ANTI-PLASMODIAL EFFECTS OF *POLYALTHIA LONGIFOLIA* (VAR.) LEAF EXTRACTS AGAINST *PLASMODIUM BERGHEI* IN MICE

BY

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ABSTRACT

Malaria is one of the most debilitating tropical parasitic diseases and the greatest cause of hospitalization and death. Recurring problems of drug resistance are reinforcing the need for finding new antimalarial drugs. In this respect, natural plant products are the main sources of biologically active compounds and have potential for the development of novel antimalarial drugs. The present study was designed to elucidate bioactive metabolite and in vivo antimalaria efficacy of crude and solvent fractions of Polyalthia longifolia against Plasmodium berghei in mice. The crude methanol extract of the plant was analysed for the presence of bioactive metabolites following standard procedure. A rodent malaria parasite, Plasmodium berghei, was used to inoculate healthy male Swiss Albino mice of age 6-8 weeks and weight 28-35 g. Crude methanol extract and the solvent fractions were administered at different doses 150, 300 and 600 mg/kg. Parasitaemia, survival time, body weight, and packed cell volume were determined using standard tests. The results indicated the presence of phytochemicals including flavonoids, phenols, tanins, and alkaloids. Saponin content (500.76±2.37 mg/100 g) was significantly higher than other phytochemicals detected. Acute oral toxicity bioassay reveals an LD₅₀ extrapolated to be above 1600 mg/kg body weight. The crude extract at doses 600 mg/kg b.wt showed appreciable antiplasmodial potency than the fraction. The crude extract prevented loss of weight and slightly affected packed cell volume. The solvent fraction also prevented loss in packed cell volume. All doses of crude extracts and fractions of P. longifolia leaf prolong the survival time of infected mice in a dose dependent pattern. The results collectively indicate that the plant has a promising antiplasmodial activity against Plasmodium berghei, which upholds the earlier in vitro findings as well as its folkloric use.

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ABBREVIATIONS

ACP	Acyl Carrier Protein
AIDS	Acquired Immunodeficiency Syndrome
ANKA	Rodent malaria model of <i>Plasmodium berghe</i> i strain
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemist
CDC	Centers for Disease Control and Prevention
DAPI	4', 6-diamidino-2-phenylindole
DDT	Dichloro-Diphenyl-Trichloroethane
FABI	Fatty Acid Biosynthesis
FASII	Type II Fatty Synthase
FUT	Federal University of Technology
GDP	Gross Domestic Product
GFP	Green Fluorescent Protein
HIV	Human Immunodeficiency Virus
HRP2	Histidine-Rich Protein 2
IRS	Indoor Residual Spraying
ITN	Insecticide Treated Net
MST	Mean Survival Time

NCDC	Nigeria Centre for Disease Control
NMCP	National Malaria Control Program
OPD	Outpatient department
PCV	Packed Cell Volume
pН	Potentials of Hydrogen or Power of Hydrogen
pLDH	Parasite Lactate Dehydrogenase
RBC	Red Blood Cell
RPMI	Roswell Park Memorial Institute
SEM	Standard Error of the Mean
UNICEF	United Nations International Children Emergence Fund
UV	Ultraviolet
WHO	World Health Organization

CHAPTER ONE INTRODUCTION

1.1

INTRODUCT

1.1 Background to the Study

Malaria, a parasitic infection caused by a protozoan of the genus Plasmodium, contributes substantially to the poor health situation in Africa. About 90 % of the world's 216 million cases and 655 000 annual deaths of malaria occur in the sub-Saharan African region (World Health Organization, (WHO), 2012). In Nigeria, more than 3 million cases are reported annually. A significant proportion (900,000) of such cases occurs in children under the age of five (WHO, 2014). Malaria has an immense effect on people of all ages but children under five years, pregnant women and immigrants from non-endemic regions are the most vulnerable because of their low immunity (Neelavathi et al., 2013). It is a major cause of absenteeism from school in endemic countries and frequent episodes of severe malaria in young children may negatively impact on their learning abilities and educational attainment. According to WHO/UNICEF (2013), about 2 % of children who recover from cerebral malaria suffer brain damage including epilepsy. In pregnant women, malaria can cause anaemia, miscarriages, stillbirths, underweight babies and maternal deaths. Malaria is therefore, a threat to human capital accumulation, which constitutes a key factor in economic development (Asante and Asenso-Okyere, 2003). The devastating impacts of malaria on adult victims are also very much disturbing. It causes considerable pain and weakness in the victims which translate into reduced working abilities. A single bout of the disease in a young adult costs an equivalent of 10 working days. It has also put an unbearable strain on household resources as malaria care can cost up to 34 % of a poor household's income (Asante and Asenso-Okyere, 2003; Fana et al., 2015). This adversely and substantially impact on gross domestic product. In endemic countries, malaria causes a negative effect on growth of tourism, investments

