ANTI-PLASMODIAL EFFECTS OF BITTER LEAF AND NEEM LEAF EXTRACTS ON *PLASMODIUM BERGHEI* INFECTED MICE

BY

ABDULRAHAMAN, Mayowa Amir

M.TECH/SLS/2017/6879

DEPARTMENT OF ANIMAL BIOLOGY

FEDERAL UNIVERSITY OF TECHNOLOGY, MINNA

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ABSTRACT

Malaria remains one of the most infectious diseases, caused by protozoan parasite of the genus *Plasmodium* and it is the most prevalent tropical disease resulting in high morbidity with its consequent economic and social loss. Currently, the control of malaria has become gradually more complex due to the spread of drug-resistant parasites. Medicinal plants are the unquestionable source of effective antimalarial agent. The present study was designed to evaluate antiplasmodial activity of crude methanol extract and alkaloidal fractions of *Vernonia amygdalina* and *Azadirachta indica* in *plasmodium berghei* infected mice. The effects of ethanolic extract of *Vernonia amygdalina* and *Azadirachta indica* on body weight changes, biochemical and haematological parameters were assessed. Also, the phytochemical constituent of the plants was analysed for the presence of bioactive metabolites and the acute lethal doses were determined using standard procedure. A rodent malaria parasite, *Plasmodium berghei*, was used to inoculate healthy Swiss Albino mice of both sexes aged 6–8 weeks and weight 28–35 g. Crude methanol extract and the alkaloidal fractions were administered at different doses 150, 300 and 600 mg/kg body weight. Parameters, including parasitemia, body weight, and packed cell volume were then determined using standard tests. The LD_{50} of the plant was extrapolated to be above 5000 mg/kg body weight. The results indicated the presence of phytochemicals including flavonoids, phenols, tanins, and alkaloids. The Methanol extract of *V. amygdalina* and *A. indica* leaves exhibited very good antiplasmodial activity as they had exhibited parasitaemia suppression of 52.23 ± 2.34 and 90.21 ± 3.94% at concentrations of 150-600 mg/kg, respectively. The body weight of all the experimental groups show initial decreases after *P. berghei* infection. However, groups of mice treated with crude and alkaloidal fraction of *V. amygdalina* and *A. indica* shows a significant ($p<0.05$) improvement in the body weight after treatments. In addition, crude extract from both plants exhibited more pronounced positive effect on the weight gain of the animals than their respective alkaloidal fraction. There was a dose dependent increase in the hematological indices when compared with the untreated control. The alkaloidal fractions of these plants show relatively low antiplasmodial activities compare to the crude extract, thus indicating a synergetic activities of phytochemicals component of each plants. Therefore, these plants can be developed as a new drug for the treatment of malaria.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Malaria remains one of the most infectious diseases, caused by protozoan parasite of the genus *Plasmodium*. *Plasmodium* parasites is a major public health problem in tropical zones including Africa, South and Central America, and Asia. According to the Malaria Report of 2016, 212 million cases of malaria occurred in 2015 with over 429,000 deaths particularly in sub-Saharan Africa, where an estimated 90% of all malaria deaths occur (World Health Organization, 2016). Each year, more than 1.5–2.7 million deaths associated with malaria are reported globally, in the past years, most of them were children (Angayarkanni *et al*., 2010; Moon, 2010). As reported by Moon (2010), more than 90 countries with an estimated population of 2,400 million people was infected with endemic malaria. According to the WHO (2013), malaria is endemic in many countries, predominantly in Africa, Asia and Latin America. About half of the world's population is living in malaria risk areas (WHO, 2009). Each year an estimated 300–400 million clinical cases of malaria occur (Onwuamah *et al*., 2010). A total of 109 countries were endemic for malaria in 2008, 45 within the WHO African region (Batista *et al.,* 2009). Evidence on Nigeria shows that malaria incidence throughout the country had been on the increase over the years ranging between 1.12 million at the beginning of 1990 and 2.25 million by the turn of the millennium 2000 and 2.61 million in 2003 (Alaba & Alaba, 2003). Nevertheless, there is a spatial and temporal variation in mortality and morbidity of malaria; particularly in semiarid and high land regions of Africa. According to Chima *et al*., (2003), the economic costs of malaria can be classified as direct and indirect. The direct one is the costs of expenditure on prevention and treatment. The indirect impact is the costs of productive labor time lost due to

malaria morbidity and mortality (Niringiye & Douglason, 2010). Despite this economic loss, there is a scarcity of therapeutic drugs in rural areas; hence cultural practices still remain important (Traore- Keita *et al.,* 2000). The financial loss due to malaria annually is estimated to be about 132 billion naira in form of treatment cost, prevention and loss of man-hours (Kabiru *et al.,* 2013). In Nigeria and the rest of the world, malaria infection continues to pose a major health challenge.

Transmission of malaria is through the bite of a female *Anopheles* mosquito infected with Plasmodium species, and it also can be acquired congenitally, through blood transfusion, sharing of contaminated needles and organ transplantation. Of all the species of Plasmodium, only four (4) infect humans. These species of plasmodium include; *Plasmodium falciparum* being the most severe causative agent and it is responsible for about 1-3 million mortalities per year mainly in children and woman (Snow *et al.,* 2005), *Plasmodium vivax* is now increasingly recognized also as a major cause of severe malaria and death (WHO, 2013b)*, Plasmodium malariae,* and *Plasmodium ovale.* (Gatie, 2010; Caraballo, 2014; CDC, 2015; Adeniyi *et al*, 2016). Additionally, in some parts of Southern Asia there are reports of *Plasmodium knowlesi* (Zoonotic malaria) a newly recognized species that cause malaria in macaques but can also infect humans (Kantele and Jokiranta, 2011).

Various factors are responsible for the epidemiology and clinical manifestation of the disease in different parts of the world, climate and other environmental conditions such as the level of immunity of the exposed human populations and susceptibility to available drugs makes the development of both the vector and parasite rapid.

Malaria can also be transmitted through blood transfusion among people living in malaria endemic areas, semi-immunity to malaria allows donors to have the parasite

without any fever or other clinical manifestations. Malaria can be transmitted through organ transplantation (i.e., congenital malaria) which can be significant in populations that are semi-immune to malaria. It is however very serious among pregnant women and children because they have less immunity. When malaria infection is not properly treated in pregnant women, it can cause anaemia and also lead to stillbirths, underweight babies and maternal deaths. Furthermore, malaria in school children is a major cause of absenteeism in endemic countries. It is estimated that about 2% of children who recover from cerebral malaria suffer brain damage including epilepsy (WHO/UNICEF, 2005).

Plants have always been exemplary sources of drug and many of the currently available drugs have been directly or indirectly obtained from plants and from time immemorial, several human diseases have been treated with medicinal plants based on traditional medical information (Haile and Delenasaw, 2007). According to the WHO, about 80% of the world population depended on herbal remedies, for their primary health care needs (Muthu *et al.,* 2006). The rapid spread of drug-resistant malaria parasites coupled with adulterated anti-malarial drugs in the market have made the treatment and control of the disease very challenging (Anyanwu and Onyesom, 2007). According to WHO, drug resistance is probably the most important factor affecting malaria control at the present time. Since its development in the forties, chloroquine has been successfully used by millions of human beings for prevention or treatment of all malaria species (Canfield, 1980). The emergence of *P. falciparum* resistance to chloroquine, mefloquine, sulphadoxine and pyrimethamine, other conventional anti-malarial drugs as well as insecticide resistance in *Anopheles* mosquito vectors have led to a dramatic resurgence of the disease. Resistance to these drugs has been reported to be as high as 40-60% in some African and Asian countries. Chloroquine resistance is now universal, and

currently the most effective combinations are based on derivatives of artemisinin, an extract from the herbal plant *Artemisia annua*. These combinations are referred to as Artemisinin-based combination therapy (ACT), and are very effective against *P. falciparum*.

In view of the resistance of the parasite to antimalarial drug therapy, development and promotion of phytomedicines may be the sustainable solution to malaria treatment (WHO, 2013) and new drugs or drug combinations are urgently required for the treatment of malaria infections from traditional medicinal plants (Hoareau and DaSilva, 1999).

Medicinal plants have always been considered to be a possible alternative and rich source of new drugs and have been used in combating several diseases, some of the antimalarial drugs in use today such as quinine and artemisinin were either obtained directly from plants or developed using chemical structures of plant-derived compounds as templates (Basco *et al.,* 1994). Some of the most potent antimalarial agents developed from plants relying on traditional knowledge include quinolines and endoperoxides/artemisinin derivative (Orwa, 2002; Waako *et al.,* 2005) and other antimalarial drugs such as piperaquine, mefloquine, Lumefantrine and naphthoquine have also been derived from plant sources (Sinclair *et al.,* 2009; Nsagha *et al.,* 2012). Plants are a rich source of nutrient and are an alternative means for proffering new antimalarial drug. Traditional medicinal plants play an important role in the medical system in Nigeria; however, plant materials remain an important resource to combat serious diseases in the world.

Some of the problems associated with malaria are the control of mosquitoes and also their resistance to a wide range of insecticides. Secondly, is the production of fake

antimalarial drugs. Thirdly, most of the *Plasmodium* parasites are resistant to most widely available and affordable drugs like Chloroquine and Fansidar (Kisame,2005). Lastly, most countries in Africa lack the necessary infrastructure and resources to manage and control malaria (WHO, 1994). The use of synthetic antimalarial drugs is among the main ways to treat malaria and are effective in controlling parasite load. However, the practical application of the majority of these therapeutic agents remains restricted owing to their limited action, pharmacokinetic properties, secondary failure rate, and accompanying side effects (Saganuwan and Onyeyili, 2011). With the increasing incidence of the disease in urban/rural population throughout the world, there is clear need for development of indigenous, inexpensive botanical sources for antimalarial crude or purified drugs.

1.2 Statement of the Research Problem

Despite advances in therapeutic agents used in the treatment and control of malaria over the past few decades, these available agents are already limited in their efficacy, while drug resistance threatens the ability to prevent and treat the infection. With the increasing incidence of the disease in urban/rural population throughout the world, there is a clear need for the development of indigenous sources for antimalarial crude or purified drugs. Disparities in development of new drugs for malaria vary due to public health importance of the disease and the amount of resources invested in developing new cures.

In the quest to search for a safe and effective anti-malarial drug, several herbal plants show promising anti-malaria activities, screening of extracts from plants used in traditional medicine have been reported to be a possible means for proffering a lasting solution aimed towards development of a new drug. Research on the bio-active ingredient identification and evaluation of these plants are not yet satisfactory. Studies

on the various therapeutic effects of *Azadirachta indica* has been reported which includes antifertility (Lal *et al,* 1986), antidiabetic (Bhargawa, 1986). Various studies have also been reported on the therapeutic effect of *V. amygdalina*. Compared to these other therapeutic activities, there has been no satisfactory report on its antimalarial tendencies. Another problem which is a major limiting factor is that the bio-active ingredients present in *Vernonia amygdalina* and *Azadirachta indica* leaf extract and their efficacy against *P.berghei* in infected mice have no yet been well evaluated. Apart from the inherent weaknesses of antimalarial drugs, there is also a challenge of availability and high cost of antimalarial drugs to those that are mostly affected, especially in developing countries, (Matowe and Odeyi, 2010).

The difficulty of creating a novel and efficient vaccines and also the adverse side-effects of the available anti-malaria drugs, beside the multiple drugs resistance to *plasmodium* species potency, highlight the urgent need to search for novel and well tolerated anti malaria drug agent from natural products (plants).

1.3 Justification for the Study

Tropical rain forest plant has been identified to have higher concentration of natural chemical defenses and in greater diversity than plant from any other biome, thus, they are potential sources of new medicines. The increase in the use of insecticide treated nets, indoor residual spraying and early treatment with Artemisinin-bases Combination Therapies (ACT's) has led to a reduction in mobility and mortality caused by malaria in some African countries (ACTwatch Group *et al*., 2017). Besides, the high cost of malaria treatment has left the poor masses of Nigeria heavily reliant on traditional practitioners and medicinal plants for the treatment of the disease (Benneth, 2008).

Chemotherapeutic agents will continue to be in demand for the complete management of malaria (Ginsburg and Deharo, 2001) and the issue of resistance means that discovering new antimalarial drugs is an urgent priority.

1.4 Aim and Objectives of the Study

The aim of this study was to evaluate the *in vivo* anti-malaria activities of crude extract and a fraction of *Azadirachta indica* and *Vernonia amygdalina* in *Plasmodium berghei* infected mice.

