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Invivo Anti-Malarial Evaluation of Leaf Extract of *Vernonia amygdalina* Del. (Asteraceae) against Plasmodium berghei

By:-Temesgen Bekele

A thesis submitted to the School of Graduate Studies, Department of Biochemistry, college of Health science, Addis Ababa University presented in partial fulfillment of the requirement for the degree of Master of Science in Medical Biochemistry.

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ADDIS ABABA, ETHIOPIA

This is to certify that the thesis prepared by Temesgen Bekele entitled*: Invivo* Anti-Malarial Evaluation of Leaf Extract of *Vernonia amygdalina* Del. (Asteraceae) against *Plasmodium berghei*, and submitted in partial fulfillment of the requirement for the degree of Master of Science in Biochemistry complies with the regulation of the university and meets the accepted standards with respect to originality and quality.

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ABSTRACT

Introduction: Malaria is one of the world's most serious infectious diseases caused by *Plasmodium* parasites. An increased drug resistance to conventional anti-malarial, increasing resistance of mosquito vectors to insecticides, challenge of having effective vaccines and adverse effects of the existing anti-malarial drugs justifies the urgent need for more effective, tolerable and affordable anti-malarial drugs.

Objective: In the attempt to search for new antimalarial drugs, the present study aimed to evaluate antimalarial activity and acute toxicity of crude extract of *Vernonia amygdalina* leaf extracts.

Methods: The leaf of *Vernonia amgdalina* extract were prepared by cold maceration technique, four day suppressive, curative and prophylaxis test in mouse model was done to cheek the effect of the plant extracts against *Plasmodium berghei* in Swiss albino mice. Extracts were administered at doses of (200, 400 and 600) mg/kg body weight of mice. Data obtained from the experiment was analyzed using paired t-test and one way ANOVA.

Results: The present study indicated that the extract did not exhibit any signs of acute toxicity up the dose of 2000mg/kg. The aqueous and hydrometanol extracts of *V. amygdalina* showed a parasitemia chemosuppression at the dose of 600 mg/kg with 68.8 and 69.2 % ($p<0.0001$) in 4-day suppressive test, 69.3 and 70.8% in curative model and 70.3 and 71.5% %($p<0.0001$) in prophylactic model as compared to normal control, respectively, with significant ($p<0.0001$) effect on survival time compared to normal control. In Curatve test of the present study, the extract had shown prevention against Packed cell volume (PCV) and weight reduction significantly ($p<0.05$), as compared to normal control, in dose dependent manner. But the 4 days suppression and prophylactic model did not prevent reduction of PCV and weight fall. In all three models, the extract did not cause significant prevention of rectal temperature decrement.

Conclusion: The extracts showed parasitemia suppression and a promising curative and prophylaxis activities in dose dependent manner.

Key words: *Antimalarial activity, V. amygdalina, P. berghei, traditional medicine, invivo.*

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Abrevation/Acronyms

ACT	Artemisinin Combination Therapy
AIDS	Acquired Immunodeficiency Syndrome
AMREF	African Medical and Research Foundation
ANOVA	Analysis of Variance
Bwt	Body weight
CDER	Center for Drug Evolution and Research
$CHCl_3$	Chloroform
CQ	Chloroquine Phosphate
CRPv	Chloroquine Resistant *Plasmodium vivax*
DDT	Dichlorodiphenyltrichloroethane
dH_2O	Distilled Water
DHA	Dehydroartemisinin
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EAAM	EurropeanAlianceAgainst Malaria
ECA	Economic Commission for Africa
EPHI	Ethiopian Public Health Institution
FMOH	Federal Ministry of Health, Ethiopia
GDP	Growth Domestic Product
Hb	Hemoglobin
HIV	Human Immunodeficiency Virus
IC	Inhibition Concentration
IDC	Intraerythrocytic Developmental Cycle
IL	Interleukin
IP	Intraperitoneal
IPTP	Intermittent Preventive Treatment for Pregnant women

IRS	Indoor Residual Spraying
ITNs	Insecticide-treated mosquito nets
LD	Lethal Dose
LLINs	Long Lasting Insecticidal Nets
MeOH	Methanol
MIC	Minimum Inhibition Concentration
MST	Mean Survival Time
MVI	Malaria Vaccine Initiative
NC	Normal Control
NIAID	National Institute of Allergy and Infectious Diseases
OCED	Organisation for Economic Cooperation and Development
PCV	Packed Cell Volume
*Pf*EMP	*Plasmodium falciparum* Erythrocyte Membrane Protein
PMI	President's Malaria Initiatives
PM	Pyrimithamine
PQ	Premaquine
RBC	Red blood cell
RITM	Research Initiative on Traditional antimalarial Method
SD	StandardDevation
SEM	Standard Error of Mean
SP	Sulfadoxine-pyrimethamine
sSA	Sub-Saharan Africa
TCM	Traditional Chinese Medicine
TNF	Tumor Necrosis Factor
UNICEF	United Nations Children's Fund
WEF	World Economic Forum
WHO	World Health Organization
IVM	Integrated Vector Management
MHC	Major Histocompateble Complex

DHPS	Dihydropteroate Synthase
DHFR	Dihydrofolate Reductase
HSP101	Heat shock protein 101
% Para	Percentage parasitaemia
% Supp	Percentage Suppression
°C	Degree Celsiu

1. INTRODUCTION

Malaria, the name, is derived from the Italian word *mal'aria,* for ''bad air". This is to describe the swampy areas in Europe in which the disease was prevalent (Amorosa *et al*, 2005). But later, the etiological agent for the disease was discovered and is known to be the protozoan parasite *Plasmodium* (Ridder *et al.*, 2008). Malaria is one of the world's most serious infectious diseases caused by *Plasmodium* parasites (Cropper *et al*., 2004). There are more than 100 species of *Plasmodium* parasite that can infect many animal species such as reptiles, birds, rodents, monkeys and humans (NIAID, 2007). Human malaria caused by genus *Plasmodium* is mainly transmitted through the bite of infected female *Anopheles* mosquitoes during blood meal (Cox, 2010; Echhoff, 2011) and other rare mechanisms including congenitally acquired, blood transfusion, sharing of contaminated needles and organ transplantation (Guerin *et al*., 2002).

Four species of genus *Plasmodium; P. falciparum, P. vivax, P. ovale and P. malarae* have long been recognized to infect humans in nature all of which are transmitted by the female anopheles mosquito. In addition, there is one species; *P. knowelesi* that naturally infects macaque monkeys has recently been recognized as a cause of *zoonotic* malaria in humans (Eede *et al*., 2009).

Malaria is the world's most important parasitic disease especially when *Plasmodium falciparum* is the cause of most of the mortality and morbidity. Malaria has been challenging human health and losing the lives of many people since long period of time. It is one of the most prevalent; devastating parasitic infectious diseases in the world (Moss *et al*., 2008). Malaria has been one of the most extensively studied parasitic infectious diseases for millennia. In 2012, there were around 627,000 malaria deaths worldwide, 90% of which were in the African region, followed by Southeast Asia (7%) and the Eastern Mediterranean (3%). Most of these deaths were due to *Plasmodium*

falciparum. However, *Plasmodium vivax* is now increasingly recognized as a cause of severe malaria and death (WHO, 2013b).

Malarial infection is still a threat to global health. It is as old as Man and is the most deadly disease in the tropics. Malaria has been associated with human beings since the history of man and the parasite themselves (Carter and Mendis, 2002). Malaria is one of the parasitic infections that cause enormous medical, economic, and emotional burden in the world. It has been estimated that more than 300–500 million people are affected by malaria throughout the world (WHO, 2013b).

Currently, it is one of the major tropical diseases adversely affecting the health of people and the economic development of many developing countries; particularly in sub-Saharan Africa (sSA) (WHO, 2003). Each year, more than 1.5–2.7 million deaths associated with malaria were reported globally, in the past years, most of them were children (Angayarkanni *et al*., 2010; Moon, 2010). As reported by Moon (2010), more than 90 countries with an estimated 2400 million people are suggested to be attacked by endemic malaria. Approximately 90% of malaria deaths occur in Africa. According to the WHO (2013), malaria is endemic in many countries, predominantly in Africa, Asia and Latin America. About half of the world's population is living in malaria risk areas (WHO, 2009). Malaria is a major public health problem in Africa. It has afflicted man and animals for over a century. Each year an estimated 300–400 million clinical cases of malaria occur (Onwuamah *et al*., 2010). Nevertheless, there is a spatial and temporal variation in mortality and morbidity of malaria; particularly in semiarid and high land regions of Africa. Africa faces the greatest economic impact of this disease, particularly in Sub Saharan countries with children and pregnant women are the main targets (Idowu *et al*., 2010).

According to Chima *et al*., (2003), the economic costs of malaria can be classified as direct and indirect. The direct one is the costs of expenditure on prevention and treatment. The indirect impact is the costs of productive labor time lost due to malaria morbidity and mortality (Niringiye and Douglason, 2010). Despite this economic loss, there is a scarcity of chemical treatment in rural areas; hence cultural practices still remain important (Traore- Keita *et al*., 2000). Malaria is distributed widely, mainly due to the multidrug resistance developed by *P. falciparum*. Of the four species of *plasmodium* parasites that cause malaria in humans, *P. falciparum* is by far the most virulent. Despite over 22 years of efforts, anti-malaria vaccine is not yet in use. The fight against this parasite has become more complex over the last few years with prevalence of multidrug resistant strains. The cases of adverse reaction induced by anti-malarial drugs such as toxicity have also been known to negatively affect the disease control (Angayarkanni *et al*., 2010). Nearly half of the world's population lives in malaria endemic areas (WHO, 2008). There is a high risk of transmission in Sub-Saharan Africa; 80% of such cases are concentrated in 13 countries, and over half in Nigeria, Congo, Ethiopia, Tanzania and Kenya (WHO, 2008), i.e., Ethiopia is one of the five main contributors to the overall African malaria burden (RBM 2009).

In Ethiopia about three quarter of the land is malarious and an estimated 68% (57.3 million people) of the populations live in this area where the risk is high and malaria could occur in epidemic form (PMI,2014). Malaria stands as the leading cause of morbidity and mortality in Ethiopia. Over the past years, the disease has been consistently reported as the first leading cause of outpatient visits, hospitalization and death in health facilities across the country (Karunamoorthi and Bekele, 2009). Annually, half a million microscopically confirmed cases of malaria are reported to the Federal Ministry of Health (FMOH) from basic health services. According to the FMOH, malaria was the leading cause of outpatient visits and health facility admissions in 2010/2011, accounting for 15% of reported outpatient visits and nearly 15% of admissions

(PMI,2014b). In Ethiopia, it is known that the vulnerability of the people to malaria has been aggravated by malnutrition and weak supply of infrastructure (Das, 2003).

In Ethiopia, malaria is the front among the health problems. Jima *et al*. (2005) indicated that about 75 % of the land mass is endemic for malaria. The FMOH estimates that there are about 12 million suspected malaria cases each year (PMI, 2014b) The FMOH reported a malaria cases from July 2011-June 2012 and 59.2% of the case are due to *P. falciparum* and 40.8% *P.vivax* (PMI, 2014b). Ethiopia reported 936 malaria deaths in 2011, according to the 2012 World Malaria Report (WHO, 2013b). *P. falciparum* is responsible for 13–28% of deaths in children under 5 years of age (Tulu *et al*., 1993). Due to climatic and geographic factors, the disease occurs in different parts of the country in epidemic form (Jima *et al*., 2005). In consequence, about 40,000 Ethiopians die from malaria every year, more than those dying from HIV and tuberculosis. Rates of morbidity and mortality increase during epidemic years that reappear at irregular intervals of 3–4 years. In 2003, from 6 million cases shot up to 16 million, and over 100,000 people died (Zeleke *et al*., 2010).

The widespread availability of cheap and effective anti-malarial drugs, particularly chloroquine and pyrimethamine sulphadoxine, has undoubtedly limited both morbidity and mortality, but it has also encouraged the development and spread of resistance (Zeleke *et al*., 2010). However, an increased drug resistance to conventional anti-malarial, unavailability and unaffordability of the drugs, increasing resistance of mosquito vectors to insecticides, challenge of having effective vaccines and adverse effects of the existing anti-malarial drugs justifies the urgent need for more effective, tolerable and affordable anti-malarial drugs (Zelalem, 2011).

Therefore majority of the Ethiopian population relies on the traditional herbal remedies for the primary health care. The wide spread use of traditional medicine in both urban and

rural population in Ethiopia could be generally attributed to acceptability from cultural perspective, efficacy, physical accessibility and economic affordability. Traditionally used herbs remains a good source of pharmacologically active compounds that can be exemplified by quinine and artemisinin derived from *Cinchona officinali* and *Artemisia annua*, respectively (Zelalem, 2011).

1.1. The Global Burden of Malaria

Malaria is one of the major diseases of poor people in developing countries and one of the leading causes of avoidable death, especially in children and pregnant women.The highest risk is found in the sub-Sahara Africa in which the populations living in this area have the highest risk of acquiring malaria of all geographical locations. On contrary, in other regions of the world, particularly Latin America and most of Asia, levels of transmission are much lower and malaria tends to affect people of all ages causing severe morbidity, but less commonly resulting in death (WHO, 2011(a); UNICEF, 2011).

The global burden of malaria is terrible. Biomedically, the burden of disease is a function of many things and various public health disciplines define it differently. Epidemiologists, on the other hand, refer to burden in terms of morbidity and mortality, while economists refer to a quantification of the costs (direct and indirect), and effects on levels of productivity, national growth, and development.Common to both of these approaches is a focus on disease and risk factors, with the aim of establishing causative linkages with broad quantitative outcomes (Jones and Williams, 2004).

In spite of promising progress in controlling the diseases, malaria remains one of the major public health problems on the Africa in that it has a heavy burden on individual families and national health systems. In many African countries 30 % or more of outpatient's visit and hospital admissions due to malaria (PMI, 2014b).

Malaria affects the economy in which it follows different channels.The direct effect is that adults are unable to work during episodes of the disease, and may be significantly weakened for a period afterward. Repeated infection with malaria is associated with anemia in children and adults (Weil, 2010).

Malaria also retards economic and social development through effects such as reduced working hours due to sickness or attending to the sick, income spent on financing health care, which in turn, lead to impacts at national level due to massive health care budgets, reduced productivity of the work force, and the like (Pierre-Louis *et al.*, 2005).

The disease subsists in more than 100 countries in different regions of the world, including India, Southeast Asia, and Central and South America, although sub-Saharan Africa is the most strongly affected (Figure.1.1). Efforts to reduce poverty and childhood mortality in those vulnerable societies will fail if this devastating disease is not adequately controlled (Alonso, 2010).

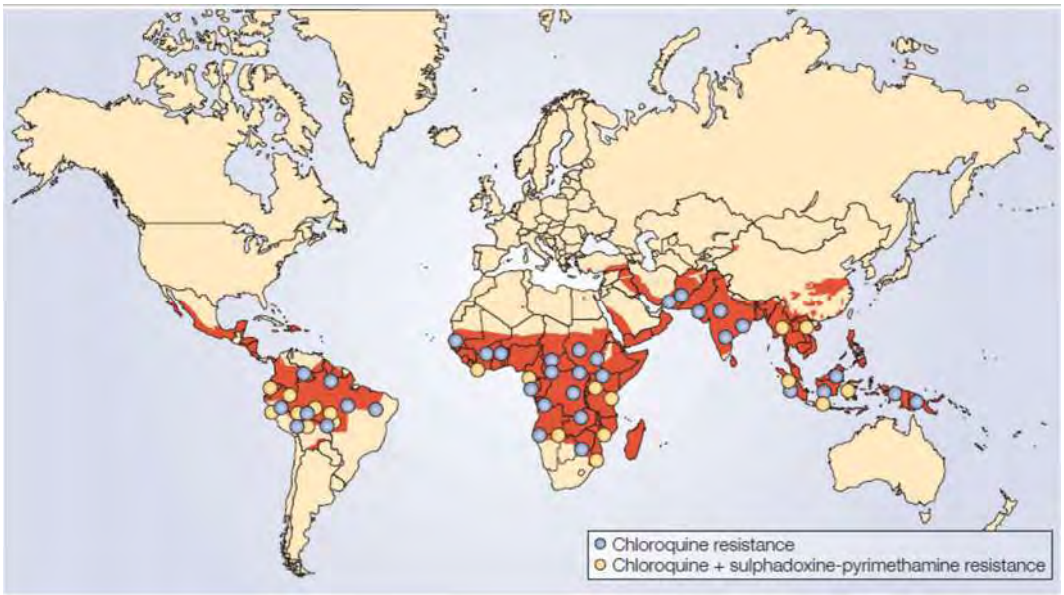

Figure 1.1 estimates of world malaria burden and resistance (Fidock et al., 2004).

Malaria also has an effect on the working population. In adults recurrent bouts of fever lower labor productivity. The transmission period of malaria coincides with the planting season, thus further lowering agricultural productivity. Lower productivity contributes to lower economic growth and the poverty levels, depth and severity that characterize most endemic Sub-Saharan Africa countries. Generally, gaining information on the burden of malaria relies on advancing the understanding of malaria epidemiology which requires investigation of the complex relationships among the malaria parasite, the vector, the host and the environment (Boland, 1993).

1.1.1. Malaria's Disease Burden in Ethiopia

Despite the moderate malaria parasite prevalence compared to many African countries. Historically, malaria has forced people to inhabit the less agriculturally productive highlands. Given that the country's economy is based on agriculture and peak malaria transmission coincides with the planting and harvesting season, this has placed a heavy economic burden on the country (PMI, 2014a).

In Ethiopia, malaria transmission is largely determined by altitude and climate as affected by Indian Ocean conditions and global weather patterns. Most of the malaria transmission occurs between September and December, after the main rainy season from June to August. Certain areas, largely in the western and eastern parts of the country, experience a second "minor" malaria transmission period from April to May, following a short rainy season from February to March (Adugna, 2014). Coinciding with major harvesting season with serious consequences for the subsistence Economy of Ethiopia's countryside, and for the nation in general. In addition, major epidemics occur every five to eight years with focal epidemics as the commonest form (UNICEF, 2011). Malaria poses no risk to in habitants at altitudes above 2000 meters, mostly in the Northern and Eastern Highland. Regionally, all of Gambella, most parts of Benishangul-Gumuz, the lower elevations of

the Northern and Eastern Highlands are alsoknown areas of malaria endemicity.The western, central and eastern highlands, as well as the highland-fringe areas along the Rift Valley areespecially vulnerable to epidemics. In all, more than half of the population lives in epidemic-prone areas (jima *et al.,* 2010).

The *Degaz*one of Ethiopia (altitude above 2,500 meters) with a mean annual temperature of 10-15 degree Celsius is malaria-free. Much of the *Woinadega* zone (Altitude 1500 – 2500 meters) is also malaria free, especially the zone in the 2000 – 2500 meters above sea level. Malaria in Ethiopia often occurs below 2000 meters, with short-lived transmission following the rains. However, malaria epidemics have been recorded up to 2400 meters during period's when increased temperature and adequate precipitation are conducive for both vector survival and parasite developments within the vector (Adhanom, 2006).

1.1.2. The Socio-Economic Impacts of Malaria in Ethiopia

Up to 2 million people die of malaria around the world annually, mostly in Africa and half ofthem children (Adugna, 2014).The disease are ranked as the leading communicable disease in Ethiopia "accounting for approximately 30% of the overall Disability Adjusted Life Years (DALYs) lost". It is estimated that over five million episodes of malaria occur each year in Ethiopia (Adhanom, 2006).

The burden of malaria has been increasing due to a combination of large population movements, increasing large scale epidemics, mixed infections of *P. vivax* and *P. falciparum*, increasing parasite resistance to malaria drugs, vector resistance to insecticides, low coverage of malaria prevention services, and general poverty. Out patient consultations, inpatient admissions and all in-patient deaths have risen by 21-23%

over the last five years. However, as 36% of the population is out of reach of the health service coverage, these figures may represent the true situation (jima *et al*., 2010).

1.2. Plasmodium Hosts

Malaria, an infectious disease associated with fever, anaemia and other pathologies, is caused by species of *Plasmodium*. This genus infects mammals, birds and lizards, and is transmitted by the bite of female mosquitoes (Anopheles species in mammals, or Culex species in birds and lizards) in which part of its life cycle is spent. Mammalian malarias are confined to antelopes, lemurs, bats, rodents and primates (including humans), and absent in felids, canids, equids or bovids, for unknown reasons (Cox-Singh and Singh, 2008).

1.3. Malaria Vectors

Anopheles arabiensis, a member of the *An. Gambiaev* complex, is the primary malaria vector in Ethiopia, with *An. funestus, An. Pharoensis* and *An. nili* secondary vectors (PMI, 2014a). The sporozoite rate for *An. Arabiensis* has been recorded to be as much as 5.4%. The host-seeking behavior of *An. Arabiensis* varies, with the human blood index collected from different areas ranging between 7.7 and 100%. *An. funestus*, a mosquito that prefers to feed on humans, can be found along the swamps of Baro and Awash rivers and shores of lakes in Tana in the North and the Rift Valley area. *An. Pharoensis* is widely distributed in Ethiopia and has shown high levels of insecticide resistance, but its role in malaria transmission is unclear. *An. nili* can be an important vector for malaria, particularly in Gambella Regional State (PMI, 2014a).

1.4. Life Cycle of Malaria

Human and other mammalian *Plasmodium* species are transmitted by anopheline mosquitoes. The parasite is injected with the saliva during mosquito feeding and first undergoes a round of merogony in the liver followed by multiple rounds of merogony in the erythrocytes (Figure 1.2). Gametogony begins within the erythrocytes of the vertebrate host and is completed within the mosquito where sporogony takes place. This life cycle exhibits the general features of other apicomplexan parasites characterized by asexual replication and the formation of invasive stages with typical apical organelles (Clark *et al.,* 2003).

The infection is initiated when sporozoites are injected with the saliva during mosquito feeding. The sporozoites enter the circulatory system and within 30-60 minutes will invade a liver cell. The sporozoites gain access to the hepatocytes by first invading and traversing a Kupffer cell. Host cell entry, as in all apicomplexa, is facilitated by the apical organelles. After exiting the Kupffer cell, the sporozoite can traverse several hepatocytes before developing into an exoerythrocytic (or pre-erythrocytic) schizont. Schizogony refers to an asexual replicative process in which the parasite undergoes multiple rounds of nuclear division without cytoplasmic division followed by a budding, or segmentation, to form progeny called merozoites. The merozoites are released into the circulatory system following rupture of the host hepatocytes. Recent observations suggest that the merozoites are released as a membrane bound aggregate, called a merosome, from the dying hepatocytes and the merosomes are delivered to the blood stream. This presumably provides protection from phagocytosis of the free merozoites by the Kupffer cells of the liver (Hyde, 2007).

