 ml/kg), TW= negative control, received 2% tween 80 (10 ml/kg), CQ=chloroquine base, 80ME = 80% methanol crude extract. Numbers after letters in the first column refer to dose in mg/kg.

5.5. Effect of Crude Extracts on Packed Cell Volume

The findings of the effect of aqueous and 80% methanol crude seed extracts of *S. molle* at different dose levels on PCV of Swiss albino mice infected with CQ sensitive *P. berghei* are summarized in Figure 3 and 4 below. Moreover, the results are expressed as the change in mean PCV between the d0 and d4 for each group in reference to the change in mean PCV of the respective negative control mice treated with the vehicle.

The change of the mean value of PCV showed reduction in mice treated with crude extracts and in those that were treated with the vehicle on d4 as compared to d0 (figure 3 and 4). However, significant protection against reduction of PCV ($p<0.05$) was exhibited in both the aqueous and 80% methanol crude extracts compared to their respective negative controls. Moreover, the crude extracts improved mean PCV to different levels. The highest protection against reduction of PCV was exhibited by 80% methanol crude seed extract at 400 mg/kg/day (Figure 3) while the lowest protection was exhibited by aqueous crude seed extract at 100 mg/kg/day (Figure 4). Nevertheless, the effect produced was less than that of the standard drug, CQP which showed the highest protection from infection induced reduction in PCV. When compared among themselves, different dose levels in each crude seed extract, exhibited statistically significant ($p<0.05$) difference in protection from infection induced reduction in PCV in this study. Moreover, the higher the dose of each crude extract given, the better the protection from infection induced reduction in PCV exhibited.

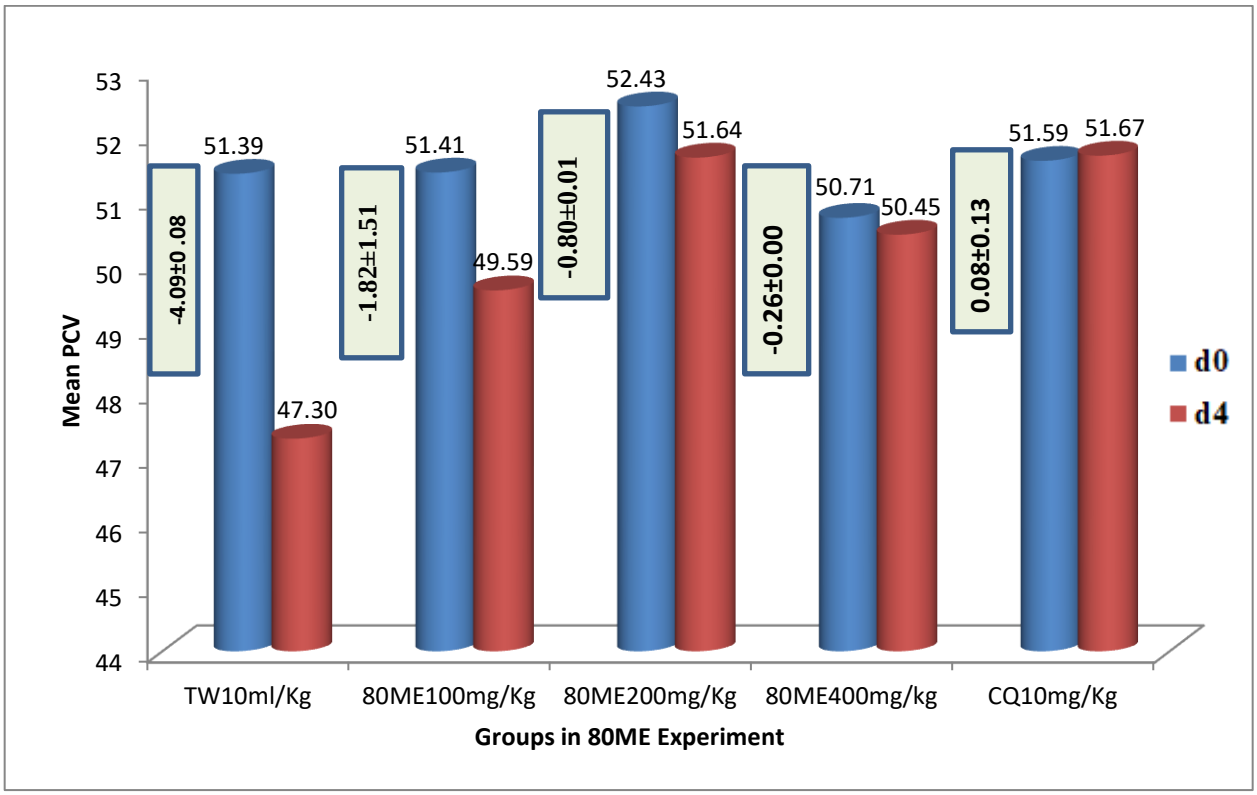

Figure 3: The effect of 80% methanol crude seed extract of *S. molle* on packed cell volume of *P. berghei* infected mice. Data are expressed as mean ± SEM (n=6); compared to the negative control and among the doses, the difference in mean change in PCV was significant at $p<0.05$; TW, negative control, 2% tween 80; CQ, positive control, chloroquine base; 80ME, 80% methanol crude seed extract; PCV, packed cell volume; d0, pre-treatment value on day 0; d4, post-treatment value on day four. The numbers in rectangles before graphs show the change in mean PCV between d0 and d4.

