Study on the *In Vivo* Antimalarial Activity of Solvent Fractions of the Leaf of *Vernonia amygdalina (*Asteraceae) against *Plasmodium berghei* in Mice

By

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This is to certify that the thesis prepared by Temesgen Bihonegn, entitled: "**Study on the *In Vivo* Antimalarial Activity of Solvent Fractions of the Leaf of *Vernonia amygdalina* (Asteraceae) against *Plasmodium berghei* in Mice"** and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Pharmacology complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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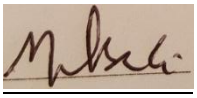
Signature:  Date: 13/01/2016

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LIST OF ABBREVIATIONS/ ACRONYMS

µm	Micrometer
µl	Microliter
AF	Aqueous Fraction
BF	Butanol Fraction
CF	Chloroform Fraction
CQ	Chloroquine
D	Day
FMoH	Federal Ministry of Health
FY	Fiscal Year
GC	Gas Chromatography
H_2SO_4	Sulfuric Acid
HDMECs	Human Dermal Microvascular Endothelial Cells
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
HRP2	Histidine-Rich Protein 2
IC_{50}	Half Maximal Inhibitory Concentration
ICAM-1	Intercellular Adhesion Molecule 1
IRBCs	Infected Red Blood Cells
LC	Liquid Chromatography
MCP	Malaria Control Program
MDGs	Millennium Development Goal

MDR	Multidrug Resistance
MIS	Malaria Indicator Survey
ml	Milliliter
MS	Mass Spectroscopy
MST	Mean Survival Time
OECD	Organization for Economic Co-operation and Development
P. falciparum	*Plasmodium falciparum*
P. vivax	*Plasmodium vivax*
P. malariae	*Plasmodium malariae*
P. ovale	*Plasmodium ovale*
PCV	Packed Cell Volume
PfCRT	*P. falciparum* Chloroquine-Resistance Transporter
Pgh	P-Glycoprotein Pumps
pLDH	Plasmodium Lactate Dehydrogenase
PMI	President's Malaria Initiative
pRBCs	Parasitized Red Blood Cells
RBCs	Red Blood Cells
RDTs	Rapid diagnostic tests
TCC	The Carter Center's
TLC	Thin Layer Chromatography
UK	United Kingdom
V. amygdalina	*Vernonia amygdalina*
WHO	World Health Organization

ABSTRACT

Malaria is a mosquito borne infectious disease caused by a protozoan of the genus Plasmodium. Around 44% of world's population is at risk from malaria. Malaria is one of the leading causes of morbidity and mortality in Ethiopia. The most important problem associated with the management of malaria are resistant to or are developing resistance to the most widely available, affordable and safest first line treatments. The study was under taken to evaluate the *in vivo* antimalarial activity of solvent fractions of the leaves of *Vernonia amygdalina* against *P. berghei* infection in mice using four day suppressive test and subsequently body temperature, weight, packed cell volume, parasitemia and mean survival time were determined. The plant has been extracted using cold maceration. 80% methanol crude extract of *V. amygdalina* was subjected to chloroform, butanol and aqueous fraction. Tween 80 2% and distilled water as negative control and chloroquine 25 mg/kg were used as positive control. Acute oral toxicity test showed that both the aqueous and hydroalcolic extracts and solvent fractions of the leaves of *Vernonia amygdalina* revealed no mortality and signs of toxicities up to 2000mg/kg. The present study indicated that the percentage suppression of hydroalcoholic extract was 32.47%, 35.4% and 37.67% at 200, 400 and 600mg/kg of the extract, respectively and the percentage suppression of aqueous extract was 21.22%, 22.20% and 24.52% at 200 400 and 600mgmkg of the extract respectively. All doses of crude extracts of *V. amygdalina* prolonged the survival time, shown prevention against weight loss and prevent PCV reduction in a dose dependent manner. Except aqueous fraction, all doses of chloroform and butanol fractions were suppressed parasitemia significantly. The percentage suppression of chloroform fraction was 21.68%, 23.72% and 33.85 % at 100, 200 and

400mg/kg of the fraction, respectively. The 100 and 200 mg/kg of butanol fraction resulted in moderate anti-plasmodial activity (18.12%, and 21.03% %, respectively), followed by the 400 mg/kg (26.88%).The mean survival effects of both chloroform and butanol fractions were capable of significantly increasing survival time at all doses compared to negative control. All the tested doses of chloroform and butanol fractions exhibited reduction in rectal temperature of *Plasmodium berghei* infected mice statistically significant ($p < 0.05$) in a dose-dependent manner. All doses of chloroform, butanol and aqueous fractions had shown prevention against weight loss ($p<0.001$). The results of the present study indicated that the *in vivo* administration of both extracts and solvent fractions of the leaf of *V. amygdalina* possess antimalarial activity and were capable of suppressing parasitemia. This confirms their use in ethnomedicine in the treatment of malaria in local communities. Therefore, there is a need to advance the current status of *Vernonia amygdalina* to an antimalarial lead drug level through isolation and characterization active antiplasmodial components in the extracts and solvent fractions. Further studies should carried out to determine the mechanism of action(s) responsible for the antimalarial activities.

Key words: Antimalarial activity, Four day suppressive test, *In vivo, P.berghei*, Solvent fractions, Mice, *V. amygdalina*.

1. INTRODUCTION

Malaria is a mosquito borne infections disease caused by a protozoan of the genus Plasmodium (Odeh and Usman, 2014). Around 44% of world's population is at risk from malaria. An estimated 3.2 billion people were at risk of being infected with malaria and developing disease in 2013. Of this, 1.2 billion people are at high risk (>1 case per 1000 population) of malaria. Over half of all the countries in the world are affected by malaria (RBMP, 2015).

It is widespread in tropical Africa and Sub tropical regions including part of America, Asia and Africa (Odeh and Usman, 2014). In 2013, worldwide, there were an estimated 198 million cases of malaria. Most of these cases (82%) were in the WHO African Region, followed by the South-East Asia Region (12%) and the Eastern Mediterranean Region (5%). About 8% of estimated cases globally are due to *P. vivax*, although the proportion outside the African continent is 47% (WHO, 2014).

Of the estimated 584,000 malaria deaths that occurred worldwide in 2013. 528,000 of these deaths, or 90%, were in the African Region, with 7% in the South-East Asia Region, and 2% were in Eastern Mediterranean Region. About 0.27 % people die every day from malaria; more than 0.24% of those people are in Africa. About 453,000 malaria deaths were estimated to occur in children under 5 years of age, or 78% of the global total. Over 1,200 children die every day from malaria, which is equivalent to 50 children dying every hour (RBMP, 2015).

An estimated 437,000 of deaths occurred in children under 5 years of age in the WHO Africa Region, accounting for 83% of the total malaria deaths in the Africa Region, and 96% of total global under 5 malaria deaths. About 80% of malaria deaths in 2013 are estimated to occur in just 16 countries: Nigeria, Democratic Republic of the Congo, India, Angola, United Republic of Tanzania, Uganda, Ghana, Niger, Chad, Mozambique, Burkina Faso, Ethiopia, Côte d'Ivoire, Mali, Guinea, and Cameroon (RBMP, 2015).

The parasites are spread to people through the bites of infected Anopheles mosquitoes, called "malaria vectors" (Singh, 2011).There are four parasite species that cause malaria in humans which are *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale.* *P. falciparum* and *P. vivax* are the most common species clinically but *P. falciparum* is the most deadly leading to many fatal complications including cerebral malaria (Singh, 2011).

Malaria is one of the leading causes of morbidity and mortality in Ethiopia. An estimated 55.7 million people (68% of the population) are at risk for malaria and approximately 80% of the 736 woredas (districts) in Ethiopia are considered "malarious". Protective immunity in Ethiopian populations is relatively low due to unstable transmission and, unlike large parts of sub-Saharan Africa; all age groups are at risk of infection and disease. *P. falciparum* accounts for 65-75% of infections, while *P. vivax* accounts for 25-35%. *P. ovale* and *P. malariae* are rare (The Carter Center's (TCC) Malaria Control Program (MCP), 2013).

The World Health Organization (WHO) estimates that approximately 80% of the world's inhabitants rely on traditional or herbal medicines for their primary health care and

plants have long formed the basis of sophisticated traditional medicine systems and purportedly provide excellent leads for new drug developments (Pravi, 2006; Akinjogunla *et al*., 2009). However, the increasing problems of multi-drug resistant (MDR) is of great concern to both the clinicians and pharmaceutical industries and this has made it significant to search for newer drugs that are highly effective, affordable, acceptable and available (Akinjogunla *et al*.. 2010).

In developing countries where modern medicine is expensive, most of the indigenes people rely on indigenous plants for the treatment of various ailments (Qureshi *et al*., 2009). Traditional medicine has maintained its popularity in all regions of the developing world and its use is rapidly spreading in ten industrilized countries (Kassaye *et al.,* 2006).

Phytochemicals differ from phytonutrients in that they are not a necessity for normal metabolism and absence will not result in deficiency disease. Phytochemicals are not required for the functioning of the body, but they are of benefit on health and play an active role in the treatment of diseases (Audu *et al*., 2012).

2. LITERATURE REVIEW

2.1. Global epidemiology of malaria

Epidemiology of malaria is important for clear understanding of the distribution and transmission pattern of the disease. This is also relevant for the control of malaria at large. Several epidemiological studies have shown the degree, and intensity of transmission is varied within continents (Snow, 2005; Guerra *et al*., 2006).

An estimated 3.2 billion people were at risk of being infected with malaria and developing disease in 2013. Of this, 1.2 billion people are at high risk (>1 case per 1000 population) of malaria. In 2013, there were 97 countries and territories with ongoing malaria transmission, and 6 countries in the prevention of reintroduction phase, making a total of 103 countries and territories, 196 internationally recognized countries affected by malaria. There were 198 million cases of malaria worldwide in 2013, with 82% of these cases occurring in Africa. In 2013, 584,000 people died from malaria worldwide, with 90% of these deaths occurring in Africa (WHO, 2014).

Malaria occurs mostly in poor tropical and subtropical areas of the world. In many of the countries affected by malaria, it is a leading cause of illness and death. In areas with high transmission, the most vulnerable groups are young children, who have not developed immunity to malaria yet and pregnant women, whose immunity has been decreased due to pregnancy (CDC, 2015).

P. vivax is regarded as the most cosmopolitan of the human malaria and the public health burden more significant that it causes severe morbidity and death. However, *P.*

falciparum is the most serious intimidation to the world at a very scale. It causes more than 90% of death due to malaria. Four thousand years on, *P. falciparum* remains widespread in Africa. This may be due to optimal environmental conditions for Anopheline mosquito vectors, amid sustained poverty (Snow, 2015). The fluctuating pattern of malaria epidemiology throughout the world could be characteristics of complex interaction between environment, vector, the human host and the parasite species.

2.2. Epidemiology of malaria in Ethiopia

The 2011 Malaria Indicator Survey (MIS) shows that 1.3% of all age groups were positive for malaria using microscopy and 4.5% were positive for malaria using RDTs below 2,000 meters. *P. falciparum* constituted 77% of these infections (Malaria Operational Plan FY, 2014). 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. Malaria also was among the ten leading causes of inpatient deaths among children less than five years of age (Malaria Operational Plan FY, 2014).

About 75% of the geographic area of the country has significant malaria transmission risk (defined as areas <2,000 m), with about 68% of the country's total population living in these areas. The FMOH estimates that there are about 12 million suspected malaria cases each year. The FMOH reported a total of 3,384,589 malaria cases from July 2011-June 2012, with 1, 793, 832 (53.0%) of these laboratory confirmed, with 1,061,242 (59.2%) *P. falciparum* and 732,590 (40.8%) *P. vivax*. Ethiopia reported 936 malaria deaths in 2011, according to the 2012 World Malaria Report (Malaria Operational Plan FY, 2014).

2.3. Etiology

Malaria, the disease caused by protozoan parasites of the genus Plasmodium (Jennifer *et al*., 2005).All malaria is transmitted by female mosquitoes of the genus Anopheles. Humans are mainly infected by four species of Plasmodium: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*, although human infections with the monkey malaria parasite, *P. knowlesi* have also been reported recently in the forested regions of Southeast Asia (Kantele and Jokiranta, 2011). The majority of all human malaria cases are caused by *P. falciparum* and *P. vivax* (Stainesl and Krishna, 2012).

Anopheles gambiae the most aggressive among the more than 60 mosquito species that transmit malaria to people in sub-Saharan Africa and bears partial responsibility for millions of human deaths per year (Vizioli *et al.*, 2000; Dunavan, 2005 and Legoff *et al*., 2006). *P. falciparum* and *P. vivax* are the two dominant parasite species with relative frequency of 60% and 40%, respectively in Ethiopia (Gezahegn, 2004).

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2.4. Life cycle

The malaria parasite has a complex, multistage life cycle occurring within two living beings, the vector mosquitoes and the vertebrate hosts (Fakhreldin *et al*., 2003; Brian *et al*., 2008).The parasite passes through several stages of development such as the sporozoites, merozoites, trophozoites and gametocytes (sexual stages) and all these stages have their own unique shapes and structures and protein complements. The surface proteins and metabolic pathways keep changing during these different stages that help the

parasite evade the immune clearance, while also creating problems for the development of drugs and vaccines (Fakhreldin *et al*., 2003).

The sporozoite form of the parasite is inoculated into humans when bitten by an infected female Anopholes mosquito (Figure 1). The parasites go through several host cells by breaching their plasma membrane before infecting a final hepatocyte (Dunavan, 2005). Sporozoites rapidly enter the liver cells where they multiply to form thousands of merozoites. These then enter the bloodstream where they invade red blood cells and multiply to form new merozoites. Infected red blood cells burst, releasing merozoites that infect new red blood cells. This is referred to as the asexual blood stage, the stage of the plasmodial life cycle that causes the clinical signs and symptoms of malaria. Some merozoites that invade the red blood cells develop into gametocytes, the sexual stages of the parasite. Gametocytes are ingested by the mosquito when it takes a blood meal. In the mosquito gut, the gametocytes develop into gametes and fuse to form a zygote. After fertilisation, the zygote transforms into a motile ookinete, which penetrates the mosquito stomach wall and becomes an oocyst. The oocyst divides to produce sporozoites, which move into the salivary glands, from where another human can be infected when the mosquito takes a blood meal from (Stainesl and Krishna, 2012).

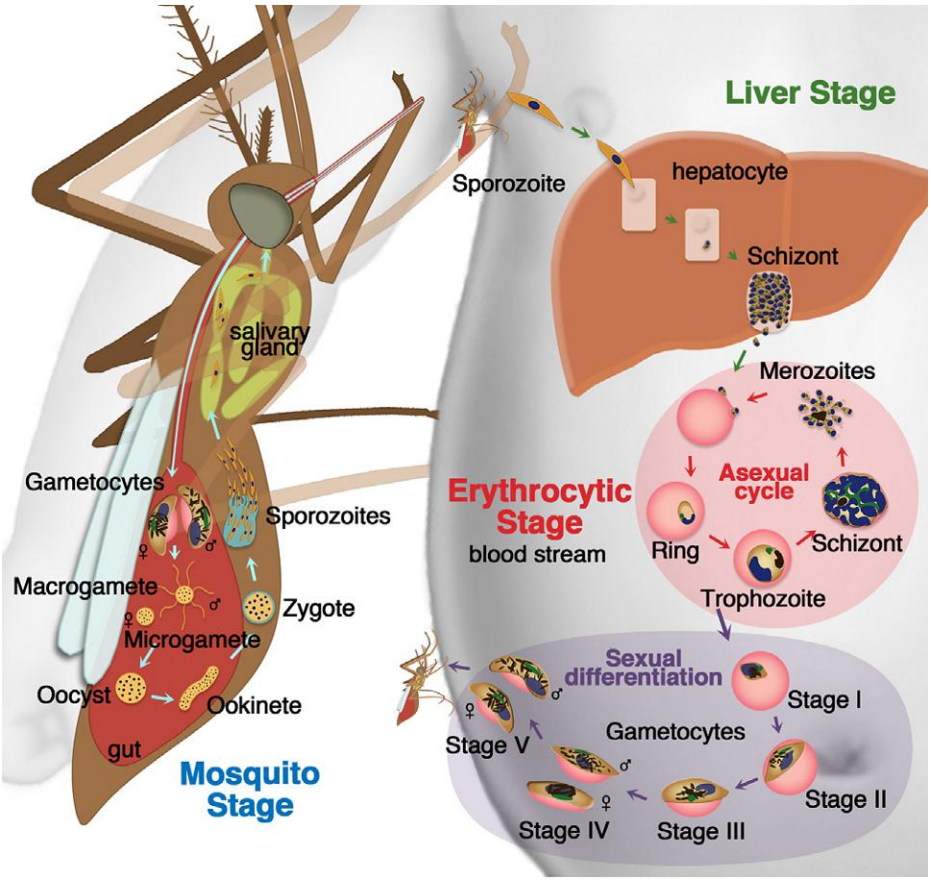


Figure 1. Plasmodium life cycle (Biamonte *et al*., 2013).