The objectives of the study were to determine the:

i. Qualitative phytochemical composition of the crude methanol extract of *Azadirachta indica* and *Vernonia amygdalina*
ii. Acute toxicity of the crude methanol extract of *Azadirachta indica* and *Vernonia amygdalina.*
iii. Antiplasmodial potency of the crude methanol extract and a fraction of *Azadirachta indica* and *Vernonia amygdalina*
iv. Effect of the crude methanol extract and fractions of *Azadirachta indica* and *Vernonia amygdalina* on body weight, hematological indices and some liver parameters of *Plasmodium berghei* infected mice.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1.0 Plasmodium

Plasmodium is a genus of unicellular eukaryotes that are obligate parasites of vertebrates and insects. The life cycles of Plasmodium species involve development in a blood-feeding insect host which then injects parasites into a vertebrate host during a blood meal. Parasites grow within a vertebrate body tissue (often the liver) before entering the bloodstream to infect red blood cells. The ensuing destruction of host red blood cells can result in a disease, called malaria. During this infection, some parasites are picked up by a blood-feeding insect, continuing the life cycle (Centre For Disease Control, 2015).

Plasmodium is a member of the phylum Apicomplexa, a large group of parasitic eukaryotes (Figure 2.1). Within Apicomplexa, *Plasmodium* is in the order Haemosporida and family Plasmodiidae. Over 200 species of *Plasmodium* have been described, many of which have been subdivided into 14 subgenera based on parasite morphology and host range. Evolutionary relationships among different *Plasmodium* species do not always follow taxonomic boundaries; some species that are morphologically similar or infect the same host turn out to be distantly related.

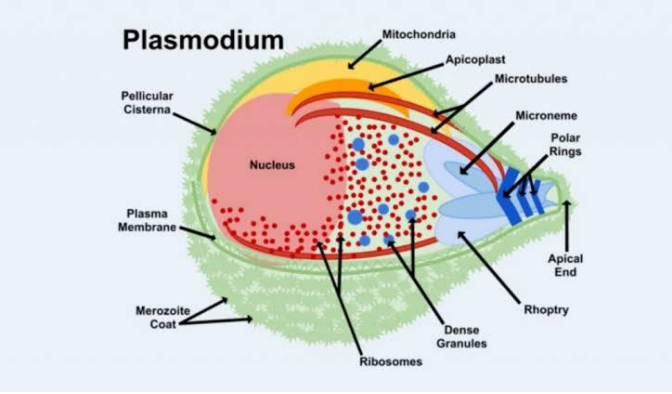

Figure 2.1: Shows a protest, the plasmodium which is an eukaryote of the phylum Apicomplexa

Source: (CDC, 2016)

2.1.1 Taxonomical Classification of Plasmodium

Kingdom	Protista
Subkingdom	Protozoa
Phylum	Apicomplexa
Class	Sporozoasida
Order	Haemosporida Eucoccidiorida
Family	Plasmodiidae
Genus	*Plasmodium*
Species	*falciparum, malariae, ovale, vivax*

2.2 The Global Burden of Malaria

Malaria is one of the major diseases of poor people in developing countries and one of the leading causes of avoidable death, especially in children and pregnant women. The people living in the sub-Sahara Africa have the highest risk of acquiring malaria. On the contrary, in other regions of the world, particularly Latin America and most part of Asia, levels of transmission are much lower and malaria tends to affect people of all ages causing severe morbidity, but less commonly resulting in death (United Nations Children's Fund, 2011). The global burden of malaria is terrible and the disease is caused by many factors. This global burden of malaria has been defined differently by various individuals in the field. Epidemiologists defined this burden based on morbidity and mortality while economists refer to it as a quantification of the costs (direct and indirect), effects on levels of productivity, national growth, and development. Common to both of these approaches is a focus on disease and risk factors, with the aim of establishing causative linkages with broad quantitative outcomes (Jones and Williams,

2004). In spite of promising progress in controlling the diseases, malaria remains one of the major public health problems in Africa and is responsible for the heavy burdens on individual, families and the nation as a whole.

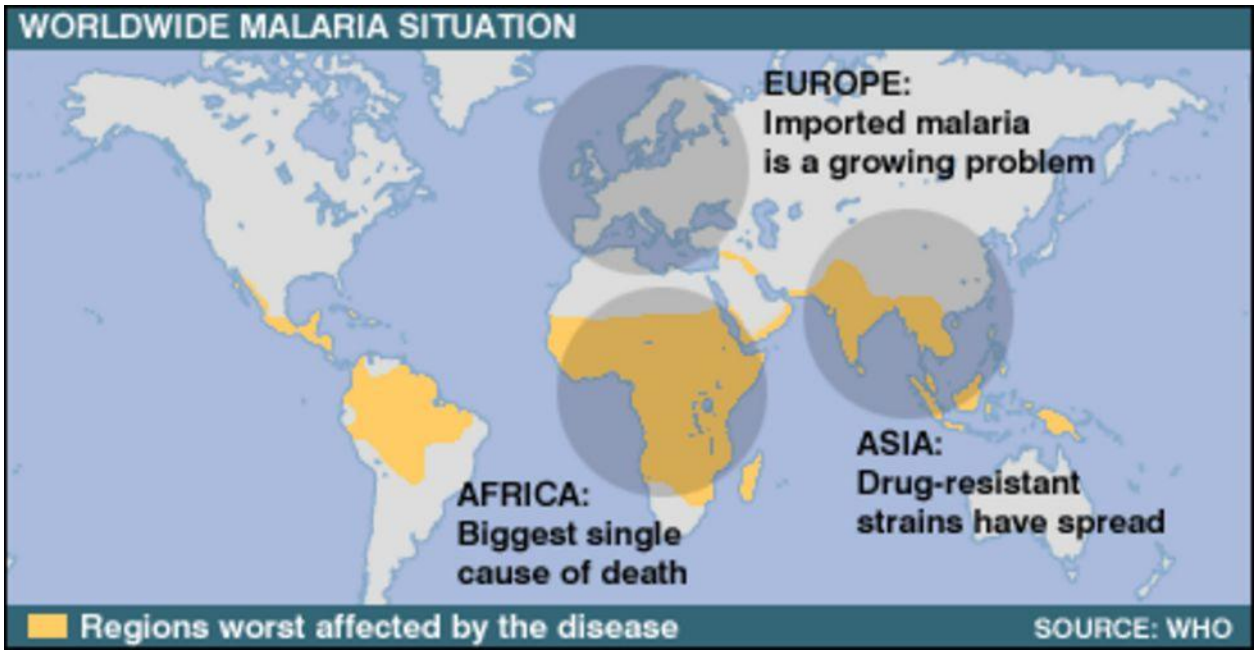

Figure 2.2: Figure above shows the region worst affected by malaria

Source: **(WHO, 2009)**

Malaria affects the economy in different way and the above picture in Figure 2.2 shows the regions worst affected by the disease. The direct effect is that adults are unable to work during episodes of the disease, and may be significantly weakened for a period afterward. Repeated infection with malaria is associated with anaemia in children and adults (Weil, 2010). Malaria also retards economic and social development through effects such as reduced working hours due to sickness or attending to the sick, income spent on financing health care, which in turn, lead to impacts at national level due to massive health care budgets, reduced productivity of the work force, and the like (Pierre-Louis *et al*., 2005). Efforts to reduce poverty and childhood mortality in those vulnerable societies will fail if this devastating disease is not adequately controlled

(Alonso, 2010). The most infectious form of the parasite, *Plasmodium falciparum* is responsible for as many as 365 million clinical malaria cases and more death in children in Africa in a single year.

In Sub-Saharan Africa, malaria is the leading cause of death for children under five. Infection during pregnancy, particularly among new mothers increases the risk of maternal mortality, neonatal mortality, and low birth weight (WHO, 2014). According to the World Health Organization, an estimated 198 million cases of malaria occurred globally in 2013 and the disease led to 584,000 deaths, representing a decrease in malaria case incidence and mortality rates of thirty and forty-seven percent since 2000, respectively. The burden is heaviest in Sub-Saharan Africa, where an estimated 90% of all malaria deaths occur, and in children aged under 5 years account for 78% of all deaths. In addition to loss of life, malaria places an economic burden on African nations. The burden is carried mostly by poor, rural families that have less access to current prevention and treatment services. Despite the devastation caused by malaria, increased international attention and funding for prevention and treatment is saving lives. Between 2000-2013, malaria incidence was cut by 30% globally and 34% in Africa and that malaria mortality decreased by 47% worldwide and 54% in Africa.

2.3 Malaria Situation in Nigeria

In Nigeria, malaria is endemic throughout the country. Nigeria is greatly affected with the burden of malaria as shown in Figure 2.3, with approximately 10 million cases and 300,000 deaths reported annually, while 97% of the total population (approximately 173 million) is at risk of infection (WHO, 2014). Moreover, malaria accounts for 60% of outpatient visits to health facilities, 30% childhood death and 11 % maternal mortality (USEN, 2011; Dawak *et al*., 2016).

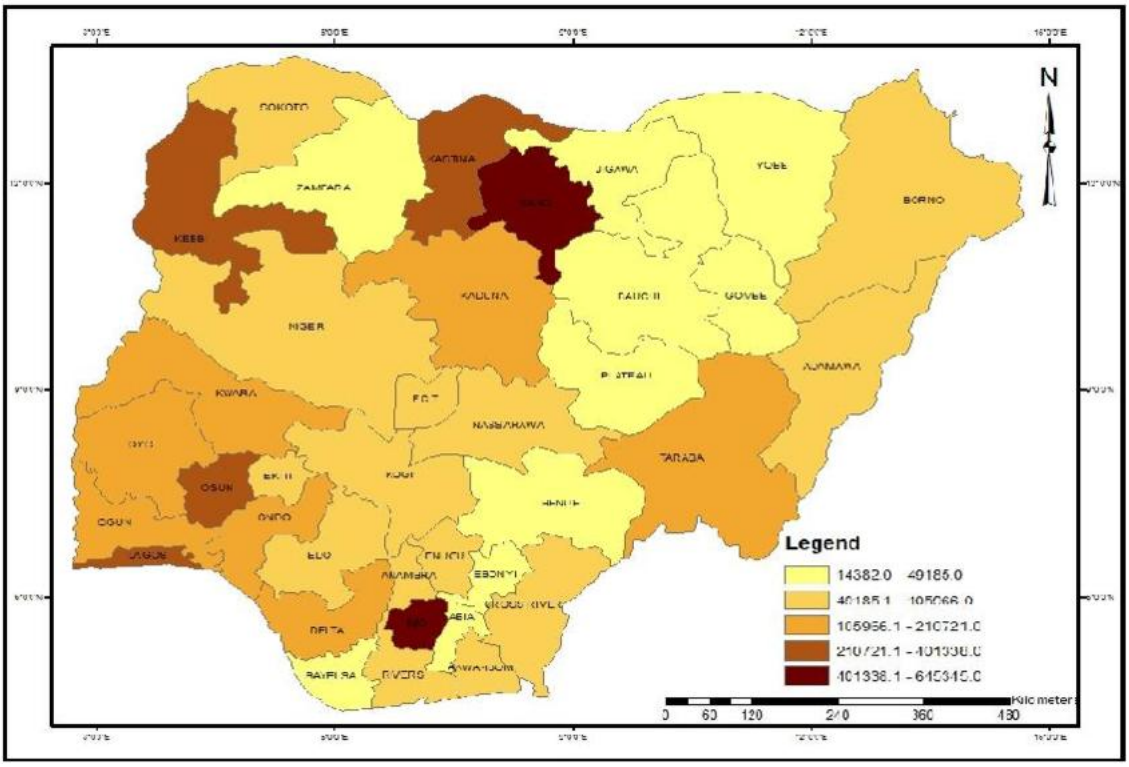

Figure 2.3: Situation of Malaria in Nigeria **Source: (Osayemi, 2014)**

2.4 Life cycle of Malaria

2.4.1 Pathophysiology

In humans, malaria is caused by four species of the plasmodium protozoa (single celled parasites) – *P. falciparum, P. vivax, P. ovale* and *P. malariae*. Of these species *P. falciparum* accounts for the majority of infections and is the most lethal.

2.4.2 Pre-erythrocytic schizogony

When an individual is bitten by the female anopheles mosquito, sporozoites are injected by the mosquito into the subcutaneous tissue (less frequently directly into the bloodstream) and travel to the liver either directly or through lymphatic channels. They reach the liver in 30-40 minutes. Approximately 8-15 (up to 100) sporozoites are injected and therefore only a few hepatocytes are infected, therefore this stage of the infection causes no symptoms. Recent evidence indicates that sporozoites pass through

several hepatocytes before invasion. Within the hepatocyte, each sporozoite divides into 10,000-30,000 merozoites. This phase is called pre-erythrocytic schizogony, meaning the development of forms of the parasite before reaching the red blood cells. This phase takes about 10-15 days in *P. vivax, P. malaria* but in *P. falciparum* malaria it takes about 7-10 days.

2.4.3 Erythrocytic schizogony

At the completion of the pre-erythrocytic schizogony, the mature schizonts rupture the liver cells and escape into the blood, wherein they infect the red blood cells. These infective forms are called merozoites and they continue their growth and multiplication within the red blood cells. In *P. vivax* malaria, the young red blood cells are predominantly infected, while in *P. falciparum* and *P. malaria*, red blood cells of all ages are affected. Thus the infective load and severity of infection are more in case of *P. falciparum* malaria. The growth and multiplication cycle within the RBCs (erythrocytic schizogony) takes about 48 hours for one cycle (72 hours in case of *P. malariae*). Each merozoite divides into 8-32 (averagely 10) fresh merozoites. The merozoites grow in stages into rings (trophozoites) and divides to release more merozoites. At the end of this cycle, the mature schizonts rupture the RBCs and release the new merozoites into the blood, which in turn infect more RBCs.