In *P. vivax* and *P. ovale* some of the sporozoites do not immediately undergo asexual replication, but enter a dormant phase known as the hypnozoite. This hypnozoite can

reactivate and undergo schizogony at a later time resulting in a relapse. Relapse has a specific meaning in regards to malaria and refers to the reactivation of the infection via hypnozoites. Recrudescence is used to describe the situation in which parasitemiafalls below detectable levels and then later increases to a patent parasitemia (Hyde, 2007).

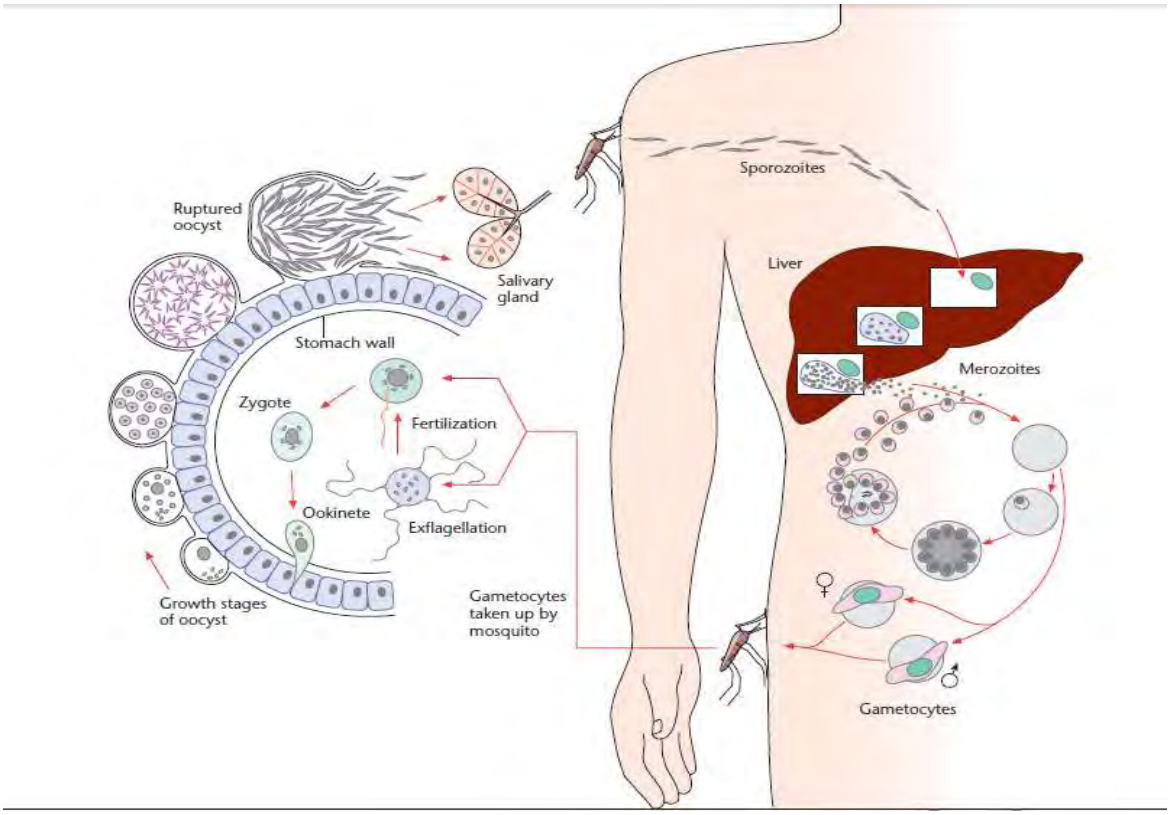

Figure 1.2: Life cycle of malaria parasite (Hyde, 2007).

In Blood Stage, Merozoites released from the infected liver cells invade erythrocytes. The merozoites recognize specific proteins on the surface of the erythrocyte and actively invade the cell in a manner similar to other apicomplexan parasites. After entering the erythrocyte the parasite undergoes a trophic period followed by an asexual replication. The young trophozoite is often called a ring form due to its morphology. As the parasite increases in size this 'ring' morphology disappears and it is called a trophozoite. During

the trophic period the parasite ingests the host cell cytoplasm and breaks down the hemoglobin into amino acids. A by-product of the hemoglobin digestion is the malaria pigment, or hemozoin. These golden-brown to black granules have been long recognized as a distinctive feature of blood-stage malaria parasites. Nuclear division marks the end of the trophozoite stage and the beginning of the schizont stage. Erythrocytic schizogony consists of 3-5 rounds (depending on species) of nuclear replication followed by a budding process. Late stage schizonts in which the individual merozoites become discernable are called segmenters. The host erythrocyte ruptures and releases the merozoites. These merozoites invade new erythrocytes and initiate another round of schizogony. The blood-stage parasites within a host usually undergo a synchronous schizogony. The simultaneous rupture of the infected erythrocytes and the concomitant release of antigens and waste products accounts for the intermittent fever paroxysms associated with malaria. Blood stage schizogony in *P.falciparum* differs from the other human malarial parasites in that trophozoite- and schizont-infected erythrocytes adhere to capillary endothelial cells and are not found in the peripheral circulation. This sequestration is associated with cerebral malaria and other complications (Idro, 2005).

As an alternative to schizogony some of the parasites will undergo a sexual cycle and differentiate into either micro- or macrogametocytes. The factors involved in the induction of gametocytogenesis are not known. However, commitment to the sexual stage occurs during the asexual erythrocytic cycle that immediately precedes gametocyte formations since the daughter merozoites from a particular schizont will develop into either all asexual forms or all sexual forms. Gametocytes do not cause pathology in the human host and will disappear from the circulation if not taken up by a mosquito. Gametogenesis, or the formation of micro- and macrogametes, is induced when the gametocytes are ingested by a mosquito. After ingestion by the mosquito, the microgametocyte undergoes three rounds of nuclear replication. These eight nuclei then become associated with flagella that emerge from the body of the microgametocyte. The

macrogametocytes mature into macrogametes. The highly mobile microgametes will seek out and fuse with macrogametes. Within 12-24 hours the resulting zygote develops into an ookinete. The ookinete is a motile and invasive stage which can transverse both the peritrophic membrane and the midgut epithelium of the mosquito. Traversing the pertrophic membrane probably involves secretion of chitinases. Penetration of the midgut epithelium involves invading and exiting several epithelial cells before emerging on the basal side of the epithelium. The invasion process is similar to other apicomplexa except that the ookinete does not have rhoptries and does not form a parasitophorous vacuole after invading the host cell (Greenwood, 2008).

After reaching the extracellular space between the epithelial cells and the basal lamina, the ookinete develops into an oocyst. Oocysts undergo an asexual replication, called sporogony, which culminates in the production of several thousand sporozoites. This generally takes 10-28 days depending on species and temperature. Upon maturation the oocyst ruptures and releases the sporozoites which cross the basal lamina into the hemocoel (body cavity) of the mosquito. These sporozoites are motile and have an ability to specifically recognize the salivary glands. After finding the salivary glands the sporozoites will invade and transverse the salivary gland epithelial cells and come to lie within its lumen. These sporozoites will be expelled into the vertebrate host as the mosquito takes a blood meal, and thus reinitiate the infection in the vertebrate host. Although the hemocoel and salivary gland sporozoites are morphologically similar, they are functionally distinct. Salivary gland sporozoites efficiently invade liver cells, but cannot re-invade the salivary glands, whereas the hemocoel sporozoites are inefficient at invading liver cells (Greenwood, 2008).

1.5. Pathogenesis of Malaria

Pathology associated with all malarial species is related to the rupture of infected erythrocytes and the release of parasite material and metabolites, hemozoin (i.e., malaria pigment) and cellular debris (Clark, 2003). In addition, there is an increased activity of the reticuloendothelial system as evidenced by macrophages with ingested infected and normal erythrocytes and hemozoin. In particular the liver and spleen are often enlarged during malaria. Except for *P. falciparum*, the pathology associated with malaria tends to be benign with little mortality (Idro, 2005).The proinflammatory cytokines, and especially tumor necrosis factor alpha (TNF-α), are believed to play a role in the disease manifestations. Higher levels of TNF-α and other proinflammatory cytokines are also associated with severe anemia, cerebral malaria, and respiratory distress. However, it is not clear the precise role proinflammatory and anti-inflammatory immune responses play in the resolution of the disease and its pathogenesis. Cytoadherence and sequestration are also believed to contribute to the pathology of severe falciparum malaria. Sequestration refers to the cytoadherence of trophozoite and schizont-infected erythrocytes to endothelial cells of deep vascular beds in vital organs, especially brain, lung, gut, heart and placenta. Sequestration provides several advantages for the parasite and contributes to the high parasitemias. In addition, this cytoadherence and sequestration can have pathological effects on the specific organs, most notably the brain. Receptor-ligand interactions between protein ligands expressed on the surface of trophozoite and schizont-infected erythrocytes and various receptors found on endothelial cells mediate cytoadherence (Clark, 2003).

As the parasite matures, parasite proteins are transported and inserted into the erythrocyte membrane. The high molecular transmembrane protein *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) is the most important ligand for cytoadherence (Magowan, 1988). Under febrile conditions, which enhance expression, *Pf*EMP1

mediated cytoadhesion begins at approximately 12 h of parasite development, 50% of the maximum effect is obtained at 14–16 h, and adherence is highly effective in the second half of the parasite life cycle (Udomsangpetch *et al.*, 2002). *Pf*EMP1 is encoded by the highly variable *VAR* gene family, comprising around 60 genes. The high switch rate between these genes gives rise to a new variant *Pf* EMP1 in 2% of the parasites every new cycle, and this clonal antigenic variation helps the parasite escaping the immune system (Roberts *et al*., 1992). *Pf* EMP1 is expressed on the surface of 'knobs', which can be identified electron-microscopically as protrusions from the erythrocyte membrane acting aspoints of attachment to the vascular endothelium. Other surface proteins that might play a role in cytoadherence are rifin and sequestrin (Ockenhouse *et al*., 1991).

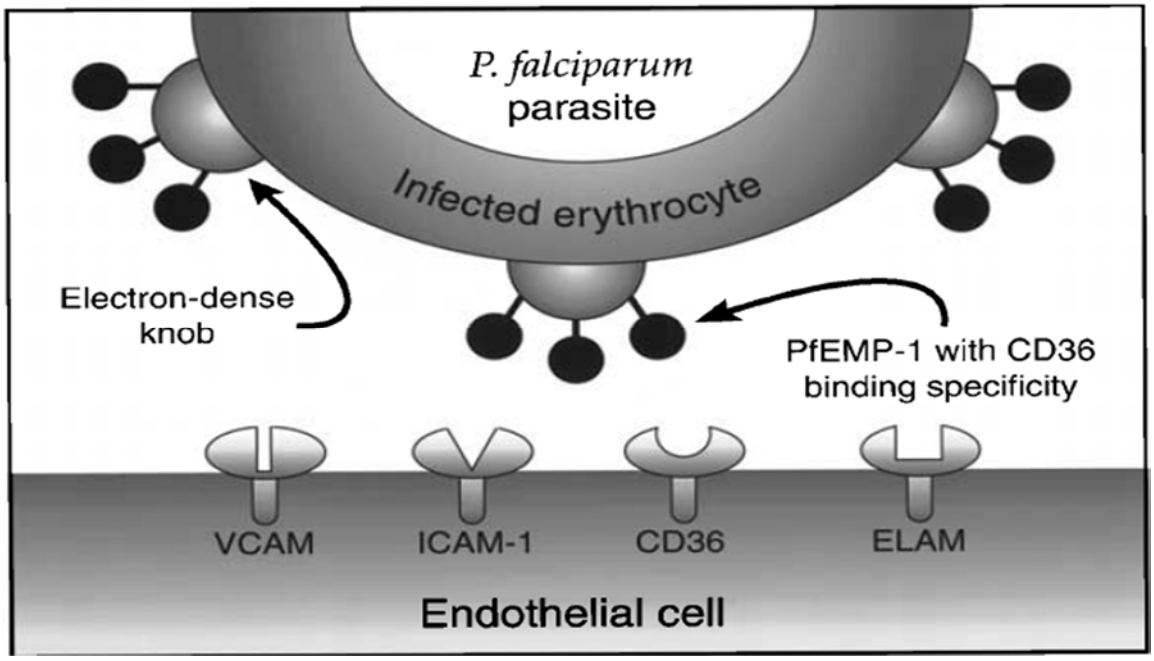

Figure 1.3: Schematic of a P. falciparum-infected erythrocyte expressing a PfEMP-1 with anti CD36 binding capacity. Other endothelial surface molecules represented are intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule (VCAM), and endothelial leukocyte adhesion molecule (ELAM).

The intercellular adhesion molecule 1 (ICAM-1) is the most important receptor on brain endothelium (Turner *et al*., 1994) and its expression is upregulated by the pro-inflammatory cytokine TNF-α. Electrostatic forces are probably important in addition to steric factors in the binding of *Pf*EMP1to its receptors. Surface potential spectroscopy of 'knobs' has revealed that knobs are positivily charged, whereas the endothelial plasmamembranes and receptors have a negative surface charge (Aikawa *et al.,* 1996). This could contribute to the promiscuity of *Pf*EMP1 for a great variety of receptors. Recently it has been suggested that platelets, which express CD36, might serve as a sticky bridge between infected erythrocytes and the endothelium, which could be particularly important in the brain microvasculature lacking CD36. Cytoadherence causes sequestration of parasitized erythrocytes in the microcirculation, mainly capillaries and post-capillary venules (Wassmer *et al*., 2004).

Rosetting is another adhesive phenomenon exhibited by *P. falciparum* infected erythrocytes which may play a role in pathogenesis. Some infected erythrocytes bind to multiple uninfected erythrocytes resulting in a large clump of erythrocytes, or a rosette. These clumps could restrict microvascular flow in a similar fashion as cytoadherence to endothelial cells and thus contribute to the pathology (Clark, 2003).

1.6. Clinical Features of Malaria

Understanding the pathogenesis of malaria requires investigation of mechanisms for parasite invasion and host defense. The parasite life cycle illustrates the interplay of parasite and host interactions. Pathogenesis of *Plasmodium falciparum* is the area of greatest study; since this species causes the most severe clinical disease. Infection with the five *Plasmodium* species has many clinical features in common. These are related to the liberation substances, especially during schizogony of fever-producing (Normark, 2008).

Plasmodium infection can exhibit non-specific symptoms a few days before the first febrile attack. These symptoms are usually described as flu-like, and include headache, slight fever, weakness, diarrhea, nausea, muscular discomfort and malaise and they tend to correlate with increasing numbers of parasites. These symptoms are followed by febrile attacks known as the malarial paroxysm, which is most notable for its periodicity; occurring every 24, 48 or 72 h. The regularity of the fever is due to the synchronous development of the malaria parasite, where the onset of fever corresponds to the rupture of pRBC at the end of the intraerythrocytic developmental cycle (IDC). The fever is believed to the cause of released proinflammatory cytokines, such as tumor necrosis factor (TNF) (Nilsson, 2011).

As Chiang *et al*., (2006) described the *P. falciparum* infection often leads to the development of multi-organ damage including cerebral malaria, renal failure, and pulmonary dysfunction. In contrast, the *P. vivax* type is called benign malaria because it is relatively non-lethal, but is often accompanied by debilitating conditions. High fever is usually accompanied with headache, shivering, intense perspiration and other symptoms such as abdominal pain, diarrhea, slight jaundice, cough, enlarged liver and spleen, and vomiting.In non-immune individuals, the clinical manifestation is more patent and a minority of these can become severe and life threatening (Arama, 2012).

Pregnant women are particularly vulnerable in both high and low transmission settings, with severe anemia, hypoglycaemia, coma, and pulmonary oedema as common features. In all patients with severe malaria metabolic acidosis is a frequent finding and is important to assess since it has a strong prognostic significance. Acidosis is mainly, but not entirely, caused by increased lactic acid production as a result of anaerobic glycolysis (Daily *et al.,* 2006).

1.7. Malaria prevention and control

WHO define malaria control as "reducing malaria morbidity and mortality to a lower level through deliberate efforts using the preventive and curative tools available today". Globally, different methods that target both the vector and parasite are used to overcome problems associated with malaria. According to WHO, (2006 and 2008), the control strategies of malaria rely on both preventive and case management. The tools used for prevention are long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS), intermittent preventive treatment for pregnant women (IPTP), malaria vaccine and antimalarial drugs (WHO, 2006a).

Controlling the diseases has always been a challenging and discouraging task, and new challenges make its control a difficult enterprise. The development of insecticide resistance in mosquito vectors and drug resistance in malaria parasite are known problems and the focus has been on the development of an effective and safe insecticide, and new drugs (Kant, 2011). Strategies against malaria infection and disease require an integrated approach, such as control of the mosquito vector, use of chemotherapeutic agents and control using insecticides, use of chemoprophylaxis and prompt, appropriate case management (Nyakeriga, 2005).

Malaria is a focal disease which differs in its characteristics from country to country and even within the same country. No single strategy is applicable for all situations, and implementing any of these may be problematic in an area: there has to be a regular assessment of each country's malaria situation. There are a variety of factors that should be taken into account, including (i) the biological, anthropological, cultural, and social characteristics of the population; (ii) the intensity and periodicity of malaria transmission; (iii) the species of malaria parasites and their sensitivity to antimalarial drugs; (iv) the species of the mosquito vector, their behavior, and their susceptibility to insecticides

,nearly 70 different species of mosquitoes are thought to be capable of malaria transmission, each with its own ecological requirements and behavioral characteristics; (v) the presence of social and ecological change; and (vi) the characteristics of the existing health services (Philips, 2001).

Current malaria control programs are mainly aimed by FMOH : (i) malaria patients, (ii) exposed individuals, and (iii) the mosquito vector, through parasite diagnosis and prompt treatment, largely by Artemisinin-based combination therapy, exposure prophylaxis with long-lasting insecticide-treated nets, and vector control by indoor residual spraying, respectively (PMI, 20140b).

1.7.1. Vectorcontrol

Vector control remains the most generally effective measures to prevent malaria transmission, and as such it is one of the four basic technical elements of the Global Malaria Control Strategy. There are basically two kinds of mosquito vector control. Those directed against the adult and those against the aquatic stages. As a process for managing vector populations to reduce or interrupt transmission of disease, WHO recommends integrated vector management (IVM). IVM is a systematic approach to f a range of interventions of proven efficacy, separately or in combination for the implementation of locally cost-effective control (WHO, 2005).

About 500 different species of Anopheles exist, up to 60 of which transmit the disease. Therefore, the vector distribution determines the malaria distribution and endemicity. Vector control is usually achieved using environmental management, biological methods, or insecticides which are either directly sprayed indoors or applied to bed nets (WHO, 2012). Treatment directed towards mosquito larvae consists of destroying larvae nests using any of the following methods: i) Environmental management methods comprising of filling breeding sites, lining water sources and canals, physical wetland drainage,

biological wetland drainage, impoundment planning, deepening and narrowing of old drains, vegetation manipulation, synchronized cropping and intermittent irrigation, biological controls using larvivorous fish such as the *Gambusia* fish is a voracious eater of mosquito larvae and saltwater flooding; ii) Larvicidal agents: bacterial larvicides, methoprene, temephos,and molecular films and oils (Gillies and Coetzee 1987).

Biological methods have recently attracted growing attention, certainly due to their relatively low cost and their assumed safety, compared to insecticides. In this regard, several options have been envisaged, including the use of refractory mosquitoes and paratransgenic organisms. In fact, most species of mosquitoes do not transmit malaria, and even among species that do, many individuals seem incapable of transmitting the disease (Marshall and Taylor, 2009).

1.7.2. Vaccination

Vaccination is an attempt to mimic certain aspects of an infection leading to an immune response that will protect the individual from that infection (Tripathi *et al*., 2005).With an increase in both insecticide and antimalarial-drug resistance, the development of a malaria vaccine carries huge expectation (Guerin *et al*.,2002). An ideal malaria vaccine encompasses mainly three essential characteristics; (a) it is multi-stage, incorporating antigenic characteristics at multiple stages of *Plasmodium* specieslife cycle, (b) it is multivalent, containing multiple epitopes restricted by different MHC molecules, which would help in overcoming the genetic restriction and allelic and antigenic variations, and (c) it is a multi-immune, inducing more than one type of immune response, comprising both cell-mediated and humoral immunity. Such a multi-component vaccine would increase the probability of a more sustainable and effective host response (Tripathi *et al*., 2005).

A vaccine to provide immunity comparable to that naturally acquired in areas of endemicity would have to be targeted at the asexual intra-erythrocytic stage of the infection, because it is this stage which is responsible for the pathology and clinical symptoms of the disease; prolonged protection by this vaccine would depend on regular boosting by reinfection (Philips, 2001).The search for a vaccine against malaria has been long, partly due to the complex life cycle of the parasite, an incomplete understanding of the mechanisms of effective immunity, and a lack of surrogate measures of protection and of adequate animal models. Various molecules from the pre-erythrocytic, asexual blood and sexual stages have been characterized and some may be promising vaccine candidate (Alonso *et al*., 1998).

In addition, Alonso, (2010) indicated that one of the greatest problems with malaria, which accounts for the extreme difficulty of developing a vaccine, is that how individuals develop immunity against the disease is not yet understood. Currently, most of the potential vaccine candidates in development are either pre-erythrocytic or blood stage vaccines because their immediate benefits to individuals promote strong commercial interests (Koram and Gyan, 2010).

The original roadmap contained a 2015 landmark goal for a modestly efficacious malaria vaccine yielding reductions in morbidity and mortality, which remains unchanged, and could be achieved by the RTS,S vaccine currently undergoing Phase testing (WHO, 2014).

Safety and efficacy data from the recently completed Phase 3 trial of RTS,S (reported as the reduction inincidence of first or only episode of clinical malaria), following 12 month follow up in both age groups, has been published in recent years. In 2013, the18-month follow up data was reported at the Multilateral Initiative on Malaria (MIM) conference in Duan, South Africa. Children aged 5-17 months at first vaccination with RTS,S experienced 46% fewer cases of clinical malaria. Severe

malaria cases were reduced by 36% and malaria hospitalizatios were reduced by 42%. Infants aged 6-12 weeks at first vaccination with RTS,S had 27% fewer cases of clinical malaria. The reduction of severe malaria cases and malaria hospitalizations by 15% and 17%, respectively, were not statistically significant. Data from 32 months follow-up from the Phase 3trial, including the impact of a fourth 'booster' dose given 18 months after the initial three doses, are expected to become available by early 2015 (WHO, 2014).