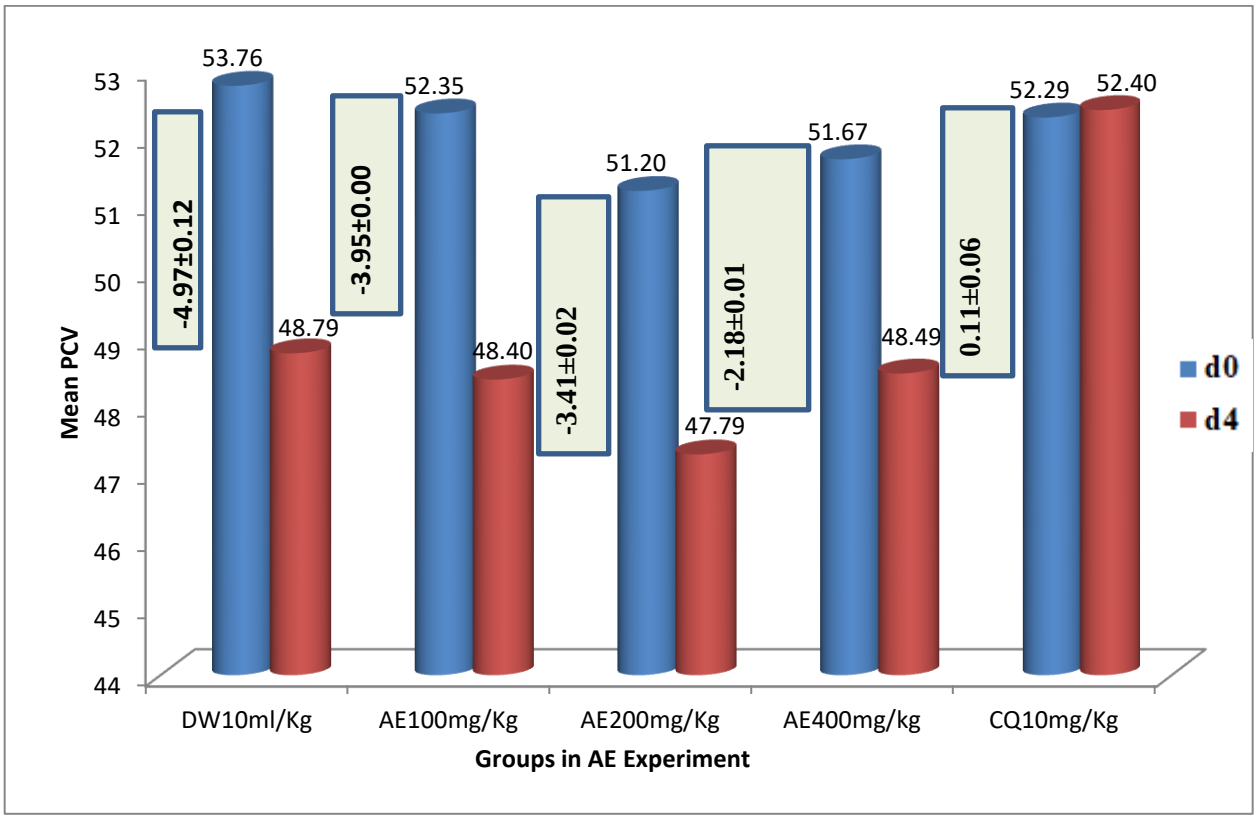

Figure 4: The effect of aqueous crude seed extract of *S. molle* on packed cell volume of *P. berghei* infected mice. Data are expressed as mean ± SEM (n=6); compared to the negative control and among the doses, the difference in mean change in PCV was significant at $p<0.05$; DW, negative control, distilled water; CQ, positive control, chloroquine base; AE, Aqueous crude seed extract; PCV, packed cell volume; d0, pre-treatment value on day 0; d4, post-treatment value on day four. The numbers in rectangles before graphs show the change in mean PCV between d0 and d4.

5.6. Effect of Fractions on Parasitemia and Survival Time

The effect of the three fractions of 80% methanol crude seed extract of *S. molle* at different dose levels on parasitemia and survival time of Swiss albino mice infected with CQ sensitive *P. berghei* are summarized in Table 4. Furthermore, the results are expressed as the PPS and MST in days in reference to the respective negative control mice.

Significant inhibition of parasitemia ($p<0.05$) was exhibited by all the fractions compared to their respective negative controls. Moreover, all the fractions reduced parasitemia to different levels. The highest inhibition (55.60%) was exhibited by the chloroform fraction of the 80% methanol crude seed extract at the highest dose given, 400 mg/kg/day while the lowest effect (15.64 %) among the three fractions was exhibited by the aqueous fraction at the least dose given, 100 mg/kg/day. However, none of them totally cleared the parasite from test mice. Instead, the standard drug, CQP cleared the

parasite to undetectable level in all the fractions.

When compared among themselves, different dose levels in each fraction, exhibited statistically significant ($p<0.05$) difference in reducing parasite load at all dose levels evaluated in the study. Furthermore, the higher the dose of each fraction given, the higher the percentage inhibition of parasitemia exhibited. More specifically, the increase in effect was consistent as the dose was increased from lower to higher level at all dose levels evaluated regardless of the fraction tested.

Comparison of the results of the chemosuppressive effect of fractions to the crude extracts showed difference in the level of parasite suppression. Accordingly, the highest parasitemia inhibition exhibited by the chloroform fraction is lower than the effect produced by the 80% methanol crude extract which was mentioned in section 5.3. Nonetheless, its effect is higher than the aqueous crude seed extract. Except the aqueous fraction, the other two fractions also showed greater level of parasitemia suppression as compared to aqueous crude seed extract. Hence, the aqueous fraction showed the least parasitemia suppression compared to any other fraction or crude extracts at any dose given in this particular investigation.

The test on survival time of mice treated with different doses of the fractions of 80% methanol crude seed extract of *S. molle* indicated that treated mice survived for more days than the respective negative control groups in general (Table 4). Moreover, the fractions prolonged the survival time of infected mice to different levels. Furthermore, the highest survival time was recorded in mice that received the highest dose of the fractions. The chloroform fraction was the one that exhibited the longest mean survival time (8.33 days) at the highest dose given. The maximum effect exerted by the highest dose of chloroform fraction was; however, lower than that of CQP in which none of the mice showed death in the follow up period (>30days). Significant prolongation of survival time was not exhibited by the aqueous fraction at all dose levels given when compared to the respective negative control mice ($p>0.05$).

When doses of each fraction were compared among each other, the lowest and the middle doses did not show significant difference in prolonging the survival time in both the chloroform and butanol fractions ($p>0.05$). The aqueous fraction that showed no significant survival time prolongation as compared to negative control mice did not also show any significant difference among each of its doses ($p>0.05$).

Comparison of the findings of the effects of the fractions of 80% methanol crude seed extract of *S. molle* on the survival time of mice infected with *P. bergie* to the crude extracts showed difference in the level of survival time. Accordingly, the highest survival time exhibited by the chloroform fraction was lower than the effect produced by the 80% methanol crude extract which was mentioned in section 5.3. Nonetheless, its effect is higher than the aqueous crude seed extract. Butanol fraction also showed greater survival time as compared to aqueous crude seed extract. Hence, the aqueous fraction showed the least survival time compared to any other fraction or crude extracts at any dose given in this particular investigation.

Table 4: The effect of solvent fractions of the 80% methanol crude seed extract of *S. molle* on parasitemia and survival time of *P. berghei* infected mice.

Group	% Parasitemia	%Suppression	Survival Time (day)
TW	55.74±1.06	0.00	6.33±0.42
CF100	37.52±1.10	32.69^{a*,c*,d*,e*}	8.33±0.33^{a*,d*,e*}
CF200	34.02±0.93	38.97^{a*,b*,d*,e*}	9.17±0.31^{a*,d*,e*}
CF400	24.75±1.37	55.60^{a*,b*,c*,e*}	12.17±0.48^{a*,b*,c*,e*}
CQ10	0.00±0.00	100.00^{a*}	>30.00±0.00 ^{a*}
TW	52.88±2.20	0.00	6.17±0.31
BF100	36.23±1.32	31.49^{a*,c*,d*,e*}	8.17±0.40^{a*,d*,e*}
BF200	33.00±1.2	37.59^{a*,b*,d*,e*}	8.67±0.49^{a*,d*,e*}
BF400	29.28±1.67	44.63^{a*,b*,c*,e*}	10.67±0.67^{a*,b*,c*,e*}
CQ10	0.00±0.00	100.00 ^{a*}	>30.00±0.00 ^{a*}
DW	51.59±1.64	0.00	6.33±0.33
AF100	43.52±.92	15.64 ^{a*,c*,d*,e*}	6.50±0.43^{e*}
AF200	41.51±1.33	19.54 ^{a*,b*,d*,e*}	6.83±0.48^{e*}
AF400	38.05±1.59	26.25 ^{a*,b*,c*,e*}	7.33±0.49^{e*}
CQ10	0.00±0.00	100.00 ^{a*}	>30.00±0.00 ^{a*}

Data are expressed as mean ± SEM (n = 6); a, compared to either DW or TW; b, compared to 100 mg/kg; c, compared to 200 mg/kg; d, compared to 400 mg/kg; e, compared to CQ10; *p<0.05. CF=chloroform fraction, BF=butanol fraction, AF=aqueous fraction, DW= negative control, received distilled water (10 ml/kg), TW= negative control, received 2% tween 80 (10 ml/kg), CQ=chloroquine base. Numbers after letters in the first column refer to dose in mg/kg.