All the clinical features of malaria are caused by these developments in the blood. The growing parasite progressively consumes and degrades intracellular proteins, principally haemoglobin, resulting in formation of the 'malaria pigment' and haemolysis of the infected red blood cell. This also alters the transport properties of the red blood cell membrane, and the red blood cell becomes more spherical and less deformable. The rupture of red blood cells by merozoites releases certain factors and toxins (such as lycosylphosphotidylinositol anchor of a parasite membrane protein, phospholipoprotein,

RBC membrane products, protease sensitive components with hemozoin, malarial toxins etc.), which could directly induce the release of cytokines such as tumor necrosis factor (TNF) and interleukin-1 from macrophages, resulting in chills and high grade fever. This occurs once in 48 hours, corresponding to the erythrocytic cycle. In the initial stages of the illness, this classical pattern may not be seen because there could be multiple groups (broods) of the parasite developing at different times, and as the disease progresses, these broods join and the synchronous development cycle results in the classical pattern of alternate day fever. It has been observed that in primary attack of malaria, the symptoms may appear with lesser degree of parasitemia or even with submicroscopic parasitemia. However, in subsequent attacks and relapses, a much higher degree of parasitemia is needed for onset of symptoms.

2.4.4 Exo-Erythrocytic schizogony

In *P. vivax* and *P. ovale*, some exoerythrocytic forms remain as single-celled dormant forms called hypnozoites. This helps them to survive in temperate countries. These hypnozoites can get re-activated once in 3-6 months to cause 'relapses'. This phase of the infection is called exoerythrocytic schizogony. In *P. falciparum* and *P. malariae* infections, relapses from the liver do not occur; however, the blood infection may remain chronic and, if untreated, may remain for years in case of *P. falciparum* and decades in case of *P. malariae*. Some of the merozoites in the blood transform into sexual forms called gametocytes. These appear in the peripheral blood after 7-10 days of the infection in *P. vivax* and 10-20 days in *P. falciparum* infection. When anopheles mosquito bites an infected individual, these gametocytes enter the mosquito and continue their sexual phase of development within the gut wall of the mosquito. This completes the asexual-sexual cycle of the malaria parasite.

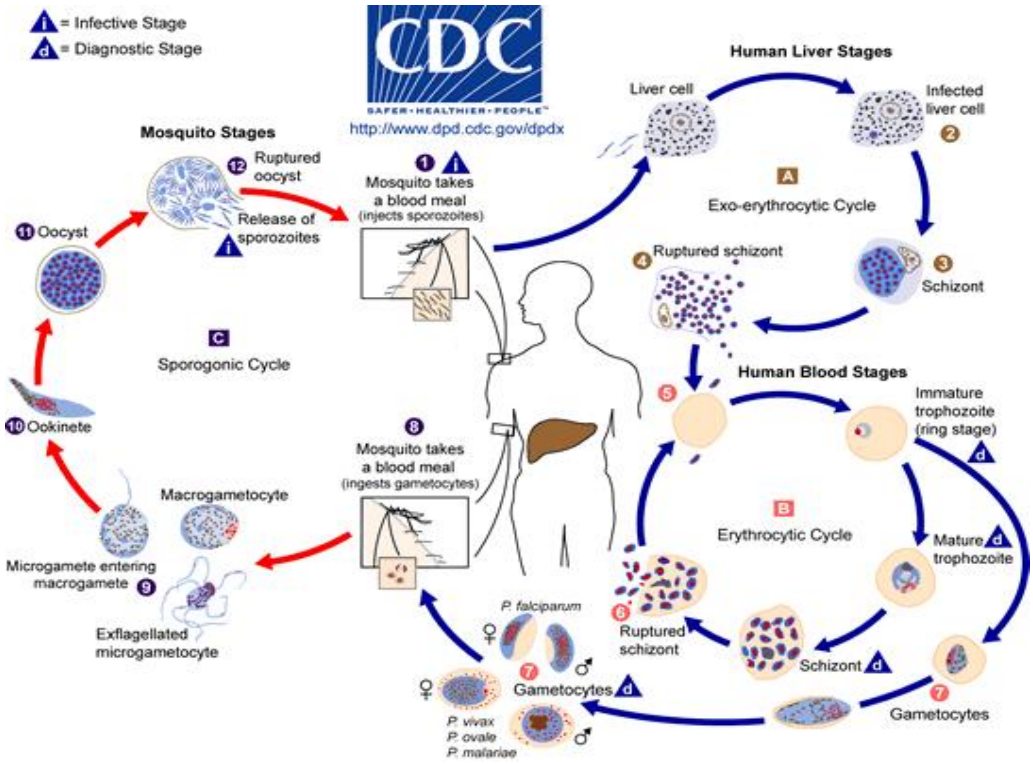

Figure 2.4: Life Cycle of Malaria Parasite **Source: (WHO, 2006)**

2.5 Signs and Symptoms of Malaria

The early signs and symptoms of malaria tend to be non-specific, characterized by fever, chills, headache, loss of appetite and body aches in adults or fever plus any other symptom in children. *P. falciparum* malaria as shown in figure 2.4 may progress to severe disease, sometimes very rapidly. Symptoms of malaria including fever, headache, and vomiting, usually appear between 10 and 15 days after the mosquito bite. If not treated, malaria can quickly become life threatening by disrupting the blood supply to vital organs. In a given area, the pattern of severe malaria varies with age; Severe anaemia predominates in the youngest ages, 15–24 months while Coma is more common in the older ages, 36–48 months and between areas with differing intensity of transmission; Age distribution of severe malaria shifts downward with increased intensity and relative proportion of severe anemia to coma increases with increased intensity.

Hence, among young children, frequent episodes of severe malaria may negatively impact on their learning abilities and educational attainment. This is a threat to human capital accumulation, which constitutes a key factor in economic development. The debilitating effects of malaria on adult victims are very much disturbing. In addition to time and money spent on preventing and treating malaria, it causes considerable pain and weakness among its victims. The mother may have placental parasitemia, peripheral parasitemia, or both, without any fever or other clinical manifestations. Causes of death in malaria have been reported including cerebral malaria, acute hemolysis and severe anemia, and vital organ damage and failure (English and Newton, 2002; López Del Prado *et al.,* 2014). Malaria associated with liver and renal damage occur between 2-5% of hospitalized patients with a mortality that can reach up to forty-five % (Mishra *et al.,* 2008; Saya *et al.,* 2012).

Untreated malaria is associated with co-morbidity in the form of anaemia. *P. malariae* also causes nephropathy and nephrotic syndrome (Ashley *et al*., 2014). Severe malaria manifestations cover a broad spectrum from prostration to unrousable coma (WHO, 2000). The mortality rate associated with treated cerebral malaria is twenty percent (20%) in non-pregnant adults and fifteen (15%) in children. Cerebral malaria during pregnancy carries a 50% mortality risk even with treatment. Symptomatic pregnant women are also at risk of fever induced contractions leading to abortion or premature delivery. Adults with cerebral malaria are typically comatose. Cranial nerve abnormalities are uncommon. Tone may be increased, decreased or normal and reflexes brisk or depressed. The abdominal reflexes are invariably absent. Fixed jaw closure and teeth grinding (bruxism) may be observed. Seizures are common and may be focal or generalised. A severe metabolic acidosis is often present in severe malaria secondary to

tissue hypoxia and is a major cause of death. Acute renal failure is more likely to occur in adults.

2.6 Malaria Diagnosis

Malaria must be recognized promptly in order to treat the patient in time and to prevent further spread of infection in the community via local mosquitoes. Malaria should be considered a potential medical emergency and should be treated accordingly.

There are different ways malaria can be diagnosed. They are:

i. **Clinical diagnosis:** this is based on the patient's symptoms and on physical findings at examination.

ii. **Microscopic diagnosis:** malaria can be identified by examining under the microscope, a drop of patient blood spread out as "blood smear**"** on a microscope slide, prior to examination, the slide is stained with mostly Giemsa stain to give parasite a distinctive appearance.

iii. **Antigen detection:** various test kits are available to detect antigens derived from malaria parasites. These Rapid Diagnostic Tests (RDTs) offer a useful alternative to microscopy in situations where reliable microscopic diagnosis is not available.

iv. **Molecular diagnosis:** parasite nucleic acids are detected using polymerase chain reaction (PCR). This is slightly more sensitive than smear microscopy. It is the most useful for confirming the species of malaria parasite after the diagnosis has been established by either smear microscopy or RDT.

v. **Serology:** serology detects anti bodies against malaria parasites, using either indirect immunofluorescence (IFA) or enzyme-linked immunosorbent assay

(ELISA). Serology does not detect current infection but rather measures past exposure.

2.7 Prevention of Malaria

Currently, several malaria control interventions exist which are proven to be efficacious, safe and cost effective. These include insecticides treated nets (ITNs), indoor residual spraying (IRS), intermittent preventive treatment and artemisin-based combinations for malaria. In addition, intermittent preventive treatment in pregnant women and infants are promising strategies which has been delivered to pregnant women and infants in various settings with considerable success in reducing malaria transmission and malaria related anemia. The roll back malaria (RMB) partnership endorsed “Scale Up For Impact” (SUFI) as approach to rapidly increase access to and use of malaria control intervention. SUFI is predicted on the rapid deployment of a package of proven malaria interventions and high levels of coverage to quickly achieve the optimal health effects based on evidence from control trial.

2.8 Treatment of Malaria

Malaria is treated with antimalarial medications; the ones used depend on the type and severity of the disease. While medications against fever are commonly used, their effects on outcomes are not clear. Uncomplicated malaria may be treated with oral medications. The most effective treatment for *P. falciparum* infection is the use of artemisinin in combination with other antimalarial (known as artemisinin-combination therapy, or ACT), which decreases resistance to any single drug component. These additional antimalarials include: amodiaquine, lumefantrine, mefloquine or sulfadoxine/ pyrimethamine. Another recommended combination is dihydroartemisinin and piperaquine. ACT is about 90% effective when used to treat uncomplicated malaria. To treat malaria during pregnancy, the WHO recommends the use of quinine plus

clindamycin early in the pregnancy (1st trimester), and ACT in later stages (2nd and 3rd trimesters). In the 2000s (decade), malaria with partial resistance to artemisins emerged in Southeast Asia. Infection with *P. vivax, P. ovale or P. malariae* is usually treated without the need for hospitalization. Treatment of *P. vivax* requires both treatment of blood stages (with chloroquine or ACT) and clearance of liver forms with primaquine.

Recommended treatment for severe malaria is the intravenous use of antimalarial drugs. For severe malaria, artesunate is superior to quinine in both children and adults. Treatment of severe malaria involves supportive measures that are best done in a critical care unit. This includes the management of high fevers and the seizures that may result from it. It also includes monitoring for poor breathing effort, low blood sugar, and low blood potassium.

2.9 Drug Resistance in Malaria

Antimalarial drug resistance has been defined as the "ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject". This definition was later modified to specify that the drug in question must "gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action". Most researchers interpret this as referring only to persistence of parasites after treatment doses of an antimalarial rather than prophylaxis failure, although the latter is a useful tool for early warning of the presence of drug resistance. This definition of resistance requires demonstration of malaria parasitaemia in a patient who has received an observed treatment dose of an antimalarial drug and simultaneous demonstration of adequate blood drug and metabolite concentrations using established laboratory methods (such as high performance liquid chromatography) or *in vitro* tests. In practice, this is rarely done with *in vivo* studies. *In vivo* studies of drugs

for which true resistance is well known (such as chloroquine) infrequently include confirmation of drug absorption and metabolism; demonstration of persistence of parasites in a patient receiving directly observed therapy is usually considered sufficient**.** Some drugs, such as mefloquine, are known to produce widely varying blood levels after appropriate dosing and apparent resistance can often be explained by inadequate blood levels.

2.10 Traditional Herbal Treatment of Malaria

In malaria endemic countries, traditional herbal medicine is the most frequent option to treat malaria with an average of a fifth of patients (Willcox *et al*., 2004)**.** It has been used to treat malaria since ancient time and surely if it was not effective, malaria would have devastated Africa **(**Idowu *et al*., 2010**)**. The World Health Organization (WHO) defines herbal medicines to include herbs, herbal materials, herbal preparations and finished herbal products that contain active ingredients parts of plants, or other plant materials, or combinations thereof (WHO, 2002).

There are 1200 plant species from 160 families used to treat malaria (Willcox *et* al., 2004)**.** In 1977, the World Health Assembly (WHA) drew attention to the potentials, efficacy and utilisation of herbal medicine in the national health care systems of member countries (Gyasi and Mensah, 2011). According to World Health Organization, about 60% of the world's population use herbal medicine for treating their sicknesses. In Ghana, Mali, Nigeria and Zambia, the first line of treatment for 60% of children with high fever resulting from malaria is the use of herbal medicine at home.