2.5. Pathogenesis

Proliferation of the parasite within the host's erythrocyte takes place using hemoglobin as predominant source of nutrition. The malaria parasite digests hemoglobin within the digestive vacuole through the sequential metabolic processes involving multiple proteases (Tekwani and Walker, 2005). Malaria pathogenesis could be explained by *Plasmodium falciparum* erythrocyte membrane protein mediated sequestration of parasitized erythrocytes. This protein helps the parasite infected RBC adhere to blood elements including noninfected erythrocytes, leukocytes, and wall of endothelial cells of microcirculation. These binding events enable parasitized erythrocytes to sequester and

avoid clearance by the spleen and also contribute to disease by causing microvascular inflammation and obstruction (Rogerson *et al*., 2004; Fairhurst and Wellems, 2006) . Cytoadherance of IRBCs on human dermal microvascular endothelial cells (HDMECs) is responsible for pathogenesis of malaria. IRBCs were observed to tether, roll, and adhere on resting HDMECs, rossets will be formed causing clogging of capillaries. IRBCs interact synergistically with multiple adhesion molecules on vascular endothelium (Bryan *et al*., 2000).

Different factors participate in the neuropathogenesis of malaria. They seem to include abnormally high production of cell-derived cytokines such as tumor necrosis factor (TNF) and IFN- induced by infected erythrocytes. These cytokines may play an important role in causing certain pathological changes, by up-regulating the expression of cell surface markers like ICAM-1 and chondroitin sulfate A, thus leading to the sequestration of infected erythrocytes, leukocytes and monocytes in the cerebral capillaries. The pathogenesis of severe malaria therefore involves a cascading interaction between parasite and red cell membrane products, cytokines and endothelial receptors, leading to inflammation, activation of platelets, hemostasis, a procoagulant state, microcirculatory dysfunction and tissue hypoxia, resulting in various organ dysfunctions manifesting in severe malaria (Henri *et al*., 2006).

2.6. Clinical manifestations

The clinical features of malaria vary. The most characteristic symptom is the occurrence of paroxysm which include fever with temperatures up to 40 - 41ºC at regular intervals –every 48 or 72 hours (tertian or quartan), alternating with good periods of no

fever. This is preceded by headache, lassitude, loss of appetite, muscle pain and chills, resulting in uncontrollable shivering with teeth chattering. This is accompanied by thirst, nausea, vomiting. Severe and complicated malaria causing renal failure, hypoglycemia, anemia, pulmonary oedema, shock and coma can have fatal consequences, leading to death (Singh, 2011).

Nonimmune people, children and pregnant women who live in endemic regions are at highest risk of complications from malaria. Complications generally involve the central nervous, pulmonary, renal and hematopoietic systems. Hypoglycemia occurs because of parasite consumption of glucose and treatment with quinine. Acidosis is another common metabolic derangement. Severe anemia, acute renal failure, respiratory failure, intravascular hemolysis, coagulopathies, and shock may also develop (Kathryn *et al*., 2004).

One of the most serious complications is cerebral malaria, manifested by altered level of consciousness, focal neurologic findings and seizures (Kathryn *et al*., 2004). Mortality is high (15% to 25%), and survivors may have residual neurologic deficits. Although semi-immune people and those living in endemic regions tend not to experience severe malaria, they may still experience complications from recurrent infections. In children, severe anemia is the most common complication of chronic malaria, with hematocrits approaching 15% (Kathryn *et al*., 2004). The predominant manifestations of severe malaria in African children are cerebral malaria and severe anemia. *P. falciparum* slightly damages blood brain barrier (Gitau and Newton, 2005). Malaria is not only a direct cause

of death but also contributes indirectly to death due to respiratory infections, diarrhea and malnutrition by impairing immunity (Kager, 2002).

2.7. Diagnosis

2.7.1. Light microscopy

In addition to providing a diagnosis with a high degree of sensitivity and specificity when performed well, microscopy allows quantification of malaria parasites and identification of the infecting species. It is considered to be the "field standard" against which the sensitivity and specificity of other methods must be assessed. A skilled microscopist is able to detect asexual parasites at densities of fewer than 10 per µl of blood, but under typical field conditions, the limit of sensitivity is approximately 100 parasites per µl (WHO, 2010).

A high degree of suspicion and rapid diagnosis are essential to optimize outcome. Thick and thin peripheral blood smears, stained with Giemsa stain (or, alternatively, Wright's or Field's stains), remain the "gold standard" for routine clinical diagnosis. Malaria smears permit both species identification and quantification (expressed as a percentage of erythrocytes infected or as parasites per microlitre) of parasites but malaria should not be excluded until at least 3 negative blood smears have been obtained within 48 hours (Kathryn *et al*., 2004).

2.7.2. Rapid diagnostic tests

Rapid diagnostic tests are immunochromatographic tests that detect parasite-specific antigens in a finger-prick blood sample. Some tests detect only one species (*P. falciparum*), others detect one or more of the other species of human malaria parasites (*P. vivax, P. malariae* and *P. ovale*). They are available commercially in different formats, e.g. dipsticks, cassettes or cards. Cassettes and cards are easier to use in difficult conditions outside health facilities. Plasmodium lactate dehydrogenase *(pLDH) or pan-specific aldolase.* These antigens have different characteristics, which may affect suitability for use in different situations, and these should be taken into account when developing RDT policy (WHO, 2010).

Current tests are based on the detection of histidine-rich protein 2 (HRP2), which are specific for *P. falciparum*, pan-specific or species-specific Plasmodium lactate dehydrogenase (pLDH) or pan-specific aldolase. These antigens have different characteristics, which may affect suitability for use in different situations, and these 2 should be taken into account when developing RDT policy (WHO, 2010).

The most practical of rapid malaria tests are the rapid antigen detection tests (RDTs), which detect parasite proteins in finger-prick blood samples. RDTs can identify only *P. falciparum* and *P. vivax*. Important shortcomings of RDTs include their inability to quantify parasitemia and suboptimal test performance with low-level parasitemia. Furthermore, some RDTs are unreliable as tests of cure because antigenemia may persist for prolonged periods even after treatment. But their simplicity may make them attractive and useful alternatives to blood smears, particularly in laboratories where expertise in

reading blood films is lacking or in centers where malaria is infrequently encountered. Based on clinical studies involving both travelers to and residents of endemic areas, the overall sensitivity and specificity of RDTs for the detection of falciparum malaria are over 90%. However, sensitivity falls dramatically with low level parasitemia, and at present RDTs cannot be used alone to exclude malaria (Kathryn *et al*., 2004).

2.7.3. Polymerase chain reaction (PCR)

Techniques to detect parasite deoxyribonucleic acid (DNA), based on the polymerase chain reaction, are highly sensitive and very useful for detecting mixed infections, in particular at low parasite densities. They are also useful for studies on drug resistance and other specialized epidemiological investigations, but they are not generally available for large-scale field use in malaria endemic areas (WHO, 2010). However, most PCR assays do not have sufficiently rapid turnaround times to be clinically useful; therefore, PCR remains largely an investigational tool (Kathryn *et al*., 2004).

2.8. Treatment of malaria

Management of malaria includes general measures to be taken to save life of the person and prevention of recrudescence using drugs and other supportive measures (Kathryn *et al*., 2004). Treatment of malaria depends on the infecting plasmodia species, the geographic area of acquisition (which affects the likelihood of drug resistance) and severity of infection (Kathryn *et al*., 2004; Sean and Sweetman, 2005). Falciparum malaria in nonimmune person is a medical emergency and requires rapid initiation of therapy (even in cases where the species cannot be immediately identified, the patient

should be assumed to have drug resistant falciparum malaria until proven (Kathryn *et al*., 2004).

Once diagnosed and confirmed, malaria is treated with one or more drugs that have been licensed to be used as antimalarial agents. The currently available antimalarial drugs are grouped into four classes according to their chemical structure and biological activity: (i) quinoline based antimalarials which include quinine and its derivatives such as chloroquine, amodiaquine, premaquine, and mefloquine (ii) antifolate compounds (pyrimethamine , proguanil, dapsone, and sulfadoxine), (iii) artemisinin and derivatives (artemisinin, artesunate, artemether, arteether, dihydroartemisinin), and (iv) hydroxynapthoquinone (atovaquone) (Na-Bangchangb and Karbwang, 2009).

The first-line treatment of uncomplicated *P. falciparum* malaria is artemether lumefantrine administered 2 times a day for 3 days. For infants less than five kg of body weight and pregnant women, oral quinine 8mg/kg administered 3 times a day for 7 days is the first line treatment (for the treatment of malaria caused by *P. vivax*, *P. malariae* or *P. ovale*, the drug of choice is chloroquine). In malaria-free areas and where compliance can be insured, in order to eliminate hypnozoite forms (relapsing stages) of *P.vivax* from the liver and to bring about radical cure, primaquine may be administered daily for 14 days starting after chloroquine treatment is completed. However, in malarious areas where there is a high risk of re-infection, and where the main purpose of treatment is to bring about clinical cure rather than radical cure, administration of primaquine is therefore, not recommended (Gezahegn , 2004).Second-line treatment is oral quinine if condition of the patient permits. Otherwise intravenous (IV) or intramuscular (IM)

administration of quinine should be given (Gezahegn, 2004). In treatment of severe malaria, supportive therapy in reducing hyperpyrexia, controlling convulsions, maintaining fluid balance and correcting hypoglycaemia should be done (Sean and Sweetman, 2005).

Along with these measures for management of other complications in severe malaria, loading dose of intramuscular or slow intravenous quinine should be used. Maintenance dose should be followed twelve hours after the start of the loading dose until the patient can take oral medication. Artemether-lumefantrine or oral quinine could be administered if intramuscular or slow intravenous quinine is not available (Gezahegn , 2004). Radical treatment and curative treatment comprise main aspects of treatment. Various pharmacological options available for this purpose are chloroquine, mefloquine, quinine, primaquine, pyremethamine, artemisinin derivatives like artesunate, artemether, arteether and amino alcohols like lumefantrine and halofantrine along with tetracycline, doxycyclines and sulfadoxime etc (Bahekar and Kale , 2013).

2.9. Antimalarial resistance

Parasite resistance to antimalarial medicines is a major threat to achieving malaria control and eventual elimination. Antimalarial resistance in *P. falciparum* parasites results in an enormous public health and economic burden (Stainesl and Krishna, 2012). Antimalarial resistance spreads when parasites are exposed to the selective window of drug concentrations that are sufficient to kill sensitive but not resistant parasites. Drugs with longer terminal elimination half-lives have the advantage of providing a longer post-treatment prophylactic effect, which appears to be important for their action in

intermittent preventive therapy (IPT) in high-risk groups such as pregnant women, infants and young children. However, these long acting antimalarials have the disadvantage of residual concentrations inhibiting sensitive parasites far longer than resistant parasites, thus fuelling the spread of resistance. The window of selection is prolonged with an increase in resistance or in the terminal elimination half-life (unless these terminal concentration are too low even to kill sensitive parasites) (Stainesl and Krishna, 2012). Antimalarial resistance spreads because gametocyte carriage and infectivity to mosquitoes is consistently higher in patients infected with drug-resistant compared with drug-sensitive parasites. An increase in gametocyte numbers has been identified as the first indication that an antimalarial is beginning to fail and emphasises the need for the treatment policy implemented to include drugs that will kill the sexual stages. Combining antimalarials with differing modes of action is expected to reduce the probability of a resistant (mutant) parasite surviving treatment. Despite their mismatched elimination half-lives, ACTs are preferred to other combination therapies given their potential to reduce malaria transmission – due to their rapid clearance of asexual parasites together with their partial gametocidal activity (Stainesl and Krishna, 2012). Some of the selected antimalarial drugs and its mechanisms of resitance described as depicted in table 1.

Table 1. Selected antimalarial drugs and its mechanisms of resistance.

Drug	**Mechanism of Resistance**
Chloroquine	Decreased intraparasite accumulation of chloroquine. Probably predominantly mediated by mutations in PfCRT, a transmembrane protein in the parasite phagolysosome, resulting in increased efflux of chloroquine from the lysosome (Warhurst, 2001)). Mutations in multidrug resistance (MDR) P-glycoprotein pumps (Pgh) encoded by Pfmdr1and Pfmdr2may also contribute Warhurst, 2001).
Mefloquine, halofantrine, quinine	Mefloquine and halofantrine resistance may be related to amplification of Pfmdr1, increased expression of Pgh1, mutations in pfcrt, and increased efflux of drug (Milhous and Kyle.,1998). The details of quinine resistance have not been established, although quinine and mefloquine resistance often correlate (White, 1998).
Cycloguanil, chlorcycloguanil, pyrimethamine	Resistance mediated through point mutations in dihydrofolate Reductase (dhfr) and dihydrofolate Synthase (dhps) genes, although more efficient use of available folate may also contribute (White, 1998).
Sulfonamides and Sulfones (sulfadoxine, dapsone)	
Atovaquone	Point mutations in mitochondrially encoded cytochrome b gene (Srivastava, 1999).

2.9.1. Determinants of drug resistance and treatment failure

Clinically relevant resistance to antimalarial drugs emerges primarily through increases in the prevalence of resistance-conferring gene mutations in an environment of selective drug pressure. Parasites survive the presence of drugs through mutation. Mutations occur at random because of replication errors. Many of those mutations are lethal and the parasite will die. Occasionally, a mutation will confer on a parasite a survival advantage when a given drug is present. This parasite, provided it is not cleared by the host's immune system, will proliferate and its progeny carry that mutation. Additional survival advantage could be gained by further mutations. This process generates populations of parasites with different abilities to survive a drug. Over time, the parasite populations with the greatest survival advantage will predominate (Rosenthal, 2001).

The range of naturally occurring drug susceptibilities within a parasite population is a function of the size of the circulating population of parasites (the "biomass" or "parasite burden"); a larger parasite biomass increases the chances for parasite mutation, broadening the distribution of parasite susceptibilities within that population. If a mutation does not compromise other biologic functions or confers a net survival advantage in the presence of a given drug, the possibility exists, provided the parasite carrying that mutation escapes the host's immune defenses, that the parasites with the mutation will be selected for and transmitted (Rosenthal, 2001).

2.9.2. Factors involved in the generation of drug resistance

2.9.2.1. Pharmaco-biological factors

Pharmacological factors that influence the rate of development of resistance include the drug's pharmacokinetic and pharmacodynamic characteristics, as well as the drug's intrinsic propensity to generate resistance. Drugs with a long residence time in the organism and a slow rate of reduction of the parasite "biomass" are more vulnerable to resistance. Equally vulnerable are drugs against which resistance develops through single-point mutations in the target molecule (also referred to as single nucleotide polymorphism (SNP), such as the antifolates and atovaquone. By contrast, the quinolines (chloroquine [CQ], quinine) have enjoyed a longer therapeutic life-span because resistance to these drugs is apparently multigenic-the greater the number of genes involved, the slower resistance will evolve (Rosenthal, 2001).

2.9.2.2. Epidemiological factors

One determinant of resistance development is the self-fertilization that occurs between male and female gametocytes when they are picked up by a mosquito during a blood meal. Such reassortment determines the mutations that are carried by the ensuing generation of parasites that are transmitted to the next individual(s). In this process, the intensity of transmission plays a critical, yet undetermined, role, which is principally related to the number of parasite clones carried by each individual (Rosenthal, 2001).

The intensity of transmission may influence resistance indirectly, via mechanisms such as immunity and drug use. Intense, continuous transmission favors the early acquisition of

immunity, at the cost of increased mortality. This should, in theory, confine use of antimalarial drugs-thus limiting the ensuing selection pressure on parasites-to the younger age groups (primarily, children under 5 yr). In practice, this is not always true, and antimalarials are often used by all age groups to treat fever. Additionally, high rates of transmission increase the probability that parasites will be exposed to sub therapeutic drug levels, especially drugs with long half-lives. People living in areas where transmission occurs at lower rates have fewer malaria attacks but, because the level of acquired immunity is far less, remain at greater risk of severe malarial illness for their entire lives(Rosenthal, 2001).

2.9.2.3. Operational and behavioral factors

The way that drugs are used by both health care providers and patients plays an important role in determining drugs' useful life-spans. Of particular concern are the manner in which drug policies are formulated and implemented and the extent to which official policy can influence practice: specifically, whether drugs are available only with a physician's prescription or whether they are readily available on the open market; whether antimalarials are prescribed only to patients with a proven malaria infection or whether they are prescribed on the basis of a clinical suspicion alone; the extent to which providers (whether formal or informal) and users of antimalarial drugs adhere to official recommendations; and whether the cost or complexity of the recommended regimen might encourage incomplete dosing (Rosenthal, 2001).