With regard to a potentialmalaria vaccine, however, partsof the scientific community are in agreement that these high standards will not be met. Other scientists, though, conclude that even a rather ill performing vaccine such as RTS,S could accomplish a massive reduction of malaria lethality. Hopefully, RTS,S will particularly contribute to a reductionin child mortality, as it may provide infants with more time to develop a form of clinical immunity. Nonetheless, almost all the scientific reviews about the currently most advanced vaccine candidate RTS,S come to a clear consensus; the formidable task of malaria disease eradication will not be accomplished with this vaccine (Veronique *et al.,*2014).

1.8. Antimalarial drugs and drug resistance

Plasmodium parasites can be killed by antimalarial drugs at both asexual and sexual stages. This is why strategies to eliminate malaria are spearheaded by antimalarial drugs. Historically, ethno medicinal plants have always been sources of antimalarial drugs, (Wink, 2012). Two important antimalarial precursors' compounds were identified and isolated from ethnomedicinal plants. The first one is quinine where quinine based antimalarial drugs such as mefloquinine and chloroquine. Quinine was isolated from a group of *Chinchona* species from Peru (Achan *et al.*, 2011). The second is artemisinin. This is the parent compound were artemisinin based drug such as; artemether, artesenuate

were modeled on, (White, 2008). Classification of antimalarial drugs is based on their core functional chemical structures and therapeutic responses to the malaria parasites. Based on their therapeutic responses there are two groups namely; the blood schizonticides, these act on the asexual erythrocytes (blood stage) and partly on sexual stages of the parasites, (Saifi *et al.*, 2013). The second group is the tissue schizonticides and these act on the hepatic stage (liver stage). Based on core chemical structures, there are three main groups; quinolines, sesquiterpenes and naphthaquinones.

Quinine based drugs

Quinine and its derivatives are the first pharmaceutical antimalarial drugs in the history of malaria treatment (Duffy & Hobbs, 2010; Daily, 2006) described quinines as bitter tasting alkaloids that belong to the aryl alcohol group. Quinine derivatives are such as chloroquine, mefloquinie and amodiaquinine.

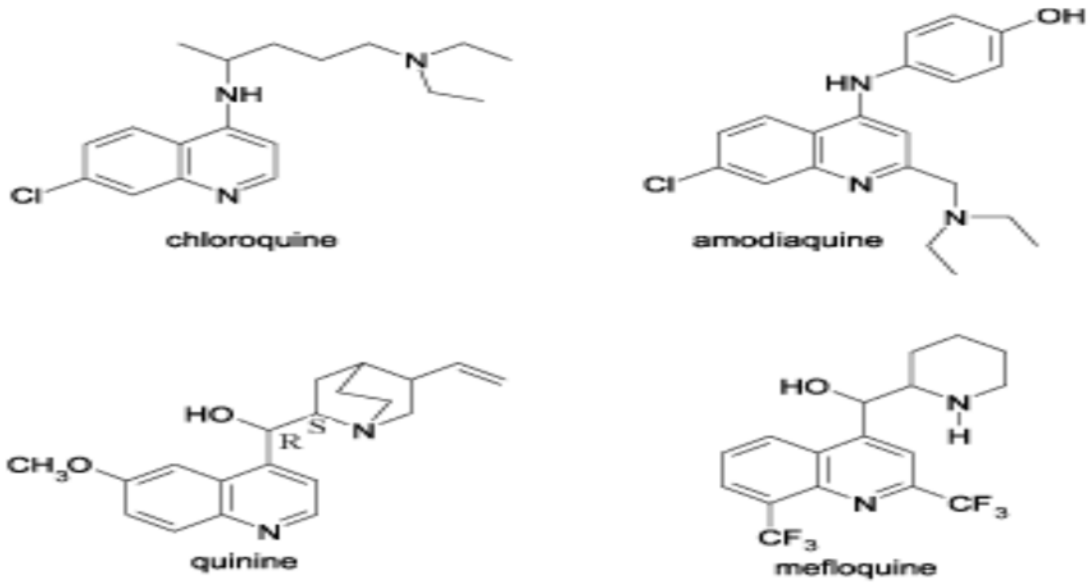

Figure 1.4: Chemical structure of quinine antimalarial drugs (Delfino *et al.*, 2002).

Since its discovery until 2005, quinines and derivatives (figure 1.4) were recommended to be used in combination with other antimalarial drugs as firstline treatment for uncomplicated and severe malaria (Achan *et al.,* 2011). The use of quinine as monotherapy had been banned by the WHO, (Bloland *et al*,1993), although some African countries continue to use quinine as monotherapy for cost reasons (Achan *et al.,* 2011).

The quinolines act mostly during the blood stage of the parasite's life cycle, but some are believed to act at the liver stage as well. Nevertheless, *Plasmodium* parasites have developed resistance to quinine as from the 1950's in South America and Asia, (Wernsdorfer *et al.,* 1980). Currently, it has spread throughout the world (Baird, 2005).

Sesquiterpenes based drugs

The second group is artemisinin and its derivatives used in combination with other antimalaria drugs. These are now the first line treatment of uncomplicated treatment worldwide. White, (2008) stated that artemisinins belong to sesquiterpenes lactones. Many derivatives such as; artesunate, dihydroartemisinin, artemotil (known as arteether) and artemether (figure 1.5) were later synthesized from the lead compound.In 2006, the WHO recommended four ACTs namely; artesunate-sulfadoxin pyrimethamine, artesunate-amodiaquine, artesunate-mefloquine and artemether-lumefantrine for uncomplicated malaria treatment, (White, 2008; WHO, 2006). Parenteral artesunate is used for severe malaria treatment in adults, (Dondrop *et al*, 2010; WHO, 2010). Additionally, in 2010, the WHO added dihydroartemisinin-piperaquine to the existing list of four recommended ACTs, (Garner, 2013). Artemether-lumefantrine, commercially known as Coartem, is recommended for uncomplicated malaria treatment in Ethiopia, (FMOH Malaria Strategic Plan 2010-2016). The efficacy and safety of ACTs depend largely on the partner drug, (Ngasala *et al*, 2009). "The artemisinins produce rapid clearance of parasitaemia and rapid resolution of symptoms, by reducing parasite

numbers from 100- to 1000-fold per asexual cycle of the parasite (a factor of approximately 10 000 in each 48-hours asexual cycle)" (WHO, 2010).

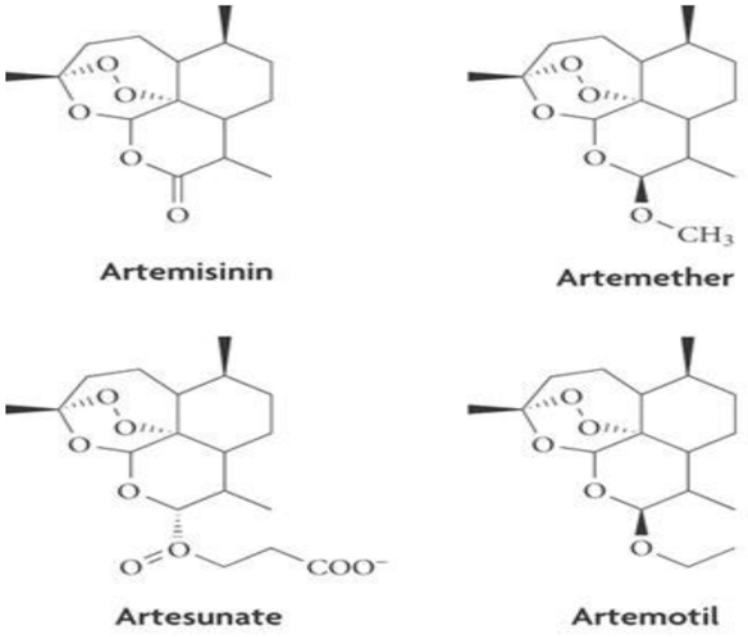

Figure 1.5: Chemical structure of artemisinins based drugs (Dondrop *et al*, 2010).

Antifolates

Antifolates are not derived from plants but rather designed by combination of medicinal chemistry and cell biology. Antifolates are mainly used as chemoprophylaxis for travelers. According to Nzila (2006), antifolate agents used in the treatment of malarial infection are subdivided into two classes: inhibitors of dihydropteroate synthase (DHPS), known as class I antifolates and class II anti-folates are inhibitors of dihydrofolate reductase (DHFR). The combination of DHFR and DHPS inhibitors is synergistic, hence their use in combination in the treatment of malaria. Their basic mode of action is the inhibition of the synthesis of folate co-factors that are required for nucleotide synthesis and amino acid metabolism (Cunha-Rodrigues *et al.,* 2005).

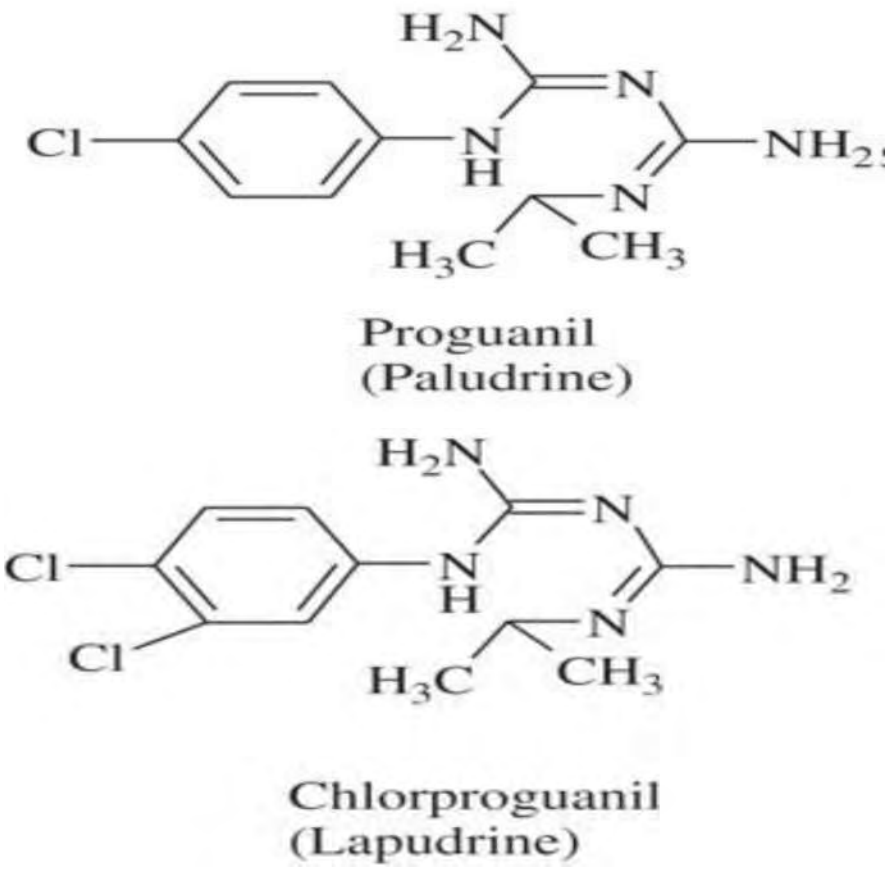

Figure 1.6: Chemical structures of antifolates (Nzila, 2006).

Atovaquone/proguanil combination

Atovaquone was discovered from high throughput screening of chemical libraries, most of which were hydroxynaphthoquinone. Atovaquone is used as a fixed-dose combination with proguanil (Malarone) for treating children and adults with uncomplicated malaria or as chemoprophylaxis for preventing malaria in travelers. Atovaquone is a hydroxynaphthoquinone that inhibit mitochondrial electron transport and collapse mitochondrion membrane potentials, (Shanks *et al,* 1998).

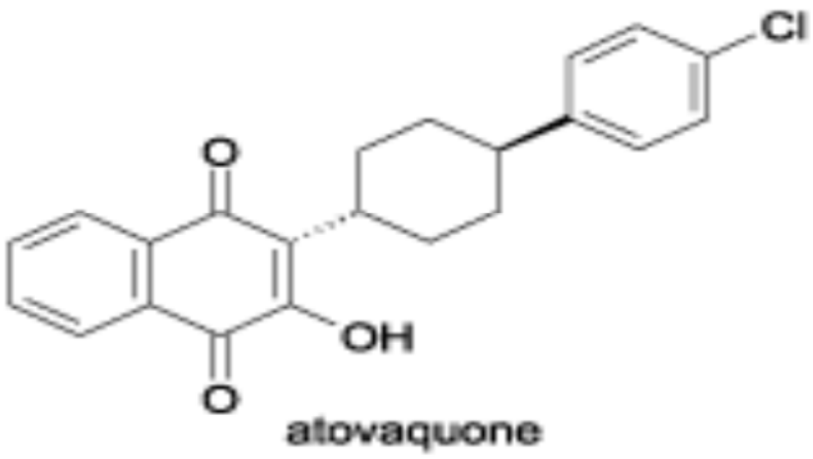

Figure 1.7: Chemical structure of atovaquone antimalarial drugs (Delfino, et al, 2002).

1.8.1. Drug resistance

For decades, drug resistance has been one of the greatest obstacles in fighting malaria. To date, drug resistance has been reported in three of the five *Plasmodium*s pecies that is, *P. falciparum*, *P. vivax* and in *P. malariae* which are the causative agents for human malaria WHO, (2000-2010). Drug resistancewas initially outlined by WHO in 1967 as the ability of a parasite strain to survive or reproduce regardless of the administration and absorption of a drug when it is given indoses that are equal to or higher than those usually recommendedbut within the tolerance range of the given subject (WHO. 1967). This was later modified by Bruce-Chwatt *et al.,* (1986) toinclude ‘‘the amount of the drug which is active against a given parasite must be able to gain access to the parasite or the infected erythrocyte for the length of the time necessary forits natural reaction’’. Drug resistance usually leads to a delayor failure to clear asexual parasites from the peripheral blood that eventually enable production of gametocytes which are responsible for transmission of

the resistant genotype. After the official recommendation by the WHO in (2001), foruse of artemisinin-based combination therapies (ACTs) asthe first-line treatment of *P. falciparum* malaria, it was seen after 2005 that there was a substantial decline in outbreak of this disease (WHO, 2012). However, parasites that are drug resistant to artemisinin and its derivatives have recently emerged in various parts of Southeast Asia, which threaten, all prior success of malaria control strategies, treatment and elimination efforts (Dondorp *et al*., 2011). At present, current antimalarial drugs act on a limited number of biological targets (Vial, 2013). Therefore, the next challengeis to identify new classes of drugs that will attack novel molecular targets, with sufficient therapeutic lifespans that will not be compromised by the rapid development of resistance, and to develop novel technologies, that will effectively clear the parasite with maximum precision, thus minimizes the risk of drug resistance (Cowman *et al.,* 2000).

Of the various antimalarial drugs available, the aminoquinoline chloroquine was the agent of choice for many decades because of its safety, efficacy and affordability. However, parasite resistance to this drug was initially observed in Thailand in 1957 and then on the border of Colombia and Venezuela in 1959. By the late 1970s, resistance reached East Africa and by the mid-1980s had become a major problem in several areas of the continent (Wernsdorfer and Payne 1991). At present, chloroquine remains effective only in some parts of Central America, Africa and South America where clinical studies have confirmed it as an effective drug (Londono, 2009). However, recent data on the prevalence of chloroquine-resistant genotypes inthese areas present an alarming situation for health officials. Amodiaquine has been observed to be more effective than chloroquine mainly in areas of persistent chloroquine resistance. As a result, amodiaquine in combination with artesunate was adopted as the first-line treatment by several countries. Parasite strains that are highly resistant to amodiaquine have however, been reported in Tanzania, which may additionally compromise the use of artesunate-amodiaquinein Africa. Another antimalarial, sulfadoxine-pyrimethamine has been widely

used by several countries to treat chloroquine-resistant malaria. Nonetheless, the treatment failure rate of this combination has been found to be low in several countries of South America and Central and Middle East Asia as compared to the failure rate in eastern Africa (Hyde, 2002).

Presently, resistance to mefloquine continues to be a concern in the Greater Mekong sub-region, in particular in Thailand and Cambodia, where artesunate-mefloquine is still used as first line treatment (Satimai *et al*., 2012). In order to maximize the effectiveness of artemisinin and its derivatives and to protect them from the development of resistance, WHO has repeatedly recommended that they can be combined with other drugs that have different mechanisms of action and longer half-life. As a result, five combinations are currently recommended: artemether-lumefantrine, artesunate-amodiaquine, artesunatemefloquine, artesunate-sulfadoxine-pyrimethamine and dihydroartemisinin-piperaquine (WHO, 2010). However, remarkable failure rates of these combinations have been observed in several African countries where resistance to onedrug has been previously encountered, like in the case of artemether lumefantrine. Artemether-lumefantrine remains highly effective in most parts of the world, with the exception of Cambodia. This combination mostly shows failure rates less than 10% (WHO, 2000-2010). However, resistances to most of these combinations will probably led to a global epidemic outbreak of malaria.

1.8.2. Traditional Medicine and Anti-Malarial Drugs from Medicinal Plants

In developing countries traditional medicine still plays an important role in local health care systems (Xu *et al*., 2011). The WHO estimates that up to 80% of some Asian and African countries depend on traditional medicine for their health care needs (Xu, 2011; WHO, 2008). It is difficult to assign one definition to the broad range of characteristics and elements of traditional medicine. The WHO thus defines traditional medicine as the

health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral-based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illness or maintain well-being (WHO, 2008). Chan, (2005) concludes that traditional healing is "holistic" in nature. It does not focus on symptoms or diseases. Instead, it deals with the total individual. Healing focuses on the person, not just the illness and healing does not follow written guidelines, that is, different people may receive the same medication or treatment even if they suffer from different diseases, in contrast to modern medicine where different people may receive different treatments even if they suffer from the same disease. Plants have been a source of medicine throughout the history of medicine. For thousands of years natural compounds, mostly from plants, have been the manifestation of traditional medicine (Ginsburg and Deharo, 2011).

Traditional medicine has remained as the most affordable and easily accessible source of treatment in the primary health care system of resource limited communities and the local therapy is the only means of medical treatment for such communities (Yineger and Yewhalaw, 2007).

WHO has stated that 74% of 119 plant-derived pharmaceutical medicines applied in modern ways resembles the method used by native cultures. Utilization of plants as primary source of medicinal agents by the majority of the population is not only due to the high cost of Western pharmaceuticals, but also because the traditional medicine is generally more acceptable from a cultural and spiritual perspective. Even in the Western world, the use of herbal medicines is steadily growing with approximately 40% of the population reporting use of herbs to treat medical illness (Kirkpatrick, 2002).

In malarial regions, affordable treatments against malaria are mainly based on the use of traditional herbal remedies. Indeed, indigenous plants play an important role in the

management of the disease, and they seem to be the most convenient solution because of their accessibility and diversity in tropical and sub-tropical regions (Phillipson and Wright, 1991). According to several reports, up to 80% of world's populations still rely on traditional medicine mainly on herbal remedies as primary source of medicinal agents for the treatment of diseases including malaria (Kirkpatrick, 2002). Malaria ranks as the most important disease treated with herbal remedies in ethno medical practices (Phillipson and Wright, 1991).

Plant based drugs provide an outstanding contribution to the modern therapeutics (Ginsburg and Deharo, 2011). It is estimated that at least 25% of all modern medicines are derived either directly or indirectly from medicinal plants primarily through the application of modern technology to traditional knowledge. In the case of certain classes of pharmaceuticals such as antitumoral and antimicrobial medicines, this percentage may be as high as 60% (Sucher, 2008). The development of useful and widely used drugs like digoxin and digitoxin from digitalis leaves, quinine from the cinchona bark, reserpine from *Rauwolfia serpentine*, morphine from *Papaver somniferum*, cocaine from *Erythroxzion coca* and the anticancer *vincristiner* and *vinblastine* from *Cartharathus troseus* of Madagascar and again the anticancer compound *bruceatin* from the Ethiopian plant *Brucea antidysentrica* are just to name a few examples of the contributions of traditional medicinal plants to the modern therapeutics agents (Bekele,2007). Many plant species continue to be used in traditional medicine for the treatment of malaria and many people depend up on such remedies as they cannot afford or do not have access to standard antimalarial drugs. Recently, the WHO estimated that 80% of people worldwide rely on herbal medicines for some part of their primary health care (WHO, 2010c), especially developing countries where malaria is endemic which depends strongly on traditional medicine as a source for inexpensive treatment of this disease. In Ethiopia it is estimated that about 80% of the Ethiopian population is still dependent on traditional medicine, which essentially involves the use of plants (Zelalem, 2011). However,

scientific data to validate the antimalarial properties of these herbal remedies are scarce (Ramalhete *et al*., 2008).

In Ethiopia, traditional medicine still constitutes an important method of health care especially among those with poor access to modem health care services. The earliest documented encounter of Ethiopians to modern health care services dates back to the reign of Atse Lebnedengel (1508-1540) when a member of a Portuguese diplomatic mission gave modem medical service to the court of the Emperor. Ethiopians have used traditional medicines for many centuries, the use of which has become an integral part of the different cultures in Ethiopia (Mesfin *et al*., 2009).

Different *invitro* and *invivo* studies have been conducted to evaluate the antimalarial effects of Ethiopian traditionally used plants. For instance, Dikasso *et al*., (2006) revealed that the extracts from the roots and aerial parts of *Asparagus africanus* were observed to inhibit *Plasomodium berghei* parasitaemia in the Swiss albino mice by 46.1% and 40.7% respectively. Kersala (2008), indicated that crude aqueous extract, crude hydroalcoholic extracts, of roots of *Clerodendrum myricoides* have antimalarial activity at much lower doses than the LD_{50} hinting the safe and efficacious nature of the plant.

In addition, there are huge numbers of plant species used by the people to treat malaria in different parts of the country such as *Carica papaya* in Asendabo district, Jimma zone (Parvez and Yadav, 2010). In the northwestern Ethiopia, *Aloe* sp, *Azadirachta indica, Calpurnia aurea, Carica papaya, Croton macrostachyus, and Zehneriascabra* are also used (Giday *et al.,* 2007).

Mengistie *et al*., (2012) has also described that the extracts of *Dodonaea angustifolia* leaf and *Bersama abyssinica fresen* significantly inhibited parasitemia and prevented PCV fall ($P<0.05$) dose-dependently. They increased the survival time of the infected mice. Likewise, Getie, (2010) evaluated the antimalarial activity of seeds of *Dodonaea*

angustifolia and leaves of *Entada abyssinica* against *Plasmodium berghei* in Swiss albino mice and found highest parasite suppressions (86.21%) at 600mg/kg and marginal efficacy were obtained from the methanol extracts of *D.angustifolia* at 100mg/kg.