The dependence of people on indigenous herbal medicine are influenced by factors such as acceptability, less communication means, poverty, cost-effectiveness, ignorance, accessibility and unavailability of modern health facilities. Until now, the world has

relied on plants for the best malaria drugs: chloroquine from Cinchona tree; and Artemisinin from Chinese salad plant, *Artemisia annua* (Willcox *et* al., 2004).

The malaria parasite has developed resistance against most malaria drugs including the popular Artemisinin-based Combination Therapy (ACT) in some region. The rapid spread of resistance parasite, makes it necessary to search for more effective herbal antimalarial compounds. The herbal knowledge of indigenous communities for malaria treatment can play an important role in identification of any new antimalarial plants that is yet to discover. Pictures of *Azadirachta indica* (Neem) and *Vernonia amygdalina* (Bitter leaf) are shown in the diagram below (plate 2.1 and 2.2). They are well known medicinal plants and several biologically active compounds have been isolated from the plants. Neem is an evergreen tree, cultivated in various parts of the world. All parts of the plant are useful and have been used in treatment of diseases ranging from teeth decay, swollen liver, ulcers, dysentery, diarrhea, malaria and other bacterial infections (Allameh *et al*., 2002 and Mossini *et al*., 2004).

The bitter constituents of leaf, trunk, bark and seed oil are nimbin, nimbinin and nimbidin. It is well known that neem and its products have variety of uses such as antipyretic (Okpanyi & Ezenkwu, 1981), antifertility (Lal *et al*., 1986), antidiabetic (Bhargawa, 1986), spermicidial (Sinha *et al*., 1984), wound healing drug (Bhargawa *et al*., 1985). It has also been reported that the leaf extract is effective as analgesic, anthelminthic, antibacterial, antifungal, antihyperglycemic, anti-inflammatory, antiviral, antimalarial, antipyretic, insecticidal, hypercho-lesteremic and hypoglycemic agents (Maragathavalli *et al*., 2012). Thus, this study was to document the knowledge and the uses of medicinal plants used by the indigenous communities for malaria treatment in minna, which can be a part of an initiative to document baseline data for future pharmacological and phytochemical studies.

Plate 2.1: ***Azadirachta indica***

Plate 2.2: ***Vernonia amygdalina***

These plants in plate 2.1 and 2.2 has also been shown to contain appreciable quantities of ascorbic acid and caroteinoids (Ejoh *et al*., 2007). Calcium, iron, potassium, phosphorous, manganese, copper and cobalt have also been found in significant quantities in neem (Ejoh *et al*., 2007; Eleyinmi *et al*., 2008). In Nigeria, it is known variously as "Ewuro" in Yoruba language, "Onugbu" in Igbo language, "Oriwo" in Bini language, "Ityuna" in Tiv, "Chusar doki or fatefate" in Hausa, while it is known as "Etidot" in Cross River State of Nigeria. The leaves are used as green leafy vegetable and may be consumed either as a vegetable (leaves are macerated in soups). Enhanced antimalarial effect of chloroquine by aqueous *V. amygdalina* leaf extract in mice infected with chloroquine resistant and sensitive *P. berghei* strains has been demonstrated with a dose-dependent efficacy being observed (Iwalokun, 2008; Oboh *et al.,* 2018).

2.10.1 *Azadirachta indica*

It is an evergreen dense tree that is widely cultivated in Asia and Africa. The bitter constituents of leaf, trunk, and bark and seed oil are nimbin, nimbinin and nibidin. It is

well known that it's product has varieties of uses such as antipyretic, anti-fertility, anti-diabetic and wound healing drug. A decoction of leaves and bark is a bitter tonic and an alternative in chronic malarial fever (Dwivedi, 2004). *A. indica* has been extensively reported as being effective in the treatment of malaria caused by various strains of *Plasmodium* parasite including those resistant to traditional anti-malaria (Nwafor *et al.,* 2003; Ibrahima *et al*., 2012).

2.10.2 *Andrographis paniculata*

It is an annual, erect, quadrangular herb, often cultivated for medicinal value. The plant is febrifuge, antihelmintic and bitter tonic commonly used as a liver stimulant. Experiment has shown anti-typhoid, antipyretic and antibiotic activity (Rastogi and Mehrotra, 2004). The tribal and rural people widely use decoction of whole plant for malaria with remarkable success (Patel, 2006).

2.10.3 *Artemisia annua*

Artemisia annua named (A-3) is an herb which helps in the prevention and treatment of malaria and it can be grown by small scale farmers and by health centers and hospitals in their medicinal garden. The leaves are used to make Artemisia tea, and it can also be used for the treatment for malaria (Chopra *et al.,* 1956). It was gathered that, following treatment with Artemisia tea, most patients claim the interval before the next malaria attack becomes much longer, compared with modern pharmaceuticals, and even with Artemisinin based drugs, the tea contains a wider range of antimalarial components (Chopra *et al.,* 1956). This reduces the danger of resistance and so developing *Artemisia annua* is also helpful with many other diseases and complaints.

2.10.4 *Enantia chlorantha*

Enantia chlorantha is an herb whose safety has been ascertained. The stem bark of this plant is also known as African yellow wood and it is well distributed in the Southern part of Nigeria, West Cameroun, Gabon, Angola and Congo. The study on the cumulative effects to using *Enantia chlorantha* decoction to treat malaria fever by researchers confirmed that it was effective against malaria fever. It was later concluded that the usage of the plant for treatment of an ailment for a short period of time was safe considering that there were no significant pathological changes observed in blood parameters in the experiment carried out (Moody *et al.,* 2007).

2.10.5 Sorghum

Sorghum is an important staple food crop in African, South Asia and Central America, it is the fifth major cereal crop in the world after wheat, rice, maize and barley (Jeevani *et al.,*2011). It is also grown in the United States, Australia, and other developed nations for animal feed. Sorghum is most extensively cultivated in the drier Northern Guinea, Sudan Savannah and Greenland of African, Plains of India and the Great Plain United State of America (Egbere *et al.,*2007). It resembles maize in its vegetative character but different in having narrow leaves and a waxy bloom covering the leaves and stem. It also has a well-developed root/stem which is twice as efficient as that of maize although its leaf area is only half that of maize (Gbadamosi *et al.,*2011).

There is a huge potential benefit, both economic and health-wise if this food crop is developed as a natural food colorant in Nigeria. The stem has been used in the preparation of local medicine to treat anaemia and other related blood ailment. It has been reported to be used as an anti-malarial and anthelminthic.

2.10.6 *Vernonia amygdalina*

Vernonia amygdalina is a perennial herb belonging to the *Asteraceae* family, *V. amygdalina* is a tropical plant that grows up to 3 meters in height. It has a short life cycle and can be harvested twice per month for up to seven years. Extracts of the plant have been used in various folk medicines as a remedies against helminthic, protozoal and bacterial infections with subsequent scientific supports to these claims. It commonly called bitter leaf (English), Ndole (French), ewuro (Yoruba), shiwaka (Hausa), awonwon (Twi) and onugbu (Igbo). The leaves are dark green coloured with a characteristic odour and bitter taste.

The findings of Abosi and Raseroka, 2003; Tona *et al*., 2004 indicates that *V. amygdalina* leaf extract holds a promise as an antimalarial agent despite its inability to clear parasitaemia completely. Phytochemicals such as saponins and alkaloids, terpenes, steroids, coumarins, flavonoids, phenolic acids, lignans, xanthones, anthraquinones, edotides and sesquiterpenes have been extracted and isolated from *V. amygdalina*. This has been shown to contain significant quantities of lipids (Ejoh *et al*., 2007; Eleyinmi *et al*., 2008), proteins with high essential amino acid score (Ejoh *et al*., 2007; Eleyinmi *et al*., 2008) that compare favorably with values reported for *Telfairia occidentalis* and *Talinum* triangulare (Ijeh *et al*., 1996), carbohydrates (Ejoh *et al*., 2007) and fiber (Ejoh *et al*., 2007; Eleyinmi *et al*., 2008). Both the leaves and the roots of *V. amygdalina* plant are used traditionally in phytomedicine to treat fever, hiccups, kidney disease and stomach discomfort.

2.11 Eradication of Malaria

i. Malaria can be eradicated by intense efforts of governments and health organizations.

ii. Genetic method of using sterile male technique can be employed.

Also, antimalarial drugs should be improved from the common schizodoncidal level as this only affects the sporozoites and merozoites in the bloodstream. The hypnozoites that ensures recurrence of the disease and the gametocytes that aids the transmission are not being tackled. If these stages are combated by new improved drugs, the disease can be totally eradicated.

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Materials

3.1.1 Experimental Animals

Sixty-five (65) inbred male and female (non-pregnant) albino mice (were purchased from the animal house of the Nigerian Institute for Trypanosomiasis Research (NITR) Kaduna weighting 13 - 24g (Plate I). The animals were observed under twelve hours light and dark cycles in aerated cages inside a well-ventilated rodent cubicle and handled according to the Institutional ethics and guidelines for the protection of human and animal welfare. They were fed with pellet diet and water ad libitum. The mice were used in accordance with the NIH guide for the care and use of laboratory animals; NIH publication (no. 83-23) revised (1985). The animals were grouped into 15 groups of 3 animals each. They were acclimatized to the new environment for four weeks during which the weight of each of the mice was monitored.

Plate I: Experimental animals enclosed in a cage

3.1.2 Feed Composition

The feed used in the course of this research was VITAL FEED (growers pellet feed 25kg). It was produced by Grand cereal limited km 17, Zawani roundabout, Jos Plateau state. The feed contains all the essential micro and macro nutrients needed for growth and proper body development, they are; cereals, vegetable proteins, premix (vitamins and minerals), essential amino acids, salt, anti-oxidants, anti-toxins, prebiotics and enzymes.

3.1.3 Parasite Strain (*plasmodium berghei* Nk-65).

A chloroquine sensitive strain of *Plasmodium berghei* (NK-65) was obtained from the institute of Malaria Research Laboratory (IMRAT) of the Department of Basic Medical Science, University of Ibadan, Oyo state, Nigeria. The parasites were kept alive by serial inoculation into other mice.

3.1.4 Drug

Chloroquine was obtained from a pharmaceutical outlet and was used as a positive control drug to evaluate the *in vivo* efficacy of both plant extract.

3.1.5 Assays Kits

The assay kits used in the experiments namely; ALT, ALP and AST are products of Randox Laboratory Limited, Antrim, United Kingdom. They were purchased from a retail dealer. The assays were carried out one after the other on peculiar tissue homogenates to assay for the enzyme's concentration and activities.

3.1.6 Apparatus

The following apparatus were used during the course of the experiments; Test tubes, beakers, mortar and pestle, slides, spectrophotometer, cuvette, water bath, centrifuge,

rotary evaporator, gloves, micro-pipette, syringe (1ml, 2ml and 5 ml), measuring cylinder (of various volumes), weighing balance, dissecting kits,

3.1.7 Reagents

Methanol, sodium chloride, distilled water, sodium hydroxide, sodium carbonate, sodium hydrogen carbonate, magnesium sulphate, para-nitrophenyl phosphate (PNPP), sucrose, and giemser powder.

3.2 Methods

3.2.1 Plants used

The plants used for this research is Vernomia *amygdalina* (Bitter leaf) and *Azadirachta indica* (Neem)

3.2.2 Plant collection and authentification

The leaf of *Vernomia amygdalina* (Bitter leaf) and *Azadirachta indica* (Neem) leaves were collected from Bosso Local Govt. Area, Minna, Niger state in November 2018. The plant was authenticated by Dr Dawud, a botanist in the Department of Plant Biology, Federal University of Technology, Minna, Niger State.

3.2.3 Plant extract preparation

The leaf samples of the plants were dusted and air-dried under shade at room temperature for one week, with adulterant picked out of the leaves. The leaves were thereafter pulverized; The powdered plant material was weighed using SCIENTECH Mode No SL 3100D Rev-c accuracy class (II). 1kg of both *Vernomia amygdalina* (Bitter leaf) and *Azadirachta indica* (Neem) was measured as shown on the following page.

Plate II: Collected plant samples

Plate III: Dried plant samples

Plate IV: Dried plant samples

Plate V: The pulverized sample of the plants

3.3 Preparation of Crude Extract and Fractionated

3.3.1 Preparation of Crude Extract

Two hundred grams (200g) of the powdered *Azadirachta indica* and *Vernonia amygdalina* plant sample will be percolated in 1600 ml of absolute methanol and kept in shade for 48 hours after which it will be filtered. The filtrate will be collected in a beaker and the solvent will be removed under reduced pressure using rotary evaporator (Adebayo *et al*., 2003).