The way that people use antimalarial drugs greatly affects the degree of selective drug pressure; many behaviors result in exposure of parasites to inadequate or sub therapeutic drug levels, which, in turn, facilitate development of resistance. One example is the interplay

between people's perceptions of illness and the likelihood of completing a full treatment course. Because people from many cultures perceive illness in terms of symptoms rather than causes, community perceptions of illness and their beliefs and practices related to treating those symptoms can differ greatly from Western biomedical definitions of malaria-related illness. Once the symptoms are gone, the illness is perceived to be gone as well. Patients' treating themselves with drugs that produce rapid relief of symptoms, such as artemisinin compounds apparently do, may be more likely to stop before the complete regimen is completed, thereby exposing parasites to a sub therapeutic dose (Rosenthal, 2001).

2.9.3. Clinical consequences of drug resistance and treatment failure

2.9.3.1. Reduced treatment efficacy

The most obvious consequence of antimalarial drug resistance is a failure of the drug to produce a rapid and complete cure. This consequence can be manifested in a variety of ways (Rosenthal, 2001).

2.9.3.2. Delayed initial therapeutic response

This is considered by many to be the first sign of resistance. The rate of parasite and fever reduction is influenced not only by parasite susceptibility but also by host factors and the drug's pharamacokinetic and pharmacodynamic characteristics (Rosenthal, 2001).

2.9.3.3. Parasitologic recrudescence and return of clinical symptoms

In some settings, recrudescent infections tend to be clinically silent. Nonetheless, the return of clinical symptoms associated with recrudescent parasitemia is probably the most common clinical consequence of failed treatment (Rosenthal, 2001).

2.9.4. Public health consequences of drug resistance

2.9.4.1. Increases in malaria transmission

At the simplest level, poor therapeutic efficacy fails to remove parasites from infected individuals, thereby maintaining a larger population of parasitemic individuals in a given area and maintaining a larger biomass of parasites contributing to malaria transmission. Drug resistance is also more directly associated with a potential for increased transmission by enhancing gametocyte carriage. This can be a result of longer parasite clearance times, which are associated with increase gametocyte carriage, or increased frequency of recrudescent infections, which are twice as likely to carry gametocytes compared with primary infections (Rosenthal, 2001).

2.9.4.2. Frequency of severe illness

On an individual basis, the primary concern with failed malaria treatment is progression to severe, potentially life-threatening or fatal illness. Crude estimates of the number of clinical attacks of malaria among African children range from 1 to 5 per year; of these, an estimated 2% of these attacks are severe. Malaria morbidity and mortality has nonetheless increased

because of the number of severe malaria cases that develop because of ineffective first-line treatment (Rosenthal, 2001).

2.9.4.3. Mortality rates

Estimates of the impact of effective treatment on the mortality rate of malaria range from a 50-fold decrease in probability of mortality among uncomplicated malaria cases (from about 5% to 0.1%) to a 5-fold decrease in probability of mortality among patients with severe illness (from nearly 100% to 15–20%) (58). As treatment fails, the overall case-fatality rate undoubtedly rises (Rosenthal, 2001).

2.10. Immunity against malaria

During its complex, multi-stage life cycle, the malaria parasite not only expresses a great variety of proteins at different stages, but these proteins also keep changing often. As a result, a natural infection with malaria parasites leads to only a partial and short lived immunity that is unable to protect the individual against a new infection (Doolan *et al*., 2009).

2.10.1. Natural or innate immunity

Natural or innate immunity to malaria is an inherent refractoriness of the host that prevents the establishment of the infection or an immediate inhibitory response against the introduction of the parasite. The innate immunity is naturally present in the host and is not dependent on any previous infection. Alterations in the structure of hemoglobin or in certain enzymes have been found to confer protection against either the infection or its severe manifestations and these traits are often found in areas of high malaria transmission. Duffy

negativity in red cells protects against *P. vivax* infection. Certain thalassemias (50% reduction in infection), homozygte hemoglobin C (90% reduction), hemoglobin E, and ovalocytosis carrier status have been reported to confer protection against *P. falciparum* or *P. vivax*. Glucose 6 phosphate dehydrogenase deficiency (50% protection) and sickle cell hemoglobin (90% protection) confer protection against severe malaria and related mortality (Doolan *et al*., 2009).

2.10.2. Acquired or adaptive immunity

Acquired or adaptive immunity against malaria develops after infection and its protective efficacy varies depending on the characteristics of the host, place of stay, number of infections suffered etc. It has been graded as anti-disease immunity (that protects against clinical disease), anti-parasite immunity (protects against high parasitemia), and sterilizing immunity (protects against new infections by maintaining a low-grade, asymptomatic parasitemia; also called premunition), with a considerable overlap between these. Following infection with malaria parasites, a nonimmune individual commonly develops an acute clinical illness with very low levels of parasitemia and the infection may progress to severe disease and death. After a couple of more infections, anti-disease immunity develops and causes suppression of clinical symptoms even in the presence of heavy parasitemia and also reduces the risk of severe disease. Frequent and multiple infection slowly lead to the development of anti-parasite immunity that results in very low or undetectable parasitemia. Sterilizing immunity, though never fully achieved, results in a high degree of immune responsiveness, low levels of parasitemia, and an asymptomatic carrier status. Premunition

suggests an immunity mediated directly by the presence of the parasites themselves and not as much the result of previous infections (Doolan *et al*., 2009).

The acquisition of immunity against malaria is, therefore, very slow and not very effective and remains species specific and strain specific. People living in unstable endemic areas tend to acquire only partial immunity (Doolan *et al*., 2009). The acquired anti malaria immunity does not last long. In the absence of re-infection for about 6 months or 1 year, as may happen when the person leaves the malarious area, the acquired immunity turns ineffective and the individual becomes vulnerable to the full impact of a malarial infection once again (Doolan *et al*., 2009).

2.11. Prevention of malaria

The current approach to manage the disease includes vector control to disrupt transmission from mosquito to human, prevention of infection and treatment after infection (Tripathi *et al*., 2005). Prevention of the disease includes vector control to disrupt transmission from mosquito to human which can be achieved using insecticide treated nets and genetically engineered mosquitoes (Tripathi *et al*., 2005;). But nowadays high insecticide resistance resulting from insensitive acetyl cholinesterase (AchE) has emerged in mosquitoes (Weill *et al.,* 2004).

2.11.1. Avoiding the bite

The best way to avoid getting bitten by a mosquito is to avoid mosquitoes entirely. Mosquitoes are most active at dusk and dawn, so avoiding outdoor activity at those times limits exposure to the insect. Wearing protective clothing, particularly light-colored clothing

that radiates infrared light much less than darker clothes, helps to avoid a mosquito's bite (Marcus, 2009).

2.11.2. Eliminating breeding grounds

Other means of avoiding mosquitoes involve limiting their opportunities to breed and find you when you are vulnerable. Mosquitoes can also breed in swimming pools, birdbaths, fountains, animal watering troughs, roof gutters, and even in carelessly discarded cans and beverage containers. Denying mosquito's access to such objects, by keeping them dry whenever possible, or removing litter where water can accumulate, limits the opportunities of mosquitoes to reproduce. In cases where water must stand, as in swimming pools, chemicals such as chlorine can be added to the water to kill mosquito larvae. In some cases, mosquito fish (*Gambusia affinis*) can be introduced into water (Marcus, 2009).

2.11.3. Using protective barriers

Protective barriers are anything that physically blocks a mosquito's access to its prey. By reducing the number of mosquitoes that enter a house, window screens do reduce the risk of malaria to the people inside it. Another protective barrier that can work quite effectively is bed netting. Bed netting can be even more effective when impregnated with insect repellents or, better still, insecticide. In Ethiopia, in particular, the Carter Center has been providing people with bed netting impregnated with pyrethrum, a natural insecticide extracted from chrysanthemums (Marcus, 2009).

2.11.4. Using repellents

Certain areas such as deep woods or swamps have mosquito populations that may be so dense and/or hungry that the mosquitos may be active at all hours, including in bright sunlight. Moreover, there are times when people have to be out at dusk or dawn. In such cases, chemical mosquito repellents can be used to prevent bites. The repellent makes the person wearing it seem too unappealing to bite (Marcus, 2009).

2.11.5. Using chemoprophylaxis

Antimalarial drugs which act at different stages of malaria parasite can be used for prophylaxis or treatment of malaria. They are classified as blood schizonticides, tissue schizonticides and gametocides (Michel *et al*., 2002). Mass chemoprophylaxis cannot be done for all people in malaria endemic areas. However, it can be done for those at high risk of malaria like children and pregnant women, particularly primigravidae, though costly (*Kathryn et al*., 2004 and Sean and Sweetman, 2005). Special risk group exposed to malaria such as long term travelers, children, pregnant women, aircrew, migrants to visit malarious areas need prophylaxis (Shanks and Edestein, 2005).

Malaria related morbidity and mortality can be reduced in children less than 5 years of age by either intermittent or continuous chemoprophylaxis. Chemoprophylaxis during pregnancy increases infant birth weight and survival, although this effect is largely limited to primi gravidae. Travelers to malaria endemic areas are also recommended to take drugs to prevent malaria (Kathryn *et al*., 2004). Chemoprophylaxis should be started 2 weeks before departure and continued for four weeks after return from the malarious area. For non-immune travelers visiting malarious areas for a period of 2- 3 months, weekly mefloquine administered at 5

mg/kg is the recommended drug for chemoprophylaxis (Michel *et al*, 2002; Fairhurst and Wellems, 2006).

2.12. *Vernonia amygdalina:* The experimental plant

Medicinal plants are various plants thought by some to have medicinal properties, but few plants or their phytochemical constituents have been proven by rigorous science or approved by regulatory agencies such as the United States Food and Drug Administration or European Food Safety Authority to have medicinal effects. A medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for the therapeutic purposes or which are precursors for the synthesis of useful drugs (Audu *et al*., 2012).

2.12.1. Brief description

V. amygdalina Del, commonly called bitter leaf in English and 'Girawa' in Amharic, is a perennial shrub of 2-5m in height that grows throughout tropical Africa. It has a rough bark with dense black straits, and elliptic leaves that are about 6 mm in length. The leaves are green and have a characteristic odor and bitter taste (Ijeh and Ejike, 2011).

Figure 2. Picture of *V. amygdalina* leaf

2.12.2. Scientific classification of Vernonia amygdalina

Kingdom: Plantae

Division: Angiosperms

Order: Asterales

Family: Asteraceae

Genus: Vernonia

Species: *V. amygdalina*

Botanical Name: *Vernonia amygdalina* (Audu *et al*., 2012).

2.12.3. Habitat and distribution

Vernonia amygdalina (Del.) commonly called bitter leaf is the most widely cultivated species of the genus Vernonia which has about 1,000 species of shrubs (Munaya, 2013).It is grown in many countries, in savannah zones and cultivated. Although most popularly used for food, it has also, been traditionally used for its medicinal properties. True to its name, bitter leaf is bitter to taste but surprisingly delicious in meals (Abosi and Raseroka, 2003). It is found in wide range of bush land, wood land and forest habitat 1200-3000 mean above sea level (masl) in Bale, Wollo, Gondar, Gojam, Wellega, Shewa, Illubabor, Kefa, Hararge and Gamo Gofa floristic regions (Mesfin, 2004).

2.12.4. Medicinal uses

V. amygdalina Del. is probably the most used medicinal plant in the genus Vernonia (Erasto *et al*., 2006). The observation that an apparently sick wild chimpanzee chewed *V. amygdalina* Del. and seemed to return to normal activity after a while. Traditional

medicine, practitioners use the plant as an anti-helminth, anti-malarial, and as a laxative. Others use it as a digestive tonic, appetizer, febrifuge and for the topical treatment of wounds (Ijeh and Ejike,2011).

V. amygdalina Del. has antibacterial, antiplasmodial/antimalarial, amoebicidal, antifungal, antileishmanial, antischistosomial, wound management, venereal disease management, anti-cancer/tumor, antioxidant, hypoglycemic/antidiabetic, hepatoprotection, nephroprotection, serum lipid modulation, gastric secretion, analgesic, anti-fertility and insecticidal properties (Ijeh and Ejike,2011) . Its Leaf decoction is used to treat fever, malaria, diarrhea, dysentery, hepatitis and cough as a laxative and as fertility inducer. They are also used as medicine for scabies, headache, and stomach ache. traditional Root extract are also use as treatment against malaria and gastrointestinal disorders. It is also useful as a control agent against disease in plant. The ash from burnt branches is used to control seed-borne fungi (Aspergillus, Fusarium and Penicillium spp) (Odeh and Usman, 2014).

In Ethiopia *Vernonia amygdalina* is used for the treatment of internal worms, stomach ache, malaria and 'mich' in Amaro Woreda (Fisseha *et al*., 2014), malaria, ascarisis and around in Hawassa city, southern Ethiopia (Reta, 2013), flariasis and ascariasis (Tolosa, 2007). Elsewhere in Ethiopia used against menstruation pain, as purgative and vermifuge, in wound dressing and against urinary inflammations and against malaria, evil eye and diarrhoea (Debela, 2001).

2.12.4.1. Antimalarial properties of *V. amygdalina Del.*

Malaria is said to be responsible for approximately one million infant deaths every year in sub-Saharan Africa (Abosi and Raseroka, 2003). What is worrisome is that the parasite is becoming resistant to a number of the current drugs for malaria treatment available in the market. Abosi and Raseroka (2003) reported that the ethanolic extract of the leaves and root-bark of VA suppressed parasitemia (induced by inoculation with *Plasmodium berghei*) in mice by 67% and 54%, respectively in four days. The aqueous extract of the leaves of the plant has also been shown to reduce the load of *P. berghei* in mice by 73% when given intraperitoneally for 4 days (Njan *et al*., 2008). It is thought that the flavonoids, saponins and alkaloids (Sayed *et al*., 1987) and sesquiterpene and steroidal constituents (Phillipson *et al*., 1993) are responsible for the antiplasmodial properties of VA (Ijeh and Ejike,2011).

The aqueous extract of *Vernonia amygdalina* leaves exhibit antimalarial activity on *Plasmodium falcifarum, Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae* even though some of these strains are resistant to conventional antimalarial drugs, they were susceptible to this plant especially at higher concentration (Odeh and Usman , 2014). So, in traditional practices ,*Vernonia amygdalina* (bitter leaf) is used in the management of parasitic infection most importantly, Malaria fever which is the most rampant of the parasitic infection(Odeh and Usman , 2014).

The ethanol, petroleum ether, dichloromethane, ethyl acetate, acetone-water and isoamyl alcohol extracts of *V. amygdalina*, showed antimalarial activity against *Plasmodium falciparum* (Dd2) *in vitro* (Masaba, 2000; Tona *et al*., 2004). The root

extract of *V. amygdalina* displayed mild activity against chloroquine-sensitive *P. falciparum* with IC_{50} of 19 µg/ml but no activity against the chloroquine-resistant strain.

This antimalarial effect of *V. amygdalina* is contributed by its active compounds, or more specifically sesquiterpene lactones such as vernolepin, vernolin, vernolide, vernodalin and hydroxyvernodalin which exhibited antiplasmodial activity of IC_{50} value lower than 4 µg/ml (Tona *et al*., 2004). Besides, Masaba (2000) discovered that acetone-water extract from *V. amygdalina* leaves showed lower IC_{50} value (25.5 µg/ml against *P. falciparum* than water extract (76.7 µg/ml) after 48h.

Furthermore, Iwalokun (2008) showed that this aqueous leaves extract (62.5, 125 mg/kg) was able to work synergistically with chloroquine (5 and 30 mg/kg) against both chloroquine-sensitive and resistant *P. berghei* to shorten the parasite clearance time, prolong the recrudescent times and improve curing rate. The study has also suggested that administration of *V. amygdalina* ethanol extract 1 h prior to chloroquine intake can avoid the reduction in chloroquine bioavailability (Igboasoiyia *et al*., 2008).

2.12.5. Phytochemical constituents of V. amygdalina Del.

A wide array of phytochemicals (including anti-nutritional factors) has been shown to be present in *V. amygdalina*. A summary of the phytochemicals present in *V. amygdalina*. Stigmastane-type saponins such as vernoniosides A1, A2, A3 (Jisaka *et al*., 1992), A4, B2, B3 (Jisaka *et al*., 1993a), C, D and E (Ohigashi, 1994) have been shown to be present in the leaves. The A-series saponins have been shown to be responsible for the bitter taste of *V.*

amygdalina. Other steroidal saponins have been identified in the plant. Sesquiterpene lactones are another class of phytochemicals found abundantly in the leaves of *V. amygdalina*. Some of the identified Sesquiterpene lactones are vernolide, vernodalol,vernolepin, vernodalin and hydroxyvernolide flavonoids luteolin, luteolin 7-O-β-glucoroniside and luteolin 7-O-β-glucoside are found in the leaves of *V. amygdalina*. Other researchers have confirmed the presence of flavonoids in the plant. Other phytochemicals present in the leaves of *V. amygdalina* are terpenes, coumarins, phenolic acids, lignans, xanthones and anthraquinones (Ijeh and Ejike, 2011).