5.7. Effect of Fractions on Body Weight and Rectal Temperature

The effect of the three fractions of 80% methanol crude seed extract of *S. molle* at different dose levels on body weight and rectal temperature of Swiss albino mice infected with CQ sensitive *P. berghei* are summarized in Table 5. Furthermore, the results are expressed as the change in mean body weight and rectal temperature between the d0 and d4 for each group in reference to the change in mean values of the respective negative control mice treated with the vehicle.

The mean value of the body weight and rectal temperature showed reduction in mice treated with solvent fractions and in those that were treated with the vehicle on d4 as compared to d0 (Table 5). However, significant increment of weight and rectal temperature ($p<0.05$) was exhibited in in all the three solvent fractions compared to their respective negative controls. Moreover, all the fractions protected against infection induced reduction in body weight and rectal temperature to different levels between the d0 and d4 compared to respective negative control mice treated with the vehicle. The highest increment in both weight and temperature was exhibited by the chloroform fraction at the highest dose given, 400 mg/kg/day (Table 5) while the least increment among the three fractions was exhibited by the aqueous fraction at the least dose given, 100 mg/kg/day (Table 5). Nevertheless, the effect produced was less than that of the standard drug, CQP which showed the highest protection from parasite-induced body weight loss and reduction in rectal temperature.

When compared among themselves, different dose levels in each fraction, exhibited statistically significant ($p<0.05$) difference in protection from infection induced body weight loss and reduction in rectal temperature in this study. Furthermore, the higher the dose of each fraction given, the better the protection from infection induced body weight loss and reduction in rectal temperature. Moreover, the increase in effect was consistent as the dose was increased from lower to higher level valuated regardless of the parameter evaluated and fraction tested.

Comparison of the effects of fractions of the 80% methanol crude seed extract of *S. molle* on body weight and rectal temperature of mice infected with *P. bergie* to the crude extracts showed difference in the level of protection from infection induced body weight loss and reduction in rectal temperature. Accordingly, the highest protection from infection induced body weight loss and reduction in rectal temperature exhibited by the chloroform fraction is lower than the effect produced by the 80% methanol crude extract which was mentioned in section 5.4. Nonetheless, its effect is higher than the aqueous crude seed extract. Except the aqueous fraction, the other two fractions also

showed greater level of protection against reduction in body weight and rectal temperature as compared to aqueous crude seed extract. Hence, the aqueous fraction showed the least protection from infection induced body weight loss and reduction in rectal temperature compared to any other fraction or crude extracts at any dose given.

Table 5: The effect of solvent fractions of the 80% methanol crude seed extract of *S. molle* on body weight and rectal temperature of *P. berghei* infected mice.

Group	Weight (g)			Temperature (°C)		
	d0	d4	Change	d0	d4	change
TW	28.41±0.86	24.82±0.57	-3.59±0.85	36.81±0.35	34.48±0.32	-2.33±0.06
CF100	28.44±0.80	26.15±0.79	-2.29±0.01 a*c*d*e*	36.95±0.16	35.29±0.15	-1.66±0.01a*c*d*e*
CF200	28.05±0.68	26.63±0.69	-1.42±0.02a* b*d*e*	36.99±0.29	36.15±0.30	-0.84±0.00 a*b*d*e*
CF400	28.18±0.64	27.64±0.63	-0.54±0.01a*b* c*e*	36.89±0.29	36.39±0.27	-0.50±0.01 a*b*c*e*
CQ10	28.59±0.88	28.83±0.86	0.24±0.04a*	37.02±0.32	37.71±0.29	0.70±0.05a*
TW	27.93±0.81	24.28±0.79	-3.65±0.09	36.97±0.81	34.66±0.80	-2.31±0.08
BF100	28.28±0.39	24.90±0.66	-3.39±0.08a*c*d*e*	37.02±0.67	35.04±0.66	-1.98±0.09a*c*d*e*
BF200	29.12±0.66	26.47±0.67	-2.65±0.02a*b*d*e*	36.87±0.66	35.74±0.67	-1.14±0.01a*b*d*e*
BF400	28.08±0.69	26.65±0.70	-1.43±0.01a* b*c*e*	37.01±0.69	36.26±0.70	-0.75±0.01a*b*c*e*
CQ10	28.24±0.61	28.43±0.60	0.19±.03a*	36.92±0.62	37.54±0.60	0.62±0.05a*
DW	28.51±0.75	24.86±0.81	-3.65	37.05±0.27	34.72±0.34	-2.33±0.10
AF100	28.33±0.68	24.72±0.67	-3.62d*e*	36.98±0.26	34.66±0.26	-2.32±0.01 d*e*
AF200	28.34±0.57	24.75±0.56	-3.59d*e*	36.96±0.26	34.61±0.27	-2.35±0.02 d* e*
AF400	28.05±0.79	24.82±.77	-3.2301a*b*c*e*	37.04±0.25	34.79±0.24	-2.25±0.01a* b*c*e*
CQ10	28.45±0.67	28.68±0.68	0.22a*	36.89±0.22	37.56±0.21	0.67±0.06a*

Data are expressed as mean ± SEM (n = 6); a, compared to either DW or TW; b, compared to 100 mg/kg; c, compared to 200 mg/kg; d, compared to 400 mg/kg; e, compared to CQ10; *p<0.05. d0 = pre-treatment value on day 0, d4 = post-treatment value on day four. CF=chloroform fraction, BF=butanol fraction, AF=aqueous fraction, DW= negative control, received distilled water (10 ml/kg), TW= negative control, received 2% tween 80 (10 ml/kg), CQ=chloroquine base. Numbers after letters in the first column refer to dose in mg/kg.

5.8. Effect of Fractions on Packed Cell Volume

The effect of the three fractions of 80% methanol crude seed extract of *S. molle* at different dose levels on PCV of Swiss albino mice infected with CQ sensitive *P. berghei* are summarized in figure 5-7. Furthermore, the results are expressed as the change in mean PCV between the d0 and d4 for each group in reference to the change in mean PCV of the respective negative control mice.