3.3.2 Extraction of the alkaloidal fractionation of extract

Alkaloid Extraction: The extraction of the alkaloid was done using the continuous extraction method using the Soxhlet apparatus. Four hundred grams (400 g) of both powdered plant was weighed and packed in a cheese cloth bag which served as an extraction thimble. The thimble was then placed into a suitable jar with cover. The sample was moistened with sufficient amount of 95 % ethanol. This was made alkaline with sufficient quantity of ammonia T.S. and mixed thoroughly. The sample in the thimble was macerated overnight, and then placed in the Soxhlet extractor on the next day. Sufficient amount of 95 % ethanol was placed in the solvent flask (4.8 liters). The sample was extracted for about 3 – 4 hours. The ethanol extract was filtered and was concentrated in a Soxhlet distilling apparatus at 60^{o}C. The crude alkaloid extract was further treated with 1.0 N hydrochloric acid. This was filtered and the filtrate was collected. The filtrate was alkalinified with ammonia T.S. and placed in a separatory funnel. Measured quantities of chloroform was added into the separatory funnel, mixed and shaken for about five times and allowed to separate into two layers. The lower layer of chloroform contained the alkaloids and the upper layer the aqueous portion. The upper layer was extracted until the last chloroform extract was found negative to

Dragendorff's reagent. The combined chloroform extract was concentrated in Soxhlet distilling apparatus at 60°C and evaporated in water bath maintained at that temperature until semi-dry. The residue was weighed and percentage yield was calculated using the formula:

$$\%\ yield\ of\ V.amygladina = \frac{weight\ of\ the\ alkaloidal\ residue\ (g)}{weight\ of\ powdered\ V.amygladina(g)} X\ 100$$

$$\%\ yield\ of\ A.indica = \frac{wieght\ of\ the\ alkaloidal\ residue(g)}{weight\ of\ powdered\ A.indica} X\ 100$$

3.4 Phytochemical Analysis

Standard screening tests for the extract were carried out for the various constituents like alkaloids, flavonoids, saponin, tannins, glycosides, phenol, anthraquinone using standard procedures using Trease and Evans (1989) and Sofowora (2008).

3.4.1 Qualitative analysis

3.4.1.1 Test for alkaloids

A portion of the extract (0.5 g) was stirred with 5 cm^3 of 1 % aqueous HCl on a steam bath. Three drops of picric acid solution were added to 2 cm^3 of the extract. The formation of a reddish brown precipitate was taken as a preliminary evidence for the presence of alkaloids (Harborne, 1973: Trease and Evans, 1989).

3.4.1.2 Test for glycoside

A portion of the extract (0.5 g) was mixed with 2 ml of glacial acetate and 1 drop of ferric chloride solution, after which 1ml of concentrated sulphuric acid were added. The reaction was observed for a brown ring formation (Sofowora, 1993).

3.4.1.3 Test for flavonoids

Few amount of the extract (1 g) was heated with 10 mL of ethyl acetate in a test tube over a steam bath for 3 minutes. The mixture was filtered and 4 mL of the filtrate was shaken with 1 ml of dilute ammonia solution. Yellow coloration was observed that indicated the presence of Flavonoids (Harborne, 1973; Sofowora, 1993).

3.4.1.4 Test for tannin

A portion of the extract (0.5 g) was boiled in 20 mL of distilled water in a test tube and filtered. 0.1 % ferric chloride ($FeCl_3$) solution was added to the filtrate. The appearance of brownish green or a blue-black colouration indicates the presence of tannins in the test samples (Harborne, 1973).

3.4.1.5 Test for saponin

Exactly (2.0 g) of the plant extract was boiled in 20 ml of distilled water in a test tube in boiling water bath and filtered. 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously to form a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously for the formation of emulsion characteristic of saponin (Obadoni and Ochuko, 2001).

3.4.1.6 Test for anthraquinone

A little portion of the extract (0.5 g) was added to 2 ml of 10 % hydrochloric acid in a test tube and boiled for about two minutes. Chloroform (5 ml) was added to the test tube and shaked twice, the chloroform layer was pipetted out and then equal volume of 10 % ammonia (5 mL) was added. A brown colour was formed in upper layer which indicated absence of anthraquinones (Trease and Evans, 1989).

3.4.1.7 Test for Phenol

A portion (0.5 g) of both plant extract was stirred with 10 mls of distilled water and then filtered. Few drops of 50% ferric chloride reagent were added to the filtrate. Blue-black or blue-green coloration or precipitation was taken as an indication of the presence of phenols. (Trease and Evans, 1989).

3.5 Acute Toxicity Test: Determination of LD_{50}

The safety of the extract was evaluated by determining its LD_{50} using the Lorke's (1983) method. Dose levels used was from 10 to 5,000 mg/kg. All the animals were kept under the same condition and individual body weights (b.w.) of mice were determined weighing between 13-24g. The study was conducted in two phases using a total of sixteen mice. In the first phase, nine mice were divided into 3 groups with 3 mice in each. The mice were left to fast overnight according to the Organization for Economic Co-operation and Development (OECD) Guidelines for the Testing of Chemicals (OECD, 2008). Animals in group 1, 2 and 3 were given 10, 100 and 1000 mg/kg body weight (b.w.) of the extract respectively to establish the range of doses producing any toxic effect. In addition, a fourth group of three mice was set up as the control group and animals in the group were not given the extract.

In the second phase, further specific doses (1600, 2900 and 5000mg/kg b.w.) of the extract were administered to three mice (one mice per dose) to further determine the correct LD_{50} value. All animals were observed frequently on the day of treatment and surviving animals were monitored daily for 2 weeks for signs of acute toxicity. Recovery and weight gain were seen as indications of having survived the acute toxicity.

3.6 Experimental Design

3.6.1 Extract administration

All oral administration of drugs and extract were carried out using orogastric tube.

3.6.2 Curative antiplasmodial assay

Evaluation of curative potential of aqueous methanol extract of *Azadirachta indica* and *Vernonia amygdalina* was done by adopting the method described by Ryley and Peters (1970). Fifty-two mice were selected and intra-peritoneally injected with 1 x 10^7 *Plasmodium berghei* infected on the first day. 72 h after, the animals were divided into thirteen groups of three (3) mice per group. These mice were treated with 150,300 and 600mg/kg/day doses of *A. indica* and *V. amygdalina* methanolic extract, alkaloid, chloroquine (5mg/kg b.wt dissolved in normal saline) and an equal volume of distilled water (negative group) for 5 consecutive days. The drug or extract was administered daily to appropriate group and the level of parasitaemia was determined using standard laboratory (Knight and Peters, 1980).

The group are listed as follows;

Group A: uninfected and untreated (positive control)

Group B: infected and untreated (negative control)

Group C: infected and treated with standard drug (standard control)

Group D: infected and treated with 150mg/kg b. wt crude methanol extract of *V. amygdalina*

Group E: infected and treated with 300mg/kg b. wt crude methanol extract of *V. amygdalina*

Group F: infected and treated with 600mg/kg b. wt crude methanol extract of *V. amygdalina*

Group G: infected and treated with 150mg/kg b. wt crude methanol extract of *A. indica* extract

Group H: infected and treated with 300mg/kg b. wt crude methanol extract of *A. indica* extract

Group I: infected and treated with 600mg/kg b. wt crude methanol extract of *A. indica* extract

Group J: infected and treated with 150 mg/kg b. wt alkaloid of *V. amygdalina* extract

Group K: infected and treated with 300 mg/kg b. wt alkaloid of *V. amygdalina* extract

Group L: infected and treated with 150 mg/kg b. wt alkaloid of *A. indica* extract

Group M: infected and treated with 300 mg/kg b. wt alkaloid of *A. indica* extract

3.7 Determination of Parasitemia

Blood samples will be collected by bleeding via the tail vein of *P. berghei* infected mice and thin blood smears will be made on microscope slides, fixed in methanol and stained with 10% Giemsa solution (Merck, Tokyo, Japan) and observed under the binocular microscope (Olympus, Japan). The percentage parasitemia will determined by counting the percentage parasitized RBC for at least four different fields (Khan *et al*., 2012).

Plate VI: Preparation of blood Smear collected from the tail of the experimental animals

3.8 Determination of Packed Cell Volume

The packed cell volume (PCV) of each mouse will be measured before infection, on day 4 after infection and day 8 after treatment. For this purpose, blood will be collected from tail end of each mouse in hapatorinized micro-haematocrit capillary tubes up to 3/4th of their length. The tube will be sealed and placed in a microhaematocrit centrifuge with the seal end out ward. The blood will be centrifuge at 12,000rpm for 5 minutes. Then the tube will the result will be read using microhaematocrit and the volume of erythrocytes will be measured (Bantie *et al*., 2014)

3.9 Hematology

Each mouse was sedated by ether suffocation and blood was pooled from mice in each group, blood was collected by cardiac puncture into heparinized tubes for haematological studies - red blood cell (RBC) counts, haemoglobin (Hb) concentration, total white blood cell (WBC) counts, neutrophils and lymphocytes and platelets counts according to methods described by Dacie and Lewis (1991). Red blood cell indices such as mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were calculated (Jain, 2010).

3.10 Determination of Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and Alkaline Phosphatase (ALP)

Blood from cardiac puncture was dispensed into plain bottles and allowed to clot after which it was centrifuged at 1372 g-force for 10 minutes and the clear sera aspirated off. Then, sera will be thawed and assayed for the levels of ALT and AST respectively using the method of Reitman and Frankel (1957). The activity of alkaline phosphatase (ALP) will be assayed by the method of Ekanem and Yusuf (2008) as outlined in Randox kits, UK.

3.11 Determination of Mean Survival Time

Mortality will be monitored daily and the number of days from the time of inoculation of the parasite up to death will be recorded for each mouse. The mean survival time (MST) will be calculated as follows:

$$Mean\ survival\ time = \frac{sum\ survival\ time\ for\ all\ mice\ in\ a\ group\ (days)}{Total\ number\ of\ mice\ in\ the\ group}$$

3.12 Data Analysis

All quantitative data obtained from the study was summarized and expressed as mean ± standard error of mean (SEM). Data analysis was performed with Statistical Package for Social Science (SPSS), version 19 using One-way analysis of variance (ANOVA) coupled to Duncan (post-hoc test) was used to determine statistical significance for comparison of parasitaemia, % inhibition, body weight, PCV, survival time, haematological and biochemical parameters among groups. Significant differences between means was assessed at 95% level of significance that is, probability values (p-value) less than 0.05 ($p < 0.05$) will be considered significant.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Results

4.1.1 Phytochemical composition

Results in Table 4.1 shows the phytochemical composition of crude methanolic leaf extracts of *Vernonia amygdalina* and *Azadirachta indica.* Flavonoids, Tannins, Alkaloids, Cardiac Glycoside, Phenols and Terpenoids was found in both the crude methanol leaf extracts of *Vernonia amygdalina* and *Azadirachta indica.* Phlobatannins was absent in *Azadirachta indica* while steroids and phlobatannins were not detected in *V. amygdalina.*

Table 4.1: Phytochemical Composition of Crude Methanol Leaves Extract of *Vernonia amygdalina* and *Azadracta indica*

Phytochemicals	***V. amygdalina***	***A. indica***
Flavonoids	+	+
Tannins	+	+
Alkaloids	+	+
Steroids	-	+
Phlobatannins	-	-
Cardiac glycosids	+	+
Total phenol	+	+
Terpenoids	+	+

+ presence, -- absence

4.1.2 Acute Oral Toxicity

The acute oral toxicity properties of both crude methanolic leaf extracts of *Vernonia amygdalina* and *Azadirachta indica* are presented in Table 4.2. Both the crude methanolic leaves extracts of *Vernonia amygdalina* and *Azadirachta indica* demonstrated LD_{50} > 5000 mg/kg b.w. as no mortality and no physical and behavioural changes was observed in animals administered with the extract up to 5000 mg/kg b.w throughout the 14 days.

Table 4.2: Acute Oral Toxicity of Crude Methanolic Leaf Extracts of *Vernonia amygdalina* and *Azadracta indica*

Dosage (mg/kg bw)	*V. amygdalina*	*A. indica*
	Phase 1	
10	0/3	0/3
100	0/3	0/3
1000	0/3	0/3
	Phase II	
1600	0/3	0/3
2900	0/3	0/3
5000	0/3	0/3
LD_{50} (mg/kg bw)	> 5000	> 5000

4.1.3 Antiplasmodial Activities

4.1.3.1 Parasitemia

Antiplasmodial Effect of Crude Methanolic Extract and Alkaloidal Fraction of *Azadirachta Indica* (Neem Plant) In *Plasmodium berghei* Infected Mice

The crude extract and alkaloidal fraction of *Azadirachta indica* (Neem Plant) exhibited progressive inhibition of *Plasmodium berghei* replication in mice in a dose dependent

manner as shown in (Table 4.3). Infected untreated animals show progressive increase in parasitaemia count throughout the study period. Rats treated with crude extract showed better activity than the alkaloidal fraction. Mice treated with the crude methanol extract of *A. indica* exhibited 52.23 ±2.34, 73.51±4.24 and 84.62±3.75% at 150, 300 and 600 mg/kg b.w respectively, while mice treated with the alkaloidal fraction exhibited 58.46±4.35 and 64.54±3.76 curative effect at 150 and 300 mg/kg b.w respectively.