2.12.6. Toxicity and Safety of V. amygdalina

Toxicology studies had been carried out via both *in vitro* and *in vivo* systems on various subjects to confirm the toxicity of *V. amygdalina*. As indicated in Table 2, *V. amygdalina* only induced mild toxic effect when administrated at very high concentration. More importantly, safe consumption dosage needs to be identified for women at different stages or vitality of pregnancy, to avoid abortion since it may induce uterine contraction (Yeap *et al*., 2010).

Table 2. *In vitro* and *in vivo* toxicity of *V. amygdalina*.

Animal	Extract	Route	LD_{50} (mg/kgb.w.)	Remark
Mice	Aqueous (500-2000 mg/kg/day for 14 consecutive days)	Oral	-	No signs of toxicity or adverse toxicological effects at all doses except for decrease of red blood cell count and dose dependent increase of serum bilirubin (Njan *et al*., 2008).
	25% of *V. amygdalina* dry powder (or equivalent amount of ethanol extract or crude/purified saponins) for 2 weeks	Oral	-	Reduction of body and liver weights and increase of urinary and fecal output associated with stomach and small intestines enlargement (Igile *et al*., 1995a).
	Aqueous (62.5 and 125 mg/kg)	Oral		Serum glutamate oxaloacetate transaminase (sGOT),serum glutamate pyruvate tansaminase (sGPT) and lactate dehydrogenase (LDH) level rose around 6-33%. Increase of serum enzyme markers level was more severe when it was consumed with antimalarial drug chloroquine (Iwalokun, 2008).
	Aqueous (87.53 to 92.57g/kg)	Oral	-	No change in organ damage, blood count and liver enzyme profile (AST and ALT) (Amole *et al*., 2006).
	Aqueous (50 and 100 mg/kg)	IP	-	No change of liver function diagnostic enzymes level (total bilirubin, conjugated bilirubin, unconjugated bilirubin,alanine aminotransferase, aspartate aminotransferase and alkaline phoosphatase) (Ojiako and Nwanjo, 2006).
	Ethanol (up to 1000 mg/kg for 1 month)	Oral	-	No change in liver (ALT, AST, ALP, total and conjugated bilirubin) and kidney (creatinine) enzymes marker level (Ekpo *et al*., 2007).
	Cold water (for 24 h)	IP	500 to 1265.22	(Nwanjo, 2005; Ojiako and Nwanjo, 2006).
Rat	Aqueous (250 and 500 mg/kg/day for 5 consecutive days)	Oral	-	Reduction of spermatozoa mobility and viability in dosage dependent mode. Hypoplasia of the seminiferous tubules was also observed in the treated rats. (Oyeyemi et al.,2008).
	Aqueous extract of *V.amygdalina*, *Ocimum gratissimum* and *Gongronema latifolia* (ratio 1:1:1 at 16 g/kg b.w. (p.o) and 2.5 g/kg b.w. (i.p.)	Oral,IP	-	No significant change in general behaviour (Iroanya *et al*.,2010).
	Methanol (50, 100 and 200 mg/kg)	Oral		Diarrhea and abortion (Awe *et al*., 1999) due to cathartic effect through weak contractile effect on smooth muscle.

	Powder in standard food (25-75%)	Oral		Skin of the rat turned lighter without alteration of tissues architecture and cellular morphology. (Ibrahim et al., 2001).
Rabbit	Aqueous	IP	1112	(Akah and Okafor, 2006)
	Aqueous (0.3 mg/ml)	injection		Increase of uterine, intestine and jejunum contraction which sustained for 30 minutes with elevated concentrations used (Caiment-Leblond, 1957; Kamatenesi-Mugisha *et al*., 2005).
Guinea pig	Aqueous (10 and 100 mg/kg)	oral		Increase of uterine and mammary gland contraction amplitudes and thus increase milk production and help in infant's delivery (Ijeh et al., 2008). This supports the traditional use of *V. amygdalina* as an oxytocic plant in assistance of child birth traditionally. (Ganfon *et al.,* 2008)
Murine macrophages J774	Lipophilic extract	*In vitro*	IC50 6.48 µg/ml	
Allium cepa root tip	Cold water extract	*In vitro*	1% after 8 h of incubation	Induced mitodepressive effect. Higher concentration of the extract or extensive incubation caused sticky effect on chromosomes during cell division and protein denaturation lead to nuclear disintegration and cell death. Presence of sesquiterpene lactones were suggested as the major contributors to this effect (Ene-Obong and Amadi, 1987).

Generally, *V. amgydalina* is safe to consume and is good for health unless it is consumed in very large quantities and the potential danger of taking this plant is much lower than that of other common vegetables (Ojiako and Nwanjo, 2006). *V. amygdalina* may cause adverse effect over the male reproductive system without controlled regimen (Yeap *et al*., 2010).

2.13. Rationale of the study

Compounds effective in *in vitro* screening tests (i.e. those with IC_{50} < 1 µM) are taken up for *in vivo* evaluation (Kalra *et al*., 2006) (figure 4). *In vivo* screening of antimalarial compounds Plasmodium species that cause human disease are essentially unable to infect non-primate animal models (with the exception of a complex immunocompromised mouse model that has been developed to sustain *P. falciparum*-parasitized human erythrocytes *in vivo*. So, *in vivo* evaluation of antimalarial compounds typically begins with the use of rodent malaria parasites. Of these, *P. berghei* has been used extensively in drug discovery and early development (Fidock *et al*., 2004) (figure 3).

Rodent models have been validated through the identification of several antimalarials for example, mefloquine, halofantrine and more recently artemisinin derivatives. In view of their proven use in the prediction of treatment outcomes for human infections, these models remain a standard part of the drug discovery and development pathway. Individual species and strains have been well characterized, including duration of cycle, time of schizogony, synchronicity, drug sensitivity and course of infection in genetically defined mouse strains (Fidock *et al*., 2004).

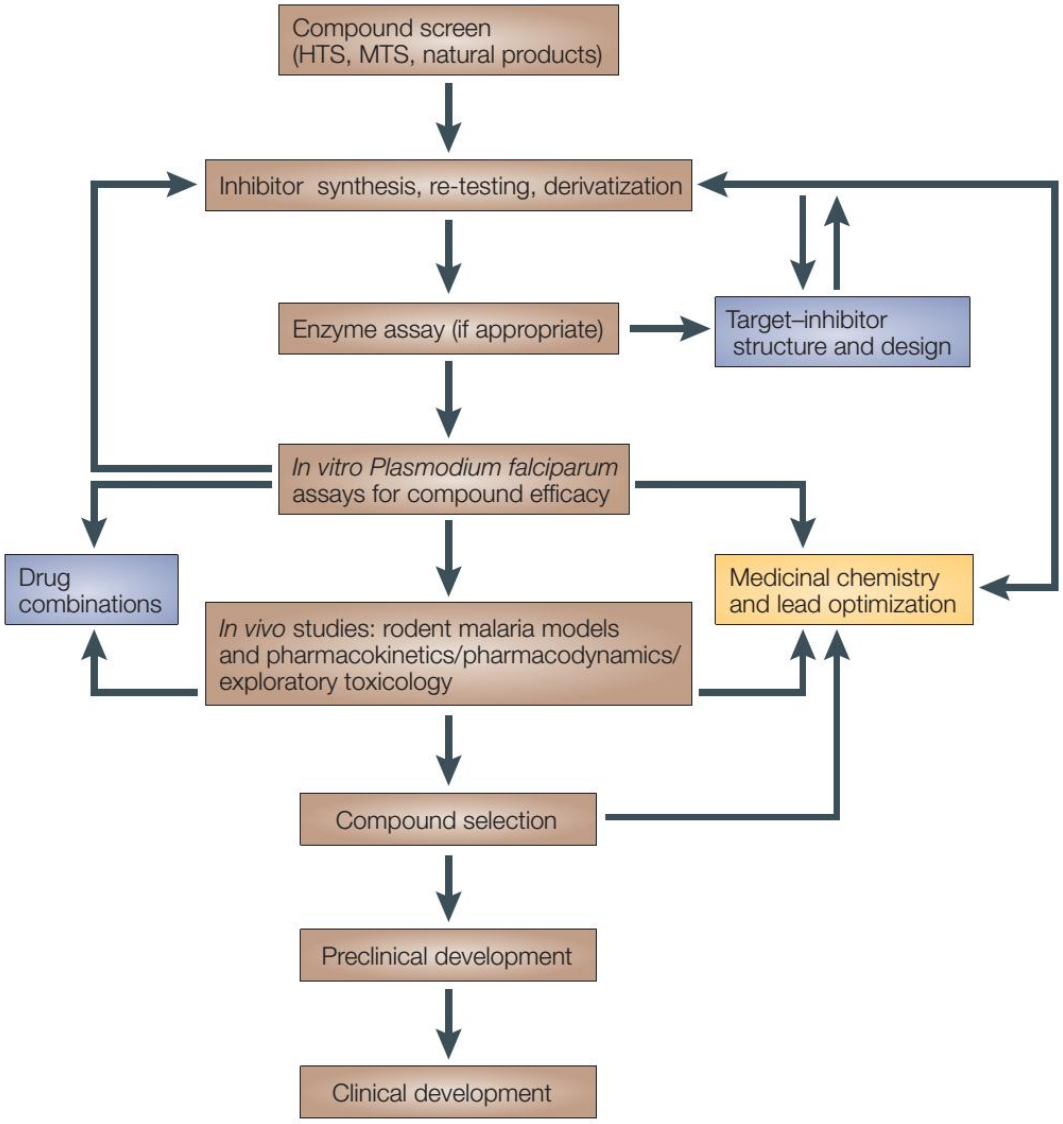


Figure 3. Example of a critical path for antimalarial drug discovery(Fidock *et al*., 2004).

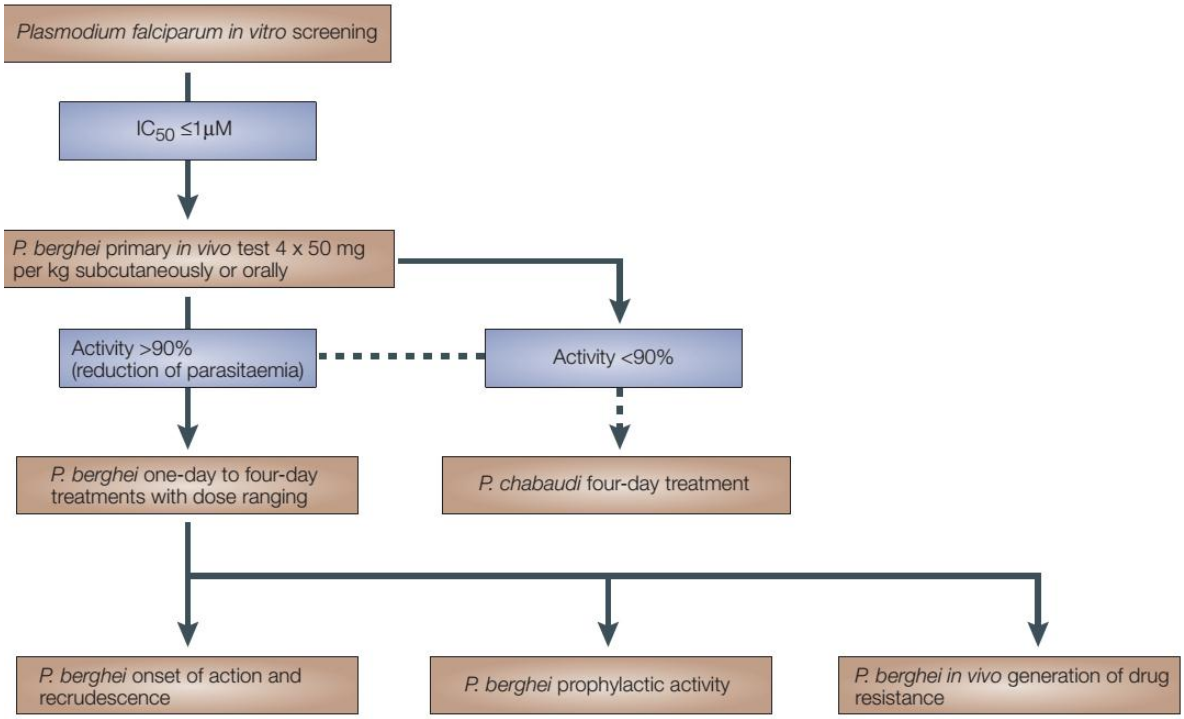


Figure 4. Flow chart of one scenario for *in vivo* screening for antimalarial activity in rodent malaria models (Fidock *et al*., 2004).

In vitro and *in vivo* studies are directed towards compound selection. Medicinal chemistry and lead optimization constitute an essential and iterative component of this part of the critical path (figure 3). Secondary *in vivo* tests, not a requisite component of a critical path but useful for detailed compound evaluation, include dose-ranging, onset of activity and recrudescence, prophylaxis, and screening for drug resistance (Fidock *et al*., 2004). Moreover, the *in vivo* test most closely reflects actual clinical situation that is the therapeutic response to the drug of currently circulating parasites infecting the actual population in which the drug will be used. This approach offers the best information on the efficacy of antimalarial drugs. Therefore,this enables evaluation of compound efficacy against the correct enzymatic target in an *in vivo* setting.

2.14. Statement of the problems

Of the approximately 3.4 billion people worldwide who are exposed annually, 1.2 billion are at high risk (WHO, 2013). Apart from taking a huge number of lives every year, malaria has lifelong effects on cognitive development and education levels through the impact of chronic malaria-induced anemia and time lost or wasted in the classroom due to illness (John and Jeffrey, 2001). Malaria also contributes significantly to anemia in children and pregnant women, adverse birth outcomes such as spontaneous abortion, stillbirth, premature delivery and low birth weight, and overall child mortality. The disease contributes approximately 1.3% annual reduction of in economic growth in the region (WHO, 2005). In addition, the changing clinical manifestations with multi-organ involvement in *P. falciparum*, emerging trends of complications in *P. vivax* malaria, and burden of malaria in pregnancy are other important issues that merit attention and formulation of suitable intervention strategies (Ashwani, 2007).

The most important problem associated with the management of malaria is that the parasites which cause malaria are resistant to or are developing resistance to the most widely available, affordable and safest first line treatments such as chloroquine and sulphadoxine-pyrimethamine (Sendagire *et al*., 2005). Secondly, the overall control of the mosquitoes which transmits malaria is made difficult by their resistance to a wide range of insecticides. The third, which is a new and rapidly developing problem, is the widespread production of fake antimalarial drugs. For example, in mainland Southeast Asia 38% and 53% of “artesunate” blister packs sampled contained no active ingredient

(Newton *et al*., 2006). Owing to the widespread suffering and death caused by malaria and the failure of the safest and most affordable antimalarials to treat the disease because of drug resistance, there is an urgent need to develop new drugs or vaccines for the treatment, management, prevention and control of malaria (Waako *et al*., 2005).

Selection of candidate drugs for clinical trials in man and the design of clinical protocols are based upon consideration of data from a battery of preclinical test systems.Hence, the search for new drugs which are effective against all strains of plasmodium and that act at all stages of the parasite is the only choice left without option to fight the war opened by this disease on human beings and rescue the millions of lives lost to malaria every year.

2.15. Significance of the study

In Ethiopia up to 80% of the population uses traditional medicine due to the cultural acceptability of healers and local pharmacopeias, the relatively low cost of traditional medicine and difficult access to modern health facilities. The vast majority of Ethiopia's population lives in rural areas where the health care coverage is low and where existing public sector resources are being stretched to the limits (Deribe *et al*., 2006; Kassaye *et al.*, 2006).

The rapid spread of resistance parasite to existing drugs, the limitation of vaccine and vector control makes it necessary to search for more effective herbal antimalarial agents (Rosenthal, 1998).Since there are so many potential antimalarial medicinal plants in the country, the antimalarial traditional plants should be screened for their antiplasmodial activity scientifically both *in vitro* and *in vivo*. The phytochemical constituents of those active plants must be screened to predict which chemical is responsible for their

antimalarial activity and toxicological assessments and standardizations should be done for traditionally accepted and potentially active antimalarial remedies.