The mean value of the PCV showed reduction in mice treated with solvent fractions and in those that were treated with the vehicle on d4 as compared to d0. However, significant protection from infection induced reduction in PCV ($p<0.05$) was exhibited in all the three solvent fractions compared to their respective negative controls. Moreover, all the fractions protected against infection induced reduction in PCV to different levels. The highest protection was exhibited by the chloroform fraction of the 80% methanol crude seed extract at the highest dose given, 400 mg/kg/day (Figure 5) while the least protection among the three fractions was exhibited by the aqueous fraction at the least dose given, 100 mg/kg/day (Figure 5, 6,7). Nevertheless, the effect produced was less than that of the standard drug, CQP which showed the highest protection from infection induced reduction in PCV. When compared among themselves, different dose levels in each fraction, exhibited statistically significant ($p<0.05$) difference in protection from infection induced reduction in PCV. Furthermore, the higher the dose of each fraction given, the better the protection from infection induced reduction in PCV exhibited.

Comparison of the results of the effects of fractions of 80% methanol crude seed extract of *S. molle* on PCV of mice infected with *P. bergie* to the crude extracts showed difference in the level of protection from infection induced reduction in PCV. Accordingly, the highest protection exhibited by the chloroform fraction is lower than the effect produced by the 80% methanol crude extract which was mentioned in section 5.4. Nonetheless, its effect is higher than the aqueous crude seed extract. Except the aqueous fraction, the other two fractions also showed greater level of protection against as compared to aqueous crude seed extract. Hence, the aqueous fraction showed the least protection from infection induced reduction in PCV compared to any other fraction or crude extracts at any dose given. This means, the rank order of protection from infection induced reduction in PCV was 80% methanol crude extract > chloroform fraction > butanol fraction > aqueous extract >aqueous fraction.

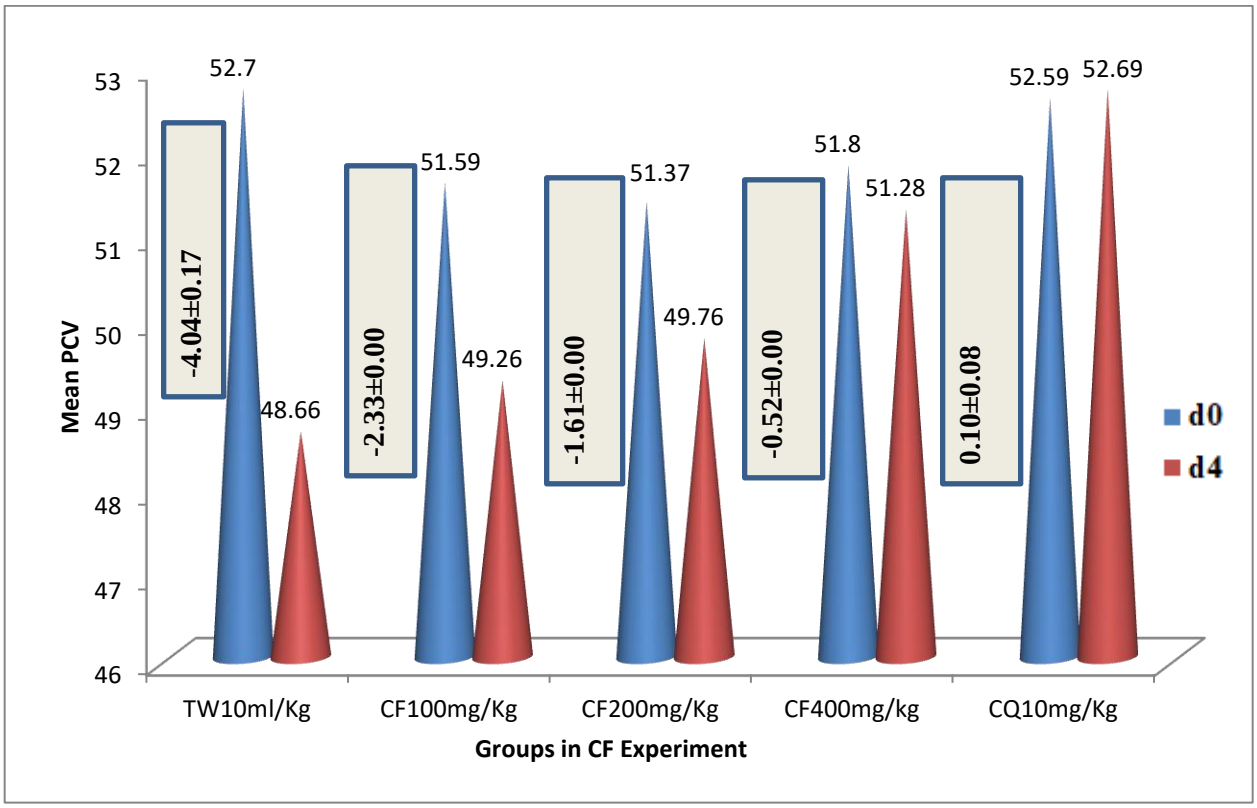

Figure 5: The effect of chloroform fraction of *S. molle* on packed cell volume of *P. berghei* infected mice. Data are expressed as mean ± SEM (n=6); compared to the negative control and among the doses, the difference in mean change in PCV was significant at $p<0.05$; TW, negative control, 2% tween 80; CQ, positive control, chloroquine base; CF, chloroform fraction; PCV, packed cell volume; d0, pre-treatment value on day 0; d4, post-treatment value on day four. The numbers in rectangles before graphs show the change in mean PCV between d0 and d4.