4.1.3.2 Antiplasmodial Effect of Crude Methanolic Extract and Alkaloidal Fraction of *V. amygdalina* in *Plasmodium berghei* Infected Mice

Effect of crude extract and alkaloidal fraction of *V. amygdalina* on parasitaemia count in *Plasmodium berghei* infected mice is shown in Table 4.4. and 4.5, the extract as well as the alkaloidal fraction significantly reduced the parasitaemia count when compared with the infected untreated animals which shows progressive increase in parasitaemia count (Table 4.4). Mice treated with the crude methanolic extract of *V. amygdalina* exhibited 75.73±3.42, 68.39±5.76 and 90.21±3.94% at 150, 300 and 600 mg/kg b.w respectively while mice treated with alkaloidal fraction exhibited 85.42±5.46 and 87.84±4.32 curative effect at 150 and 300 mg/kg bw respectively.

Table 4.3: Antiplasmodial Effect of Crude Methanolic Extract and Alkaloidal Fraction of *Azadirachta indica* In *Plasmodium berghei* Infected Mice

Extract Treatment	Dose (Mg/kg bw)	Day 3	Day 4	Day 5	Day 6	Day 7
Crude	150	35.23 ± 0.23^{a}	37.32 ± 0.19^{b}	33.25 ± 0.32^{c}	21.23 ± 0.12^{d}	22.11 ± 0.48^{f}
	300	38.12 ± 2.22^{a}	37.12 ± 0.78^{b}	22.45 ± 1.15^{b}	18.98 ± 1.23^{c}	12.25 ± 1.15^{c}
	600	36.32 ± 0.18^{a}	28.13 ± 0.04^{b}	15.35 ± 1.12^{a}	9.31 ± 0.45^{b}	7.11 ± 0.23^{b}
Alkaloid	150	39.32 ± 3.11^{a}	36.21 ± 1.22^{b}	22.14 ± 1.31^{b}	20.12 ± 0.68^{d}	19.21 ± 0.31^{e}
	300	35.41 ± 0.23^{a}	35.10 ± 0.31^{b}	20.65 ± 1.21^{b}	$18.67 \pm 0.^{c}$	16.41 ± 1.12^{d}
Chloroquine	5	33.23 ± 0.41^{a}	30.14 ± 0.04^{a}	15.31 ± 1.63^{a}	6.23 ± 0.12^{a}	1.02 ± 0.11^{a}
Negative Control		34.12 ± 0.01^{a}	36.76 ± 0.11^{b}	38.41 ± 1.45^{d}	44.13 ± 0.32^{e}	46.25 ± 0.23^{g}

Values are mean ± SEM of 3 determinations. The values along the same row with different superscripts are significantly different ($p<0.05$).

Table 4.4: Antiplasmodial Effect of Crude Methanol Extract and Alkaloidal Fraction of *V. amygdalina* (Bitter Leaf) in *Plasmodium berghei* Infected Mice

Extract treatment	Dose (mg/kg bw)	Day 3	Day 4	Day 5	Day 6	Day 7
Crude extract	**150**	14.21±1.3^{a}	31.02±1.32^{d}	31.01±1.23^{d}	24.14±0.56^{e}	10.22±1.32^{c}
	300	16.71±032^{a}	24.22±1.31^{c}	18.12±2.14^{c}	15.13±0.16^{d}	13.31±0.54^{d}
	600	13.23±4.12^{a}	32.34±0.15^{d}	14.32±0.87^{b}	10.23±1.22^{c}	4.12±1.32^{b}
Alkaloid	**150**	13.14±3.85^{a}	24.32±1.23^{c}	28.14±0.12^{c}	8.12±1.67^{c}	6.14±3.22^{b}
	300	12.23±4.45^{a}	16.21±1.23^{b}	13.12±0.13^{b}	6.14±0.67^{b}	5.12±0.12^{b}
Chloroquine	**5**	15.12±1.2^{a}	8.71±1.01^{a}	4.53±0.12^{a}	1.15±0.32^{a}	1.98±0.00^{a}
Negative Control	-	13.32±1.14^{a}	18.28±1.02^{a}	29.14±1.2^{d}	34.12±1.22^{f}	42.12±1.45^{e}

Values are mean ± SEM of 3 determinations. The values along the same row with different superscripts are significantly different ($p<0.05$).

Table 4.5 Curative Effect of Crude Extract and Alkaloidal Fractions of *Azadirachta indica* and *Vernonia amygdalina* In *Plasmodium berghei* Infected Mice

Extract Treatment	Dose (mg/kg bw)	*Azadirachta indica*	*V. amygdalina*
Crude extract	**150**	52.23 ±2.34[a]	75.73±3.42 [b]
	300	73.51±4.24 [c]	68.39±5.76 [a]
	600	84.62±3.75 [d]	90.21±3.94 [cd]
Alkaloid	**150**	58.46±4.35 [a]	85.42±5.46 [c]
	300	64.54±3.76 [b]	87.84±4.32 [c]
Chloroquine	5	97.36±2.45 [e]	95.29±6.43 [d]

Values are mean ± SEM of 3 determinations. The values along the same column with different superscripts are significantly different ($p<0.05$).

4.1.3.3 Body weight

Effect of crude and alkaloidal fraction of *V. amygdalina* and *Azadirachta indica* on body weight changes in *P. berghei* infected mice are shown in Table 4.6 and 4.7 respectively. The body weight of all the experimental groups show initial decreases after *P. berghei* infection. However, groups of mice treated with crude and alkaloidal fraction of *V. amygdalina* and *A. indica* shows a significant ($p<0.05$) improvement in the body weight after treatments. In addition, crude extract from both plants exhibited more pronounced positive effect on the weight gain of the animals than their respective alkaloidal fraction. In the infected untreated mice, there was a continuous decrease in their body weight throughout the study period.

Table 4.6: Weight Change in *P. berghei* Infected Mice Treated With Crude and Alkaloidal Fraction Of *V. amygdalina*

Extract	Dose (mg/kg bw)	Day 0	Day 4	Day 7
Crude	**150**	20.12 ± 0.12^{a}	21.12 ± 1.13^{a}	21.45 ± 1.02^{c}
	300	21.07 ± 0.11^{a}	22.41 ± 1.12^{a}	22.68 ± 0.12^{c}
	600	20.13 ± 0.67^{a}	22.13 ± 1.06^{a}	22.41 ± 0.02^{c}
Alkaloid	**150**	19.13 ± 0.18^{a}	18.12 ± 1.12^{a}	19.65 ± 0.04^{b}
	300	22.14 ± 0.09^{a}	21.12 ± 0.34^{b}	22.45 ± 0.03^{c}
Positive control		21.45 ± 0.15^{a}	20.12 ± 0.12^{b}	21.12 ± 0.12^{c}
Negative control		20.02 ± 1.12^{a}	20.14 ± 1.21^{b}	18.21 ± 0.15^{a}

Values are mean ± SEM of 3 determinations. The values along the same column with different superscripts are significantly different ($p<0.05$)

Table 4.7: Weight Change in *P. berghei* Infected Mice Treated With Crude and Alkaloidal Fraction Of *A. indica*

Extract	Dose (mg/kg bw)	Day 0	Day 4	Day 7
Crude	**150**	21.23±0.32^{a}	20.35±0.57^{a}	21.78±0.96^{bc}
	300	21.03±0.86^{a}	20.34±0.83^{a}	22.21±0.35^{c}
	600	22.34±0.83^{a}	21.23±1.90^{a}	23.95±0.84^{c}
Alkaloid	**150**	21.23±0.32^{a}	20.13±1.01^{a}	20.34±0.35^{b}
	300	24.03±0.54^{a}	22.93±0.98^{b}	23.97±0.32^{c}
Positive control		21.45±0.15^{a}	20.12±0.12^{b}	21.12±0.12^{c}
Negative control		20.02±1.12^{a}	20.14±1.21^{b}	18.21±0.15^{a}

Values are mean ± SEM of 3 determinations. The values along the same column with different superscripts are significantly different ($p<0.05$).

4.1.4 Hematological parameters

The mean haemoglobin (Hb), packed cell volume (PCV), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), white blood cells (WBC) and platelet counts in *P. berghei* infected mice were significantly reduced ($p<0.05$) when compared with normal control group and also with the extract treated groups (Table 4.8 and 4.9). Treatment of the infected mice with crude extract and alkaloidal fraction of *A. indica* (Table 4.8) and *V. amygladima* (table 4.9) caused a significant ($p<0.05$) and dose dependent increase in the hematological indices when compared with the untreated control group. Crude extract of both plants caused higher increase in platelet count when compared with their respective alkaloidal fractions.

Table 4.8: Effect of Crude and Alkaloidal Fraction of *A. indica* (Neem*)* on Hematological Parameters in *P. Berghei* Infected Mice

Extract	Dose	HB (g/dl)	MCV (FL)	MCH (PG)	RBC ($X10^{12}$/L)	PLC ($X10^{12}$/L)	WBC ($X10^{9}$/L)
Crude	**150**	6.23 ± 1.23^{a}	35.16 ± 1.22^{b}	13.18 ± 0.44^{a}	6.01 ± 0.12^{b}	800.19 ± 0.01^{e}	6.12 ± 0.01^{c}
	300	8.13 ± 1.22^{b}	41.12 ± 0.99^{c}	14.05 ± 0.32^{b}	6.98 ± 0.08^{b}	933.12 ± 0.12^{f}	5.19 ± 0.03^{b}
	600	11.12 ± 0.31^{c}	39.23 ± 0.09^{c}	13.15 ± 0.31^{a}	7.98 ± 0.14^{c}	789.23 ± 0.11^{d}	7.85 ± 0.04^{d}
Alkaloid	**150**	12.13 ± 0.04^{d}	43.12 ± 2.13^{c}	12.65 ± 0.14^{a}	7.63 ± 0.31^{c}	696.23 ± 0.03^{c}	6.19 ± 0.04^{c}
	300	14.41 ± 0.15^{e}	41.18 ± 1.32^{c}	14.14 ± 0.41^{b}	7.81 ± 0.31^{c}	645.16 ± 0.12^{b}	6.03 ± 0.11^{c}
Standard		12.32 ± 0.21^{d}	43.21 ± 2.14^{c}	14.25 ± 0.96^{b}	7.23 ± 0.04^{c}	635.38 ± 0.11^{b}	6.38 ± 0.12^{c}
Negative		6.16 ± 0.07^{a}	29.31 ± 1.11^{a}	4.12 ± 1.27^{a}	5.11 ± 0.05^{a}	532.12 ± 0.23^{a}	3.41 ± 0.13^{a}
Positive		11.95 ± 3.21^{c}	41.45 ± 3.12^{c}	13.38 ± 0.05^{a}	8.23 ± 0.12^{c}	631.38 ± 0.11^{b}	7.32 ± 0.07^{d}

Values are mean ± SEM of 3 determinations. The values along the same row with different superscripts are significantly different ($p<0.05$). Haemoglobin (Hb), packed cell volume (PCV), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), white blood cells (WBC), platelet count (PLC).

Table 4.9: Effect of Crude and Alkaloidal Fraction of *V. amygdalina* (Bitter leaf) on Hematological Parameters in *P. berghei* Infected Mice

Extract	Dose	HB (g/dl)	MCV (FL)	MCH (PG)	RBC ($X10^{12}$/L)	PLC ($X10^{12}$/L)	WBC ($X10^{9}$/L)
Crude	150	7.21 ± 1.23^{a}	38.21 ± 0.12^{b}	14.25 ± 0.12^{b}	6.58 ± 1.21^{b}	812.07 ± 0.12^{d}	5.67 ± 0.51^{b}
	300	8.12 ± 1.12^{a}	39.00 ± 0.98^{b}	13.43 ± 0.11^{a}	7.11 ± 0.07^{c}	934.18 ± 0.11^{e}	5.01 ± 0.04^{b}
	600	12.11 ± 0.14^{b}	40.12 ± 0.32^{b}	14.12 ± 0.31^{b}	8.12 ± 0.11^{c}	811.12 ± 0.31^{d}	7.21 ± 0.06^{d}
Alkaloid	150	13.41 ± 0.05^{b}	42.34 ± 0.31^{c}	13.71 ± 0.21^{a}	7.11 ± 0.21^{c}	701.28 ± 0.12^{c}	6.14 ± 0.31^{c}
	300	14.52 ± 0.12^{b}	43.44 ± 0.14^{c}	15.12 ± 0.36^{b}	7.21 ± 0.06	648.12 ± 1.11^{b}	5.23 ± 0.22^{b}
Standard Negative		13.11 ± 1.12^{b}	43.98 ± 0.98^{c}	14.12 ± 0.08^{b}	6.19 ± 0.11^{b}	645.78 ± 1.62^{b}	6.11 ± 1.10^{c}
Untreated Control		6.25 ± 1.21^{a}	30.12 ± 0.31^{a}	13.23 ± 0.09^{a}	5.12 ± 0.03^{a}	540.12 ± 4.51^{a}	3.01 ± 0.04^{a}
Normal		14.68 ± 0.15^{b}	44.00 ± 0.06^{c}	14.22 ± 0.12^{b}	8.75 ± 0.11^{c}	612.10 ± 3.19^{b}	7.12 ± 0.02^{d}

Values are mean ± SEM of 3 determinations. The values along the same row with different superscripts are significantly different ($p<0.05$). Haemoglobin (Hb), packed cell volume (PCV), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), white blood cells (WBC), platelet count (PLC).