Despite their wide use in the traditional health care, the work that has been done to evaluate the safety and efficacy of Ethiopian traditional medicinal plants is not extensive. Previous studies have shown the anti-malarial activities of *Vernonia amygdalina* and *Withania somnifera in vitro* against *P. falciparum* (Zelalem, 2011) and activities of these plants against *P. berghei* in mice.

The organic extracts of leaves of *V. lasiopus* show significant antimalarial activity (Erasto *et al*., 20o6). The chemical compounds isolated from alcoholic extracts of dried aerial parts of *V. lasiopus* are the elemanolides, epivernodalol and lasiopulide. These are carbon-10 epimers of the sesquiterpene lactones vernodalol and demethyl acroylated vernodalol, which have been found present in other species of *Vernonia*, such as *Vernonia amygdalina*.

The crude Water and ethanol extract of *V. amygdalina* was found to show a significant inhibitory activity *in-vitro* were tested on trophozoites, mainly ring forms, and there was a significant reduction in the number of parasitized cells relative to control ($P > 0.05$) and showed the highest antimalarial activity of 78.1 %, with $IC_{50}$of 11.2 µg/mL. The water extract had the parasite growth inhibition of 74.0 % and $IC_{50}$of 13.6µg/mL (sha'a *et al*., 2011).

Melariri *et al*., (2011) has also described that the extracts of *In vitro* and *in vivo* antiplasmodial activities of extracts of *Cymbopogon citratus* Staph and *Vernonia amygdalina* Del. by petroleum ether, ethyl acetate, and methanol extracts of *C. citrates* showed IC_{50} of 9.1µg/mL, 12.1µg/mL, 15.9µg/mL respectively while the petroleum ether, ethyl acetate, and methanol extracts *V. amygdalina* recorded IC_{50s} of 14.1µg/mL,

10.7µg/mL and >50µg/mL respectively. Water extracts of both plants showed IC_{50} > 50µg/mL. The dichloromethane extracts of the two plants recorded the greatest antiplasmodial activity *invitro.* Simultaneously, the *in vivo antimalarial* activitiesshowed a marked growth inhibition of parasites with values of 87.2% and 95.8% by the dichloromethane extracts of *C. citratus* and *V. amygdalina*, respectively at a dose of 800 mg/kg.

It was found that the Ethanolic, aqueous, and hydroethanolic extracts both *In vitro* and *in vivo* antiplasmodial activity from *Vernonia amygdalina* Leaves showed that the ethanolic extract of the plant leaves had the highest ($p<0.05$) antiplasmodial activity (IC_{50} = 9.83 ug/mL) and cytotoxicity (IC_{50} = 60.33) with moderate selectivity index of 6.14 when compared with the other extracts and the ethanolic extract was also significantly active in vivo against *P. berghei* in a dose-dependent manner with maximum activity observed at 1000 mg/kg (% inhibition of 82.3 %)(Omoregie *et al.,* 2010).

Recent *Invitro* study by Odeh *and* Usman, (2014) of Antimalarial activity and phytochemical analysis of aqueous leaf extract of *Vernonia amygdalina* showed at different concentration ranging from 20 mg/mL, 40mg/mL, 60mg/mL, 80mg/mL and 100mg/mL. It was observed that the extract showed a higher zone of inhibition (15.0±0.58) at 100mg/mL concentration on *P. malariae* and (14.00± 1.00) on *P. falciparum. P. ovale* showed a moderate zone of inhibition of (14.00±0.58) while *P. vivax* showed the least zone of inhibition (13.00±0.57).The analysis was controlled with Coartem (14.3±0.58).The phytochemical screening of the aqueous leaf extract of *Vernonia amygdalina* was investigated which showed the presence of Saponins, Tannins, Alkaloids, Cardiac glycosides, Glycosides, Steroids and Anthraquinones which have an antimalarial activity on *Plasmodium* parasites.

Najan *et al*., (2008) found thatthe analgesia and toxicity of the aqueous extract of *V. amygdalina* extract caused a significant ($p< .05$) and dose-dependent reduction in mean parasitemia in mice infected with *P. berghei* in comparison to Chloroquen(CQ) (5 mg/kg). The extract caused a parasitemia reduction of 52% with 50 mg/kg, 64% with 100 mg/kg, and 73% with 200 mg/kg (i.p.). All the mice in the saline group died within 15 days throughout the 30-day observation period of the experiment, while the remaining mice recovered fully.

Ethanolic leaf and root-bark extracts of *V. amygdalina* were found to possess significant antimalarial activities against drug-sensitive *P. berghei* in mice *in-vivo* (Abosi and Roseroka, 2003).

Moreover, Iwalokun, (2008) reported that prophylactically, chloroquine (5mg/kg) in combination with *V.amygdalina* extracts achieved a dose-dependent (57.2 – 72.7%) suppression of parasitaemia due to CQ sensitive and resistant *P. berghei* strains in the experimental animals. Therapeutically, chloroquine (30mg/kg for 3 days) combined with *Vernonia* to dose-dependently shorten the parasite clearance times (2.6 – 4.4 vs. 4.8 days; $p<0.05$ for CQ-V62.5/125 combination), prolong the recrudescent times (8.9 – 18.9 vs. 7.2 days; $p< 0.05$) and improve day 14 curerate (66.7 – 100 vs. 58.3%) in the treated *P. berghei* infected mice compared to CQ monotherapy. Whereas CQ monotherapy failed, resolution of parasitaemia due to the CQ resistant parasite with day 14 cure rates of 25 – 100% were also observed with these combinations. In therapeutic terms, the potencies of CQ-V125 combination were comparable to those of CQ-chlorpheniramine (0.25mg/kg, 12hourly, 7 days) in the infected animals. Toxicity testing indicates that these combinations elicited mild to – moderate increases in the liver enzymes measured when administered orally to mice for 7 days.

Currently Abay *et al*., (2015) also reported that Ver-H2O reduced the *P. berghei* macrogametocyte density in mice by about 50% and Ver-EtOH reduced *P.berghei* oocyst prevalence and density by 27 and 90%, respectively, in *An. stephensi* mosquitoes. Ver-EtOH inhibited almost completely (>90%) early sporogonic stages (ESS) development *invitro* at 50µg/mL.

Madaki, (2015) also indicated that the ethanol extract of *Vernonia amgdalina* were showed that the extract contained alkaloid, tanin, saponin, glycoside, terpenoid, flavonoid, while the LD_{50} of the extract was 2236mg/kg. The % chemo-suppression was within the range of 17.15% at 200mg/kg to 66.65% for the standard drug group. There was no significant difference ($p<0.05$) in the % suppression between the group administered 600mg/kg (58.24%) and the standard drug group. The mean survival time of mice treated with standard drug (21.33 days) was significantly ($p>0.05$) higher than those in other treatment groups and normal saline group (2.27 days). On the 5th day, the PCV of mice administered 600mg/kg (44.63±1.67 %) showed no significant ($p<0.05$) different compared to those in the standard drug treated group (47.92±1.75%).

The phytochemical analysis indicated that the presence of antioxidant agents (sesquiterpene) such as saponins, flavonoids, oxalates, alkaloids and vernoniosides (glucosides) in the methanolic leaf extract (Eleyinmi *et al*., 2008). However, in aqueous leaf extract vernodalin, vernolide, hydroxyvernolide, and glucosides (vernonioside) in related species *(V. amygdalina)* (Osinubi, 2008). Nwanjo (2006) in his study also showed the presence of tannins in addition to alkaloids, saponins, flavonoids, and glycosides chemical constituents in the fresh leaf of related plant *(V. amygdalina)*. According to Diwan *et al*., (2000) the presence of saponin in the extract can cause from mild to severe diarrhea. The availability of active ingredient in methanolic leaf extract that can induce enlargement of spleen has also indicated in *V. scorpioides* (Pagno *et al*., 2006).

Adbayo *et al*., (2014*)* showed the phytochemicals tested were found to be present in both the methanolic and ethanolic leaf extracts of *Vernonia amgdalina* leaf. The aqueous extract showed the presence of saponins, reducing sugars and anthracenosides. Glycosides, flavonoids, saponins and alkaloids were the only groups of phytochemicals found in the petroleum ether extract. The methanolic extract showed the greatest amount of saponins (14.23%), flavonoids (2.15%), alkaloids (7.49%), tannins (5.4%), terpenes (10.20%) and phenolics (8.24%).

Petros, (2011) indicated that traditional herbal remedies have wide acceptance from cultural and spiritual perspective, and a number of effective drugs have been isolated from plants for malaria treatment, majority of the population relies on it. Hence, there is a need to undertake antimalarial activity evaluation and toxicological assessment of claimed medicinal plants and formulate standardized herbal preparation in Ethiopia.Therefore, this study was designed to substantiate the scientific relevance of the claimed medicinal plants, that is, the leaves of *Vernonia amygdalina* as anti-malarial treatments.

1.9. Description of the Study Plant

The largest family of flowering plants that contains about 900 genera and some 13,000 species. Members are herbs, shrubs or rarely small trees or climbers. Leaves are alternate or opposite, simple or variously divided (Hutchinson, 1963). Members are known to store carbohydrate as polyfructose, notably inulin and commonly producing polyacetylenes which are borne in the resin-canals. Polyacetylenes are characterized by the presence of cyclic, aromatic or heterocyclic end-group; bitter sesquiterpenes (especially sesquiterpene lactones), terpenoid volatile essential oils and often one or another sort of alkaloids, notably the pyrrolizidine alkaloids, but without tiridoid compounds (Cronquist, 1981). Asteraceae are known to contain pyridine, quinoline alkaloids and occasionally

diterpenes. Glycosides, saponins, flavonoids and tannins are their chief constituents and flavonoids are used as taxonomic markers (Evans, 1999).

1.9.1. The Genus *Vernonia* Shrub

The genus has about 60 members that are trees, shrubs or woody climbers. They are characterized by involucral bracts with colored or whitish appendages; capitula 1.3-3cm or more diameters; achenes often black and ribbed; pappus-setae more or less uniform. They are usually deciduous and buff in color and leaves are often toothed (Hutchinson, 1963).

They are classified in according to Sanogo (2005) as follows:
Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Asterales
Family: Asteraceae
Genus: Vernonia
Species: *Vernonia amygdalina* Del.

1.9.2. The Species: *Vernonia amygdalina* Del.

Vernonia amygdalina commonly known as bitter leaf (English), grawa (Amharic), Eebicha (Afaan Oromo) is a tropical shrub that grows up to 3 meters high in the African tropics and other parts of Africa particularly Nigeria, Cameroon, Zimbabwe and Ethiopia. The leaves are dark green colored with a characteristics odor and a bitter taste. It is reputed to have several health benefits. It is effective against amoebic dysentery, gastrointestinal disorder and has antimicrobial and anti-parasitic activity (Erasto *et al*., 2006). *Vernonia amygdalina* is a perennial herb belonging to the Asteraceae family. The

species is indigenous to tropical Africa and is found wild or cultivated all over sub Saharan African. The leaves are eaten after crushing and washing thoroughly to remove the bittern. However, almost all parts of the plant are pharmacologically useful, both the root and the leaves are used in phyto-medicine to treat fever, hiccups, kidney disease and stomach discomfort among others (Nwanjo, 2006; Erasto *et al*., 2006).

Vernonia amygdalina has been ascertained to provide various culinary and medicinal properties, these medicinal properties exert bacteriostatic and bacteriocidal effect on some bacteria. Antihelmitic and Antimalarial properties (Abort and Raserika, 2003) as well as antitumourigenic properties (Izevbigie *et al*., 2004) have also been reported for extracts from the plant.

Furthermore, other studies have demonstrated hypoglycemic and hypolipidaemic effect of the leaf extract in experimental animals (Nwanjo, 2006). Many herbalist and native doctors in Africa recommend its aqueous extract for their patients for the treatment of varieties of ailment ranging from emesis, nausea, diabetes, loss of appetite, dysentery and other gastrointestinal tract problems to sexual transmitted diseases and diabetes mellitus among others. *V.amygdalina* and its relatives are good sources of sesquiterpenes lactones. Compounds have been isolated and identified from the plant leaves including vernolide, vernodalin and hydroxyvernolide (Kraft *et al*., 2003). The occurrences of steroidal saponins, terpenes, coumarins, tannins, alkaloids and flavonoids have also been reported (Erasto *et al.,* 2006). The phytochemicals present in the plant have been suggested to play a role in its biological activity (Erasto *et al*., 2006).

Therefore, this study was primarily undertaken to confirm the acclaimed anti plasmodium properties of *Vernonian amygdalina* based on their ethno medicinal use in Ethiopia.

Figure 1.8: Vernonia amygdalina Del. (Captured, 2014)

1.10. Statement of the Problem

Malaria remains one of the most serious world health problems and the major cause of morbidity and mortality in the endemic areas (WHO, 2008). Malaria is highly endemic in Ethiopia, where, according to the WHO, 67 per cent of the populations are at risk of malaria transmission. In 2010 in Ethiopia, there were 4,068,764 probable and confirmed cases of malaria (World Malaria Report, 2011). Malaria is also a significant burden on child mortality, accounting for 2 per cent of all under-five deaths in Ethiopia in 2010 (Malaria Consortium, 2013). Annually, half a million microscopically confirmed cases of malaria are reported to the Federal Ministry of Health (FMOH) from basic health services. According to the 2011/2012 report of the FMOH, malaria was the leading cause of outpatient visit accounting for 12% of cases (Adugna,2014; Guthman *et al*., 2007). Malaria control requires an integrate approach, including prevention (primarily vector control) and prompt treatment with effective antimalarial. However, the increasing prevalence of drug-resistant strains of *Plasmodium falciparum*, its most widespread etiological agent, to standard antimalarial drugs necessitates a continual effort to search

for new antimalarial drugs with new modes of action, used alone or in combination (wongsrichanalai *et al*., 2002).

Since, *Plasmodium* species have become resistant to the majority of today's available anti-malarial drugs and the infection is one of the most serious causes of morbidity and mortality globally, particularly in sub-Saharan Africa (SSA), there should be a need to develop new and highly efficient anti-plasmodia medicines that can be easily affordable by those poor living in the malarious endemic tropical countries. Recently, there is a focus on traditional medicinal plants and other natural products having high anti-malarial activities. In the search for new, safe and effective anti-malarial drugs, screening of extracts from plants used in traditional medicine was indicated. However several herbal plants show promising anti-malaria activities, research on their active ingredients identification and evaluation on anti-malarial activities not yet satisfied. Therefore crude extraction of *Vernonia amygdalina* extract is highly important because the extract obtained from such plants will be good components for the possible development of new, safe and effective anti-malarial (Abosi and Raseroka, 2003).

1.11. Significance of the Study

In developing countries, majority of people almost exclusively use traditional medicines in treating all sorts of diseases, including malaria. Although a range of medicinal plants with antimalarial properties have been widely used by traditional healers, therapeutic potentials of some of these medicinal plants have not been scientifically evaluated (Havagiray *et al*., 2004). Among these plants, the leaves extract of *Vernonia amygdalina* acclaimed folklore use as an anti-malarial agent; however, there is a dearth of scientific evidence to substantiate such traditional claim. Hence, this study may evaluate the antimalarial activity of this plant with a view to validate its acclaimed use by the traditional practitioners. Besides, the finding of this study might provide a clue about the

possible mechanisms of antimalarial action and may serve as a lead compound for the development of new antimalarial drugs. Furthermore, it might serve as baseline information for scientific community to further investigate the plant *Vernonia amygdalina* by initiating advanced studies on molecular mechanisms with identification the specific agent responsible for the antimalarial effect of the extract.

1.12. Research Hypothesis

Vernonia amygdalina possess chemo-suppressive, curative and prophylactic features. Thecrude Hydrometanol and aqueous extracts of the plants have anti-malarial activities that support their traditional uses in the management of parasitic diseases.

2. OBJECTIVES

2.1. General objective

- To evaluate the antimalarial activity of *Vernonia amygdalina* leaf extracts against *Plasmodium berghei* in Swiss albino mice.

2.2. Specific objectives

- To assess acute oral toxicity of crude extracts of the plant material in mice.
- To determine the extent of parasitic suppression by the leaf extracts of *Vernonia amygdalina* in *Plasmodium berghei* infected mice
- To determine the curative effects of the leaf extract of *Vernonia amygdalina* in *Plasmodium berghei* infected mice.
- Evaluate the prophylactic effects of the leaf extract of *Vernonia amygdalina* against *Plasmodium berghei* infected mice

3. MATERIALS AND METHODS

3.1. Plant Material Collection and Extraction

The fresh leaves of *Vernonia amgdalina* plant (Figue 1.9) were collected based on Ethno-botanical description and with the help of local traditional healers around Bossa kito area, Jimma Town, Oromiya National Regional State, 365 km away from Addis Ababa. December 2014. The plants were identified and authenticated by Dr. Mirutse Giday, botanist, at Addis Ababa University, Institute of Pathobiology and the specimen was deposited in the National Herbarium of Addis Ababa University, Addis Ababa, Ethiopia with voucher number (Temesgen 01/14). The Fresh leaves of *Vernonia amgdalina* were cleaned from extraneous materials, air-dried under shade at room temperature then cut and reduced to appropriate size by grinding with an electric mill (IEC, 158VDE0660, Germany) in Biochemistry Laboratory, school of Medical science, Addis Ababa University for two weeks. The powdered plant material was weighed (1kgs) using SCIENTECH Mode No SL 3100D Rev-c accuracy class (II) (Debella, 2002).

3.2. Aqueous Extract Preparation

Powdered materials (500 g) obtained from the *Vernonia amgdalina* were prepared by cold maceration technique; soaking the plant powder in 1**:**8 (w/v) of distilled waterfor 24 hours with intermittent agitation by Orbital shaker DS-500. Then, the supernatant part of agitated materials was separated from the un-dissolved portion of the plant (marc). The supernatant portion filtered with 0.1mm^2mesh gauze and 15 cm Whatman grade1 filter paper. The filtrate of the *Vernonia amgdalina* was lyophilized with lyophilizer (CHRIST, 3660 sterode/harz/, France)at lower temperature (-41to - 51^oC) and lower pressure (133 x 10^{-3}mbar) to form crude extract.The extracts were stored in screw cap glass bottle at -20^oC until they were applied in the experiment (Debella, 2002).

3.3. Methanol Extract Preparation

Powdered *Vernonia amgdalina* (500g) was macerated with 80 % methanol for 72 hours with intermittent agitation by Orbital shaker at 120 rpm. The supernatant part of agitated material filtered with 15 cm Whatman grade1 filter paper two times. The filtrate of *Vernonia amgdalina* was then concentrated using Rotary evaporator (BÜCHI R-250,Switzerland) at 40^{o}c to remove methanol and further dried using in a lyophilizer (CHRIST, 3660Osterode/harz/, France) to remove water and the extract were kept at −20°C until used (Debella, 2002).

3.4.3. Phytochemical Analysis

In the quest to study the distribution of secondary metabolites in the different extracts obtained, a phytochemical analysis was conducted (Annex II) to test for either the presence or absence of them. Thus, phytochemical tests for the presence of alkaloids, sterols, coumarins, flavonoids, tannins, reducing sugars as general test for glycosides, saponin, and glycosides were carried out. Tests were carried out on the aqueous and the methanolic extracts. The standard methods employed for the tests are as described in literature (Sofowora 1993; Evans 2002).

3.4.4. Experimental Animals Preparation

The animals employed for this study were adult male and female 8-12 weeks of Albino Swiss mice (25- 35g). The mice were obtained from the Animal Facility Centre, Faculty of Life Sciences of Addis Ababa University and purchased from Ethiopian Public health institute (EPHI), Addis Ababa, Ethiopia. The mice were acclimatized to a laboratory condition for a week before the commencement of the experiment. Finally, all mice were housed in plastic cages under 25±2^{0}C. They had unrestricted access to a standard pellet

diet and water *ad libitum*. The animals were maintained under 12 hrs light-dark cycle throughout the duration of the study.

For *invivo* anti-malarial assays of plant extracts, the mouse-infective chloroquine sensitive strain of *P. berghei* (ANKA) obtained from Ethiopian Public health institute (EPHI) was kept alive by continuous intraperitoneal (i.p.) serial blood passage from mice to mice on a weekly basein Aklilu Lemma Institute of Pathobiology, Addis Ababa University.

3.5. *In Vivo* Acute Toxicity Tests

Acute oral toxicity of hydromethanolic and aqueous leaf extract of *Vernonia amygdalina* was evaluated. Healthy female Swiss albino mice aged of 8-12 weeks maintained under standard laboratory conditions were used for acute toxicity test according to Organization for Economic Cooperation and Development (OECD) guidelines No 425 (OECD, 2001).

The limit test dose of 2000 mg/kg was orally administered sequentially to five female mice and mortality was observed in all mice. According the OECD up and down procedure guideline, the dose was tapered to 3000 mg/kg and administered and mice were observed for 24 hrs and then for 14 days. They were also observed for toxicity signs like changes in physical appearance, behavioral change, motor and feeding activities hair erection, lacrimation, reduction in motor .And other signs of acute toxicity and mortality after administration of the dose were also observed.

3.6. *In vivo* Evaluation of the Anti-malarial Activity of Plant Extracts

3.6.1. Activity on early infection (four-day suppressivetest)

In screening of the plant extracts, the standard four-day suppressive method was used (Petere, 1967).45 Male Swiss albino mice weighing 25-35 were infected with $1x10^7$ infected RBC (infected with *P. berghei*) and randomly divided into respective groups of five mice per cage. The infected mice were randomly divided into three test groups and two controlled groups for each extract Chloroquine as (CQ) a standard drug and distilled water (dH_2O) as a normal control (for aqueous extract treated group) and 20% DEMSO (for hydromethanol treated group). The test extracts were prepared in three different dosed; (200mg, 400mg, 600) mg/kg, Chloroquine at (10 mg/kg) in a volume of 0.2 ml and vehicles at 0.2 ml/mouse. Each extract was administered as a single dose per day. All the extracts and the drug were given through intragastric route using standard intragastric tubeto ensure safe ingestion of the extracts and the drug (OECD, 2001). Treatment were started after 3 hours of infection on day 0 and continued daily for four days (i.e. from day 0 to day 3). On the fifth day (Day-4), 24 h after the last dose (i.e. 96 h post-infection), blood sample were collected from tail snip of each mouse (Kalra *et al.,* 2006).