Previous study done by Abosi *and Raseroka,* 2003 demonstrated the *in vivo* antimalarial activity of ethanolic leaf extract of *V. amygdalina* produced 67% suppression of parasitaemia in the four day test. 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). The aqueous extract of *Vernonia amygdalina* leaves exhibit antimalarial activity on *P. falcifarum, P. vivax, P. ovale* and *P. malariae* even though some of these strains are resistant to conventional antimalarial drugs, they were susceptible to this herbal plant especially at higher concentration Odeh and and Usman, 2014). The *in vivo* ethanol and aqueous extract of the *V. amygdalina* leaves demonstrated proven antimalarial activities but there were no prior efforts at isolating the pure compounds responsible for the observed effects. Hence, as bioassay guided fractionation is one research approach where an active lead compound could be developed, this study was attempt to determine which fraction(s) of the crude extract responsible for antimalarial activity of the plant. Furthermore, knowing the *in vivo* antimalarial activities of solvent fractions of the leaf of *V. amygdalina* can be a part of an initiative to document baseline data for future pharmacological studies. The study was aimed to provide sound evidence that encourages the traditional use of the plant by communities which live in areas where modern health facilities did not reach due to geographic and socio-demographic challenges.

2.16. Hypothesis

The crude extracts of *V. amygdalina* have antimalarial activity. The active phytoconstituents of *V. amygdalina* could be used for the management of malaria.

3. OBJECTIVES OF THE STUDY

3.1. General objective

- ✈ The general objective of the study was to evaluate the antimalarial activity of solvent fractions of the leaf of *Vernonia amygdalina* against *P. berghei* infection in mice.

3.2. Specific objectives

- ♣ To determine acute toxicity effect of each fractions of *Vernonia amygdalina* in mice.
- ♣ To evaluate *in vivo* antimalarial activity of solvent fractions of *Vernonia amygdalina* against *P. berghei* in mice on percentage of parasitemia, mean survival time, rectal temperature, body weight and PCV using four day suppressive test.
- ♣ To perform preliminary phytochemical screening of solvent fractions of the leaves of *Vernonia amygdalina.*

4. MATERIALS AND METHODS

4.1.Materials

4.1.1. Chemicals, reagents and drugs

The following chemicals, reagents, and drugs were used in the present study: absolute methanol (Cheshire, UK), chloroform, (British Drug Houses (BDH), Poole, England), butanol (BDH, Poole, England), normal saline, 2 % lead acetate,10% ammonia solution, ethyl ether(BDH chemicals Ltd, England), Geimsa stain (BDH Chemicals Ltd, England) 10% at pH 7.2, Trisodium citrate (BDH chemicals Ltd, England), Tween 80 (BDH Laboratory Supplies, England), Dragendrof's reagent. Mayer's reagent, 10% ethanolic ferric chloride, 2% lead acetate, concentrated sulfuric acid, glacial acetic acid, 5% ethanolic ferric chloride, sodium chloride, 1% gelatin and standard chloroquine (Ethiopian Pharmaceutical Manufacturing, Addis Ababa, Ethiopia). All the chemicals were analytical grade.

4.1.2. Parasites

Chloroquine- sensitive *Plasmodium berghei* was obtained from Ethiopian Public Health Institute (EPHI) and was used for the experiment. Chloroquine sensitive *P. berghei* strain was maintained at Pharmacology animal house, School of Medicine, College of Health Sciences (CHS), Addis Ababa University (AAU) by subsequent passage of blood from infected mouse to healthy mouse every 5 days.

4.1.3. Collection of plant materials

Fresh leaves of *V. amygdalina* were collected in January 2015 from Kombolcha, South Wollo, Amhara Regional State, 376 North/East of Addis Ababa. It has a latitude and longitude of 11°5′N 39°44′E with an elevation between 1842 and 1915 meters above sea level. The leaves were identified as *V. amygdalina* by a taxonomist and its specimen plant was deposited with Voucher No. TB001 at National Herbarium, College of Natural Sciences, Addis Ababa University for future reference.

3.1.4. Experimental mice

Male Swiss albino mice (age 6-8 weeks and weight of 22-30g) were obtained from Ethiopian Public Health Institute and used in the study. The mice were kept at room temperature (20^0C) in relative humidity (50-70%) and were exposed to 12 hours light and 12 hours darkness in the animal house of Pharmacology Department of School of Medicine, (CHS), AAU under standard environmental conditions.The mice were allowed free access to food and water *ad libitum* throughout experimental period. Good hygiene was maintained by constant cleaning and removal of feces from cages daily. The mice were maintained and cared for according to the international guidelines for the use and maintenance of experimental animals (OECD, 2001). Animals were acclimatized for one week to the experimental environment. The mice were allowed to acclimatize to the laboratory environment under a room temperature of 20^0C and at optimum (50-70%) relative humidity for three days before being used for the experiment (Dikaasso *et al*., 2006).

4.2. Methods

4.2.1. Crude extract preparation

The collected fresh plant leaves were brought to Pharmacology laboratory, School of Medicine, CHS, AAU where the extraction was conducted. The leaves were cleaned with tap water and air dried under shade at room temperature for two weeks and coarsely powdered using a grinding mill. The powdered leaves were packed in plastic bag and stored at ambient temperature until extraction. A total of 900 gm of coarsely powdered leaves were weighed by sensitive digital weighing balance (Mettler Toledo, Switzerland) and extracted by cold maceration technique with 80% methanol (200g in 1600 ml) i.e in the ratio of 1:8 in Erlenmeyer flask and 500gm powdered leaves macerated with distilled water for 72 hrs at room temperature (figure 5). The extraction process was facilitated using shaker (Bibby Scientific Limited Stone Staffo Reshire, UK) at 120 rpm. After 72 hrs the extract was first filtered using gauze from the marc (the residue left after the extraction) and then further filtered by Whatman filter paper No.1 with pore size 150mm diameter (Wagtech international Ltd, England).

The marc was macerated twice using the same volume of 80% methanol and distilled water to exhaustively extract the plant material. After exhaustive extraction, the hydroalcohol (methanol 80%) was removed by evaporation under reduced pressure by rotary evaporator (Buchi Rota vapor, Switzerland) in distillation flask at 80 revolutions per minute (rpm) and 40^0C to obtain the crude extract of plant. The extract was further concentrated to dryness by freeze drying using lypholizer (Operon, Korea vacuum limited, Korea) and a total of 128.5gm (14.23%) was obtained. Whereas the aqueous

extract, the filtrate was frozen in deep freezer overnight and then freeze dried using lypholizer (Operon, Korea vacuum limited, Korea) and a total of 51gm (10.2%) was obtained. All the extracts were stored in screw cap vials in refrigerator at -20^{0}C until used (Innocent *et al*., 2009; Tiwari *et al*., 2011).

The portion of the crude extracts were used to evaluate dose response toxicity and for confirmation of the antimalarial activity in *in vivo* model (four day supresive test) and the remaining extract was subjected to chloroform, butanol and aqueous fractionation. The water and hydroalcoholic extracts were dissolved in distilled water for use in the tests.

4.2.2. Solvent fractionation of V. amygdalina hydroalcoholic extract

Hydroalcoholic crude extract of *V. amygdalina* had better antiplasmodial activity and was subjected further to fractionation using chloroform, butanol and aqueous solvent technique (figure 5). A total of 90gm of 80% methanol crude extract of *V. amygdalina* was dissolved using separatory funnel in 350 ml distilled water. The dissolved hydroalcoholic extract was partitioned with 3 ×240 ml chloroform. The filtrate was concentrated in a rotary evaporator (Buchi Rota vapor, Switzerland at 80 rpm and 40^{0}C to obtain chloroform fraction and 20.29 grams (22.54%) was obtained. The aqueous residue was further partitioned with 3x 240ml n-butanol. The butanol filtrate was concentrated similarly as chloroform fraction to have butanol fraction and 14gm (15.6%) was obtained. The remaining aqueous residue was frozen in deep freezer overnight and then freeze dried with a lyophilizer (Operon, Korea vacuum limited, Korea) and a total of 26gm (28.9%) of aqueous fraction was obtained. All fractions were kept in tightly closed containers in refrigerator at -20^{0}C until used for *in vivo* test. The butanol and aqueous

fractions were dissolved with distilled water whereas chloroform fraction was dissolved in 2% Tween 80 for use in the tests.

4.2.3. Preliminary phytochemical screening

Both extracts and solvent fractions of *V. amygdalina* leaves were screened for the presence of different chemical constituents to relate the antimalarial activity of the plant with the presence or absence of these constituents following standard procedure (Trease and Evans, 1989; Jones and Kinghorn, 2006).

4.2.3.1.Test for phenolic compounds

One ml of test solution was treated with 10% ethanolic ferric chloride and a color change to dark blue is affirmative of a positive test.

4.2.3.2. Test for alkaloids

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

4.2.3.3. Saponins test

For butanol and aqueous fraction: 0.5 g of butanol and aqueous fractions were dissolved in 10 ml of distilled water in a test tube. The test tube was stopped and shaken vigorously for 30 seconds and allowed to stand in a vertical position and observed over 30 min.

Formation of "honey comb" froth over the surface of liquid and persistence after 30 min indicates the presence of saponins (Jones and Kinghorn, 2006; Trease and Evans, 1989).

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

4.2.4. Polyphenols (Phenolic compounds)

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

4.2.5. Test for flavonoids

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

4.2.6. Test for terpenoids

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

4.2.7. Test for steroids

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

Another test was performed by mixing crude extract with 2ml of chloroform. Then 2ml of each of concentrated H_2SO_4 and acetic acid were poured into the mixture. The development of a greenish coloration indicated the presence of steroids (Yadav and Agarwala, 2011).

4.2.8. Test for tannins

About 2 ml of the extract was stirred with 2 ml of distilled water and few drops of Iron (III) Chloride/ Ferric Chloride ($FeCl_3$) solution were added. The formation of green precipitates indicates for the presence of tannins (Njoku and Obi, 2009).

4.2.9. Test for glycosides

4.2.9.1. Keller-Kiliani test

Two ml of each extract was dissolved in 2 ml of glacial acetic acid containing one drop of Iron (III) Chloride/ Ferric Chloride ($FeCl_3$) solution. The mixture was then poured into a test tube containing 1 ml of concentrated H_2SO_4. A brown ring at the interphase indicates the presence of a deoxy sugar, characteristics of cardenolides (Njoku and Obi, 2009).

4.2.10. Test for anthraquinones

One half gm of the extract was boiled with 10 ml of sulphuric acid (H_2SO_4) and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipette into another test tube and 1 ml of dilute ammonia was added. The resulting solution was observed for colour changes.

4.3. *In vivo* antimalarial screening

4.3.1. Acute toxicity test

Limit test was done using twenty five female mice for 80% methanol extract, water extract, CF, BF and AF. The mice were randomly selected, marked to permit individual identification, and kept in their cages for 7 days prior to the start of dosing to allow for acclimatization to the laboratory conditions. The mice were fasted for four hours and individual weights of animals were determined before the test substance was administered and at least weekly thereafter. Oral administration of a single dose of 2000mg/kg substance was given for one female mouse and signs of toxicity were observed for 24 hours was observed. Further four female mice were used at the dose level of 2000mg/kg and each mouse was observed for 14 days to assess safety of the extracts and solvent fractions. Acute toxicity signs were observed for gross changes such as loss of appetite, hair erection, lacrimation, tremors, convulsions, salivation, diarrhoea, mortality and other signs of overt toxicity **(**OECD, 2001**;** Dikasso *et al*., 2006). After acute toxicity test, three dose levels were chosen for each extracts: the middle dose, which is one-tenth of the maximum dose during acute toxicity study, a low dose, which is half of the middle dose, and a high dose which is twice of the middle dose.

4.3.2. Preliminary test for the crude extracts

Preliminary test of the crude extract was performed using 60 mice. Mice were grouped into five groups, six mice per group and inoculated with 0.2ml of blood infected by *P. berghei* intraperitoneally. In such a way, group I received 0.2ml distilled water, group II-IV treated with extract at doses of 200 mg/kg, 400 mg/kg and 600 mg/kg while group V treated with chloroquine 25mg/kg. Once it was ascertained that the crude extracts indeed had a biological activity as demonstrated in earlier studies (Abosi and Raseroka 2003; Iwalokun, 2008); Njan *et al*., 2008), the study on the fractions was started using 30 mice on each fraction.

4.3.3. Inoculation of parasite

Albino mice previously infected with *P. berghei* and having parasitemia level of 20-30% were used as a donor. To infect the mice, blood sample was collected from a donor mouse with a rising parasitemia of about 20-30 % (Adediji *et al*., 2012; Deressa *et al*., 2010). After determination of the percentage parasitemia and erythrocytes count, the donor mouse was sacrificed using ethyl ether as anesthesia and blood was collected in a petri-dish containing 2% trisodium citrate (BDH chemicals, England) as anticoagulant. The blood was diluted with physiological saline (0.9%) in a proportions indicated by both determinations (Okokon *et al*., 2011a). Based on parasitemia level of the donor mice and the red blood cell (RBC) count of normal mice in such a way that 1 ml blood contains 5×10^7 infected RBCs (Waako *et al*., 2005).

On day 0(before starting administering the test substance and standard), each mouse was inoculated intraperitoneally, with 0.2 ml of infected blood containing about 1×10^7

parasitized red blood cells, which was expected to produce steadily rising consistent infection of the required intensity in mice. The doses to be given for mice were determined based on the acute toxic effect of the respective extracts and fractions.

4.3.4. Test for the fractions (*The 4 day suppressive test)*

For evaluating antiplasmodial activity of *V. amygdalina* solvent fractions in four day suppressive test, *P. berghei* infected Swiss albino male mice were used and classified into five groups of six mice per group. Mice were randomly assigned in to three treatment and two control groups and inoculated as described above in section 4.3.2. Group I was negative control and treated with vehicle; 2% of Tween 80 v/v in water for CF or distilled water for AF and BF. Group II, III and IV were treated with solvent fractions of the plant extract at doses of 100,200 and 400mg/kg respectively. The doses were selected based on the preliminary test. Group V was treated with chloroquine (25mg/kg). Administration was performed via oral route using gavage. Volume to be administered was calculated based on individual mouse body weight and 0.45 ml is the maximum volume administered.

The infected mice were randomly divided into five groups as described in section 4.3.2**.**The treatment was started three hours after the mice had been inoculated with the parasite on day 0, and then continued for the next four days from day 0 to day 3 with 24 hrs time interval between the doses. After giving the treatment for four days, thin blood film was stained with 10% Gemisa from the tail of the mouse on the fifth day (D4 i.e 96 hr post- infection) to determine the level of parasitemia and percentage inhibition by

counting 4 fields of approximately 100 erythrocytes per field according to the method of David *et al*. (2004).

4.3.5. Determination of body weight

The body weights of the mice were measured using sensitive weighing balance (Mettler Toledo, Switzerland) before infection (day 0) and on day 4 in four day suppressive test to observe whether the extracts and fractions of *V. amygdalina* leaves prevent weight loss (Dikasso *et al*., 2006). Then, the average body weight was compared with the control groups over time in each group.

$$Mean\ body\ weight = \frac{total\ weight\ of\ mice\ in\ a\ group}{total\ number\ of\ mice\ in\ that\ group}$$

4.3.6. Measurement of body temperature

The daily measurement of rectal temperature of each mouse in all groups were measured daily by a digital thermometer (one hour before infection, four hours after infection) to predict the effectiveness of the extracts and fractions on temperature in four day suppressive test. It is theoretically accepted that the body temperature of mice decreased in a rapid manner with increasing parasitemia in contrary to the situation in human beings. The efficacy of the extracts and fractions were determined by observing the protective effect against the rapid fall in temperature (Dikasso *et al*., 2006).

4.3.7. Determination of packed cell volume

Packed cell volume (PCV) is a measure of the proportion of red blood cells to total blood volume, used in estimating the mean erythrocyte hemoglobin concentration. The PCV

was measured to determine the effectiveness of the extracts as well as fractions in preventing hemolysis resulting from increasing parasitemia associated with malaria using the modified Wintrobe's method to counteract PCV reduction (Gilmour and Syke, 1951). Blood was taken from the tail of the mouse with heparinized microhaematocrit tubes. The capillary tubes were filled to ¾th of their height with blood and sealed with sealing clay. The tubes were placed in micro-hematocrit centrifuge (Centurion Scientific, UK) with the sealed end outwards and centrifuged for 5 min with 12000 rpm. The tubes were then taken out of the centrifuge and PCV was determined**.** PVC was measured before inoculating the parasite and after treatment using the following formula as follow**s:**

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

This test was done just before infection (Day 0) and on day 4 after infection (Dikasso *et al*., 2006).