4.1.5 Biochemical parameters

4.1.5.1 Aspartate transaminase

Effect of crude and alkaloidal fraction of *Azadirachta indica* (Neem) and *Vernonia amygdalina* (Bitter Leaf) on aspartate transaminase activities in serum of *P. berghei* infected mice is shown in Figure 4.1. The serum aspartate transaminase activities were significantly higher in *P. berghei* infected mice when compared with the normal control. Treatment with the crude and alkaloidal fractions of *Azadirachta indica* (neem) and *Vernonia amygdalina* significantly reduced ($p<0.05$) the AST activities when compared with the untreated control. The AST activities in mice treated with crude extract of *V. amygdalina* at 150, 300 and 600 mg/kg b.w and 300 mg/kg b.w of alkaloidal fraction were comparable ($p>0.05$) with the normal control. However, the serum AST in mice treated with 150 mg/kg b.w crude of *A. indica* extract and 300 mg/kg b.w of its alkaloidal fraction were not significantly different from the untreated control.

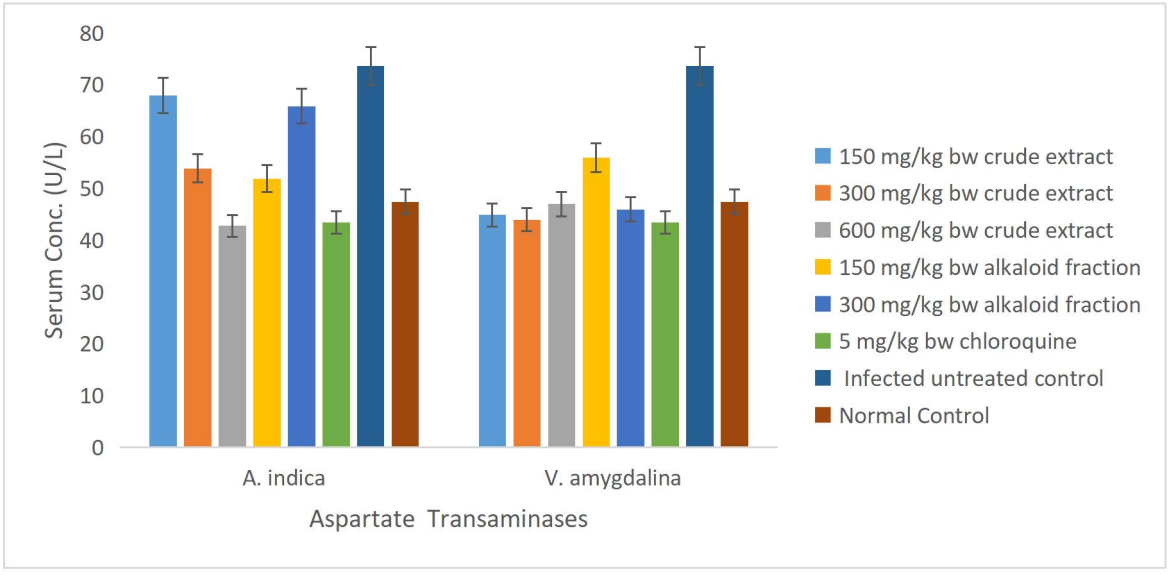

Figure 4.1: Effect of Crude and Alkaloidal Fraction of *Azadirachta indica* (Neem) and *Vernonia amygdalina* (Bitter Leaf) on aspartate transaminase activities in serum of *P. berghei* infected mice

4.1.5.2 Alanine transaminase

Effect of Crude and Alkaloidal Fraction of *Azadirachta indica* (Neem) and *Vernonia amygdalina* (Bitter Leaf) on alanine transaminase activities in serum of *P. berghei* infected mice is shown in Figure 4.2. The alanine transaminase activities in *P. berghei* infected mice were not significantly different when compared with the normal control and extract treated groups. Mice treated with the alkaloidal fractions were also comparable with the activities in those treated with chloroquine, normal control and untreated group.

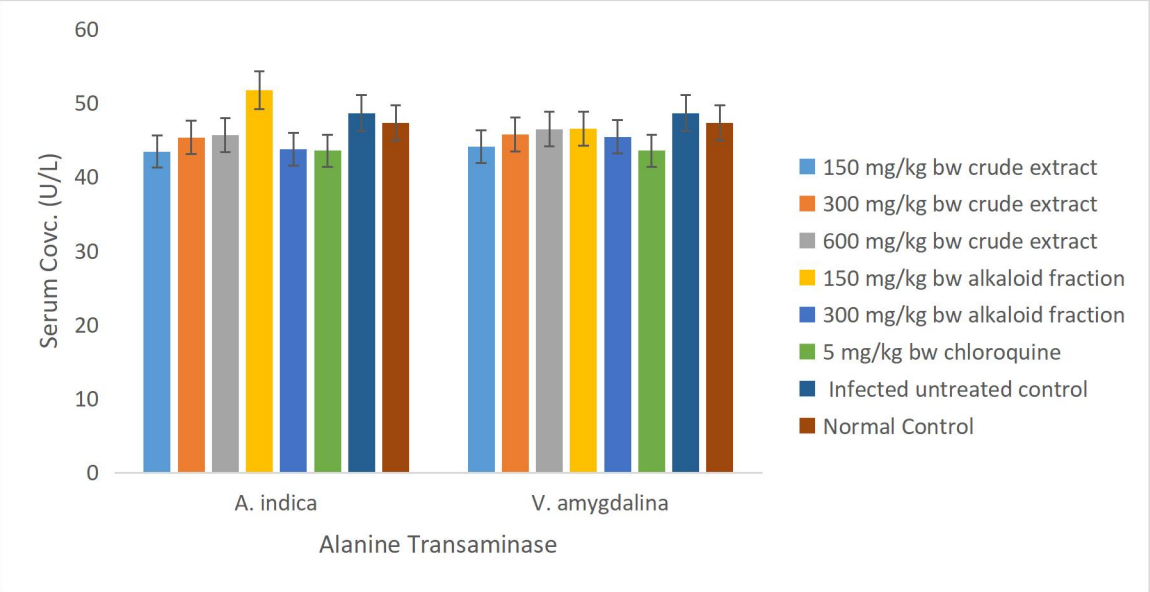

Figure 4.2: Effect of Crude and Alkaloidal Fraction of *Azadirachta indica* (Neem) and *Vernonia amygdalina* (Bitter Leaf) on alanine transaminase activities in serum of *P. berghei* infected mice.

Key: Each bar represents mean ± SEM of 3 determinants. Bars with different superscripts are significantly different ($p<0.05$).

4.1.5.3 Alkaline phosphatase

Effect of Crude and Alkaloidal Fraction of *Azadirachta indica* (Neem) and *Vernonia amygdalina* (Bitter Leaf) on alkaline phosphatase activities in serum of *P. berghei* infected mice is shown in Figure 4.3. The alkaline phosphatase activities in *P. berghei* infected mice

were significantly ($p<0.05$) higher when compared with the normal control and extract treated groups. Treatment of the infected mice with the crude and alkaloidal fractions of *Azadirachta indica* (Neem) and *Vernonia amygdalina* (Bitter Leaf) significantly reduced ($p<0.05$) the ALP activities towards their normal values in a dose dependent fashion. The ALP activities in mice treated with alkaloidal fractions of 150 and 300 mg/kg bw as well as those treated with crude extract of both plants at 300 and 600 mg/kg bw were comparable ($p>0.05$) with the normal control.

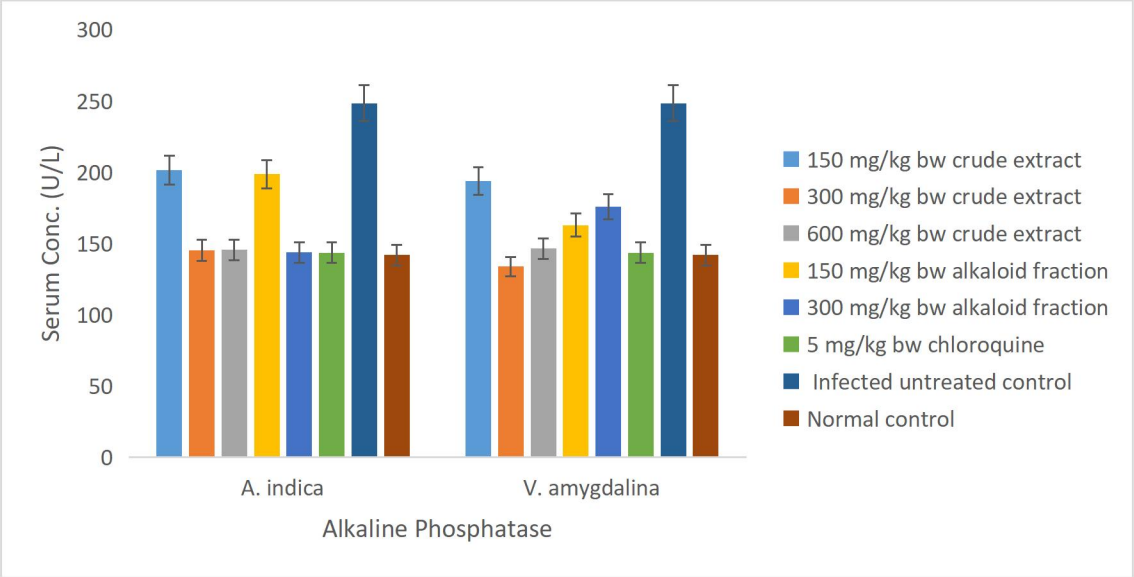

Figure 4.3: Effect of Crude and Alkaloidal Fraction of *Azadirachta indica* (Neem) and *Vernonia amygdalina* (Bitter Leaf) on alkaline phosphatase activities in serum of *P. berghei* infected mice.

Key: Each bar represents mean ± SEM of 3 determinations. Bars with different superscripts are significantly different ($p<0.05$).

4.1.5.4 Total proteins

Effect of crude and alkaloidal fraction of *Azadirachta indica* (Neem) and *Vernonia amygdalina* (Bitter Leaf) on serum total proteins concentrations of *P. berghei* infected mice is shown in Figure 4.4. The serum total proteins concentrations in *P. berghei* infected mice

were significantly ($p<0.05$) lower when compared with the normal control and extract treated groups. Treatment of the infected mice with the crude and alkaloidal fractions of *Azadirachta indica* (Neem) and *Vernonia amygdalina* (Bitter Leaf) significantly increased ($p<0.05$) the serum total proteins concentrations when compared with the untreated groups. The serum total proteins concentrations in mice treated with the crude extract of both plants at 300 and 600 mg/kg bw were higher than the ($p <0.05$) with the normal control

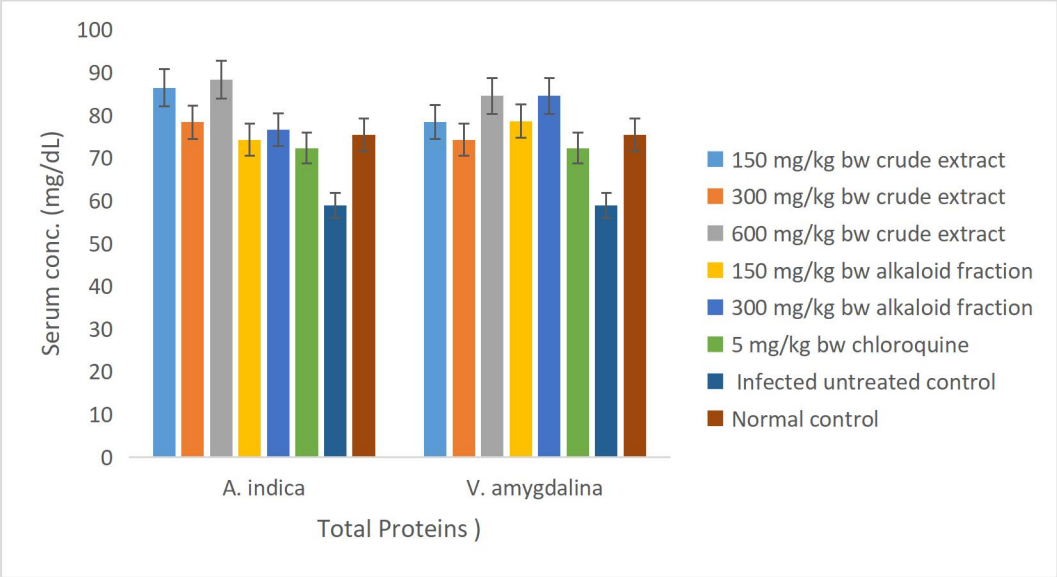

Figure 4.4: Effect of Crude and Alkaloid Fraction of *Azadracta indica* (neem*)* and *Vernonia amygdalina* on total proteins in serum of *P. berghei* infected mice.