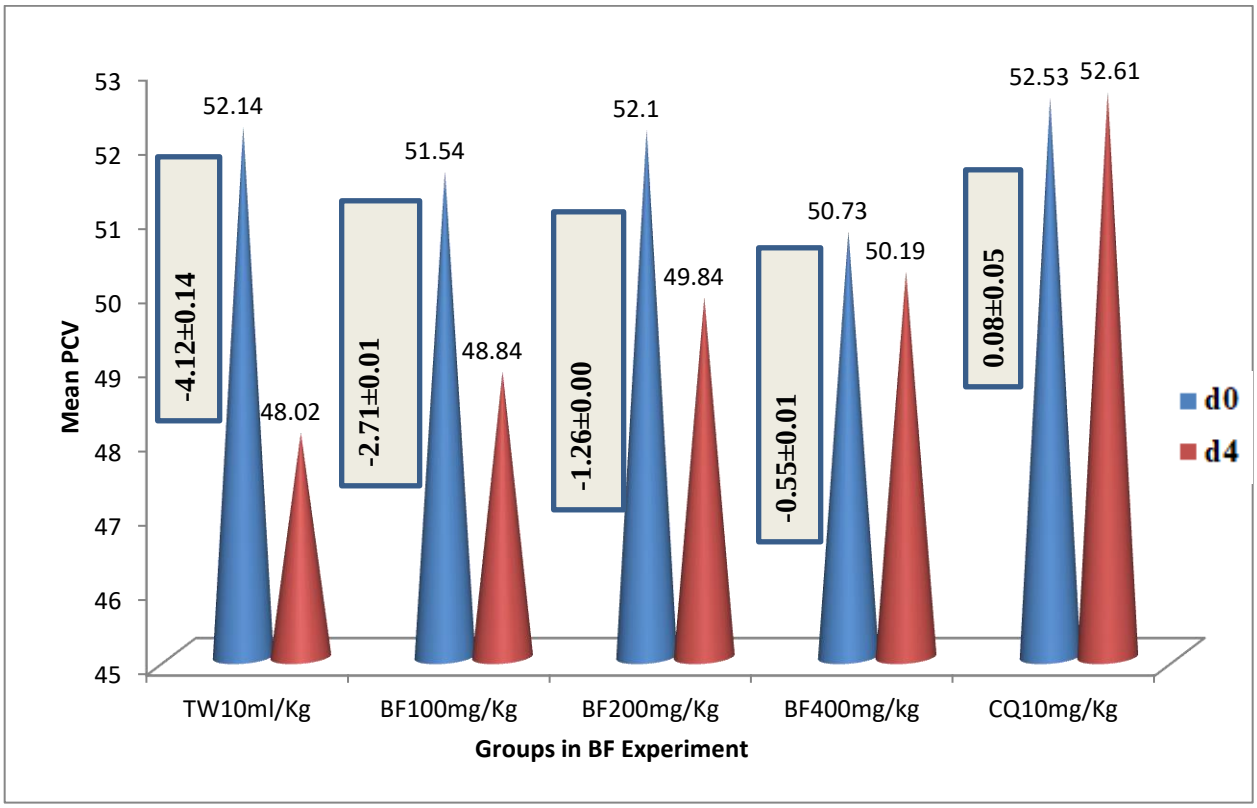

Figure 6: The effect of butanol fraction of *S. molle* on packed cell volume of *P. berghei* infected mice. Data are expressed as mean ± SEM (n=6); compared to the negative control and among the doses, the difference in mean change in PCV was significant at $p<0.05$; TW, negative control, 2% tween 80; CQ, positive control, chloroquine base; BF, butanol fraction; PCV, packed cell volume; d0, pre-treatment value on day 0; d4, post-treatment value on day four. The numbers in rectangles before graphs show the change in mean PCV between d0 and d4.

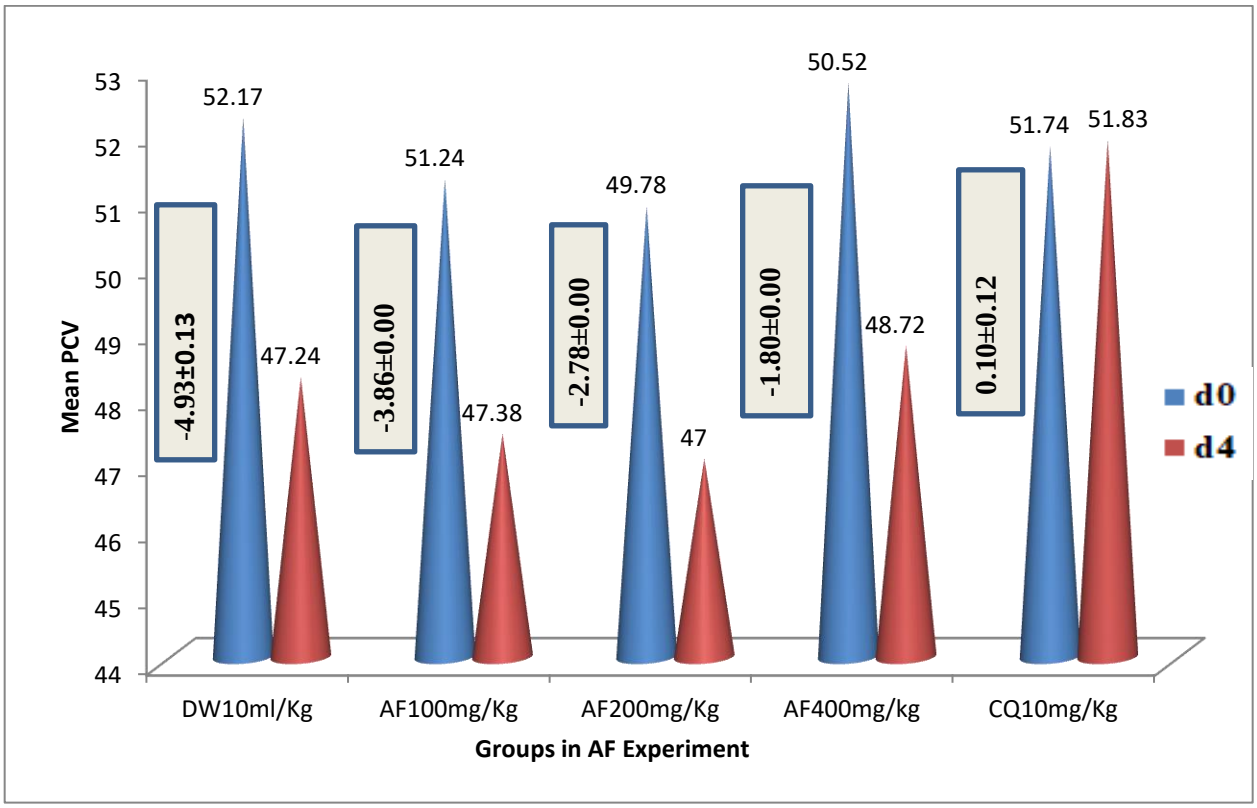

Figure 7: The effect of aqueous fraction of *S. molle* on packed cell volume of *P. berghei* infected mice. Data are expressed as mean ± SEM (n=6); compared to the negative control and among the doses, the difference in mean change in PCV was significant at $p<0.05$; TW, negative control, 2% tween 80; CQ, positive control, chloroquine base; AF, aqueous fraction; PCV, packed cell volume; d0, pre-treatment value on day 0; d4, post-treatment value on day four. The numbers in rectangles before graphs show the change in mean PCV between d0 and d4.

5.9. Phytochemical Screening

From phytochemical screening tests done on the crude extracts and solvent fractions of the seed of *S. molle*, the main phytochemical constituents were found in some while absent in others based on presence or absence of expected colour changes as shown in Table 6. Accordingly, the 80% methanol crude seed extract was positive for alkaloids, saponins, tannins, flavonoids, phenols, cardiac glycosides, steroids and terpenoids. The aqueous fraction was positive only for alkaloids, tannins and phenols. Phenols and glycosides absent in chloroform fraction were found in the butanol fraction while terpenoids and steroids contained by chloroform fraction were absent in the butanol fraction.

Table 6: The result of phytochemical screening of aqueous and 80% methanol crude seed extracts and solvent fractions of the seed of *S. molle*.