The smears were applied on microscope slides (76 × 26 mm) (Menzel-Glaser, Germany), fixed with absolute methanol for 15 min and stained with 15% Geimsa stain at pH 7.2 for 15 min. The stained slides were then washed gently using distilled water and air dried at room temperature. Then, each stained slide was examined under Olympus microscope (CHK2-F-GS, Taiwan) with an oil immersion objective of 100x magnification power to evaluate the percent suppression of each extract with respect to the control groups. The microscope had an Ehrlich's eyepiece showing about 100 red blood cells per field (Dikasso *et al*., 2006). The parasitaemia level was determined by counting minimum of five fields per slide with 100 RBC in random field of the microscope. Percent

parasitaemia and percentage of suppression was calculated using formula described in all model (Adhroey *et al*., 2011).

$$\% \text{ Parasitaemia} = \frac{\text{Number of Parasitized RBC}}{\text{Total RBC counted}} \times 100\ \%$$

$$\% \text{ Suppression} = \frac{(\text{Parasitaemia in normal control}) - (\text{parasitaemia in treated group})}{\text{Parasitaemia in normal control}} \times 100\ \%$$

3.6.1.1. Determination of Packed cell Volume

The packed cell volume (PCV) of each mouse was measured (WHO, 1980) before infection and on day 4 after infection. For this purpose, blood was collected from tail of each mouse in heparinized microhaematocrit capillary tubes up to 3/4thof their length. The tubes were sealed by crystal seal and placed in a microhematocrit centrifuge (Hettich haematokrit) with the sealed ends out wards. The blood was centrifuged at 12,000 rpm for 5 minutes. Then the tubes were taken out from the centrifuge and the result was read using microhaematocrit reader.Using the modified Wintrobe's Method (Munzer *et al*., 1980).The volume of the total blood and the volume of erythrocytes were measured and PCV was calculated as;

$$\text{PCV} = \frac{\text{Volume of erythrocytes in a given volume of blood}}{\text{Total blood volume}} \times 100$$

3.6.1.2. Determination of Mean Survival Time

Mortality was monitored daily and the number of days from the time of inoculation of the parasite up to death was recorded for each mouse in the treatment and control groups throughout the follow up period. The mean survival time (MST) for each group was calculated as the following formula described in (Mengistie *et al*., 2012).

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

3.6.1.3. Determination of Body Weight andTemperatureChange

The body weight of each mice in all the groups was measured before infection (day 0) and on day-4 in case of treatment, in the same fashion in case of acute toxicity, it was measured before and after the different doses were given by a sensitive digital weighing balance (Mettler Toledo, Switzerland). Rectal temperature (Electronic thermo meter model CR-623, china) was also measured by a digital thermometer before infection, four hours after infection and then daily (Satayavivad *et al*., 1998).

3.6.2. Activity on Established Infection (Curative or Rane'stest)

Evaluation of curative anti-malarial potential of the extract was done using a method described Ryley and Peters (Ryley and Peters, 1970) and modified by Carvalho *et al*., (1990). To assess the curative potential of the hydrometanol and aqueous extracts on established *P. berghei* infection, 45 Swiss albino male mice were each inoculated with around 1×10^7 *P. berghei*.The mice were injected by intra peritoneal on the first day and infection was allowed to be established for 72 hrs. The animals were then randomly divided into respetive groups of five mice each group. Seventy-two hours later, one of the groups of mice was orally administered with the crude hydroalcoholic and the other group received aqueous extract of *Vernonia amygdalina* leaves (200, 400, and 600) mg/kg each, for the control chloroquine (10 mg/kg) and the vehicle (0.2ml) (and 20% DEMSO in case of hydrometanol extract) was given. The groups were given the extract/control solution once daily for 4 days. Thin blood films stained with Giemsa were prepared from tail blood of each mouse on day 3 and day 7 to monitor the parasitaemia level. The animals were observed till the 30^{th} days. Rectal temperature was measured daily using digital rectal thermometer, while PCV and body weight were also measured at day 0(day 3) and day 4(day 7). Each mouse was followed after treatment and till their death and survival time was also recorded.

3.6.3. Activity on Residual Infection (Repository Test)

Evaluation of prophylactic potential of theextract was done using Peters methods with slight modification (Peters, 1967). 45 male Swiss albino mice were divided into respective groups with five mice per group (cage). Mice in the test groups were administered with the hydrometanol and aqueous extract of *Vernonia amygdalina* leaves (200,400, 600) mg/kg orally at a single daily dose for four daysprior to infection. Mice in the positive and normal control groups were treated with pyrimethamine (1.2 mg/kg), and vehicle (2mL) (20% DEMSO in the case of hydrometanol extract), respectively. On day-4, a standard inoculum of *Plasmodium berghei* infected-erythrocytes was administered by intraperitoneal route to each mouse. After 72 hr, thin blood films stained with Giemsa were prepared from tail blood of each mouse on day 8 to monitor the parasitaemia level. The animals were observed till the 30^{th} days. Rectal temperature was also measured by a digital thermometer before infection, four hours after infection and then daily. The Packed Cell Volume (PCV) was measured to predict the effectiveness of the test extracts.This test was done from each mouse just before infection and on the 8^{th} day of post infection and body weight were also measured at day 5 and day 8 and all parameter calculated based on the formula described above under suppression test. Each mouse followed after treatment till day 30 and their death and survival time also recorded.

3.7. Statistical Analyses

Data were analyzed using window software; Microsoft office Exce2013 Followed by Graphpad prsim v6.0.02. (GraphPad Software, SanDiego, CA, USA) For the antimalarial efficacy tests, statistical analysis was undertaken usingone way analysis of variance (ANOVA) followed by Tukey's multiple comparison to compare the levels of parasitaemia and survival times of the *P. berghei* infected mice between the control and extract treated groups at a fixed time. In addition, Student's paired t-test was also carried out to determine percent change of PCV and body weight of *P. berghei* infected mice

between the comparison days. The results were presented as the Mean±SEM (Standard Error of the Mean) and Mean ± SD (Standard deviation) and statistical significance was considered at a 95% confidence interval ($P<0.05$).

3.8. Ethical Consideration

After the Department Ethicsand Research Committee of the Department of Medical Biochemistry at Addis Ababa University approved the study topic, the researcher has taken letter of clearance with a protocol number MSc thesis 13/14 to be submitted to the concerned officials.

4. Result

4.1 Percent Extract Yield of the Plant Material

Percent yields of the leaves of *Vernonia amygdalina* aqueous and hydromethanol crude extracts are indicated in table 4.1. The aqueous and the hydrmetanolic extracts yielded 9.06 %(45.3g) and 12% (60g) respectively in a 1**:**8 w/v ratios of the plant powder per solvent reagents. The physical nature of each extracts was different. Whereas, the methanol extract was dried powder.

Table 4.1: Percentage yield extracts from the leaves of Vernonia amgdalina crude aqueous and hydrometanol extracts.

Plant species	**Solvent**	**Weight of powder (g)**	**Volume of solvent (mL)**	**Yield (g)**	**%of yield**
Vernonia amygdalina	**Aqueous**	500	4000	45.3	**9.06**
	80% Methanol	500	4000	60	**12**

4.2. Acute toxicity test for the plant materials

Invivo studies on the effect of hydro-alcoholic and aqueous extract from *Vernonia amygdalina,* the toxicological changes in tested animals were carried out by the present study. Before the experiment was commenced the mice were fasted overnight (OECD, 2001).The value of the *Vernonia amygdalina* hydrometanol and aqueous extract for acute toxicity given were 2000 and 3000mg/kg body weight. At the level of 2000 $mgkg^{-1}$bw, no

death and other toxicological changes were observed. At the level of 3000 rigidity and sleepy activities were observed but no death was recorded (Table 4.2.).

Table4.2: Acute toxicity test for the aqueous and hydrometanol extracts.

Signsof toxicity	**Aqueous extract**		**Hydrometanol extract**	
	Doses		Doses	
	2000mg/kg	3000mg/kg	2000mg/kg	3000mg/kg
Hyperactivity	**0**	**0**	**0**	**0**
Rigidity	**0**	++	**0**	+
Irritability	**0**	**0**	**0**	**0**
Jumping	**0**	**0**	**0**	**0**
Sleep	**0**	+	**0**	+
Sedation	**0**	**0**	**0**	**0**
Abnormal secretion	**0**	**0**	**0**	**0**
Death	**0**	**0**	**0**	**0**

+ = Signs of toxicity present; 0 = Signs of toxicity not detected

4.3. Phytochemical Screening

Phytochemical screening revealed the presence of various phytochemicals in the extracts (Table 4.3). Both Aqueous and hydromethanol leaf extracts of *V. amygdalina* were found to contain almost all the phytochemicals tested. Of all the extracts, saponins, tannins, phenol, terpenes, Flavonoids, glycosides and alkaloids were found in the extract.

Table 4.3: Phytochemical Screening of Vernonia amygdalina of Hydromethanol and Aqueous leaves Extract.

Bioactive agent	**Indication**	
	Methanol Extracts	Aqueous Extracts
Flavonoids	+	++
Saponins	++	+
Tannins	++	+
Alkaloids	++	+
Glycosides	+	+
Steroids	+	+
Terpenoids	++	+
Phenol	++	-

+ = phytochemical present; ++= abundantly present; - = phytochemical not detected

4.4. *Invivo* Evaluation of the Antimalarial Activity of Plant Extracts

4.4.1. Anti-plasmodia effect on early infection (Four day suppressive test)

Antimalarial suppressive test results of Aqueous and Hydromethanol extracts of *Vernonia amygdalina* against *P. berghei*in Swiss albino mice are summarized in (Tables 4). Both aqueous and methanol extract of the plant showed dose dependent chemo suppressive effect at all doses used. However, it did not clear the parasite completely on day four. Whereas, the positive control group (treated with 10mg/kg CQ) had very less detectable parasitaemia on D4 of post infection.

The crude aqueous extracts at dose (200,400, 600) mg/kg and CQ 10mg/kg showed percent parasitaemia levels of 20.82±4.68, 17.02±2.16, 12.44±1.52% and 7.04±0.59, respectively.

The highest suppression of parasitaemia was observed at the dose of 600mg/kg (68.8%, *p<0.0001*) followed by 400(56.5%, *p*<0.001) and 200(47.8%, *p<0.01*) while the CQ showed (82.9, *p<0.0001*) as compared to normal control (Table 4.4).

Table 4.4: Four-day suppressive test antimalarial activity and survival mean time of aqueous and hydromethanol Vernonia amygdalina leaves extract.

Treatment	**Dose**	**% paracitemia**	**% of supper -ssion**	***p*-value**	**Survival time(days)**	***p*-value**
H_2O(NC)	0.2 mL/kg	42.82±5.59	-		8±0.70	-
Aqueous extract of V. amgdalina	200 mg/kg	20.82±4.68 a^2	47.8	0.0018	13.80±1.16 a^1b^4	0.0138
	400 mg/kg	17.02±2.16 a^3	56.5	0.0002	15.80±0.86 a^3b^4	0.0003
	600 mg/kg	12.44±1.52 a^4	68.8	0.0001	20.20±1.6 $a^4b^3c^2$	0.0001
CQ	10 mg/kg	7.04±0.59 a^4	82.9	0.0001	29.00±0.45 a^4	0.0001
20%DMSO	0.2mL/kg	42.10±5.69	-	-	8.2±0.37	-
Hydroalcohol extract of V. amgdalina	200 mg/kg	22.72±2.99 a^2	42.7	0.0081	13.00±0.84 b^4e^2	0.0709
	400 mg/kg	19.26±1.39 a^2	51.4	0.0011	14.6±0.93 $a^2b^4e^1$	0.0047
	600 mg/kg	12.96±1.35 a^4	69.2	0.0001	20.8±1.86$a^4b^4d^2C^2$	0.0001
CQ	10 mg/kg	7.04±0.58 a^4	82.9	0.0001	29.00±0.45 a^4	0.0001

Values are presented as Mean±SEM (Standard Error Mean*). Statistical significance denoted by $^1p<0.05$, $^2p<0.01$ and $^3p<0.001$, $^4p<0.0001$a=compared to NC, b= to CQ10mg/kg, c=to 200mg/kg d=to 400mg/kg, e=to 600mg/kg. CQ: Chloroquinediphosphate dH2O: Distilled water NC: Negative Control*

The Percent parasitaemia of hydromethanol extract at the doses of (200, 400,600) mg/kg and CQ 10mg/kg were 22.72±2.99, 19.26±1.39, 13.96±1.35 % and7.04±0.58 %, respectively. Whereas, the normal control was 42.10±5.69. Moreover, it exhibited a significant suppression at the doses of (200,400, 600) mg/kg and CQ10mg/kg ranged 42.7 %($p<0.01$), 51.4 %($p<0.01$), 69.2 %($p<0.0001$) and (82.9, $p<0.0001$) as compared to normal control group, respectively (Table 4.4.).

Analysis of variance of the mean survival time of aqueous extract treated groups showed statistically significant survival time difference at doses of (200,400 600) mg/kgand CQ10mg/kg and lived 13.80±1.16($p<0.05$), 15.80±0.86($p<0.0001$), 20.80±1.6 ($p<0.0001$) and 29.00±0.45 ($p<0.0001$) , respectively, as compared to normal control. At doses of (200,400 600) mg/kgand CQ10mg/kgthe hydromethanol extract also showed 13.00±0.84, 14.6±0.93 ($p<0.001$), 19.8±1.86 ($p<0.0001$) and 29.00±0.45 ($p<0.0001$) days of survival time respectively, as compared to normal control (table4.4).

4.4.2. Effect of *Vernonia amygdalina* extract on Body weight and PCV determination on 4 day suppression test

In both the control and extract treated experimental mice, loss of body weight between D0 and D4 was observed. The paired t-test was conducted to compare the weights of mice before and after the treatment with both extracts and showed a statistically non-significant ($P>0.05$) change in both the respective normal control and extract treated groups although there was loss of body weight between the days. In addition, analysis of variance in comparison between the control and extract treated *P. berghei* infected mice resulted in statistically significant ($p<0.05$) difference in their body weight.

However, the methanol extract did not show significance differences as compared to control group (Table 4.5).

Table 4.5: Effect of aqueous and hydroametanol leaf extracts of Vernonia amygdalina on the body weights of P. berghei infected Swiss albino mice.

Treatment	Dose	Body weight		p-value	% Change	P-value
		Pre (D0)	Post (D4)			
dH_2O(NC)	0.2 ml/kg	31.8±3.71	29.12±3.86	0.0016	-2.76 ± 0.79	-
Aqueous extract Of *V.amgdalina*	200 mg/kg	26.7±1.7	24.54±1.66	0.0001	-2.16±0.34b[1]	0.9421
	400 mg/kg	30.36±6.2	29.60±6.7	0.2604	-0.64±1.28	0.0994
	600 mg/kg	28.5±3.1	28.14±3.6	0.3023	-0.16±0.38a[1]	0.0296
CQ	10mg/kg	35.04±1.7	35.23±3.89	0.8026	0.28± 2.35a[2]	0.0089
20%DMSO(NC)	0.2ml/kg	31.6 ± 3.5	29.1± 3.87	0.0010	-2.56± 0.79	-
Hydromethanol extractof *V.amgdalina*	200 mg/kg	28.04± 2.3	26.7± 1.45	0.0845	-2.16±0.336	0.5302
	400 mg/kg	34.2± 2.4	33.32±3.11	0.3355	-0.64±1.28	0.2851
	600 mg/kg	28.00±2.2	26.66± 1.4	0.0944	-0.16± 1.29	0.5063
CQ	10 mg/kg	35.04±1.7	35.23±3.89	0.8026	0.28± 2.35a[2]	0.0020

Values are presented as Mean±SD (Standard Deviation*). Statistical significance as compared to treatment control denoted by [1]p<0.05, [2]p<0.01, a=compared to NC b= to CQ, CQ: Chloroquinediphosphate, dH2O: Distilled water NC: Normal ControlD0 = a day infection initiated, D4 = 5th day of infection t test*

The crude aqueous extract of *Vernonia amygdalina* showed PCV reduction. Only CQ administered experimental *P. berghei* infected mice showed statistically significant (*p<0.05)* increment of PCV on D4 as compared to D0. The paired t-tests at all doses of the aqueous extract treated mice have also showed statistically non-significant (*p>0.05*) deviations (figure 4.1).

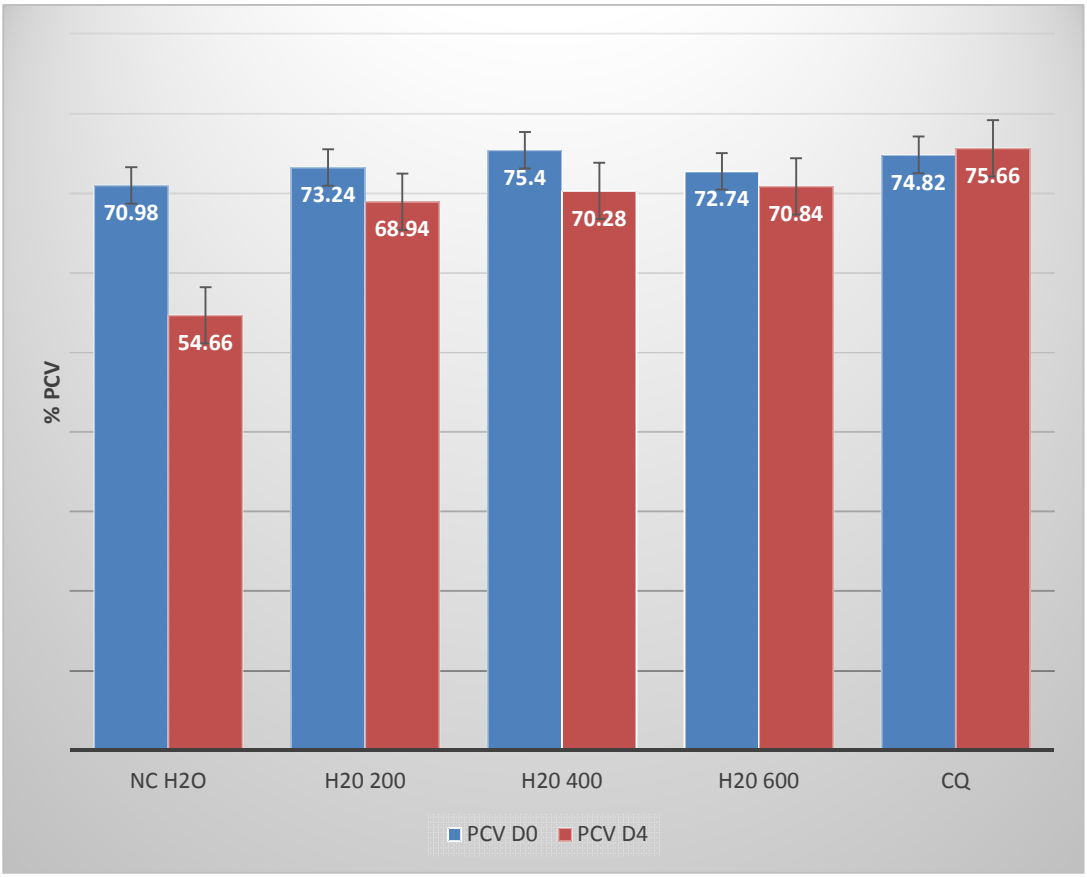

Figure 4.1: Effect of <u>Vernonia amygdalina</u> aqueous extract on PCV on 4-days suppression test.

Values are presented as Mean±SD (Standard Deviation) PCV: packed cell volume, D0: day 0, D: Day 4.

The hydromethanol extract also showed loss of PCV reduction due to parasitemia infection. However ,the current result showed that PCV increment amongCQ 10 mg/kg treated group significantly (p<0.05) as compared to normal control (figure 4.2).

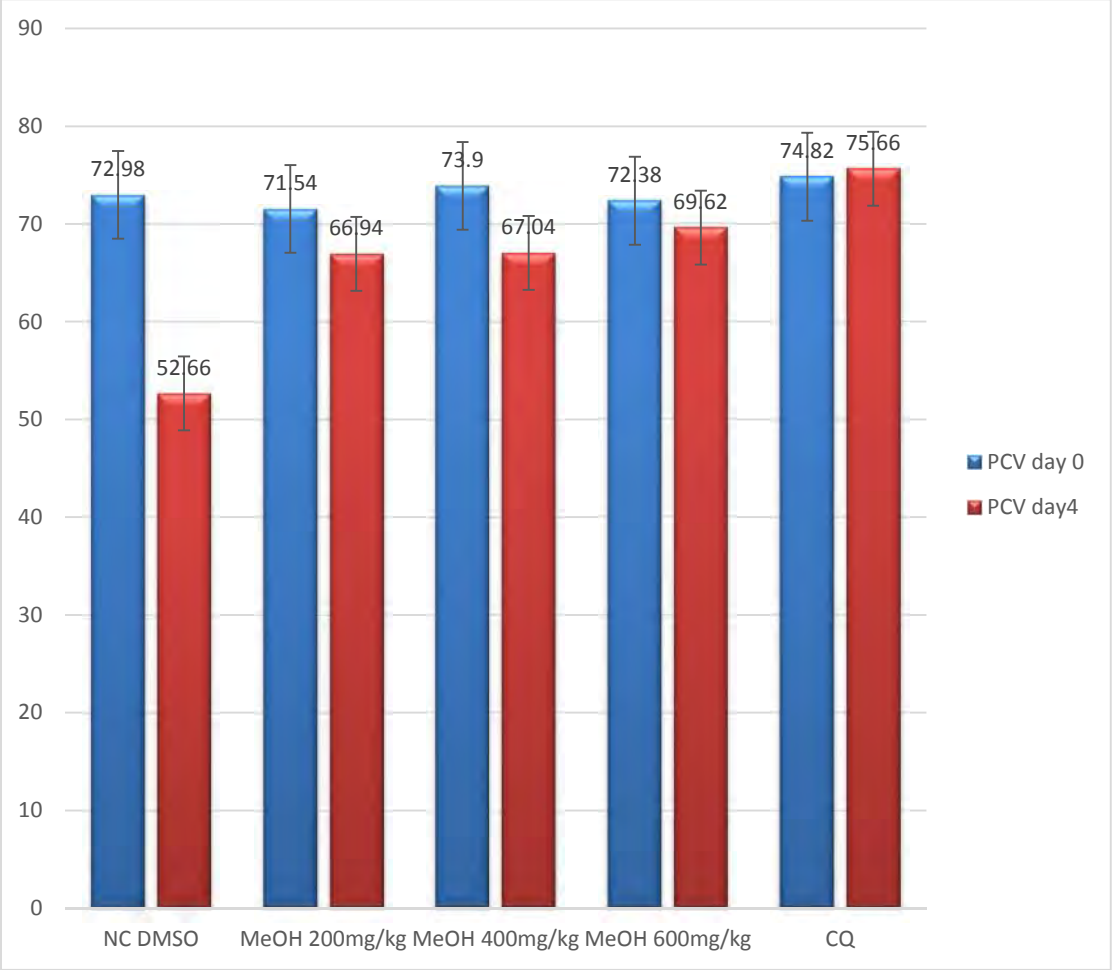

Figure 4.2: Effect of Vernonia amygdalina hydrometanol extract on PCV in 4-days suppression test.