Key: Each bar represent mean ± SEM of 3 determinations. Bars with different superscripts are significantly different ($p<0.05$).

4.2 Discussion

The existence of experimental animal models of a disease aids not only in the understanding of the pathophysiology of such disease but also in the development of drug candidate. The control of these parasitic disease using synthetic antimalarial drugs such as primaquine and chloroquine have been hindered by rapid parasite resistance to these drugs over the few decades (WHO, 2009). Following the resistance by this parasite causing disease and lack of accessibility to the available drugs has necessitated for the search for a novel antimalarial agent from natural product.

Based on the acute toxicity study, the LD_{50} of methanol extract of *V. amygdalina* and *A. indica* were found to be greater than 5000 mg/kg, indicating their wide safety margin. Previous studies on *Vernonia amygdalina* extract also showed that there are no significant changes in the body weight, wet organ weight and abnormalities in the vital organs (Akah *et al*., 2009; Ekpo *et al*., 2007). The present result is also in line with the finding of Momoh *et al.* (2015) who reported no sign of clinical symptom at 1600, 2900 and 5000mg/Kg body weight during the administration of *A. indica* which was administered orally.

This study also corroborate with the study of Adiukwu *et al*. (2012) and Anoka *et al.* (2008) who reported that *V. amygdalina* caused no clinical signs of toxicity at doses between 500 and 2000 mg/kg/day for 14 consecutive days and Njan *et al*. (2008) who reported the absence of signs of over toxicity or adverse toxicological effects at all tested doses. Amole *et al*. (2006) also found no toxic effect of the extract during his experiment conducted on the *in vivo* effect of *V. amygdalina* on mice.

Also, based on the acute toxicity study, the methanolic extract of *A. indica* was administered from high to low doses to test for the level of acute oral toxicity. Clinical signs of toxicity

and various test were carried out before and after the administration of the extract to authenticate effect of the extract. Although, there was a progressive increase in body weight in both test and control animals during the entire duration of the administration of the extract but there was no observed sign of toxicity in both groups. The effect of the bitter nature of the extract was seen when the animals became dull and lost appetite. Tulashie *et al*. (2011) also found no sign of toxic effect of *A. indica* during the experiment conducted on the toxicological analysis of the effect of neem tree extract in an organism. Generally, if LD_{50} value of the test chemical is more than three times the minimum effective dose, the substance is considered to be a good candidate for further studies in *in vivo* assays (OECD, 2008). The LD_{50} has also been used for classification of chemicals and following the WHO hazard classification system, the crude extract of *V. amygdalina*, to which the LD_{50} was greater than 5000 mg/kg, are designated as "unlikely to be hazardous (WHO, 2009). Therefore, the methanol extract of *V. amygdalina* are considered to be safe at the tested doses. The aforementioned descriptive toxicological studies also support this finding.

The 4-day suppressive test on *P. berghei* infected mice is the standard test commonly used for antimalarial drug screening (Peter, 1984) and the determination of percentage suppression of parasitaemia is the most reliable parameter. *In vivo* antiplasmodial activity can be classified as moderate, good and very good if an extract displays a percentage of parasite suppression equal to or greater than 50% at a dose of 500, 250 and 100 mg/kg/day, respectively (Deharo, 2001; Munoz *et al*., 2000; Lawal *et al*., 2015). Based on this classification, methanol extract of *V. amygdalina* and *A. indica* leaves exhibited very good antiplasmodial activity as they had exhibited parasitaemia suppression of 52.23 ± 2.34 and 90.21 ± 3.94% at concentrations of 150 -600 mg/kg, respectively. The high antimalarial

activity of *V. amygdalina* were reported in previous study authored by Temesgen *et al*. (2019). The present finding on *A. indica* disagrees with the earlier report that methanolic extracts of Neem leaf administered at 300, 500 and 1000 mg/kg displayed no activity against the *P. berghei* malaria parasite, and that the extract was not effective in preventing death due to cerebral malaria during treatment. These results also disagree with tests using Neem extracts in malaria-infected mice and chicks that were uniformly disappointing (Willcox *et al*., 2004). Despite the fact that an ethanolic extract of Neem leaf had an IC_{50} value of 5 µg/ml against *P. falciparum* in culture (Rochanakij *et al*., 1985). The same authors also reported that Neem ethanolic extract and aqueous decoctions did not show any activity in a 4-day test against *P. berghei* in mice administered either orally or sub-cutaneously at levels up to 746 mg/kg/day (Rochanakij *et al*., 1985). On the other hand, the *in vitro* anti-malarial activity of Neem was previously reported by El-Tahir *et al* in 1999, where they reported that an aqueous extract of Neem leaf showed an IC_{50} value <5 µg/ml against *P. falciparum* (El Tahir *et al*., 1999). Similar results were obtained by Alshawsh and colleagues in 2007, when they reported that aqueous Neem extracts inhibited the development of the ring stage of *P. falciparum* (Alshawsh *et al*., 2009).

The discrepancies in the antimalarial activity reported in previous studies compared with the present finding might be related to the difference in geographical distribution, time of collection (seasonal variation), stage of development and/or extraction processes which can independently or in concert affect the quality and quantity of phytochemical constituents (Lawal *et al*., 2014). It is crystal clear that several exogenous (altitude, day length, collection season, soil condition and allelopathy) and indigenous factors (morphological variety and biochemical diversity) and post cultivation factors possibly affect the quality and

quantity of bioactive secondary metabolites. Interestingly, the presence of significant activity on body weight, and haematological indices which are also important parameters of an antimalarial assay, should be considered while treating malaria.

The overall antimalarial activity of the methanol extract of *V. amygdalina* and *A. indica* leaves in this investigation might be due to the presence of bioactive secondary metabolites contained in the plant including terpenoids, alkaloids, phenols, flavonoids and tannins. Several phenolic compounds, flavonoids and tannins are also reported to have antiplasmodial activities (NjomnangSoh *et al*., 2012). Terpenoids also play an ecological role in the 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, molluscicidal and herbicidal properties. For example, the anticancer drug, taxol and the antimalarial drug artemisinin are widely known terpene-based drugs (Ijeh and Ejike, 2011). Alkaloids are known for their antimalarial activity (Ijeh and Ejike, 2011; Toyang *et al*., 2013). Alkaloids exert their antimalarial effect by disrupting the parasite ability of detoxifiying heme into non-toxic malaria pigment, which by doing so exposes the parasite to toxic heme compounds (CorreaSoares *et al*., 2009). However, in the present study, crude methanolic extract of *V. amygdalina* and *A. indica* leaves exhibited higher antiplasmodial activity than their respective alkaloidal fractions.

Results of this investigation on the leaves of *V. amygdalina* revealed reduced parasitaemia suppression as compared to that of Challand and Willcox (2009).The antiplasmodial effect exhibited by the *V. amygdalina* was also higher than the results of a previous study (Dawet *et al*., 2014) conducted on other plants. The ethanol and aqueous stem bark extracts of *Pseudocedrela kotschyi* demonstrated a parasitaemia suppression of 39.43% and 28.36%,

respectively, at dose of 200 mg/kg, and methanol and water extracts of *Aloe debrana* produced parasitaemia suppression of 30.21% and 23.53%, respectively, at a dose of 200 mg/kg.

With regard to other parameters of *in vivo* antimalarial bioassays, MST is important to evaluate the antimalarial activity of plant extracts (Peter, 1975). Except for the untreated control, all doses of the *V. amygdalina* and *A. indica* as well as their respective alkaloidal fractions prolonged the survival time of mice which could be due to suppression of parasitaemia and reduction of the overall pathologic effect of the parasite on the mice under study (Basir *et al*., 2012). The different secondary metabolites present in the plant might have played a role in prolonging the survival time of the infected mice.

Weight decrease in malaria-infected mice has been associated with decreased food intake, disturbed metabolic function and hypoglycemia (Basir *et al*., 2012). Antimalarial agents obtained from plants are expected to prevent body weight loss in infected mice due to overall pathological effect caused by the parasite. In this study, all doses of the of *V. amygdalina* leaves and *A. indica* as well as their alkaloidal fractions significantly prevented weight loss in a dose-dependent manner due to their parasitaemia suppressing activity. This indicates that the extracts had suppressed *P. berghei* and thereby reduced anaemia and the overall pathological effect of the parasite on the test groups (Atkinson *et al*., 2000).

The extracts at all doses reduced AST, ALT and ALP activities (Fig. 4.1-4.3). An increase in the activities of AST and ALP in the blood serum of parasitized untreated mice may be as a result of liver injury and altered hepatocyte integrity caused by the *Plasmodium* infection and the consequent release of the enzymes into the blood stream. This result is in agreement with the findings of Patrick-lwuanyanwu *et al*. (2011). Also, this result is in consistent with

other studies which reported that majority of malaria patients show elevation in serum activities (AST, ALT and ALP) indicating liver damage (Fabbri *et al*., 2013). Activities of ALT rises in diseases associated with death of hepatocytes like viral hepatitis (George *et al*., 2011). AST on the other hand is not found to be specific for liver damages but has been found to be a cardiac marker as it is found in cardiac and skeletal muscles (Ujowundu *et al*., 2011). The serum ALP is related to the function of hepatic cell and increase in serum level of ALP may be due to increased synthesis of the enzymes in presence of increasing biliary pressure (Emeka & Obidoa, 2009). Generally, an increase in this enzyme indicates injury or toxicity to the organ. Several African medicinal plants have been shown to have hepatoprotective effects (Emeka & Obidoa, 2009). This hepatoprotection is possibly due to flavonoids and other chemical composition which exert membrane-stabilizing action that protects the liver cells from injury (Emeka & Obidoa, 2009)

Haematological parameters can be used to determine extent of infection and effects of medicinal plant extracts on the blood constituents of an animal (Berinyuy *et al*., 2015). Such analysis is relevant to risk evaluation because changes in the hematological system are highly predictive for human toxicity, when data are translated from animal studies (Umar *et al*., 2019). *P. berghei* infected mice suffer from hemolytic anemia because of RBC destruction, due to either parasite multiplication or spleen reticuloendothelial cell action as the presence of many abnormal RBC stimulates the spleen to produce many phagocytes (Chinchila *et al*., 1998). This massive destruction leads to a decrease in erythroid precursors and erythropoiesis inhibition, usually resulting in the death of the patient. Anemia is a preventable cause of death in malaria-infected children under five years and pregnant women (WHO, 2006). This necessitates hematological analysis to evaluate the effectiveness

of test materials in preventing hemolysis. In this study, it was noted that the Mean haemoglobin (Hb), packed cell volume (PCV), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), white blood cells (WBC) and platelet counts in *P. berghei* infected mice where significantly lowered ($p<0.05$) when compared with normal control and extract treated groups (Table 4.8 and 4.9). Treatment of the infected mice with the crude extract and alkaloidal fraction of *Azadracta indica* (Table 4.8) and *V. amygladima* (table 4.9) caused a significant ($P<0.05$) and dose dependent increase in the hematological indices when compared with the untreated control. This could be due to antiplasmodial effect of the crude extracts and the alkaloids and as a result of sustaining the availability of new RBCs produced in the bone marrow. It could also be an indication of haematopoietic properties of the plant extract as reported by Lawal *et al*., (2015). However, crude extract of both plants caused higher increase in platelet count when compared with their respective alkaloidal fractions. This further strengthened the initial claim that alkaloid is not the only anti-plasmodial agents in these plants extract, a synergetic activity of the phytochemicals in these plants extract is therefore implicated in the higher ant-plasmodial activity recorded.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The result of the in vivo acute toxicity study of *A. indica* and *V. amygdalina* in mice revealed no signs of toxicity indicating a good safety profile of the plant material. The results indicated that *A. indica* and *V. amygdalina* possessed promising antimalarial activity. A key factor that makes this work unique is the fact that previous work did not consider the alkaloidal fraction of both plant extract and this alkaloidal fractions of both plants show a relatively low antiplasmodial activities compare to the crude extract, thus indicating a synergertic activities of phytochemicals component of each plants. Thus these plants might contain potential lead compound(s) for the development of a new drug for the treatment of malaria. This supports the acclaimed traditional use of this plant by local communities in Nigeria to treat malaria.

5.2 Recommendation

Based on the results obtained from this study the following recommended were suggestions.

1. Further toxicological studies such as sub-acute, sub-chronic and chronic toxicities should be done in order to assess the long term effect of the plant extracts
2. Further studies should be done to isolate, purify and identify pharmacologically active principle (s) responsible for antiplasmodial activity.

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