Phytochemicals	80% Methanol crude extract	Aqueous crude extract	Chloroform fraction	Butanol fraction	Aqueous fraction
Alkaloids	+	+	+	+	+
Tannins	+	+	+	+	+
Saponins	+	+	+	+	-
Flavonoids	+	+	+	+	-
Terpenoids	+	-	+	-	-
Steroids	+	-	+	-	-
Phenols	+	+	-	+	+
Glycosides	+	-	-	+	-

-, indicates absence; +, indicates presence of corresponding phytochemical constituent.

6. Discussion

As indicated by the findings of the acute toxicity test, none of the test mouse died or showed signs of acute toxicity within 24h and the next 14 days of treatment with crude extracts and fractions at a single dose of 2000mg/kg. Furthermore, the study suggested that the oral medial lethal dose (LD50) of the extract could be greater than 2000 mg/kg body weight of the extract as per OECD guideline No 425 [OECD, 2008]. The experimental determination of lack of acute toxicity at the dose of extract/fraction up to 2000 mg/kg body weight of mice would justify the use of plant extracts. Therefore, the results could justify the safe folkloric use of the seed of *S. molle* for the treatment of malaria by the local people in Ethiopia [Giday *et al*., 2006; Kenea and Tekie, 2015]. If the LD50 of the extract is 3 times more than the minimum effective dose investigated, the extract is a good candidate for further study. Oral dose of 2000mg/kg body weight used in this test is 20 times more than the minimum effective dose used in this test (100mg/kg). Perhaps, the LD50 of this plant could be greater than 2000mg/kg, but the minimum effective dose could not be greater than the minimum dose used in this study. Hence, greater than 2000mg/kg body weight is more than 20 times the minimum effective dose, and justifies its safety for further investigation of the plant [Dikasso *et al*., 2006]. The female mice were used for toxicity study as opposed to male counter parts because, generally, female mice are more sensitive to toxins than male ones and better opted for such studies [OECD, 2008].

As observed from the results of this study, significant inhibition of parasitemia was exhibited in both the aqueous and 80% methanol crude seed extracts and all the three fractions of the seed of *S. molle* compared to their respective negative controls. Moreover, the crude extracts and solvent fractions reduced parasitemia to different levels. The highest inhibition was exhibited by 80% methanol crude seed extract though the effect produced was less than that of the standard drug. The alkaloids, tannins, saponins, flavonoids, terpenoids, steroids, phenols and glycosides abundantly present in this crude extract as revealed in phytochemical analysis could be responsible for its antimalarial activity which could synergistically inhibit the parasite [Bantie *et al*., 2014]. The higher doses in each crude extract and fraction showed better parasitemia suppression because of the higher concentration of the active components in the higher doses given. The present study is in line with the one conducted on *Croton macrostachyus* by Bantie *et al.* [2014] where the 80% crude extract showed the highest parasite suppression. On the contrary, it is not in line with the study conducted on *Strychnos mitis* by Fantahun *et al.* [2017] where the aqueous crude extract was reported to exhibit the highest

parasitemia suppression.

Among the solvent fractions evaluated, the highest parasite inhibition was exhibited by the chloroform fraction at the highest dose given while the lowest effect was exhibited by the lowest dose of aqueous fraction. However, none of them totally cleared the parasite from test mice after a consecutive four days treatment. The highest inhibition by chloroform fraction could be attributed to the localization of many of the phytoconstituents in sufficient concentration except glycosides and phenols. The butanol fraction which showed more suppressive effect next to chloroform fraction exhihibited equal number of type of phytoconstituents. But, the absence of steroids and terpinoids in butanol fraction and the lesser intensity of color with some other constituents depicted by this fraction could be responsible for its lower effect. The chloroform and butanol fractions showed better chemosuppressive effect indicating the possible localization of active ingredients in these two fractions [Bantie *et al*., 2014]. This finding is consistent with other studies in which both the chloroform and butanol fractions showed higher chemosuppressive activity than the aqueous fraction [Asnake *et al*., 2015; Mengiste *et al*., 2012] even though the plants are different. Moreover, this study suggests that nonpolar to semi polar compounds found in these two fractions that act individually or synergistically could be responsible for the chemosuppressive activity of the plant.

The parasitemia suppression in ascending order was as follows: aqueous fraction, aqueous crude extract, butanol fraction, chloroform fraction and 80% methanol crude extract. The lower parasitemia suppression exhibited by the aqueous crude extract and fraction may suggest that highly polar compounds could not be the main factors for the chemosuppressive activity supporting the results of chloroform and butanol fractions. Furthermore, the least parasitemia suppression exhibited by aqueous fraction was consistent with least number of phytoconstituents observed from the phytochemical screening test results and these could be trace amounts left over during extraction and fractionation. This is also consistent with other studies where aqueous fraction was found to be the least in chemosuppressive activity [Bantie *et al*., 2014]. Furthermore, the aqueous fraction is found to be inactive as its parasite suppression was less than 30% even at the highest dose administered [Krettli *et al*., 2009: Nureye *et al*., 2018]. If antimalarial activity of a compound displayed a percent growth inhibition of > 50% at a dose of 500-250, 250-100 and <100 mg/kg/day, literature grades it as moderate, good and very good, respectively [Munoz *et al*., 2000; Nureye *et al*., 2018]. Therefore, the seed of *S. molle* has a good antimalarial activity.

Although the mechanism of chemosuppressive activity of the crude extracts and solvent fractions of the seed of *S. molle* is not shown, the reduction of parasite load in treated mice might be due to the presence of phytochemical constituents such as alkaloids, saponins, tannins and terpenoids. Previous studies have indicated the potential of these phytoconstituents for antimalarial drug development [Asnake *et al,* 2015; Mengiste *et al*., 2012; Nureye *et al*., 2018]. Moreover, the chemosuppressive effect could be via indirect boosting of the immune system or inhibition of other target pathways which are not fully realized [Dikaso *et al*., 2006]. The phytosteroids, terpenoids, phenolic compounds and flavonoids observed in this plant have been proved to possess potential immunomodulatory, antinflammatory and antioxidant activity [Abi *et al*., 2016; Silva-Júnior *et al*., 2015]. Furthermore, the plant constituents may target the previously discovered targets in the pathogenesis and life cycle of the malaria parasite but with unique or similar mechanism of action at molecular level [Bantie *et al*., 2014].

From the chemosuppressive test discussed above, it can be deduced that the study plant has antimalarial activity and its use by the local community for malaria treatment is logical specially in areas where the modern health service is scarce [WHO, 2013]. Moreover, its antimalarial activity has been further ascertained by the *in vitro* study done on the plant in other area with MIC50<10 μg/ml [Abdel-Sattar *et al*., 2010]. However, it has to be standardized by scientific community by conducting further investigations on the plant. In addition, previous studies conducted on the plant also found that the plant has antibacterial [Mehani and Segni, 2013] and antiprotozoal [Quintanilla-Licea *et al*., 2014] activities. Conventional antimalarial drugs such as tetracyclines which possess both antibacterial and antiprotozoal activity has been used for the treatment of malaria suggesting further the antimalarial activity of the plant [Bantie *et al*., 2014; WHO, 2015].