4.3.8. Determination of parasitemia

Thin smears of blood films were made from the tail of each mouse in the test and control groups on day four of experiment using microscopic slides. After air drying, the smears on the microscopic slides were fixed with absolute methanol and stained with 10% Giemsa stain at pH 7.2 for 15 min. The stained slides were washed gently using tap water and air dried at room temperature. With little drop of oil immersion, the number of pRBC were counted using light microscope (Olympus N-120A, Philippines) with the objective lens of 100x magnification power. Five fields of approximately 100-200 cells in each slide were counted (the numbers of infected and uninfected red blood cells)

(Krettli *et al*., 2009; Basir *et al*., 2012) and taking the average count, the mean parasitemia was calculated using the formula (Hilou *et al*., 2006).

$$\text{Parasitemia} = \frac{\text{Total number of parasitized red blood cells}}{\text{Total number of Red blood cells (No of parasitized RBC+ No of Uninfected RBCs)}} \times 100\%$$

Finally, percent parasitemia suppression of the fraction was compared with respect to the controls. Parasitemia suppression was calculated using the following formula (Devi *et al*., 2001):

$$\text{suppression} = \frac{\text{mean parasitemia of negative control} - \text{mean parasitemia of treated group}}{\text{Mean parasitemia of negative control}} \times 100$$

4.3.9. Determination of mean survival time

Survival time was recorded to observe the effect of the extracts and fractions for improvement in survival days. The animals were fed *ad labitum* and observed for about 28 days. Any death that occurs during this period was noted for each mouse in the treatment and control groups to determine the mean survival time (Eulifoye and Agbedahunsi, 2004).

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

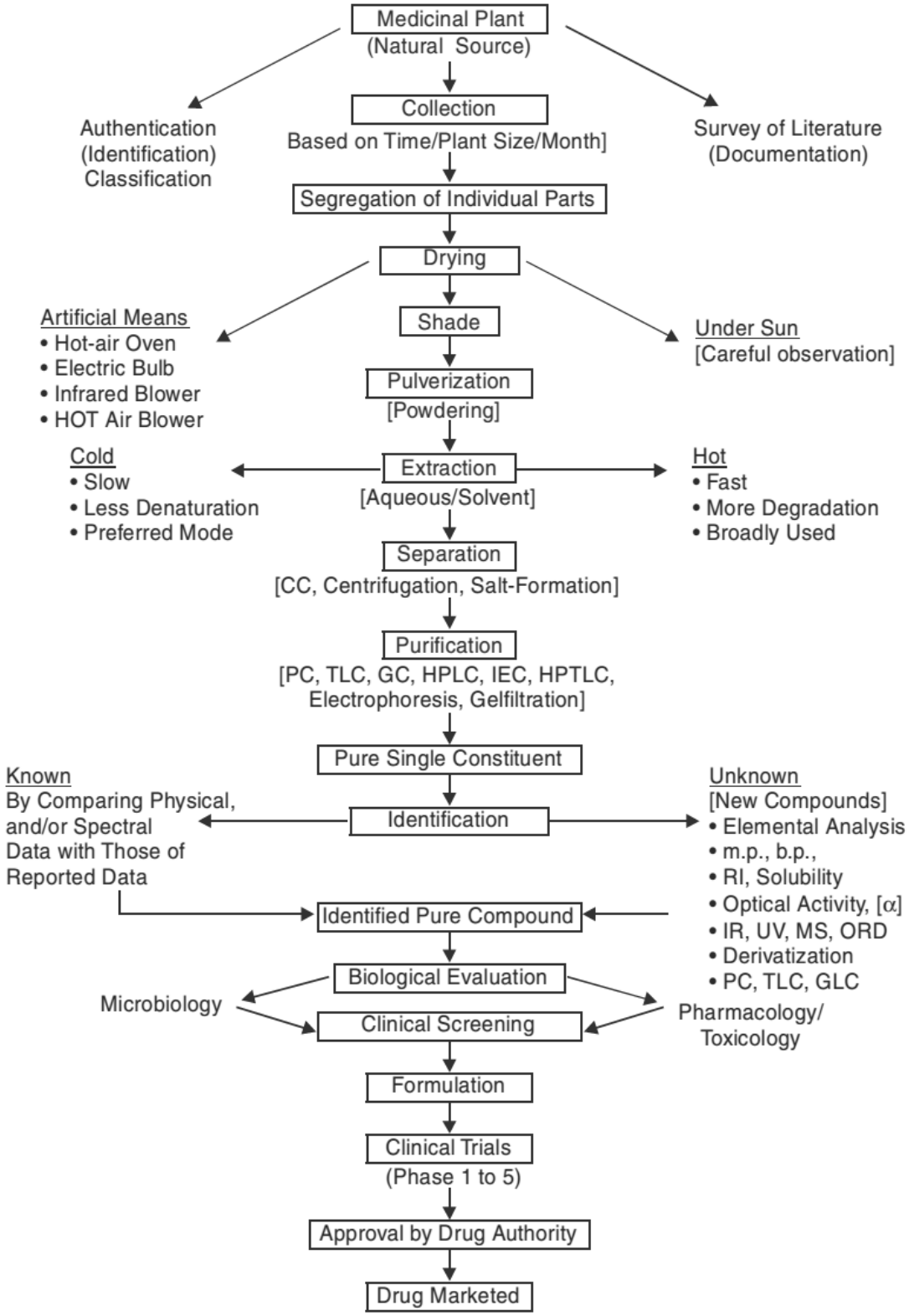


Figure 5. Schematic Development of a 'Drug' from a 'Medicinal Plant'(Kar ,2007).

3.4. Data analysis

All the results obtained during the experiment were expressed as mean ± standard error of mean (SEM) for each group. The data were analyzed using IBM Statistical Package for the Social Sciences (SPSS) Version 20.0 software. One way analysis of variance (ANOVA) was performed followed by Tukey's HSD post-hoc to test compare the significance among and within group differences of parasitemia, mean survival time, and changes in body weight, PCV, rectal temperature of the *P. berghei* infected mice between the control and treatment groups. The data were analyzed at 95% confidence interval ($\alpha = 0.05$) and p value < 0.05 was considered as statistically significance.

5. RESULTS

5.1. Acute oral toxicity test

Acute oral toxicity test showed that both the aqueous and hydroalcoholic extracts and solvent fractions of the leaves of *Vernonia amygdalina* revealed that no mortality and signs of toxicities such as loss of appetite, hair erection, lacrimation, tremors, convulsions, salivation, diarrhoea, mortality and other signs of overt toxicity observed for 14 days in mice after oral administration of the extracts and solvent fractions of the leaves of *V. amygdalina* at dose level of 2000 mg/kg signifying that the oral LD_{50} was greater than 2000 mg/kg (thus the experimental doses used 100, 200, 400 and 600mg/kg were within the safety margin).

5.2. *In vivo* antimalarial activity of the leaf of *Vernonia amygdalina* on four days suppressive test

5.2.1. Effects of crude extracts of the leaf of V. amygdalina on percentage of suppression of parasitemia and mean survival time

The present study indicated that after four day suppressive test the percentage suppression of hydroalcolic extract was 32.47%, 35.4% and 37.67% at 200mg/kg/day, 400mg/kg/day and 600mg/kg/day of the extract, respectively and percentage suppression of aqueous extract was 21.22%, 22.20% and 24.52% at 200mg/kg/day, 400mg/kg/day and 600mg/kg/day of the extract, respectively. At the dose of 600 mg/kg, hydroalcoholic extract produced chemosuppression of 37.67 % which is higher than that of aqueous extract (24.52%) at 600mg/kg indicating moderate antimalarial potential.

Following Peters four days chemosuppressive activity test for both the hydroalcoholic and aqueous leave extracts of *V. amygdalina* produced a dose dependent chemosuppression activity. The highest suppression of parasitaemia was observed at the dose of 600mg/kg body weight of mice. Percentage suppression was observed to increase as extract concentration increased. The antimalarial activity produced by all doses of the hydroalcolic and aqueous extract were statistically significant ($P < 0.05$) when compared to the negative control as shown in the table 3. The mice treated with CQ were completely free from the parasites on day four.

Both extracts were also capable of significantly increasing survival time at all doses compared to controls, but the effect was significantly lower than CQ25mg/kg. Comparison among the dose themselves indicated that 600 mg/kg resulted in significantly higher survival time when compared to 200 mg/kg ($p < 0.05$) as shown in table 3.

Table 3. Antimalarial activity of the hydroalcoholic and aqueous extracts of the leaf of *V. amygdalina* in the 4 day suppressive test on percentage of suppression and mean survival time of infected mice.

Treatment	**% Parasitemia**	**% Suppression**	**MST**
CON	50.00±0.58	0.00	6.50±0.22
ME200 mg/kg	33.77±1.33	32.47±2.65^{a3b3}	9.50±0.42^{a2b3e1}
ME400 mg/kg	32.30±1.57	35.40±3.14 ^{a3b3}	10.17±0.79^{a3b3}
ME600 mg/kg	31.17±1.25	37.67±2.50 ^{a3b3}	12.00±0.58^{a3,b3}
CQ25 mg/kg	0.00	100	29.67±0.21^{a3b3}
CON	37.92±2.37	0.00	6.33±0.21
AE200 mg/kg	30.28±4.10	21.22±10.39 ^{a1b3}	8.00 ±0.52^{a1b3e1}
AE400 mg/kg	29.50±0.22	22.20±0.59 ^{a2b3}	9.17±0.30^{a3b3}
AE600 mg/kg	28.68±1.50	24.52±3.94 ^{a1b3}	9.83±0.48 ^{a3b3}
CQ25 mg/kg	0.00	100.00	29.67±0.21

Data are expressed as mean ± SEM; n = 6; a, compared to negative control; b compared to positive control, c to 200mg/kg, d to 400 mg/kg, e to 600 mg/kg; 1 $p<0.05$, 2 $p<0.01$, 3$p<0.001$; AE, aqueous extract, CON, Control, ME, Methanol extract, D0, at day 0 and D4, at day 4; MST, mean survival time.

5.2.2. Effect of crude extracts of the leaf of *V. amygdalina on body temperature*

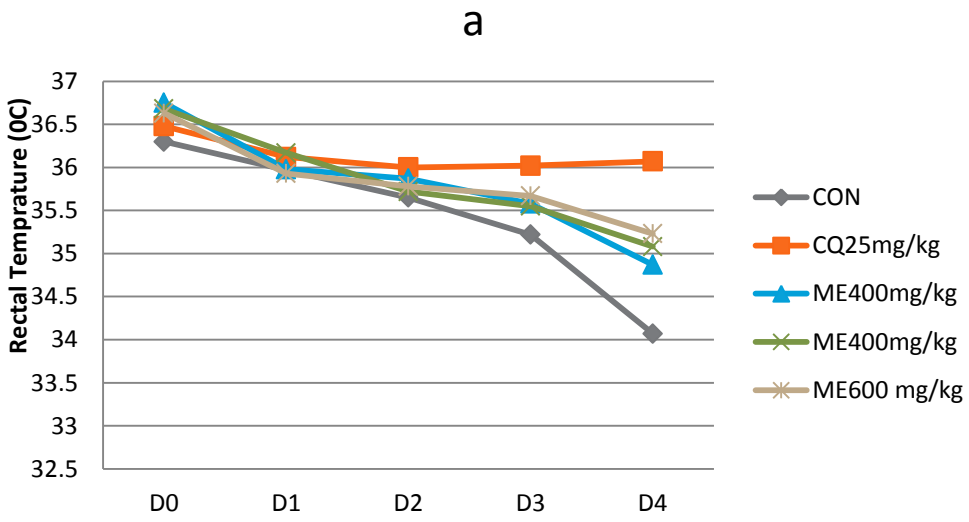
All extracts were exhibited reduction in temperature of *Plasmodium berghei* infected mice in a dose-dependent manner and was statistically significant ($p <0.05$) as compared to negative control. Analysis of rectal temperature revealed that 80% methanolic extract of the leaf of *V. amygdalina* significantly reduces temperature ($p < 0.05$) at dose of 200mg/kg and ($p < 0.001$) and ($p<0.01$) at doses of 400 and 600 mg/kg and at a dose of 600mg/kg, the effect is comparable to that of CQ (25mg/kg) as shown in table 4. Comparison among different doses of both the 80% methanolic extract and aqueous extract revealed apparent difference in prevention of temperature decline between the lowest and largest doses of hydroalcoholic extract (400mg/kg and 600 mg/kg) (p

<0.001) as well as between the lowest dose and 400 mg/kg and 600mg/kg of aqueous extract ($p < 0.05$). Chloroquine, on the other hand, significantly prevented the decline in rectal temperature as compared to all doses of both extracts (figure 6).

Table 4. Body temperature infected animals treated with crude extracts of the leaf of *Vernonia amygdalina* in the 4 day suppressive test.

Treatment	**Temperature**		
	D0	**D4**	**% change**
CON	36.30±0.15	34.07±0.23	-3.37±0.09
ME200mg/kg	36.75±0.23	34.77±0.14	-2.08±0.56^{a1b3e1}
ME400 mg/kg	36.68±0.21	35.08±0.32	-1.57±0.10^{a3b1}
ME600 mg/kg	36.63±0.24	35.23±0.29	-1.01±0.54^{a3}
CQ25 mg/kg	36.50±0.35	36.07±0.36	-0.32±0.13
CON	36.00±0.15	34.82±0.16	-3.39±0.09
AE200 mg/kg	35.67±0.30	34.83±0.25	-2.87±0.01^{a3b3d3e3}
AE400 mg/kg	35.97±0.27	35.17±0.27	-2.28±0.02^{a3b3e2}
AE600 mg/kg	35.10±0.25	34.62±0.12	-1.88±0.103^{a3b3}
CQ25 mg/kg	35.12±0.34	36.27±0.90	-0.39±0.06

Data are expressed as mean ± SEM; n = 6; a, compared to negative control; b compared to positive control, d to 400 mg/kg, e to 600 mg/kg; 1 $p<0.05$, 3$p<0.001$; AE, aqueous extract, CON, Control, ME, Methanol extract, D0, at day 0 and D4, at day 4.



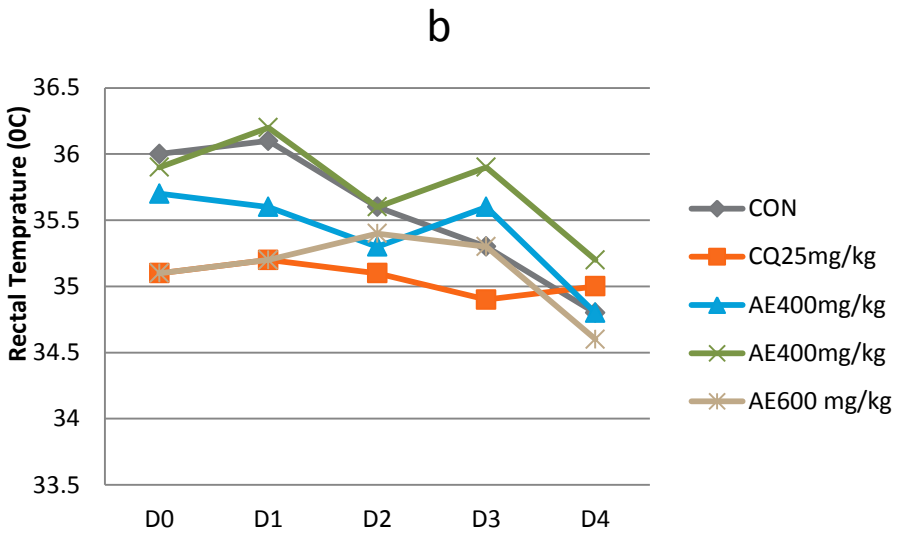


Figure 6. The effect of hydroalcoholic (a) and aqueous (b) extracts of the leaves of *V. amygdalina* on rectal temperature of *P. berghei* infected mice in four day suppressive test. Data are expressed as mean ± SEM; n = 6; AE, aqueous extract; CON, control; CQ , chloroquine; D, day; ME, methanol extract.

5.2.3. Effects of crude extracts of the leaf of V. amygdalina on body weight

In 4-day suppressive test all doses of 80% methanol and aqueous crude extracts had shown prevention against weight loss compared to negative control and weight gain was observed in all doses of the extracts. Therefore, the percentage of change in body weight of hydroalcolic and aqueous extract at all dose levels were significantly different compared to that of the negative control ($p< 0.001$) as depicted in table 5. Chloroquine had prevented body weight reduction significantly ($p<0.001$) when compared to all doses of the extracts.

Table 5. Body weight of infected mice treated with crude extract of *Vernonia amygdalina* in the 4 day suppressive test.

Treatment	**Body weight**		
	D0	**D4**	**% change**
CON	24.22±0.81	21±0.45	-15.31±2.85
ME200	24.87±1.16	26.23±0.09	5.31±0.61[a3]
ME400	22.75±0.63	24.45±0.58	6.98±0.92[a3]
ME600	22.77±0.35	24.67±0.42	7.66±0.97[a3]
CQ25	25.57±0.59	28.65±0.31	10.77±1.74
CON	24.57±1.22	21.27±1.00	-15.48±1.34
AE200	24.18±0.60	26.70±1.18	3.74±0.67[a3b1]
AE400	23.17±1.03	25.15±1.12	5.47±0.21[a3]
AE600	24.18±0.60	27.52±0.99	5.73±0.84[a3]
CQ25	20.03±0.61	27.63±0.47	9.93±1.73

Data are expressed as mean ± SEM; n = 6; a, compared to negative control; b compared to positive control, 1 $p<0.05$, 3$p<0.001$; AE, aqueous extract, CON, Control, ME, Methanol extract, D0, at day 0 and D4, at day 4.