Values are presented as Mean±SD (Standard Deviation) PCV: packed cell volume, D0: day 0, D: Day 4.

4.4.3. Effect of *Vernonia amygdalina* extract on body temperature on 4 day suppression test

In all test group, the hydromethanol and aqueous leaf extract did not cause significant (*P>0.05)* prevention of rectal temperature reduction of *P. berghei* infected mice.

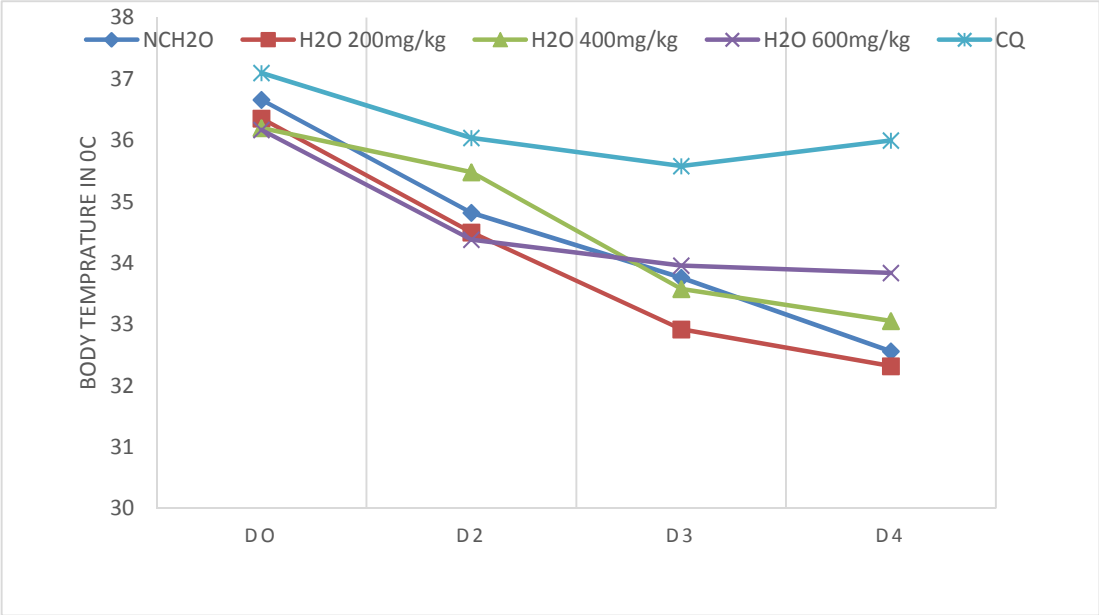

Figure 4.3: Effects of aqueous extract of V. amygdalina on rectal temperature against P. berghei infected mice on 4-days suppressive test.

The standard drug, chloroquine 10mg/kg, had showed significant (p<0.05) activity in prevention against rectal temperature reduction when compared to both extract treated and normal control (Figures 4.3 and 4.4).

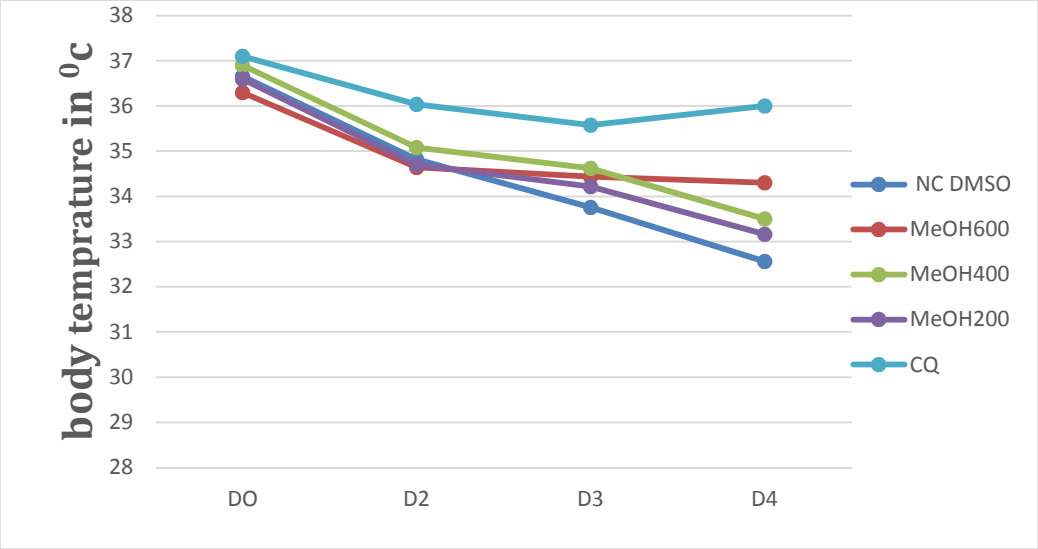

Figure 4.4: Effects of Hydmetanol extract of V. amygdalina on rectal temperature against P. berghei infected mice on 4-days suppressive test

4.5. Curative Effect of Aqueous and Hydromethanol Leaf Extract of *V.amygdalina* in *P. berghie* infected Swiss Albino Mice (Rene's test).

On established infection, both extract caused a significant reduction in comparison to normal control which showed a daily increment of parasitaemia (table4.6.).

On day 7 as compared to day 3, the mean percentage parasitaemia of the aqueous extract treated at the doses (200, 400, and 600) mg/kgand CQ 10mg/kgwere25.86±3.48%, 19.46±2.6%, 17.5±4.8% and 8.3±0.94%, respectively.

The aqueous extract at doses (200, 400 and 600) mg/kg and CQ10mg/kghad shown statistically significant ($p<0.05$) parasitemia suppression of 61.4, 64, 69.3%and 86.4%as compared to normal control group, respectively.

The crude Hydromethanol extract at dose (600 mg/kg) reduced average percent parasitaemia suppression at the seventh day (70.81%, $p<0.001$) as compared to the normal control group. The dose (400 mg/kg) of the extract reduced by (67%, $p< 0.05$) and 200mg/kg was (56.3%, $p< 0.05$) as compared to normal control group. CQ treated suppressed parasitaemia by 86.9% ($p< 0.001$) as compared to normal control group. From the investigation, it was observed that both extract exhibited a dose dependent curative effectwith a slight difference between the two solvent extract.

Table 4.6: Curative effect and survival time of aqueous and hydromethanol leaf extract of Vernonia amygdalina in P. berghei infected mice.

Treatment	Dose	% parasitemia		% suppres-sio (D7)	Survival time (days)	*p*-value
		Pre(D3)	Post(D7)			
dH2O(NC)	0.2mL	22.36±3.0	60.28±4.81	-	8.4±0.68b[4]	-
Aqueous extract of V.amgdalina	200 mg/kg	20.04±4.21	22.06±3.19a[1]	61.4	12.8±0.86	0.2731
	400 mg/kg	20.02±1.76	21.22±3.26a[1]	64	17.4±1.44a[3]	0.0004
	600 mg/kg	20.32±1.41	17.64±3.06a[1]	69.3	19.4±2.04a[4]	0.0001
CQ	10 mg/kg	21.56±2.80	8.304±0.94a[2]	86.4	29.4±0.4a[4]c[4]	0.0001
20%DMSO	0.2mL	21.86±2.75	61.66±5.55a[2]	-	8.6±0.25	-
Hydromethanol *extract of V.amgdalina*	200 mg/kg	19.94±2.22	25.86±3.48a[2]	56.34	11.2±0.37d[1]e[4]	0.8627
	400 mg/kg	21.12±1.79	19.46±2.59	67	18.0±0.84a[3]	0.0002
	600 mg/kg	21.38±1.76	17.50±4.87a[3]	70.81	21.2±21.20a[4]	0.0001
CQ	10 mg/kg	21.56±2.80	8.304±0.94a[3]	86.4	29.4±0.40a[4]c[3]	0.0001

Values are presented as Mean±SEM (Standard Error Mean*). Statistical significance denoted by [1]p<0.05, [2]p<0.01 and [3]p<0.001, [4]p<0.0001a=compared to NC, b= to CQ10mg/kg, c=to 200mg/kg d=to 400mg/kg, e=to 600mg/kg. CQ: Chloroquinediphosphate, dH2O: Distilled water NC: Negative Control, n=5; D3= Day three; D7= Day seven.*

The mean survival times exhibited at the doses (200, 400, and 600) mg/kg and CQ 10mg/kg were 12, 17, 19and 29 days for aqueous and 11, 18, 21and 29 days for hydromethanol extracts, respectively.

The MST of the 600 mg/kg concentration of both solvent extract treated group was significantly ($p< 0.0001$) prolong the survival time as compared to the normal control group. All mice in the negative control group were died within 9 days of the study. However, CQ treated survived almost (29.40±0.4, $p< 0.0001$) throughout the study duration.

4.5.1. Effects of extract on Body weight and PCV determination on curative test

In Curative test, the extract treated mice exhibited statistically significant (p<0.05) increments of body weight as compared to normal control group between day 3 and day 7(Table 4.7). CQ treated group showed significant (p<0.001) increments as compared to normal control.

Table 4.7: Curative Effect of aqueous and hydroalchol leaf extracts of Vernonia amygdalina on the body weights of P. berghei infected mice.

Treatment	Dose	Body weight		p-value	% Change	p-value
		Pre(D3)	Pre(D7)			
dH2O(NC)	*0.2mL*	33.92±2.08	31.22±0.91b[1]	0.0272	-2.70±1.78	-
Aqueous extract of V. amygdalina	*200 mg/kg*	32.24±1.40	29.86±3.15b[2]d[2]e[1]	0.0216	-2.32±3.75b[1]	>0.9999
	400 mg/kg	33.64±1.68	35.32±2.02c[2]	0.0077	1.68±0.76a[1]	0.0327
	600 mg/kg	33.20±2.73	34.58±1.03c[1]	0.0195	1.38±0.82a[1]	0.0582
CQ	*10 mg/kg*	34.96±1.51	36.20±0.49a[1]c[2]	0.0399	0.44±0.49	0.2710
20%DMSO (NC)	*0.2mL*	33.92±2.08	31.42±2.09	0.0168	-2.5±1.42	-
Hydromethanol *extract of V. aymgdalina*	*200 mg/kg*	32.40±2.82	33.96±3.32a[1]	0.0115	1.56±0.79a[2]	0.0604
	400 mg/kg	30.02±0.55	31.82±31.82	0.2575	1.8±3.05a[2]	0.0382
	600 /mg kg	33.18±2.28	34.52±4.08	0.2510	1.32±1.32a[2]	0.0931
CQ	*10 mg/kg*	34.96±1.51	36.20±0.49a[1]	0.0399	0.44±0.48a[2]	0.2710

Values are presented as Mean±SD (Standard Deviation). Statistical significance as compared to treatment control denoted by [1]p<0.05, [2]p<0.01 a=compared to NC b= to CQ10mg/kg, c=to200mg/kg d=400mg/kg e=600mg/kg CQ: Chloroquinediphosphate dH2O: Distilled water NC: Negative Control D3 = day three, D7 = day seven of infection

The effect of extract on packed cell volume is indicated in Figure4.1 and 4.2. statistically non-significant (*P>0.05)* difference were recorded in the mean PCV between on days (3)

and day(7) at all dose levels, but as compared to normal control group ,the extract showed statistically significantly *($p<0.05$)* PCV differencesin aqueous extract treated group.

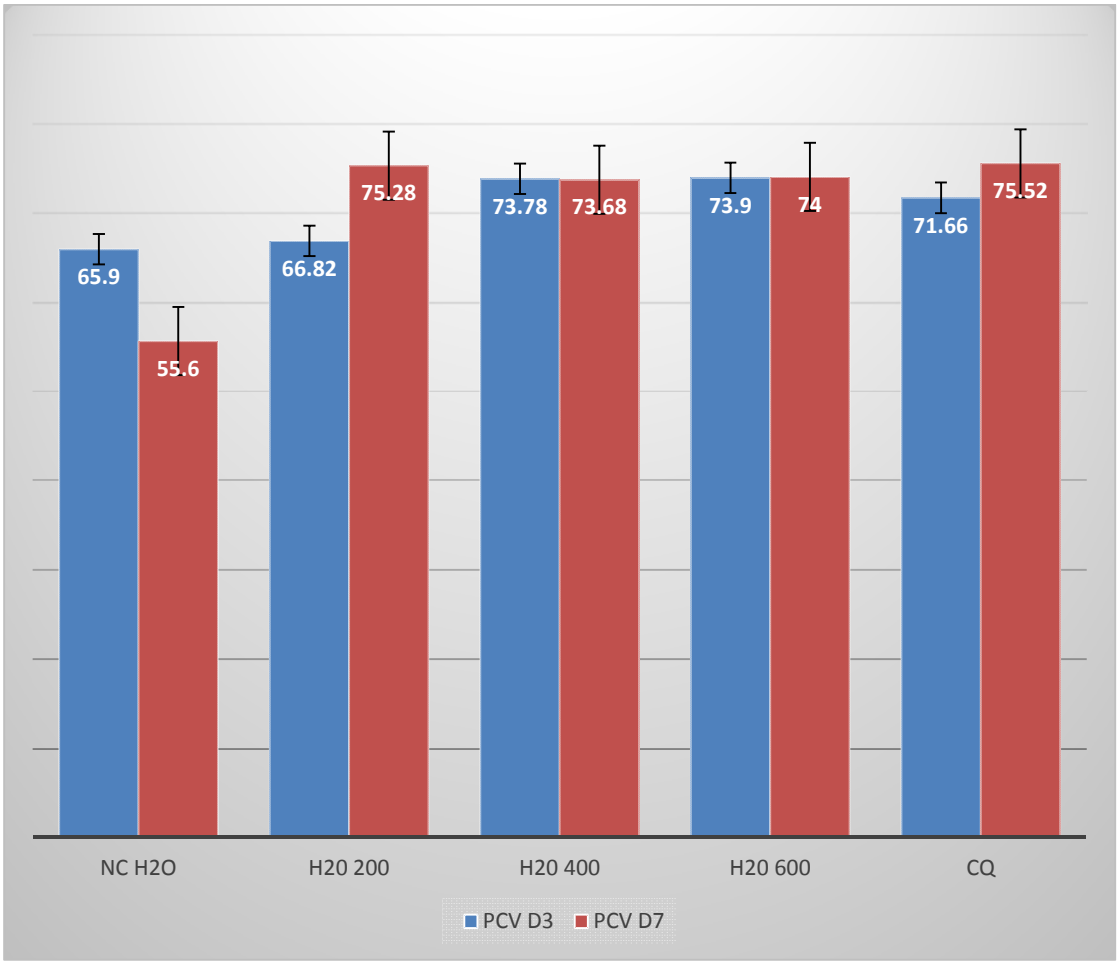

Figure 4.5: Effect of aqueous extract of Vernonia amygdalina on PCV in curative test.

Values are presented as SEM (Standard Mean ± Error Mean*) PCV: packed cell volume, D3: day 3, D7: Day 7.*

The analysis of Packed Cell Volume (PCV) on day 3 and day7 indicated that the hydrometanol extract also showed reduction in PCV (figure 4.5 and 4.6).

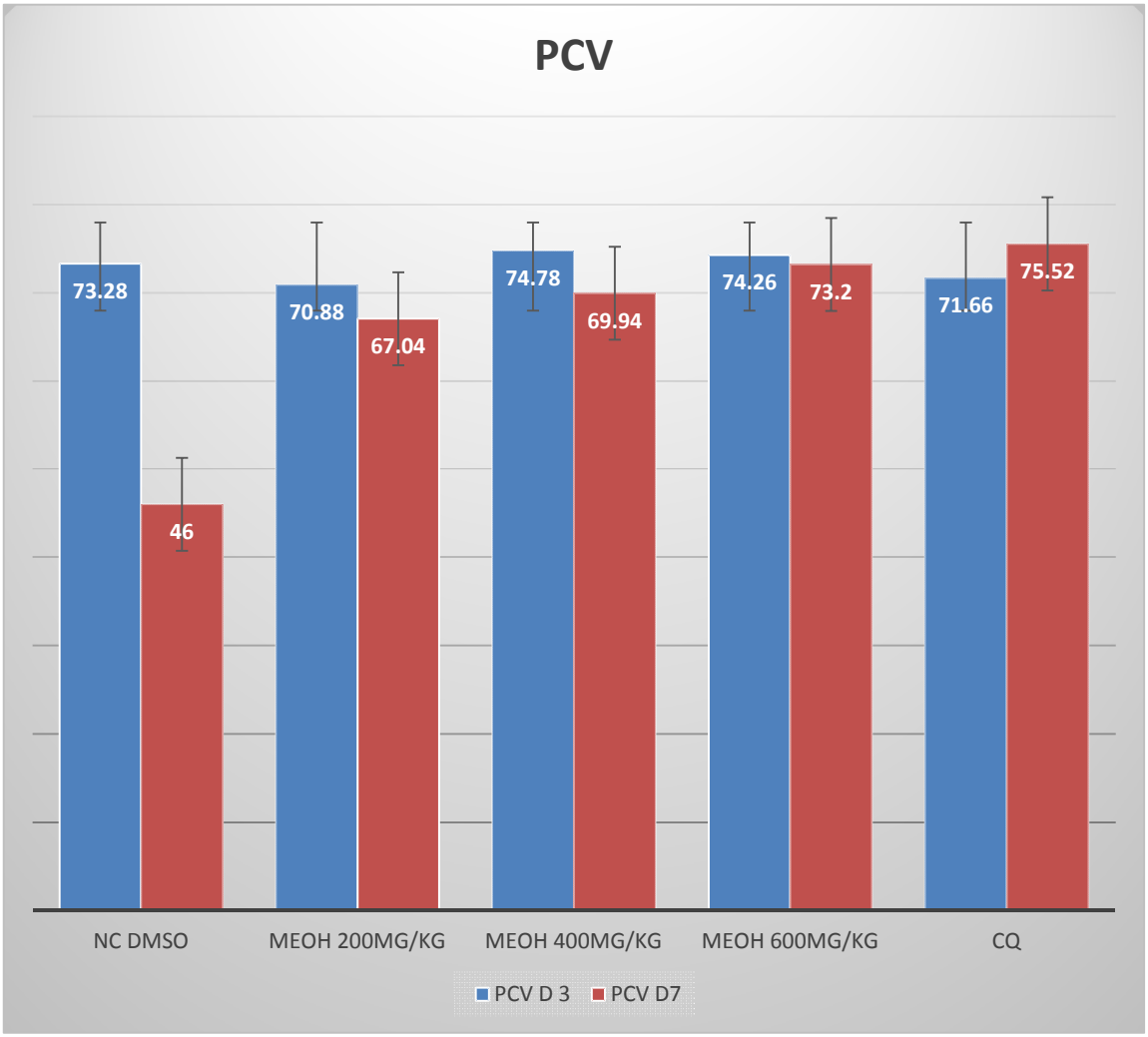

Figure 4.6: Effect of hydrometanol extract of Vernonia amygdalina on PCV in curative test.

Values are presented as Values are presented as Mean±SD (Standard Deviation*) PCV: packed cell volume, D3: day 0, D7: Day 7.*

4.5.2. Curative effect of *Vernonia amygdalina* extract on body temperature against *P. berghei* infected Swiss albino mice

Rectal temperature analysis indicated that the extract treated group observed decrement of in rectal temperature at all doses in compared to normal control (figure 4.7 and 4.7), but a significant ($p < 0.05$) increment were observed in CQ treated group.

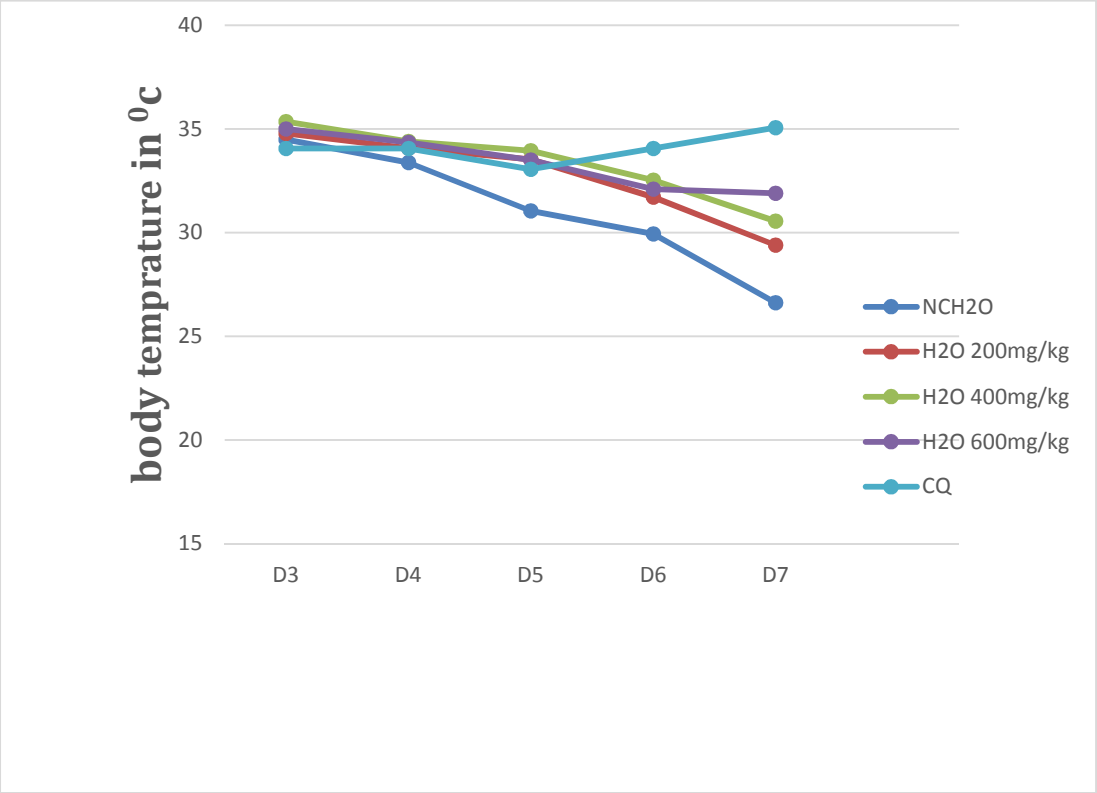

Figure 4.7: Curative Effect of aqueous leaf extracts of Vernonia amygdalina on rectal temperature of P. berghei infected Swiss albino mice.