and trade and loss of productivity on the major sectors of their economies (Perkins and Austin, 2011). In Africa, the low productivity and high mortality resulting from malaria has been estimated to cost US\$ 12 billion in lost Gross Domestic Product (GDP) and has also slowed economic growth by 1.3 % every year. Malaria control in the sub-region is key to achieving five of the eight millennium development goals (i.e. Eradicating extreme poverty and hunger, achieving universal primary education, reducing child mortality rates, improving maternal health, combating HIV/AIDS, malaria, and other diseases). Control measures of the disease include prevention of infection, treatment of infected people and control of the mosquito vector. Great efforts have been made to eradicate malaria all over the world. The WHO in 1955 launched the Global Malaria Eradication Program which aggressively employed effective treatment of the infection (with chloroquine) and control of the mosquito vectors (with DDT insecticide) (Kantele & Jokiranta, 2011). Even though this program helped to eradicate the disease in nations with temperate climates and seasonal malaria transmission, many other nations such as Indonesia, Haiti, Afghanistan and Nicaragua, recorded negligible achievements. The hyper endemic countries in the sub-Saharan Africa region were however completely excluded from this eradication campaign (WHO, 2008).

In Nigeria, malaria control has been high on the public health agenda as far back as preindependence with preventive interventions including indoor residual and aerial spraying with insecticides and the addition of Pyrimethamine to table salt (Wellems, 2002; Adams *et al.*, 2004). However, the widespread mosquito resistance to insecticides, inaccessibility of health services in the rural areas, proliferation of fake and substandard antimalarial drugs in the sub-region and the emergence of multidrug-resistant malaria parasites, has hampered the goal of these control measures. Treatment of malaria with potent, effective, available and affordable drugs nevertheless, remain crucial to the control and the eventual eradication of the disease in Nigeria and the sub-region as a whole. Many antimalarial chemotherapeutic agents have been used to treat the infection but most of these agents are now not effective due to the widespread of multidrug-resistant malaria parasites (Asante and Asenso-Okyere, 2003).

1.2 Statement of the Research Problem

Despite more than a century of efforts to control or eradicate malaria, the disease remains a major growing threat to public health and economic development of countries in the tropical and sub-tropical world. This has been largely attributed to *P. falciparum* resistance to most antimalarial drugs (Achan, *et al.*, 2011).

In Nigeria, malaria transmission occurs throughout the year round, and the country accounts for a quarter of all malaria cases in the World Health Organization (WHO), African region (WHO, 2008). Most malaria cases are caused by *P. falciparum*, although they remain unconfirmed.

Malaria endemic regions of the world are faced with an unprecedented situation in which affordable treatment options are rapidly losing therapeutic efficacy because of some degree of resistance (Batista, *et al.*, 2009). As a consequence of drug resistance, drugs like quinine, chloroquine, primaquine and mefloquine are ineffective in treating malaria in many endemic regions of the world (CDC, 2012). Another problem relating to drug resistance in *P. falciparum* is the occurrence of cross-resistance among drugs belonging to the same chemical family (WHO, 2001). Even though no clinically relevant artemisinin resistance has been reported yet, it is likely to occur since artemisinin resistance has been obtained in laboratory animals (Bayor, 2007). *Plasmodium falciparum* is reported to have reduced *in vivo* susceptibility to artesunate in Western Cambodia, historically part of a site of emerging antimalarial-drug resistance (Bhatta, *et al.*, 2011).

1.3 Justification for the Study

Some of the reasons for increase in mortality due to malaria include; *P. falciparum* resistance to most anti-malarial drugs, *Anopheles* mosquito's resistance to insecticides, environmental changes, war and civil disturbances, travels and cross border movements. The main reason, however, is parasite resistance to antimalarials which complicates the problem of treatment. In the absence of a functional, safe, inexpensive and widely available malaria vaccine, the effort to develop new antimalarial drugs from local plants traditionally reputed to cure malaria becomes profoundly important.

One of the strategies in the search for new anti-malarial compounds is the study of active constituents (metabolites) of medicinal plants. Generally, scientific information about antimalarial activity of plants traditionally acclaimed to cure malaria is very limited or does not exist in some cases. Phytochemical screenings of medicinal plants are not only used to search for bioactive agents but also help to reveal the presence of agents in plants which serve as starting products for the partial synthesis of some useful drugs. It is therefore important to screen medicinal plants for antimalarial activity in order to ascertain their potentials as sources of new anti-malarial compounds. The reputed efficacies of *Polyalthia longifolia* have been documented. However, lack of scientific proof claimed by traditional healers in Nigeria necessitates a scientific study on this plant. It is in light of this, that *Polyalthia longifolia* have been screened for antimalarial activities.