The findings of the survival time of mice treated with different doses of aqueous and 80 % methanol crude seed extracts of *S. molle* indicated that treated mice survived for more days than the respective negative control groups. The 80 % methanol crude seed extract was the one that exhibited the longest survival time at the highest dose given. On the other hand, the two lower doses of aqueous crude seed extract did not exhibit significant prolongation of survival time when compared to the respective negative control mice. The maximum effect exerted by the highest dose of the 80 % methanol crude extract was; however, lower than that of CQP where none of the mice showed death in the follow up period. The maximum effect exerted by 80% methanol crude extract could be due to the presence in abundant of the phytoconstituents which probably acted individually or syneregestically as more

constituents were found in this extract. The result is consistent with the study done on the root bark of *Gardenia ternifolia* by Nureye *et al*. [2018] but inconsistent with the study done on *Strychnos mitis* by Fantahun *et al.* [2017] where the survival time exerted was higher with aqueous crude extract instead. The highest prolongation of survival time observed by the highest doses was due to localization of the active components in high doses and also because of its best parasite suppressive effect. This is in line with the work of Bantie *et al*. [2014] and Mengiste *et al*. [2012] where the highest doses exhibited better survival time**.**

All doses of the 80 % methanol crude seed extract were capable of significantly increasing survival time at different significant levels compared to the respective negative control mice which might be because of the presence of all the phytoconstituents in sufficient concentration. When doses are compared among each other, the lowest and the middle doses did not show significant difference in prolonging the survival time may be the extent of difference in phytoconstituents was not enough to show the difference in effect. Otherwise, the upper dose exhibited significant prolongation of survival time as compared to the lowest and the middle doses for the reason mentioned above. The aqueous crude seed extract, on the other hand, significantly prolonged survival time only at the highest dose given when compared to the respective negative control mice. This could be because of the presence in low amount of phytoconstituents in the two lower doses as opposed to the highest dose [Nureye *et al*., 2018].

As observed from the results of the survival time of mice treated with different doses of the fractions of 80% methanol crude seed extract of *S. molle*, treated mice survived for more days than the respective negative control groups except the aqueous fraction. Furthermore, the highest survival time was recorded in mice that received the highest dose of the fractions. The chloroform fraction was the one that exhibited the longest survival time at the highest dose given probably due to its better chemosuppressive activity as mentioned before and its difference in phytoconstituents from the other fractions. This is not in line with the work of Nureye *et al*. [2018] on *Gardenia ternifolia* and Bantie *et al*. [2014] on *Croton macrostachyus* where butanol fractions were found to show longest survival time. The maximum effect exerted by the highest dose of chloroform fraction was; however, lower than that of CQP. Significant prolongation of survival time was not exhibited by the aqueous fraction at all dose levels given when compared to the respective negative control mice. This could be probably due to the absence or reduction of the phytoconstituents with chemosuppressive activity in this fraction [Adumanya, 2014].

Both the chloroform and butanol fractions, at all doses given, were capable of significantly increasing survival time at different significant levels compared to the respective negative control mice whereas the aqueous fraction showed no significant difference as mentioned above. When doses of each fraction were compared among each other, the lowest and the middle doses did not show significant difference in prolonging the survival time in both the chloroform and butanol fractions may be the concentration difference was not enough to cause difference in effect. However, the highest dose of the two fractions prolonged the survival time of infected mice more than the two lower doses. The aqueous fraction that showed no significant survival time prolongation as compared to negative control mice, did not also show any significant difference among each of its doses. This finding is similar with that of Nureye *et al*. [2018] on *Gardenia ternifolia* and Bantie *et al*. [2014.] on *Croton macrostachyus* where the aqueous fraction was found to be inactive in prolonging survival time as opposed to the work of Fantahun *et al*. [2017] on *Strychnos mitis* leaves where aqueous fraction was found to be active.

The highest survival time exhibited by the chloroform fraction was lower than the effect produced by the 80% methanol crude extract, which could be directly associated with the low parasite level in the crude extract treated groups [Bantie *et al*., 2014; Mengiste *et al*., 2012; Nureye *et al*., 2018]. Nonetheless, its effect is higher than the aqueous crude seed extract that showed only prolongation of survival time at the highest dose. Butanol fraction also showed greater survival time as compared to aqueous crude seed extract. Hence, the aqueous fraction showed the least survival time compared to any other fraction or crude extracts at any dose given in this particular investigation. The prolongation of survival time in descending order was as follows: 80% methanol crude extract, chloroform fraction, butanol fraction, aqueous extract and aqueous fraction and in agreement with the degree of inhibition of parasitemia. However, they did not cure the infection, this could be because of the recrudescence of the parasites after apparent cure related to the short half-lives of the active components of the extracts and fractions [Mengiste *et al*., 2012; Nureye *et al*., 2018].

Reduction in PCV, body weight loss and reduction in body temperature are the general features observed in rodents infected with malaria parasite [Bantie *et al*., 2014; Nureye *et al*., 2018]. Consequently, an ideal antimalarial agents derived from plants are expected to prevent reduction in PCV, body weight loss and reduction in body temperature due to the development of parasitemia [Adumanya *et al*., 2014]. The mean value of the body weight showed reduction in mice treated with crude extracts and fractions and in those that were treated with the vehicles on d4 as compared to d0.

These could be attributed to the inability of the extracts and fractions to completely clear the parasites from the body of the mice other than reducing to different levels [Asnake *et al*., 2015]. It could also be because of the appetite suppressants present in the extracts and fractions of the test plant [Nureye *et al*., 2018]. However, significant increment of weight was exhibited in both aqueous and 80% methanol crude extracts and the fractions compared to their respective negative controls. This improvement of weight might be because of the extracts or fractions pharmacological effect that counteract other aspects of malaria illness such as fever, immunosuppression and pain, improvement in PCV, rectal temperature and parasite clearance among extract-treated mice as shown in the result section [Adumanya *et al*., 2014; Fantahun *et al*., 2017].

Moreover, the crude extracts showed body weight increment to different levels in both the crude extracts and all the fractions between the d0 and d4 compared to the respective negative control mice. In the present study, the highest increment was exhibited by 80% methanol crude seed extract while the lowest was exhibited by aqueous fraction. The reason behind the differences in improvement of weight might be related to the differences in the type and amounts of such phytoconstituents where 80% methanol crude extract is the richest while the aqueous fraction was found to be the poorest. The result is in line with the work of Asnake *et al*. [2015], Bantie *et al*. [2014] and Nureye *et al*. [2018] where 80% methanol crude extract exhibited better improvement in weight as compared to the negative control mice. Nevertheless, the effect produced was less than that of the standard drug, CQP which showed the highest protection from parasite-induced body weight loss that is attributed to the complete eradication of the parasite to undetectable level. The higher doses exhibited better improvement in weight compared to the lower doses. This finding is consistent with other findings where the higher doses improved weight as compared to lower doses [Fantahun *et al*., 2017] as opposed to the works of Toma *et al*. [2017] where lower doses in some instances were found to be more active than the higher doses.