Hydromethanol extract of *Vernonia amygdalina* alsodid not cause prevention of rectal temperature reduction of *P. berghei* infected mice.

The standard drug, CQ 10 mg/kg had showed significant (P<0.05) activity in prevention against rectal temperature reduction when compared to both extract treatedand normal control.

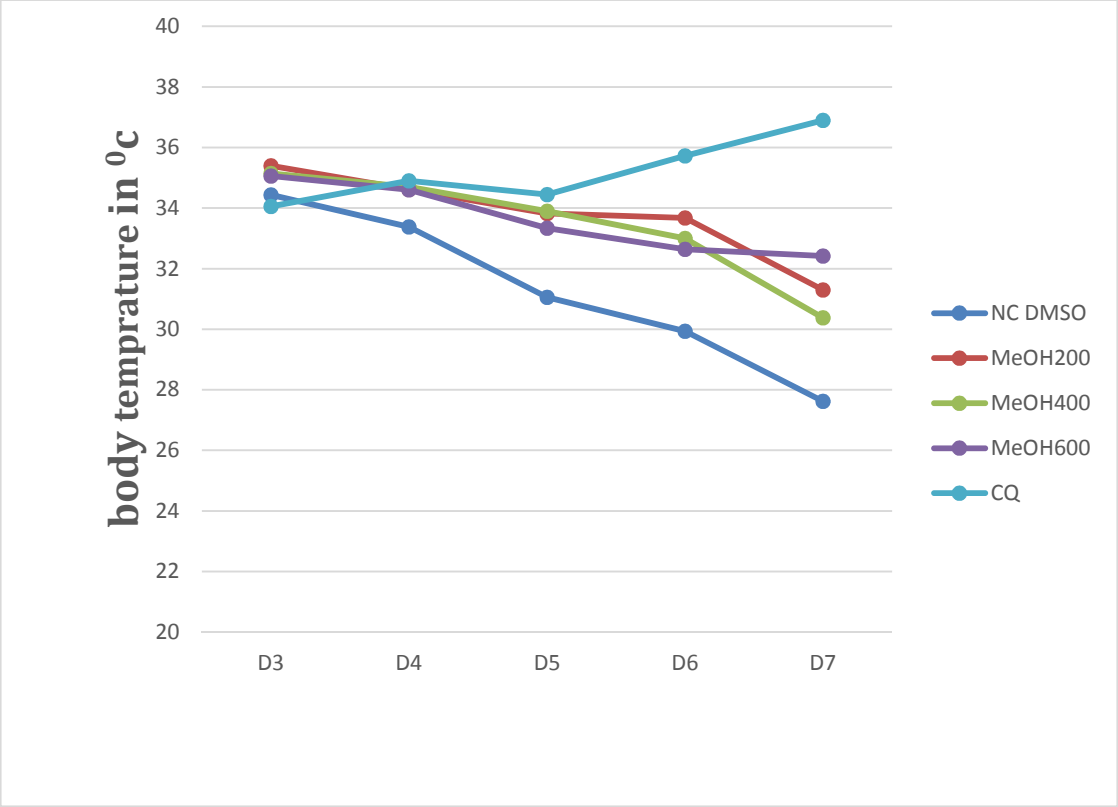

Figure 4.8: Curative Effect of hydrometanol extracts of V. amygdalina on rectal temperature of P. berghei infected Swiss albino mice.

4.5.3. Prophylactic effect of aqueous and hydromethanol leaf extract of *Vernonia amygdalina* in *P. berghei* infected mice (Anti-plasmodial effect on residual infection)

The prophylactic test wasalso conducted on the aqueous and hydromethanol extract of *Vernonia amygdalina* and exerted a dose dependent reduction of parasitaemia.

The aqueous extract exhibited different degrees of suppression at the doses (200,400 and600) mg/kg and Pyrimitamine 1.2mg/kg which were (60.7, p<0.001; 67, p<0.0001 70.3%, p<0.0001) and (87.3%, p<0.0001) respectively as compared to normal control group.

Table 4.8: Prophylactic effect and survival time of hydromethanol and aqueous extract of Vernonia amygdalina in P. berghei infected Swiss albino mice.

Treatment	Dose	% paracitemia On day 8	%suppr-ession On day 8	p-value	Survival time(days)	p-value
dH2O(NC)	0.2 mL/kg	52.14±9.210	-	-	$7.4\pm0.51b^4c^1d^4e^4$	-
Aqueous extract of *V.amygdalina*	200 mg/kg	$19.16\pm1.04a^3$	60.7	0.0003	$10.8\pm1.64b^4d^3e^4$	0.0173
	400 mg/kg	$16.28\pm2.80a^4$	67	0.0001	$15.8\pm0.58a^4b^4c^3e$	0.0001
	600 mg/kg	$14.40\pm0.76a^4$	70.3	0.0001	$18.60\pm0.51a^4b^4c^4$	0.0001
PM	10 mg/kg	$6.520\pm0.90a^4$	87.3	0.0001	$29.20\pm0.37a^4c^4d^4$	0.0001
20%DMSO (NC)	0.2mL/kg	50.60±8.47	-	-	7.6±0.51	-
Hydrometanol extract of *V. amygdalina*	200 mg/kg	$24.28\pm2.36a^2$	50.7	0.0062	$12.40\pm0.50b^4d^1e^4$	0.0002
	400 mg/kg	$17.06\pm2.76a^3$	65.97	0.0002	$15.60\pm0.81a^3b^4c^1e^1$	0.0001
	600 mg/kg	$14.52\pm2.34a^4$	71.5	0.0001	$18.80\pm1.0a^4b^4c^4d^1$	0.0001
PM	1.2 mg/kg	$6.520\pm0.90a^4$	87.3	0.0001	$29.20\pm0.37a^4c^4d^4$	0.0001

Values are presented as Mean±SEM (Standard Error Mean).*Statistical significance as compared to treatment control denoted by $^1P<0.05$, $^2P<0.01$ and $^3P<0.001$, $^4P<0.001$ a=compared to NC b= to, CQ10mg/kg, c=to 200 d=400, e=600. PM: Pyrimethamine dH2O: Distilled water NC: Negative Control, D8= Day eight.*

The crude Hydromethanol extract of the plant also showed a chemo-suppressions effect at the doses (200, 400 and 600) mg/kg and pyrimethamine 1.2 mg/kg resulted in (50.7,

p<0.01; 65.97, *p*<0.001; 71.5%,*p*<0.0001 and 87.3%,*p*<0.0001), respectivelyas compared to the normal control.

The mice treated with aqueous extract had the mean survival time at the doses of (200, 400 and 600) mg/kg and pyrimthaine 1.2 mg/kg groups with 10.8±1.64, 15.8±0.58, 18.60±0.59 and 29.2±0.37 days, respectively.Whereas, the hydromethanol extract treated group were, 12.4±0.5, 15.6±0.81, 18.8±1.02 and 29.0.37, respectively (table 4.8). The MST of the 400 and 600 mg/kg concentration of the extract treated group was significantly (*p*< 0.001) longer than that of normal control.

4.6.1. The Prophylactic effects of aqueous and hydromethanolic extract of *Vernonia amygdalina* on Body weight and packed cell volume (PCV) determination.

The effect of the extracts on the weights of the animals was also evaluate and observed that generally the weights of the normal control and all dose (200,400and 600) mg/kg levels were reduced except the standard treated group (Tables 4. 9).

Table 4.9: The effect of hydromethanoland aqueous extract of V. amgdalina on body weight of P. berghei infected mice in prophylactic test.

Treatment	Dose	Body weight		P value	% Change	p-value
		Pre (D5)	Post (D8)			
dH_2O(NC)	0.2*mL/Kg*	31.10±1.63	28.06±1.50a[1]	0.0003	-3.08±0.54d[1]	-
Aqueous extract Of *V.amygdalina*	200 *mg/kg*	31.82±4.76	28.98±4.96	0.0048	-1.04±2.53	0.5462
	400 *mg/kg*	25.92±4.19	25.18±3.63	0.2059	-0.74±1.10a[1]	0.3649
	600 *mg/kg*	31.18±2.27	30.38±2.03	0.3266	-0.68±1.81a[1]	0.3326
PM	10mg/kg	28.32±4.62	27.32±4.33	0.4254	0.60±1.51a[1]	0.0220
20%DMSO(NC)	0.2mL/kg	31.50±1.63	28.32±1.45a[1]	0.0012	-3.18±0.87e[1]	-
Hydromethanolic extract of *V.aymgdalina*	200 *mg/kg*	29.08±1.89	27.52±3.07	0.2051	-1.56±2.31	0.8002
	400 *mg/kg*	32.18±2.39	30.54±1.48	0.0937	-1.64±1.67	0.8398
	600 *mg/kg*	29.62±3.09	28.82±3.49	0.1227	-0.80±0.92a[1]	0.3432
PM	1.2*mg/kg*	28.32±4.62	27.32±4.33	0.4254	0.60±1.51a[1]	0.0170

Values are presented as SEM (Standard Mean ± Error Mean*). Statistical significance as compared to treatment control denoted by [1]P<0.05, a=compared to NC b= to, PM 1.2 mg/kg, c=to 200 d=400, e=600. PM: pyrimethamine, dH2O: Distilled water NC: Negative Control*

Both extract treated and the negative control mice had lost some of their body. Pyrimethamine had prevented body weight reduction significantly ($p<0.05$) when compared to normal control group. The extract had significant prevention at 600 mg/kg ($p<0.05$) in both solvent extract as compared to normal control (Table3.9).The weights increased gradually afterwards, with a slight decline for standard treatment group.

In prophylactic test, similar to the suppression and curative test, mice treated at doses (200,400 and 600) mg/kg did not show significant protection against the PCV reduction between day 5 and day 8 (Figure 4.9 and 4.10).

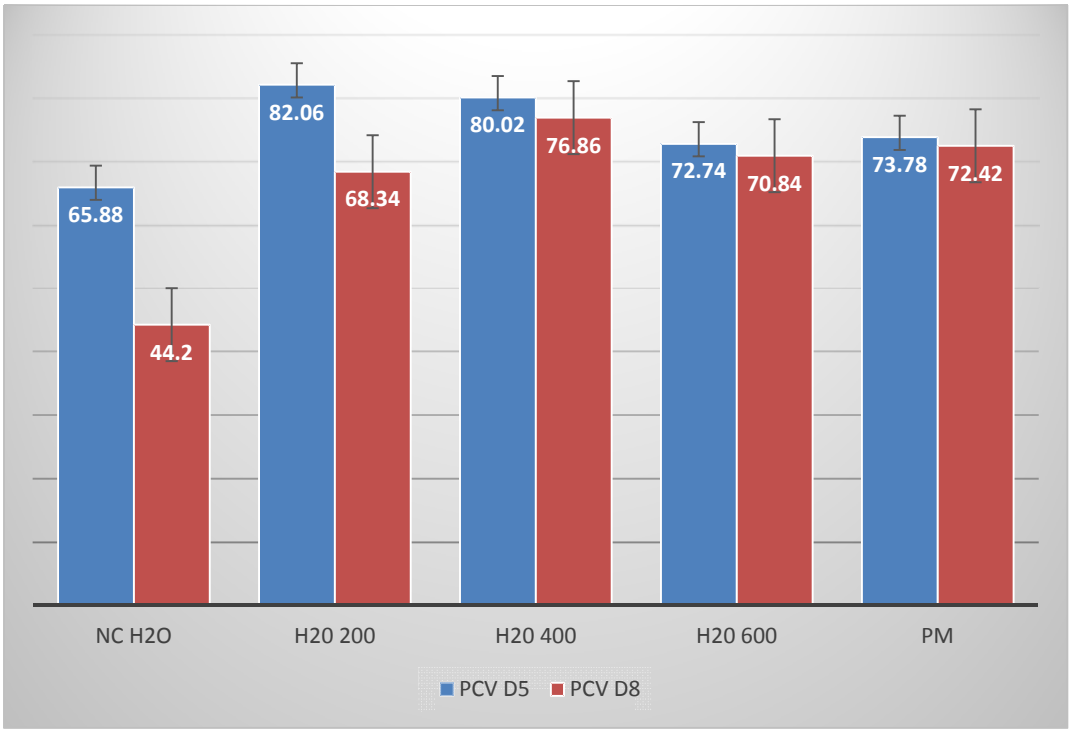

Figure 4.9: Prophylacteffect of aqueous extract of Vernonia amygdalina on PCV.

Values are presented as SEM (Standard Mean ± Error Mean*) PCV: packed cell volume, D5: day 5, D8: Day 8.*

However, as compared to normal control both extract and standard drug treated group showed significant ($p<0.05$) prevention of PCV reduction.

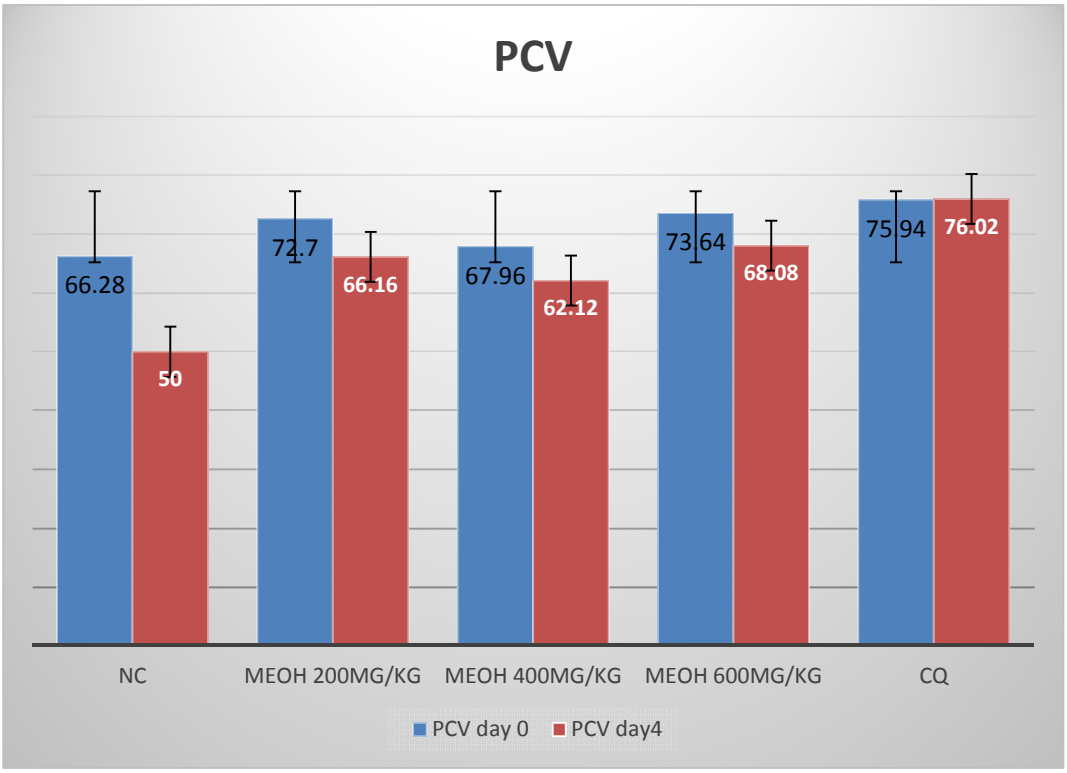

Figure 4.10: Prophylacteffect of hydrometanols extract of Vernonia amygdalina on PCV.

Values are presented as SEM (Standard Mean ± Error Mean*) PCV: packed cell volume, D5: day 5, D8: Day 8*

4.6.2. Prophylactic Effect of Hydromethanoland Aqueous Extract of *Vernonia aygdalina* Rectal Temperature

From the current result, decrement of rectal temperature in both hydromethanol and aqueous leaf extract of *Vernonia amygdalina* on *P. berghei* infected mice were observed. The standard drug, pyrimethamine 1.2mg/kg had showed significant ($p<0.05$) increment of rectal temperature as compared to both extract treated and normal control (Figure 4.11 and 4.12) of both extract.

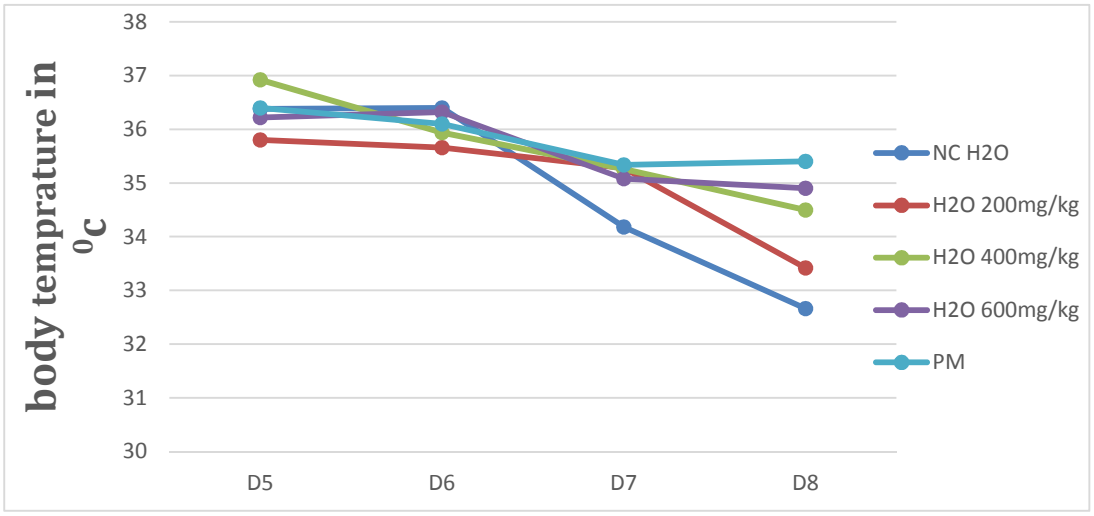

Figure 4.11: The effect of aqueous extract of Vernonia amygdalina on body temperature in prophylactic test

The normal control group showed a significant ($p < 0.05$) reduction in their rectal temperature on day 8 of infection. In the case extract treated group, they all experienced varying degrees of decline in their temperature on between day 5 and day 7 post infections.

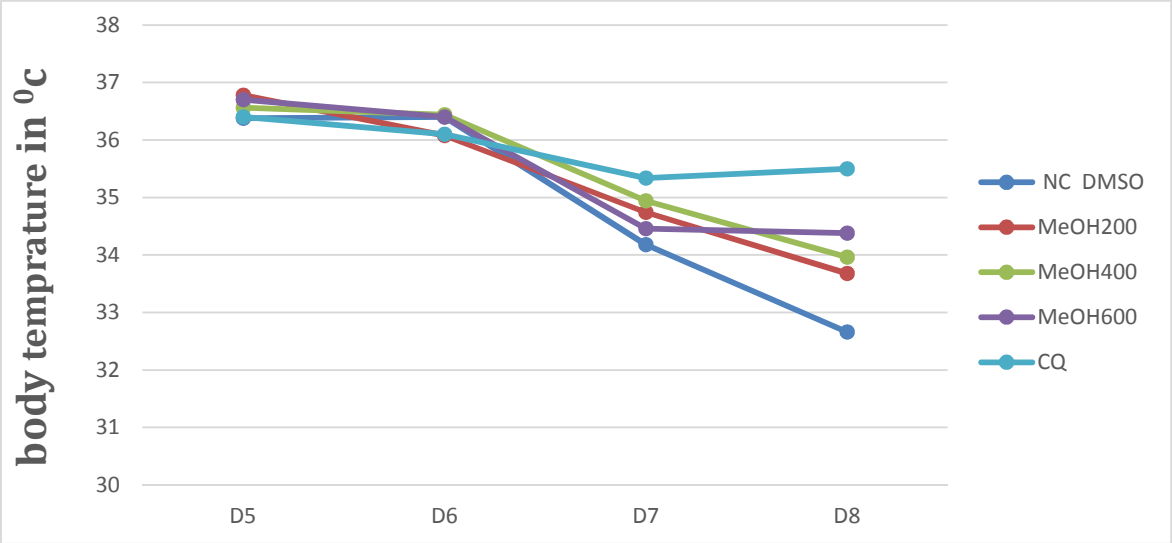

Figure 4.12: The effect of hydrmethanolic extract of *Vernonia amygdalina* on body temperature in prophylactic test

5. DISCUSSION

Plant materials remain an important source of medicine in the fight against malaria (Aderounmu, 2007). Since widely used antimalarial drugs such as quinine and artermisinin were isolated from plants (Rosenthal, 2001), and because of the increased resistance and lack of accessibility to existing affordable drugs, the pharmacognostic investigations of plants for the establishment of complementary medicine for malaria within traditional plants is necessary (Wang *et al*., 2007).

The widely used medicinal plants have formed the basis of health care throughout the world since the earlier days of humanity and have considerable importance (Ebong *et al*., 2008). The majority of Ethiopians depend on medicinal plants as their source of health care especially in rural areas. Thus, knowledge of uses and side effects of medicinal plants provide a vital contribution to human health care. *Vernonia* species are the sources of many local medicines (Amole *et al*., 2006), people living in areas, where *V. amygdalina* grows use the plant for treating malaria and malaria symptom.

The percentage yield of extracts obtained out of 500 g of leaf powder of *V. amygdalina* (Table 4.1) from the two solvent showed that, the yield of hydromethanolic was 12%. However, the aqueous extract yield (9.06%) was less than the former solvent. The variation in the yield of the hydro-alcoholic and aqueous extracts could be due to affinity of the chemical composition. And the possible reason for this variation might be due to high concentration of less polar compounds in the leaf of the plant species, which are capable of dissolving in relatively less polar solvents (Paiva *et al.*, 2010). Organic solvents were preferred to aqueous extraction as compounds have been shown to be more soluble in organic solvents (Bodekar, 2004). Bodekar (2004) further states that organic solvents are a good alternative in evaluating antimalarial plant properties as they have the ability to extract wide spectrum of chemical constituents. Extraction with pure water,

however, is seldom used for plant material as hydrophilic compounds are usually extracted with methanol-water or ethanol-water mixtures (Yrjönen, 2004).