5.2.4. Effects of crude extracts of the leaf of V. amygdalina on packed cell volume

The effect of hydroalcolic extract of *V. amygdalina* on PCV at all dose levels had shown significant activity on prevention against PCV reduction($p< 0.001$) as compared to negative control(Table 6). The effect of aqueous extract on packed cell volume at 400mg/kg ($p<0.05$) and at 600mg/kg ($p < 0.01$) had shown significant activity on prevention against PCV reduction when compared to negative control (figure 7).

Table 6. Packed cell volume of infected mice treated with crude extract of *Vernonia amygdalina* in the 4 day suppressive test.

Treatment	Packed cell volume		
	D0	**D4**	**% change**
CON	76.90±0.92	52.50±1.18	-46.92±4.23
ME200	75.35±1.73	64.52±1.24	-16.98±3.24[a3]
ME400	77.82±2.26	68.75±2.37	-13.32±1.33[a3]
ME600	76.50±1.82	67.80±2.17	-13.11±2.70[a3]
CQ25	85.27±0.84	78.63±0.88	-7.51±0.48
CON	80.45±2.52	56.25±3.57	-45.77±9.69
AE200	77.57±1.77	60.45±1.77	-28.67±3.67[b1]
AE400	75.42±1.42	62.30±2.14	-21.49±2.96[a1]
AE600	74.85±3.57	64.52±1.24	-16.98±3.24[a2]
CQ25	75.15±3.46	68.50±3.40	-7.63±0.24

Data are expressed as mean ± SEM; n = 6; a, compared to negative control; b compared to positive control, 1 $p<0.05$, 2$p<0.01$; AE, aqueous extract, CON, Control, CQ, Chloroquine, ME, Methanol extract, D0, at day 0 and D4, at day 4.

5.2.5. Effects of solvent fractions of the leaf of V. amygdalina on percentage of suppression and mean survival time

The result of 4-day suppressive test indicated that the percentage suppression of chloroform fraction was 21.68%, 23.72% and 33.85 % at 100mg/kg/day, 200mg/kg/day and 400mg/kg/day of the fractions respectively. The percentage suppression effects of chloroform fraction at the tested doses were significantly different ($p<0.05$) at 100 and 200mg/kg) compared to that of the negative control ($p<0.001$) at 400mg/kg). The result of 4-day suppressive test indicated that all dose levels of the solvent fraction of butanol produced significant ($p<0.001$) parasitemia reduction when compared to negative control (Table 7). The 100 and 200 mg/kg body weight of butanol fraction resulted in moderate anti-plasmodial activity (18.12%, and 21.03% %, respectively), followed by the 400 mg/kg body weight (26.88%).

At the dose of 400 mg/kg, chloroform fraction produced chemosuppression of 37.67 % which is higher than that of butanol (26.88%) and aqueous fraction (9.75%). Following Peters four days chemosuppressive activity test for chloroform and butanol solvent fractions of *V. amygdalina* produced a dose dependent chemosuppression activity. The highest suppression of parasitaemia was observed at the dose of 400mg/kg body weight of mice. Percentage suppression was observed to increase as solvent fractions concentration increased. The mice treated with CQ were completely free from the parasites on day four.

The mean survival effects of both chloroform and butanol fractions were capable of significantly increasing survival time at all doses compared to negative control, but the effect was significantly higher at 400 mg/kg fraction doses ($p<0.001$). Comparison among butanol fraction (9.17±0.3), chloroform fraction at 400 mg/kg body weight dose has shown statistically significant prolongation of survival time (9.83±0.40) ($p<0.001$).The survival time was prolonged and statistically significant only in aqueous fraction treated with 400 mg/kg body weight of fraction as compared to negative control($p<0.05$). However, all dose levels of the aqueous fractions had not shown significant prolongation of survival time when compared to negative control($p>0.05$). Comparison among the three fractions indicated that 400 mg/kg fraction of chloroform has shown significantly higher survival time (9.83±0.40) when compared to butanol and aqueous fraction ($p<0.001$) as shown in table 7.

Table 7. Antimalarial activity of solvent fractions of the leaf of *V. amygdalina* in the 4 day suppressive test on percentage of suppression of parasitemia and mean survival time of infected mice in the 4 day suppressive test.

Treatment	% Parasitemia	% suppression	MST
CON	47.13±4.04	0.00	6.67±0.33
CF100 mg/kg	36.88±2.54	21.68±5.41^{a1b3}	8.5±0.43^{a1b3}
CF200 mg/kg	35.90±0.89	23.72±1.92^{a1b3}	9.00±0.58^{a2b3}
CF400 mg/kg	31.15±4.27	33.85±9.08^{a3b3}	9.83±0.40^{a3b3}
CQ25 mg/kg	0.00	100	29.5±0.34
CON	49.81±3.83	0.00	6.33±0.49
BF100 mg/kg	41.60±2.45	18.12±4.82^{a3b3}	7.83±0.31^{a1b3}
BF200 mg/kg	39.33±0.31	21.03±0.62^{a3b3}	8.50±0.0.23^{a1b3}
BF400 mg/kg	36.55±0.35	26.88±0.70^{a3b3}	9.17±0.31^{a3b3f3}
CQ25 mg/kg	0.00	100	29.33±0.33
CON	57.78±3.09	0.00	6.00±0.26
AF100 mg/kg	56.42 ±2.89	5.60±3.60^{b3}	6.00±0.37^{b3e1}
AF200 mg/kg	55.33±0.83	6.72±1.74 ^{b3}	6.50±0.22^{b3}
AF400 mg/kg	52.55±2.97	9.75±4.84 ^{b3}	7.33±0.21^{a1b3}
CQ25 mg/kg	0.00	100	29.5±0.34

Data are expressed as mean ± SEM; n = 6; a, compared to negative control; b compared to positive control, e to 400 mg/kg; f, compared to Chloroform; 1p<0.05; 2p<0.01; 3p<0.001; AF, aqueous fraction; BF, Butanol fraction; CF, Chloroform fraction CON, Control; CQ, Chloroquine; D0, at day 0 and D4, at day 4; MST, mean survival time.

5.2.6. Effects of solvent fractions of the leaf of *V. amygdalina* on body temperature

All the tested doses of chloroform and butanol fractions exhibited reduction in rectal temperature of *Plasmodium berghei* infected mice in a dose-dependent manner and statistically significant ($p < 0.05$) as compared to negative control. Comparison among different doses of Chloroform fraction revealed apparent difference in prevention of temperature decline between the lowest and higher doses of the fraction (400mg/kg) ($p < 0.001$) whereas 400 mg/kg doses of butanol fraction has shown better effect in reduction of rectal temperature ($p < 0.001$) as compared to its effect at 100 and 200mg/kg(figure 8). The effect of aqueous fraction on rectal temperature at all the tested doses were

statistically insignificant as compared to the negative control ($p>0.05$) as a result did not reduce rectal temperature. Chloroquine, on the other hand, significantly prevented the decline in rectal temperature as compared to all doses of both extracts (table 8).

Table 8. Body temperature of infected animals treated with solvent fractions of the leaf of *Vernonia amygdalina* in the 4 day suppressive test

Treatment	**Temperature**		
	D0	**D4**	**% change**
CON	37.68±0.20	36.43±0.21	-3.43±0.10
CF100 mg/kg	37.08±0.29	36.12±0.29	-2.68±0.06 ^{a2b3d2}
CF200 mg/kg	35.85±0.28	35.05±0.26	-2.28±0.10 ^{a3b3}
CF400 mg/kg	36.13±0.29	35.43±0.31	-1.98±0.19 ^{a3b3}
CQ25 mg/kg	36.35±0.33	36.18±0.32	-0.46±0.09
CON	36.05±0.16	34.88±0.17	-3.35±0.13
BF100 mg/kg	35.87±0.33	34.87±0.33	-2.87±0.28 ^{a3b3d2e3}
BF200 mg/kg	36.00±0.15	35.13±0.14	-2.47±0.57 ^{a3b3e3}
BF400 mg/kg	35.75±0.12	35.03±0.10	-2.05±0.05 ^{a3b3}
CQ25 mg/kg	35.92±0.30	36.42±0.31	-0.33±0.05
CON	35.90±0.24	34.67±0.22	-3.56±0.09
AF100 mg/kg	35.75±0.39	34.57±0.37	-3.42±0.03 ^{b3}
AF200 mg/kg	35.50±0.37	34.35±0.35	-3.35±0.04 ^{b3}
AF400 mg/kg	35.53±0.29	34.38±0.27	-3.34±0.05 ^{b3}
CQ25 mg/kg	36.10±0.17	35.93±0.18	-0.47±0.06

Data are expressed as mean ± SEM; n = 6; a, compared to negative control; b compared to positive control, d to 200mg/kg; e to 400 mg/kg;1 $p<0.05$, 3$p<0.001$; AF, aqueous fraction, BF, Butanol fraction, CF, Chloroform fraction CON, Control, CQ, Chloroquine, D0, at day 0 and D4, at day 4; MST, mean survival time.

5.2.7. Effects of solvent fractions of the leaf of V. amygdalina on body weight

In 4-day suppressive test all doses of chloroform, butanol and aqueous fractions had shown prevention against weight loss compared to negative control and weight gain was observed in all doses of the fractions ($p<0.001$). The percentage of change in body weight

of chloroform at 400 mg/kg dose level was shown to have comparable effect to that of positive control ($p>0.05$) as depicted in table 9. Chloroquine had prevented weight reduction significantly ($p<0.001$) when compared to all doses of the extracts.

Table 9. Body weight of infected animals treated with solvent fractions of the leaf of *Vernonia amygdalina* in the 4 day suppressive test.

Treatment	**body weight**		
	D0	**D4**	**% change**
CON	24.70±0.67	21.17±0.60	-16.77±1.71
CF100 mg/kg	25.05±0.66	25.85±0.72	3.04±1.06^{a3b1}
CF200 mg/kg	28.00±0.28	29.03±0.54	3.48±1.01^{a3b1}
CF400 mg/kg	26.68±0.32	27.48±0.23	4.13±0.71^{a3}
CQ25 mg/kg	29.12±0.70	32.28±0.40	9.79±2.07
CON mg/kg	25.35±1.19	23.17±0.95	-9.36±2.32
BF100 mg/kg	26.35±0.32	26.92±0.32	2.10±0.29^{a3b2}
BF200 mg/kg	24.38±0.66	24.91±0.59	2.17±0.45 ^{a3b2}
BF400 mg/kg	27.98±1.29	28.68±1.30	2.46±0.15 ^{a3b2}
CQ25 mg/kg	29.32±1.80	32.47±1.51	10.08±0.15
CON	27.90±1.26	23.60±1.08	-18.25±1.50
AF100 mg/kg	24.28±1.50	24.67±1.47	1.63±0.31^{a3b3}
AF200 mg/kg	26.08±1.29	26.58±1.29	1.90±0.38 ^{a3b3}
AF400 mg/kg	26.90±1.43	27.47±1.36	2.14±0.90 ^{a3b3}
CQ25 mg/kg	27.17±1.38	30.47±1.33	11.00±0.82

Data are expressed as mean ± SEM; n = 6; a, compared to negative control; b compared to positive control,1 $p<0.05$; 2$p<0.01$; 3$p<0.001$; AF, aqueous fraction, BF, Butanol fraction, CF, Chloroform fraction CON, Control, CQ, Chloroquine, D0, at day 0 and D4, at day 4.

5.2.8. Effects of solvent fractions of the leaf of V. amygdalina on packed cell volume

The effect of chloroform and butanol fractions of *V. amygdalina* on PCV at all dose levels had shown significant activity on prevention against PCV reduction($p< 0.01$) as compared to negative control(Table 10). Aqueous fraction on packed cell volume at all

doses had not shown significant activity on prevention against PCV reduction and were not statistically significant ($p>0.05$) when compared to the negative control (figure 8).

Table 10. Packed cell volume of infected animals treated with solvent fractions of the leaf of *Vernonia amygdalina* in the 4 day suppressive test

Treatment	**Packed cell volume**		
	D0	**D4**	**% change**
CON	80.45±2.52	54.60±4.08	-50.77±10.23
CF100mg/kg	77.57±1.77	64.48±1.49	-20.37±2.04[a2]
CF200 mg/kg	75.42±1.42	63.02±1.44	-19.79±1.68 [a2]
CF400 mg/kg	75.35±1.73	64.52±1.24	-16.98±3.24 [a2]
CQ25 mg/kg	75.15±3.46	67.83±3.02	-10.78±1.59
CON	81.39±1.38	54.54±3.70	-53.21±12.28
BF100 mg/kg	81.71±1.04	67.18±2.50	-22.25±3.55[a2]
BF200 mg/kg	80.55±1.33	66.90±2.84	-21.15±3.70 [a2]
BF400 mg/kg	78.61±3.68	66.50±2.79	-18.09±1.43[a2]
CQ25 mg/kg	75.78±3.70	68.83±3.44	-10.19±1.40
CON	80.45±2.52	54.60±4.08	-50.77±10.23
AF100 mg/kg	77.57±1.77	56.33±1.74	-38.03±3.25[b1]
AF200 mg/kg	75.42±1.42	55.67±1.17	-35.59±2.05 [b1]
AF400 mg/kg	74.85±3.57	57.23±2.85	-31.15±4.61 [b1]
CQ25 mg/kg	75.15±3.46	68.10±3.21	-10.43±1.78

Data are expressed as mean ± SEM; n = 6; a, compared to negative control; b compared to positive control,1 $p<0.05$; 2$p<0.01$; AF, aqueous fraction, BF, Butanol fraction, CF, Chloroform fraction CON, Control, CQ, Chloroquine, D0, at day 0 and D4, at day 4

5.3. Phytochemical test

Phytochemical screening of the hydroalcolic and aqueous crude extracts of the leaves of *V. amygdalina* revealed that the the presence of alkaloids, tannins, saponins, flavonoids, terpenoids, cardiac glycosides, anthraquinones, and phenol as shown in table 9. Phytochemical screening of chloroform, butanol and aqueous fractions of the leaves of *V.*

amygdalina revealed the presence of alkaloids, tannins, saponins, flavonoids, cardiac glycosides, phenol and quinone as shown in Table 9.

Table 11. Phytochemical screening of the leaves of crude extracts and solvent fractions of *Vernonia amygdalin.*

Phytochemical constituents	**Crude extracts**		**Solvent fractions**			**Control**	
	80%Methanol	**Aqueous**	**AF**	**BF**	**CF**	**Dis.H_2O**	**Tween 80**
Alkaloids	+	+	+	+	+	-	-
Tannins	+	+	+	-	+	-	-
Saponins	+	+	+	+	+	-	-
Flavonoids	+	-	-	+	+	-	-
Terpenoids	+	+	-	-	+	-	-
Steriods	-	-	-	-	-	-	-
Cardiac glycosides	+	-	-	-	+	-	-
Anthraquinones	+	+	-	-	-	-	-
Phenol	+	+	+	-	+	-	-

Key: + Shows the presence of phytochemical constituent

- shows the absence of phytochemical constituent

5. DISCUSSION

Medicinal plants are important for pharmacological research and drug development because they contain undiscovered biodynamic compounds with unrealized potential for the use in modern drugs (Westh *et al*., 2004). Malaria is one of the most important infectious diseases in the world. Currently, antimalarial drug resistance has become one of the most important challenges to malaria control (Hossein *et al*., 2012). The scientific community is now underway to combat this problem by searching for new, affordable and effective antimalarial agents from medicinal plants and other sources (Gamo, 2014).

Medicinal plants, although assumed to be safe, are potentially toxic which necessitates investigation of their safety status (Ifeoma and Oluwakanyinsola, 2013).The toxicity study of *V. amygdalina* was determined based on OECD guideline 425 (OECD, 2008) of acute toxicity studies. Based on this test, the LD_{50} of *V. amygdalina* was found to be greater than 2000 mg/kg. Results from the safety tests of the extracts and solvents fractions of *V. amygdalina* showed no visible toxic effects up to 2000 mg/kg. The present result is in agreement with finding which Adiukwu *et al*. (2012) reported that *V. amygdalina* caused no clinical signs of toxicity at doses of 500-2000 mg/kg/day for 14 consecutive days and Njan *et al*. (2008) who reported no signs of toxicity or adverse toxicological effects at all doses. In addition, Amole *et al*. (2006) and Njan *et al*. (2008) found no toxicity effect in extracts of *Vernonia amygdalina* in *in vivo* rat studies. Generally, if the LD_{50} value of the test chemical is more than 3 times the minimum effective dose, the substance is considered a good candidate for further studies (Mohammed *et al*, 2014). LD_{50} can also be used for classification of chemicals. Based

on the method of WHO hazard classification, the hydroalcoholic and aqueous leaves extracts and solvent fractions of *V. amygdalina* whose LD_{50} was greater than 2000 mg/kg which is designated as 'unlikely to be hazard' (WHO, 1975). Therefore, the experimental plant was safe on doses employed.