A decrease in the metabolic rate of infected mice occurs before death and is accompanied by a corresponding decrease in body temperature [Bantie *et al*., 2014; Fantahun *et al*., 2017] a condition which is different in the case of human subjects [Dikasso *et al*., 2006]. Ideally, the rectal temperature decreases as parasite level escalates. Active compounds should prevent the rapid dropping of rectal temperature [Bantie *et al*., 2014; Mengiste *et al*., 2012]. The mean value of the temperature showed reduction in mice treated with crude extracts and fractions and in those that were treated with the vehicles on d4 as compared to do. These could be attributed to the inability of the extracts and

fractions to completely clear the parasites from the body of the mice other than reducing to different levels [Nureye *et al*., 2018]. It could also be because of the hypothermic components present in the extracts and fractions of the test plant [Bantie *et al*., 2014; Mengiste *et al*., 2012].

However, low level of decrease in temperature was significantly exhibited in both the aqueous and 80% methanol crude extracts and the fractions compared to their respective negative controls. This improvement of temperature might be because of the extracts or fractions pharmacological effect that counteract other aspects of malaria illness such as immunosuppression, pain, improvement in PCV, body weight and parasite clearance among extract-treated mice [Asnake *et al*., 2015]. Moreover, the crude extracts exhibited low level of decrease in rectal temperature to different levels where the higher doses exhibited better protection against reduction in temperature in both the crude extracts and all the fractions between the d0 and d4. This could be attributed to the fewer in amounts of the phytoconstituents in the lower doses [Fantahun *et al*., 2017; Nureye *et al*., 2018]. The highest increment was exhibited by 80% methanol crude seed extract while the lowest was exhibited by aqueous fraction in line with the findings of the weight, survival time and parasite suppression that were discussed above.

The reason behind the differences in improvement in rectal temperature might be related to the differences in the type and amount of such phytoconstituents where 80% methanol crude extract exhibited the presence of all of them while the aqueous fraction was found to be the poorest [Nureye *et al*., 2018]. Nevertheless, the effect produced was less than that of the standard drug, CQP which showed the highest protection from parasite-induced temperature reduction that is attributed to the complete eradication of the parasite to undetectable level. Overall, this activity probably indicate that the extracts ameliorate some pathological processes that cause reduction in internal body temperature and metabolic rates [Bantie *et al*., 2014; Mengiste *et al*., 2012]. This finding is consistent with the findings of Nureye *et al*. [2018] where the higher doses improved temperature as compared to lower doses as opposed to Bantie *et al*. [2014] where the increase in the temperature in some cases was more at lower doses.

After infected with malaria the host can suffer from malaria induced reduction in PCV [Asnake *et al*., 2015; Toma *et al*., 2017]. Malaria induced reduction in PCV occurs due to loss of RBCs as a result of hemolysis of infected RBCs, destruction of uninfected cells in the spleen, erythropoietic suppression, dyserythropoiesis and oxidative stress which increases membrane fragility [Bantie *et al*., 2014; Mengiste *et al*., 2012]. This effect of malaria on RBCs triggers the evaluation of extracts and

fractions protection of the cells through monitoring of PCV [Asnake *et al*., 2015]. As a result, the effect of crude extracts and fractions on the PCV was also evaluated in this test.

The mean value of the PCV showed reduction in mice treated with the crude extracts and fractions and in those that were treated with the vehicles on d4 as compared to d0. These could be attributed to the inability of the extracts and fractions to completely clear the parasites from the body of the mice other than reducing to different levels [Dikaso, 2006]. It could also be because of the saponins present in the extracts and fractions of the test plant as some studies indicated that saponins are known to cause hemolysis by increasing the permeability of plasma membrane of the red blood cells [Asnake *et al*., 2015; Bantie *et al*., 2014; Nureye *et al*., 2018]. However, significant prevention of decrease in mean of PCV was exhibited in both the aqueous and 80% methanol crude extracts and the fractions compared to their respective negative controls. The main reason for better protection of PCV level in 80% methanol crude extract treated group could be due to the chemosuppressive effect of the extract which is associated with less destruction of RBCs [Asnake *et al*., 2015].

The highest protection from infection induced decrease in mean PCV was exhibited by 80% methanol crude seed extract while the lowest was protection exhibited by aqueous fraction in line with the findings of the weight, survival time, temperature and parasite suppression that were discussed above. Moreover, the crude extracts and fractions improved change in mean PCV to different levels where the higher doses exhibited better protection against reduction in PCV in both the crude extracts and all the fractions between the d0 and d4. This finding is consistent with other findings where the higher doses better protected PCV from decreasing in as compared to lower doses [Fantahun *et al*, 2017] while inconsistent with other studies where lower doses showed better improvement in PCV due to the high concentration of saponins in higher doses [Bantie *et al*., 2014; Nureye *et al*., 2018]. The present study indicates that the extracts and fractions ameliorate some pathological processes that cause reduction in PCV and loss of RBCs. This could be due to the marked decrease in parasite load in the course of infection in mice treated with the extracts and fractions [Mengiste *et al*., 2012; Toma *et al*., 2017]. In the untreated mice, the parasite number increased and consequently destroyed more RBCs and resulted in marked decrease of hematocrit PCV [Asnake *et al*., 2015].

7. Conclusion

Acute toxicity test conducted on the crude seed extracts and solvent fractions of *Schinus molle* Linnaeus showed no signs of toxicity in mice treated up to a dose of 2000 mg/kg which could indicate the safe use of the plant by local communities. Furthermore, the findings of the present study indicate that the seed of *Schinus molle* has significant *in vivo* antimalarial activity. The highest antimalarial effect was exhibited by the 80% methanol crude extract at the highest dose tested. Among the fractions tested, the chloroform fraction was found to be the most active in suppressing the parasite indicating the possible localization of the active compounds in this fraction. Moreover, the data would provide evidence to uphold the earlier *in vitro* antimalarial investigation on the plant as well as the traditional use of the plant by the local communities for the treatment of malaria in Ethiopia. Consequently, the seed of *Schinus molle* could be used as a potential source to develop more effective and safer antimalarial drugs.

8. Recommendations

As the seed of *Schinus molle* is found to be promising source for developing more effective and safer antimalarial drugs, the following further investigations are recommended:

- Sub-acute and chronic toxicity studies should be conducted to better establish the safety status of the plant.
- Studies on the other parts of the plant ought to be done to compare the antimalarial activity with the present study.
- Bioassay guided isolation is suggested to isolate agent/s responsible for the activity of the plant.
- Elucidating the structure and the molecular mechanism of antimalarial action is suggested.
- Evaluation of the antimalarial activity of the plant against *P. falciparum* infected immunocompromised mice is suggested to better simulate the actual human malaria infection.
- Further investigations on the chloroform fraction is recommended as this fraction was found to be the most active among the fractions and might contain a potential lead molecule for the development of new drug/s to treat malaria.

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