Even though medicinal plants are assumed to be safe, many of them are potentially toxic (Ajaiyeoba *et al.,* 2006). Therefore, evaluating the safety level of the herbal medicine is necessary for the determination of the safe dose that can be used for treatment (Verma and Singh, 2008). For this reason, the oral acute toxicity test in mice, such as mortality, gross physical and behavioral changes were observed in accordance with Center for Drug Evaluation and Research (CDER) guideline for the testing of chemicals in rodents. The absence of serious acute toxic symptoms such as mortality, impaired movement, restlessness, reduced motor activity etc. within 24 hours and survival of mice after oral administration up to a dose of 2000 mg/kg body weight of the extract for a two week indicates that the estimated oral median lethal dose (LD_{50}) is not toxic and the extract at 4000 mg/kg body weight is non-lethal (CDER, 1996). This suggests that acute oral administration of the extract is safe and also explains the reason why the plant is widely used in traditional treatment of malaria in Ethiopian folk medicine (Giday *et al*., 2007).

Consequently, for the present study oral administration of the hydromethanol and aqueous extract of *Vernonia amygdalina* at the dose of 2000 mg/kg for the acute toxicity did not causesign of toxicity like hyperactivity, twitching, rigidity, irritability, jumping, sleep, sedation, abnormal secretion, death within 24 hours, while in the dose of 3000mg/kg rigidity and sleep was observed in hydro-alcohol extract but these signs did not persist and the mice recover instantly after one day for both plant extracts given in the current study. Therefore, the currents study of plants leaf can be considered as safe, according to the Organization for Economic Cooperation and Development (OECD) a guideline which recommends maximum doses for acute toxicity were 2000mg/kg (OECD, 2001).

The acute toxicity of the current study seems comparable with Madaki, (2015) who recorded the ethanol extract showed sign of toxicity at dose of 2500 mg/kg and the highest dose at which no death occurred was recorded at 2000 mg/kg.Other study by Ojiako and Nwanjo (2006) who recorded an LD_{50} of 500 mg/kg in rats administered intraperitoneally with the same plant extract. Inadition, Sha'a *et al*. (2011) also work who recorded an LD_{50} of 1950mg/kg having shown some sign of toxicity at dose of 3000mg/kg on *Vernonia amygdalina*.

Plants possess different classes of secondary substances that are responsible for antimalarial activity; the therapeutic properties ascribed to most medicinal plants have been linked to the presence of phytochemical compounds contained in them. Phytochemicals such as alkaloids, terpenes, saponins, flavonoids and so on have been reported to exhibit anti-plasmodial activity through various mechanisms. These secondary metabolites are known to be biologically active and therefore play significant roles in bioactivity andthe values of medicinal plant lies in these phytochemical compounds which produced a definite and specific action on the human body (Martin and Appel, 2010; Erasto *et al*., 2006).

The present study focused mainly on the phytochemical screening of major classes of phytochemicals with known antiplasmodial activity such as; terpenoids, alkaloids, flavonoids, as results the tests used in the extracts of *Vernonia amygdalina* leaf showed that, the plants contained Alkaloids , Saponins ,Glycosides , Terpenoids **,** Tannins, Flavonoids, Anthraquinone, Steroids,Terpenoids,Cardenolide . (Table 4.3) The result of the current study is therefore, in agreement with a study conducted by Imaga *et al.,* (2013) also detected the presence of alkaloids, tannins, saponins, cardiac glycosides and flavonoids as the most preponderant in their study on aqueous bitter leaf extracts. *Vernonia amygdalina* leaf extract was also screened to contain Sesquiterpene lactone, flavonoids, Steroids, glycoside and vernonioside.

Similarly Adbayo *et al*., (2014) also found the phytochemicals tested were found to be present in both the methanolic and ethanolic leaf extracts of *Vernonia amygdalina* leaf. The aqueous extract showed the presence of saponins, reducing sugars and anthracenosides. The methanolic extract showed the greatest amount of saponins, flavonoids, alkaloids, tannins, terpenes and phenolics also supports the present studies.

The phenol present in this plant which has antioxidant effect (Alexandru *et al.,* 2007) may also contribute to the antiplasmodial activity. Antioxidative activity can inhibit haem polymerization, as haem has to be oxidized before polymerization, and then unpolymerized haem is very toxic for the intraerythrocytic plasmodia (Ramazani *et al*., 2010).

Several bioactive flavonoids have been derived from medicinal plants growing in Africa. Even though the molecular mechanism of action of anti-malarial activitiesof flavonoids is not fully elucidated, it is believed that flavonoids act by inhibiting the fatty acid biosynthesis (FAS II) of the parasite. Some flavonoids have also been shown to inhibit the influx of L-glutamine andmyoinositol into infected erythrocytes (Ntie-Kang *et al.,* 2014).

The main antimalarial mode of action of terpinoieds, quinolines and artemisinin derivatives whose structures are totally different with those of quinoline alkaloids has been established to be their inhibitionof haemin polymerisation through their binding with haemin (Kayembe *et al.,* 2012).

The *invivo* model was employed for this study because it takes into account the possible prodrug effect and possible involvement of the immune system in eradication of infection (Waako *et al*., 2010). *P. berghei* ANKA was used in the prediction of treatment outcomes (Dikasso *et al.,* 2006) and hence it was an appropriate parasite for the study. Moreover, several conventional antimalarial agents such as chloroquine, halofantrine, mefloquine

and more recently artemisinin derivatives have been identified using rodent model of malaria (Madara *et al*., 2010).

The 4 day suppressive test, which mainly evaluates the antimalarial activity of candidates on early infections, and Rane's test, which evaluates the curative capability of candidate extracts on established infections, and prophylactic test (repository test) which evaluate the prophylactic capability of the candidate extract prior to infection to prevent the upcoming disease are commonly used for antimalarial drug screening.

The extracts were considered active when parasitaemia was reduced by > 30% (Carvalho *et al.,* 1990). Accordingly, the current study result (table 3.) showed that the hydmetanol and aqueous extracts of *Vernonia amygdalina* were a dose dependent chemosuppressive effect against *P. berghei* in all the three model of antimalarial test. In Four-day suppressive model, both the aqueous and hydromethanol leaf extract showed a significant ($p< 0.0001$) chemo suppression of 68.8% and 69.2% at the dose of 600 mg/kg respectively when compared to the normal control. Furthermore, mice treated with 200 and 400 mg/kg also showed significant parasetmeia suppression as compared with the normal control.

The result of the current study is comparable with the work of Abosi and Raseroka (2003), who reported that the ethanol leaf extracts and root bark of *V. amygdalina* suppressed parasites by 67% and 53.5%, and Madika, (2015) also indicated that the ethanol extract of *V. amygdalina* were showed 61% suppression effect at the dose of 600mg/kg bw.The current studyshowed an increment chemosupressive effect than the previous report, which might be the solvent used by the present study had good extraction effect.

Iwalokun (2008), who recorded that the *in vivo* antimalarial activity of *V.amygdalina* suppressed 79.37 and 80.71 at the dose of CQ5 + V. amygdalina 125mg/kg on both

chloroquine resistant and sensitive P. berghei, strains respectively. The possible reason for the difference may be due to in the previous finding, the aqueous extract of the plant was used to as an adjuvant with chloroquine but the present study used only the plant extract, though there is a similarity in dose dependent suppression effect.

In addition, the findings as recorded by Melariri *et al.,* (2011) who reported that 85% and 95% parasite growth inhibition at two different doses (400 mg/kg and 600mg/kg) of the combined extract of *C. citratus* + *V. amygdalina,* the possible reason for the difference may be due to the individual or synergetic effect of using polyherbal treatment.

The extracts of *V. amygdalina* suppressed the growth of *P. berghei* malaria in the Rane's test, with highest malaria suppression recorded at the dose of 600 mg/kg. The percentage decrease in parasitemia was comparable to 10 mg/kg CQ, though CQ had the highest chemosuppression. This suggests that *V. amgdalina* leaf extract can suppress parasite growth if given orally for curative purposes. The oral treatment with *V. amygdalina* on Day 7 revealed that the aqueous and hydromethanol extracts had reduced the level of parasitemia to 17.64% and 17.50%, respectively (table 4.6).

In addition, in curative test of the current study, the effect of the aqueous and hydromethanol leaf extract of *V. amygdalina on* the established malaria infection had shown a significant ($p<0.001$) parasitemia chemosuppression with maximum of 69.3 and 70.81 % atthe dose of 600mg/kg, respectively (Table 3.6).

Other study was also done on *V. amygdalina* plant against this rodent parasite, (Anoka, 2008), which reported that the aqueous leaf extract of *V.amygdalina* were found to have a significant ($P < 0.05$) and caused a 73.9% curative effect at the dose of 200mg/kg i.p (intraperitonial). The slight result difference might be due to rout of administration. Inaddition, Iwalokun (2008), which showed 83.3 and 100 % cure rate at the dose of CQ10+*V.amygdalina*62.5 and CQ10+*V.amygdalina*125 respectively. The possible reason

for the difference might be due to the synergetic effect of chloroquine with *V.amygdalina* in the previous study.

The present study also showed the prophylactic effect of aqueous and hydromethanol leaf extract of *V. amygdalina* with significant (P<0.001) parasitemia chemosuppression effect at the dose of 600 mg/kg with 70.3 and 71.5%, respectively as compared to normal control group. While the standard drug, pyrimethamine, showed the highest effect (87.3%, P<0.0001) at the dose of 1.2 mg/kg as compared to normal control group. The 400 mg/kg also showed significant (P<0.05) record with 67 and 65.97% prophylactic activity as compared to the vehicle-treated group (table 3.7).

Evenif no data obtained that has been done the repository effect on *V. amygdalina* , other report during a repository study with the use of medicinal plant extract by (Ebiloma *et al*., 2012), in the treatment of mice infected with *P. berghei* with aqueous leaf extract of *Morinda lucida*, with 70.18% at the dose of 800mg/kg activity were recorded. Study done by Petros and Daniel (2012) who also cheeked the prophylactic effect of other traditional medicinal plant extract against *P. berghie* also exhibited a significance (75.6%, P < 0.05) effect at the dose of 600mg/kg.

The longest mean survival time of the mice was strongly associated with the maximum parasitemia inhibition and this parameter used to evaluate the efficacy of antimalarial plant extract (David *et al*., 2004). In early malaria infectiontest ofthe current study showed statistically significant (P<0.001) mean survival time at the doses of 400 and 600mg/kg which lived 15.80±0.86 and 20.80±1.6 days in the case of aqueous extract as compared to normal control, respectively.At the doses of 400 and 600mg/kg hydromethanol extract the mice lived14.6±0.93&19.8±1.86 days, respectively. The normal and positive control mice lived 8.2±0.37 and 29.00±0.45 days, respectively (table

3.5). However, neither the extract nor the standard drug cured the infection on the fourth day.

In curative test, the mean survival time of mice treated with plant extract was 19 and 21 days for the hydro-alcoholic and aqueous extracts at the dose of 600mg/kg respectively and lived longer compared to the respective negative controls. Possibly the crude extracts of the study plant suppressed *P. berghei* and probably reduce the overall pathogenic effect of the parasite on the study mice on the established malaria infection. However, in those mice treated with 200 mg/kg body weight of both extract treated groups showed non-significant ($p>0.05$) difference as compared to the normal control who reported by another, *invivo* studies done on extracts of medicinal plant by Muthaura *et al*., (2007); Petros and Daniel, (2012) and Abdulelah and Zainal-Abidin, (2007).

The extracts prolonged the mean survival time in prophylactic test of the current study. At the dose of 600mg/kg in both extract,the mean survival of the mice was 18 days and exhibited significant ($p<0.001$) survival time. Even though, extract treated mice lived longer time than the ones fed with vehicle, the 200mg/kg body weight treated group still showed non-significant ($p>0.05$) difference and did not survived longer than the normal controls. According to Zelalem and Daniel (2012) this is considered as evidence for the antiplasmodial activity of the extract and there was an increase in the survival time of the mice indicating a dose dependent pharmacological activity.

Different parameters are used when evaluating antiplasmodial activity of a given plant extract in animal model. Body weight loss is one feature of rodent malaria infections (Okokon *et al*., 2005).

In 4-day suppressive test, it was only chloroquine treated mice that prevented body weight loss significantly ($P<0.001$) but all the extract treated mice had not shown significant ($P>0.05$) prevention against body weight loss when compared to negative

control. Body weight loss in extract treated mice might be possibly due to depressing effect of the crude extract on feed intake or appetite or increment of parasitemia.

Mengistie *et al*., (2012), reported that mice treated with crude extracts of *Dodonaea angustifolia and Bersama abyssinica* showed a lower body weight pattern as compared with the non-treated ones. Anemia, body weight loss and body temperature reduction are the general features of malaria-infected mice also mentioned by (Bantie *et al*, 2014).

Despite the fact that, the body weight gained in curative test at all doses was might be due to the effect of the crude extract decrease the highest parasitemia level on established infection since the inoculum was given three days prior to treatment.

Comparable observations were reported by Dikasso *et al.,* (2006) where extracts of *Asparagus africanus* prevented body weight loss of *P. berghei* infected mice.

However, the result of the present study in prophylactic model showed loss of body weight betweenat D8 than from D5. The loss of body weight in those extract treated mice might be due to decrease in appetite suppression effect of the crude extract on residual infection since it was given for four days prior to infection.

Bantie *et al.,* (2014) also reported that, the lossof weight might indicate that the plant could have appetite suppressive effect. This appetite suppressive activity might be ascribed to saponins, flavonoids, glycosides and phenolic compounds found in the crude extracts, which support the present study.

On the other hand, the effect of rodent malaria on PCV as measured by haematocrit was parasite-induced fall, which occurred approximately 48 hours post-infection (Taylor and Hurd, 2001). *P. berghei* infected mice suffer from anaemia because of RBC destruction, either by parasite multiplication or by spleen reticulo-endotelial cell action as the presence of many abnormal RBC stimulates the spleen to produce many phagocytes

(Chinchilla *et al*., 1998). In the curative test of the present study, the extract had shown prevention against PCV reduction significantly ($p<0.05$), as compared to normal control. On the other hand the 4 days suppression and prophylactic model did not prevent PCV fall, but still the extract treated group exhibited better prevention against PCV reduction.

The work of Mengiste *et al*., (2012), in which recorded the crude extracts from other species of plant such as *Dodonaea angustifolia* and *Bersama abyssinica* did not prevent reduction PCV values.

Statistically significant ($P<0.05$) differences were recorded at the doses 600mg/kg and Pyrimitamine ($P<0.001$) protective effect in preventing PCV reduction compared to normal control. A research done by Madaki, (2015) on *V.amygdalina* indicated that the mice treated with the highest dose of the extract (600mg/kgbw) and the group administered Standard drug (Chloroquine) showed a high PCV on the fifth day. This might be the extract at high dose can reverse the manifestation of the low PCV that occurred during the early stage of infection of the mice as the treatment continues i.e. with time. The significant decrease in the PCV of the normal control could be an indication of anaemic condition which may be attributed to the malarial infection.

In all three models, (4 -days suppression, curative and prophylaxis) the hydromethanol and aqueous leaf extract of *V. amygdalina* did not cause significant prevention of rectal temperature reduction of *P. berghei* infected mice. The standard drug, (chloroquine 10 mg/kg and pyrimitamine 1.2mg/kg) had shown significant ($p<0.05$) activity in prevention against rectal temperature reduction as compared to extract treated and negative control).

Comparable observation was reported with the use of other medicinal plant extract by Mengistu *et al*., (2012). And decrease in the metabolic rate of infected mice occurs before death and is accompanied by a corresponding decrease in internal body temperature. Ideally, the rectal temperature decreases as parasite level escalates and Active compounds

should prevent the rapid dropping of rectal temperature, but if not the crude extract did have protective effects against temperature reduction (Bantie*et al.,* 2014).

Mebrahtu *et al.*, (2013) also reported that the attributed to the effect of the extract as it may have less amount of hypothermic effect on the extract treated mice. The extract treated mice had shown more prevention of rectal temperature reduction than normal control even though it was not statistically significant.

In general the present study therefore, is yet evidence showing the relevance of traditional herbal medicine of Ethiopia. It is hoped that easily affordable plant based antimalarial drugs will be developed from *Vernonia amygdalina* in the days to come.

8. Conclusion

The spread of resistant malaria parasites to the available antimalarial drugs call for a new chemotherapeutic agent to control the disease. Medicinal plants are constantly screened for bioactivity in our quest for the discovery of new and effective therapeutic agents. The results of this study could help encourage more identification and validation of natural products which has shown antiparasitic properties thus facilitating the development of a new generation of antimalarials.

In conclusion, the results obtained from current study indicated that;

- Both the safety and efficacy test of the crude aqueous and hydromethanolic leaf extracts of of *V. amygdalina* suggested that the plant is not toxic and did not cause acute toxicity symptoms up to the highest dose (2000mg/kg) administered to the experimental mice. But the 3000mg/kg showed some sign of toxicity.
- The antimalarial test results of this study showed that the crude extracts of the plant material possessed a suppressive antimalarial activity. The hydromethanol and the aqueous extracts showed antimalarial effects, thus it justify the traditional usage of this plant as malaria remedy. The plant extract produced statistically significant parasitaemia suppression in dose dependent manner during a standard 4-days suppressive test and prolonged the survival time of *P. berghei* infected mice.
- The hydro-alcoholic extracts of the leaves of *V. amygdalina* shown a promising curative and prophylaxis anti-malarial activities using *Plasmodium berghei* infected mice in a dose-dependent manner.
- In general, antimalarial activities as well as the lack of toxicity of the extracts found in the present study may partly confirm the claim by traditional practitioners for the use of the plants against malaria and support the ethno-botanical use of the plant in the treatment of malaria in Ethiopia.

9. Recommendations

In view of the research findings from the present study, the following recommendations have been made.

- Further pharmacological screening with bioassay guided chemical fractionations of the crude extracts of the study plant would permit the isolation and identification of antimalarial active compounds.

- It is recommended that further study should be conducted using the other parts of the plant *Vernonia amygdalina* to find out the comparative efficacy in regulating anti-plasmodial activity.
- It is recommended that, the plant extracts should be evaluated together with some of the other antimalarial drugs currently used in order to find out synergistic effects on their ability to reverse development of resistance. Further pharmacological screening with bioassay guided chemical fractionations of the crude extracts of the study plant would permit the isolation and identification of antimalarial active compounds.
- It is also recommended that Ethiopian population should develop the habit of using bitter leaf in their culinary practices so that they can gain the benefits associated with the medicinal plant *Vernonia amygdalina*

10. Limitations of the Study

The main aim of this study was to evaluate the use of selected ethnomedicinal plants to treat malaria in traditional settings. In traditional settings, plant extracts preparation is a complex process that involves performance of rituals and the combination of different plant species and possibly other organisms to develop effective treatment concoctions. This study was limited to the therapeutic activity of single plant species and it disregards other aspects that are used in traditional settings to mix extracts. For these reasons, the conclusions drawn from this study about the efficacy of the selected plants are not a representation of the whole traditional healing system. Secondly, crude extracts comprises a mixture of compounds. Thirdly, a laboratory *P. berghie* model was used to evaluate the *invivo* antiplasmodial activity of the selected plants instead of a *P. falciparum and* other *plasmodium* species of clinical model. Hence; the conclusions about the efficacy of the selected plants do not reflect their activity in clinical settings.

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Annex I

***Plasmodium berghei* infection**

CQ-sensitive strain of the rodent parasite *P. Berghei* was kept alive by continuous intraperitoneal (i.p.) passage in mice on weekly bases. Percent parasitaemia of the donor mouse was first determined (about 20 to 30% Para) and blood was collected by gentle jugular vein puncthur from the donor mouse using surgical blade. Then, 1ml of blood was diluted with 4ml of PBS (phosphate buffer saline) and 0.2 ml of the dilution contains aproximatly10^7 of infected erythrocytes. Therefore, each mouse was infected intraperitoneally with 0.2ml dilution of infected blood (standard inoculum) containing approximately $1x10^7$ *P. berghei* parasitized red blood cells (Krettli *et al*., 2009)

Annex II

Phytochemical Analysis of the leaf extracts

The Phytochemical screening of the leaves extracts; Flavonoids, Alkaloids, Saponins, Phenol, Glycosides, Volatile Oil, Tannins, Steroids and Terpenoids, Cardenolide was analyzed using the method described by Harbourne (1976), Evan (1999), and Sofowora (1993), respectively.

Test for Flavonoids

Sodium hydroxide method was used for the test. 5g of the sample was weighed and detanned completely with acetone. The residue was extracted in warm water after evaporating the acetone on a water bath. The mixture was filtered and the filtrate was used for the test. 5ml of 10% sodium hydroxide was added to an equal volume of the detanned water extract. A yellow solution indicates the presence of Flavonoids.

Test for Alkaloids

2mls of the extract was measured in a test tube to which picric acid solution was added. The formation of orange colouration indicates the presence of alkaloids.

Test for Saponins

Froth test for saponins was used. 1g of the sample was weighed into a conical flask in which 10ml of sterile distilled water was added and boiled for 5minutes. The mixture was filtered and 2.5ml of the filtrate was added to 10ml of sterile distilled water in a test tube. The test tube was stopped and then shaken vigorously for about 30seconds. It was allowed to stand for half an hour. Honey comb froth indicates the presence of Saponins.

Test for Phenol

25ml of extract was added to 2ml of ferric chloride solution, a deep bluish green solution formed indicates the presence of phenol

Test for Glycosides

25ml of 1ml Sulphuric acid was added to 5ml of the extract in a test tube and boiled for 15minutes, cool and neutralized with 10% sodium hydroxide, and then 5ml of fehling solution A and B was added. A brick red precipitate of reducing sugars indicates the presence of Glycosides.

Test for Tannins

3g of the sample was boiled in 50ml distilled water for 30minutes on a hot plate. The mixture was filtered and a portion of the filtrate was diluted with sterile water in a ratio of 1:4 and 3drops of 10% ferric chloride solution was added. A blue or green colour indicates the presence of tannins.

Test for volatile oils

2ml of extract solution was shaking with 0.1M sodium hydroxide and a small quantity of 0.1M hydrochloric acid. A white precipitate was formed with volatile.

Test for Steroids

Exactly 2ml of acetic anhydride added to 0.5g of the extracts with 2ml of H_2SO_4. The colour changes from violet to blue indicating the presence of steroids.

Test for Terpenoids

About 0.2g extracts was mixed with 2ml Chloroform and 3ml of concentrated H_2SO_4 was carefully added to form a layer. A reddish brown coloration of the interface formed indicating the presence of terpenoids.

Statement of declaration

I, the undersigned, declare that this MSc thesis is my original work, it has not been presented for a degree in this and any other university and that all sources of materials used for this thesis have been duly acknowledged. All scholarly matter that is included in this thesis has been given recognition through citation. I affirm that I have cited and referenced all sources used in this document. Every effort has been made to avoid any plagiarism in the preparation of this thesis.

Name: Temesgen Bekele (BSc B.Phar, MSc candidate)

Date: _____

Signature: _____