The *in vivo* 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*., 2005). *Plasmodium berghei* ANKA was used in the prediction of treatment outcomes (Dikasso *et al*., 2006) and hence it was an appropriate parasite for the study. Even though the rodent malaria model, *P.berghei*, is not exactly similar to that of the human Plasmodium parasites, it is the first step to screen most of the *in vivo* antimalarial activities of test compounds (Fidock *et al*., 2004). Moreover, several conventional antimalarial agents such as chloroquine, halofantrine, mefloquine and more recently artemisinin derivatives have been identified using rodent model of malaria (Madara *et al*., 2010).

The 4-day suppressive test is the standard test commonly used for antimalarial screening. *P. berghei* infected mice are used for better prediction of antimalarial efficacy of drugs for human use in many studies in search of antimalarial drugs (Peters, 1998). Determination of percent inhibition of parasitemia is the most reliable parameter. A mean parasitemia level ≤90% to that of mock-treated control animals usually indicates that the test compound is active in standard screening studies (Peter and Anatoli, 1998). *In vivo* antiplasmodial activity can be classified as moderate, good and very good if an extract displayed a percent parasite suppression equal to or greater than 50% at a dose of 500,

250 and 100mg/kg body weight per day, respectively (Deharo *et al*., 2001 and Muñoz *et al*., 2000). Based on this classification, crude extracts and solvent fractions of *V. amygdalina* exhibited moderate antiplamodial activity, with a dose dependent inhibition against *P. berghei* infection in mice. Hence, the present study result indicated that *P. berghei* infected mice treated with the hydroalcoholic and aqueous extracts and chloroform and butanol fractions of *V. amygdalina*, the percentage of parasitemia measured changed significantly from those in the control animals.

Four-day suppressive test assesses the potential schizontocidal activity of plant extracts and solvent fractions in early infection whereby the primary attack due to malaria can be prevented or mitigated (Peters, 1975). The hydroalcoholic and aqueous extracts of *V. amygdalina* had a maximum suppression of 37.67% and 24.52% respectively. Whereas the chloroform and butanol fractions suppress 33.85 % and 26.88%), respectively. Therefore, both extracts and fractions can be considered to be active in their schizontocidal activity against malaria. The antimalarial activity of *V. amygdalina* may be due to the presence of active secondary metabolites present in these extracts and fractions. The antimalarial action being attributed to sesquiterpene lactone. The vernolides, a class of sesquiterpene lactone were identified as the most studied compounds from the genus and show interesting bioactivity in antiplasmodial, antileishmanial, antischistosomial, cytotoxicity, antimicrobial and anti-inflammatory assays. On the basis of results from a combination of *in vitro* and *in vivo* efficacy and toxicity studies reported, *Vernonia amygdalina* holds most promising for development into a pharmaceutical against diabetes and malaria (Toyang and Verpoorte, 2013). High levels of chemosupression were produced at high doses of the extracts and solvent

fractions, indicating a dose dependent effect (Abosi and Raseroka, 2003). This might be due to the presence of good concentrations of active compounds in higher doses. Tona *et al* (2004) demonstrated *in vitro* antimalarial activity of ethanol and chloroform extracts of the *V. amygdalina* leaf, while Masaba (2000) showed *in vitro* activity of an acetone water extract.

The present study indicated that the percentage suppression of hydroalcolic extract of *V. amygdalina* produced statistically significant ($p<0.001$) 32.47%, 35.40% and 37.67% at 200mg/kg/day, 400mg/kg/day and 600mg/kg/day of the extract, respectively when compared to negative control, and statistically significant percentage suppression of aqueous extract of *V. amygdalina* was also produced ($p<0.05$) 21.22%, p(<0.01) 22.20% and 24.52% at 200mg/kg/day, 400mg/kg/day and 600mg/kg/day of the extract, respectively when compared to negative control after four day suppressive test. This parasite suppression exhibited by the extracts were comparable with the results of (Dawet *et al*., 2014) done on ethanol and aqueous stem bark extract of *Pseudocedrela kotschyi reported that* 39.43% and 28.36% of suppression of parasitemia at 200mg/kg boy weight respectively; *Aloe debrana* methanol (30.21%, 44.15%, 73.95% at the dose levels of 200, 400 and 600mg/kg) and water extracts (23.53%, 33.22% and 54.36% at the dose levels of 200, 400 and 600mg/kg).

The highest chemosepressive effect of the leaf of *V. amygdalina* at the dose of 600mg/kg in hydroalcoholic and aqueous extract was 37.67% and 24.52% which is low and disagrees with the finding of Abosi and Raseroka (2003) that ethanolic leaf extract of *V. amyygdalina* at doses of 500 mg/kg (s.c.) and methanolic extracts at doses of 1000 mg/kg

(p.o.) produced 67% suppression of parasitaemia in the four-day test (Challand and Willcox, 2009), while an aqueous extract given to mice at 125 mg/kg (p.o.) caused a 63% reduction of parasitaemia (Iwalokun, 2008). The big difference in the results might be due to variability in geographical location, stage of development, method of cultivation and processing may influence the occurrence of secondary metabolites (Monitoo, 1981). Results on *in vitro*/*in vivo* bioactivity of plants reveals that the same plant species from different origin can give different activity in the same assay. These variations can be attributed to various factors including but not limited to environment, season, age of plant, part of plant, time of day the collected, postharvest handling, extraction solvent (Eloff, 1998) and sensitivity of assay (Phillipson, 1995).

Chloroform and n-butanol fractions were found to have better blood schizontocidal activity than aqueous fractions. The chloroform fraction showed good antimalarial activity among the solvent fractions. This suggests the possible segregation of active ingredients into chloroform fraction than butanol and aqueous fractions.

The parasite suppressive effect of *V. amygdalina* leaf extracts and solvent fractions might be through indirect boosting of immune system or by inhibition of other target pathways which are not fully realized (Muthaura *et al*., 2007). Likewise the parasite inhibitory effect of *V. amygdalina* leaf extracts and solvent fractions with unknown compounds might be attributed to the antiplasmodial activity of specific compound or group of compounds (Koch *et al*., 2005). Previous study reported that sesquiterpene lactones such as vernolepin, vernolin, vernolide, vernodalin and hydroxyvernodalin isolated from *Vernonia amygdalina* leaves showed an antiplasmodial activity (IC_{50}<4µg/ml)

against *Plasmodium falciparum* strains (Phillipson *et al*.,1993). The positive control eliminated the parasite to non-detectable level and as a result led to survival time of more than 28 days. The significantly lower parasitemia suppression by the extracts and solvent fractions as compared to the positive control could be due to low level of active compound(s) associated with the crude nature of the extract (Krettli *et al*., 2009). This is in agreement with other studies where plant extracts had lower effect than standards (Mengiste *et al*., 2012; Bantie *et al*., 2014).

The mean survival time is important to evaluate the antimalarial activity of plant extracts (Peters, 1975). Except aqueous fraction, all dose levels of the extracts and solvent fractions of *V. amygdalina* prolonged the survival time of mice which could be due to the suppression of parasitemia and reduced the overall pathologic effect of the parasite on the study mice. The higher doses of all extracts and solvent fractions of *V. amygdalina* recorded the longest survival time when compared to negative control could be linked to the presence of active secondary metabolites in sufficient concentration unlike that of the lower doses. This indicates that these doses suppressed *P. berghei* and thereby reduced anemia and the overall pathologic effect of the parasite on the test groups (Basir *et al*., 2012).

A decrease in the metabolic rate of infected mice occurred before death and was ccompanied by a corresponding decrease in internal body temperature (Mengiste *et al*., 2012). This implies that infected mice body temperature drops as parasite level grow rapidly. This decrement in temperature has been associated with reduction in basal metabolic rate and impact of anemia on heat production and/or heat conservation

(Dascombe and Sidara, 1994). Active compounds should prevent the rapid dropping of rectal temperature. At all dose levels of the crude extracts and the chloroform and butanol fractions did have protective effects against temperature reduction, which reflects the active constituents responsible for this effect were likely found in a good concentration that have antimalarial activity in these extracts and fractions.

Hematological abnormalities like anemia, body weight loss and body temperature reduction are the general features of malaria-infected mice (Langhorne *et al*., 2002). Weight decrement has been associated with decreased food intake, disturbed metabolic function and hypoglycemia (Atkinson *et al*., 2000; Basir *et al*., 2012). So, an ideal antimalarial agents obtained from plants are expected to prevent body weight loss in infected mice due to the rise in parasitemia. Except aqueous fractions, all dose levels of the crude extracts and solvent fractions of *V. amygdalina* significantly prevented weight loss in a dose dependent manner. This indicates that these doses suppressed *P. berghei* and thereby reduced anemia and the overall pathologic effect of the parasite on the test groups (Basir *et al*., 2012).

Rodent malaria causes parasite induced fall down of PCV, which occurred approximately 48 hours post infection (Mace *et al*., 2015). The underlying cause of anemia includes; loss of infected erythrocytes through parasite maturation, destruction of uninfected red cells in the spleen and liver by macrophages activation and/or enhanced phagocytosis, reduced erythropoiesis and dyserythropoiesis (Lamikanra *et al*., 2007). *P. berghei* infected mice suffer from anemia because of RBC destruction, either by parasite multiplication or by spleen reticuloendothelial cell action as the presence of many

abnormal RBC stimulates the spleen to produce many phagocytes (Chinchilla *et al*., 1998). All this mechanisms are accountable to malaria induced anemia in mice and human (Lamikanra *et al*., 2007). This necessitates hematocrit (packed cell volume) analysis that evaluates the effectiveness of the extract in preventing hemolysis. In this study, it was noted that both crude extracts and solvent fractions of *V. amygdalina* prevented reduction in PCV at all dose levels when compared to negative control in a dose dependent manner. This could be as a result of destructive antiplasmodial effect of the extract against the parasitized red blood cell and the causative parasite, thereby sustaining the availability of the new red blood cells produced in the bone marrow. This result is in agreement with Fantahun (2015) who reported the effect of crude extracts and fractions of *Strychnos mitis* leaves significantly prevented PCV reduction. Failure of the aqueous fraction to reverse PCV reduction could probably be related to the presence of higher concentration of saponins which are known to have strong hemolytic effects (Yang *et al*., 2005).

The therapeutic benefits of traditional remedies are often attributed to the presence of non-nutritive bioactive constituents (Ghisalberti, 2008). Many secondary metabolites of plants' origin were found to have antimalarial activity (Bero *et al*., 2009; Onguéné *et al*., 2013). As explained by Dharani *et al*. (2008), common antimalarial plants used to treat malaria in traditional medicine contain secondary metabolites, such as alkaloids, terpenoids, coumarins, flavonoids, chalcones, quinones and xanthones. Antiplasmodial screening of plants has implicated alkaloids, terpenes and flavonoids in this activity (Christensen and Kharazmi, 2001). Sesquiterpenes and monoterpenes such

as limonene have been implicated in endoperoxidation, leading to plasmodicidal activity (Hatzakis *et al*., 2007).

The preliminary phytochemical tests conducted on the hydroalcoholic and aqueous extracts as well as chloroform, butanol and aqueous fractions of *V. amygdalina* revealed the presence of alkaloids, tannins, saponins, flavonoids, terpenoids, steroids, cardiac glycosides, anthraquinones and phenol. Therefore, this phytochemical screening is in agreement with other studies (Anyasor *et al*., 2010; Akinjogunla *et al*., 2011; Audu *et al*., 2012 and Odeh and Usman, 2014).

The plant contains active secondary metabolites which have antimalarial activities. Some of the identified sesquiterpene lactones are vernolide, vernodalol, vernolepin, vernodalin and hydroxyvernolide flavonoids luteolin, luteolin 7-O-β-glucoroniside and luteolin 7-O-β-glucoside are found in the leaves of *V.amygdalina*. Other researchers have confirmed the presence of flavonoids in the plant. Other phytochemicals present in the leaves of *V.amygdalina* are terpenes, coumarins, phenolic acids, lignans, xanthones and anthraquinones (Ijeh and Ejike, 2011).

The presence of phytochemicals such as sesquiterpene lactones and flavonoids namely vernolide and vernodalol, luteolin and luteolin- 7-O-glucoside isolated from *Vernonia amygdalina* has been revealed using various solvent system for extraction and analytic techniques such as chromatography, IR and NMR for partial characterization (Audu *et al*., 2012). Meanwhile, other anti-plasmodial phytoconstituents reported include quinoline alkaloids such as cephantharin, cryptolepine, isocryptolepine and neocryptolepine, courmarins and terpenoids (Iwalokun, 2008). The last two phytoconstituents are

abundantly present in Vernonia sp. and may thus contribute to the observed anti-berghei properties of the plant (Iwalokun, 2008). Terpenoids play a role in interaction of plants with their environment and have been shown to have a broad range of biological activities such as antibiotic, cytotoxic, antimalarial, antifeedant, insecticidal, moluscidal and herbicidal properties. For example, the anticancer drug, taxol and the antimalarial drug Artemisinin are widely known terpene based drugs (Toyang and Verpoorte, 2013).

Although the mechanism of action of these extracts and solvent fractions have not been elucidated, some plants are known to exert antiplasmodial activity either by causing red blood cell oxidation (Hatzakis *et al*., 2007) or by inhibiting protein synthesis, (Kirby *et al*., 1989) depending on their phytochemical constituents. The extracts and solvent fractions could have exerted its action through either of the two mechanisms mentioned above or by some other unknown mechanism. There are several proposed mechanisms through which these phytochemicals exert their antimalarial effect. Alkaloids like chloroquine exert their antimalarial effect by disrupting the parasite ability of detoxification, which by doing so exposes the parasite to toxic heme compounds (Correˆa Soares *et al*., 2009). Several phenolic compounds, especially flavonoids and tannins are reported to have antiplasmodial activities.While the antiplasmodial activities of tannins like ellagic acid has been largely ascribed to their antioxidant activities (Soh *et al*., 2012), flavonoids have been found to exert antiplasmodial effect by inhibition of the influx of L-glutamine and myoinositol into infected RBCs that are important for parasite growth (Saxena *et al*., 2013). Terpenoids were also detected in the plants of this study and several terpenoids have been found to have antiplasmodial effect in other studies (Kaur *et al*., 2009; Onguéné *et al*., 2013). For

example, sesquiterpenes like artemisinin are believed to exert their antiplasmodial effect by alkylation of proteins like heme (Robert and Meunier, 1998) or by generation of free radicals that inhibits sarco/endoplasmic reticulum Ca^2 -ATPase (SERCA) outside the food vacuole after activation by iron (Eckstein-Ludwig *et al*., 2003). Steroidal compounds, a subgroup belonging to the class terpenoids, were also found to exert their antimalarial activity by changing the membrane of infected RBC and hence block entry of essential nutrients like amino acids into the RBCs and thereby into the parasite (Elford, 1995).

The extracts and fractions could have exerted its action through either of the mechanisms mentioned above or by some other unknown mechanism. Hence, these compounds might be acting singly or in synergy of phytoconstituents of *V. amygdalina* with one another to exert the antiplasmodial activity observed in this study.

6. CONCLUSION

The result of the *in vivo* acute toxicity study of both extracts and solvent factions of the leaf of *V. amygdalina* in Swiss albino mice revealed no signs of toxicity and showed the safety of the plant. The results of the present study indicated that the *in vivo* administration of both extracts and solvent fractions of the leaf of *V. amygdalina* possess antimalarial activity and were capable of suppressing parasitemia. Chloroform fraction was found to be relatively the most active fraction and might contain potential lead molecule for the development of a new drug for treatment of malaria.The antimalarial action of both extracts and solvent fractions has been attributed to the presence of active secondary metabolites in the leaf of *V. amygdalina*. This confirms their use in ethnomedicine in the treatment of malaria in local communities.

7. RECOMMENDATIONS

- ♥ Despite the fact that the experimental plant used in the study was considered safe, further *in vitro* and *in vivo* experiments that incorporate therapeutic and toxicology studies are required.
- ♥ Similar study should be conducted on the stem and root of *V. amygdalina* to compare the antimalarial activity with its leaf.
- ♥ Therefore, there is a need to advance the current status of *Vernonia amygdalina* to an antimalarial leas drug level through isolation and characterization active antiplasmodial components in the extracts and solvent fractions, known to contain many terpenoid and alkaloid compounds.
- ♥ Further studies should be carried out to determine the mechanism of action(s) responsible for the antimalarial activities.

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