1.4 The Aim of the Study

The aim of this study is to evaluate bioactive metabolite and *in vivo* antimalaria efficacy of crude and solvent fraction of *Polyalthia longifolia* in *Plasmodium berghei*.

1.5 Objectives

The objectives of the study are to determine:

- i. phytochemical constituent of the crude extract of *Polyalthia longifolia*.
- ii. median lethal dose (LD₅₀) of the extract (crude) of *Polyalthia longifolia*.
- iii. the antiplasmodial activities of the crude and fraction of P. longifolia
- iv. the effect of the crude and fractionated extract on body weight, packed cell volume, and haematological parameters of *P. berghei*-infected mice

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Malaria Disease Burden

Among the parasitic infectious diseases, malaria is the most serious public health problem in tropical and sub-tropical regions of the world. Malaria ranks high among the WHO listing of 'Infectious diseases of poverty' and remains a major cause of morbidity and mortality world wide. Malaria is known to exert a huge negative impact on population health and economic development in countries where the disease is endemic (WHO, 2011). Over the years, significant efforts have been made at both national and international levels to reduce the burden of malaria disease and its impact on global health (Dasari *et al.*, 2011).

In Nigeria, the malaria situation is typical of the sub-Saharan African pattern. Transmission of the disease is all-year-round with prevalence rates in the forest ecological zones being higher (51.3 %) than the coastal savannah (36.6 %) (Adzu and Salawu, 2009). From the Nigeria Health Service National Malaria Control Program (NMCP) annual report, malaria has been a major cause of poverty and low productivity accounting for about 32.5 % of all OPD attendances and 48.8 % of children under five years hospital admissions in the country (NCDC, 2016). The attempt to control malaria in Nigeria began in the 1950s. It was aimed at reducing the malaria disease burden until it is no longer of public health significance. It was also recognized that malaria cannot be controlled by the health sector alone; therefore, multiple strategies were pursued with other health related sectors. In view of this, interventions were put in place to help in the control of the deadly disease. Some of the interventions applied at the time included residual insecticide application against adult mosquitoes, mass chemoprophylaxis with Pyrimethamine

medicated salt and improvement of drainage systems. But malaria continued to be the leading cause of morbidity in Nigeria (NCDC, 2016). Malaria is really a threat to the whole world as imported malaria (infection acquired outside and brought into a national territory of a malaria-free country) and local transmission from these cases has often been reported in many countries including France, Spain, USA and UK since 2001 (WHO, 2011).



Figure 2.1 Geographical Distribution of Malaria Burden Worldwide.

(Source: WHO, 2011).

Malaria is a potentially deadly parasitic disease caused by infection of liver and red blood cells by a protozoan of the genus *Plasmodium*. It is transmitted to humans following a bite from its vector, various species of the Anopheles mosquito (Figure 2.2) or by a contaminated needle or transfusion. Five mains species of *Plasmodium* can cause malaria in humans; P. vivax and P. ovale, both causing benign tertian malaria, characterised by fever that occurs every third day; P. malariae, which causes malariae or quartan malaria, the fever classically recurs every fourth day; P. falciparum, responsible for malignant tertian malaria; and P. knowlesi which is the most recently implicated species in human malaria (White et al., 2009). So far, the most virulent of the parasites is P. falciparum, which also accounts for the majority of severe illnesses, complications and deaths from malaria. Falciparum or malignant tertian malaria is also the most prevalent in sub-Saharan Africa, where the disease burden is highest globally (Winstanley et al., 2004; Perkins et al., 2011; Dasari and Bhakdi, 2012). The symptoms of malaria include cycles of chills, fever, sweats, muscle aches and headache that recur every few days. There can also be vomiting, diarrhoea, coughing, and yellowing (jaundice) of the skin and eyes. Persons with severe falciparum malaria can develop bleeding problems, shock, kidney and liver failures, central nervous system problems, coma and death. Malaria transmission occurs primarily between dusk and dawn because of the nocturnal feeding habits of the Anopheles mosquitoes.



Figure 2.2 Anopheles Mosquito, the Vector of Plasmodium falciparum.

(Source: NCDC, 2016).

2.2 History of Malaria

Malaria is probably one of the oldest diseases known in history and it appears to have evolved together with humans as it has been known for millennia. Description of malaria dates back to the earliest medical writings in Egypt, China and India where it was mostly attributed to evil spirits. Hippocrates, in the 5th century, tried describing the clinical symptoms of the disease and by so doing differentiated quotidian and tertian fevers. Vapours and mists arising from swamps and marshes were the proposed causative agents and hence the name *malaria* (bad air) (Asase *et al.*, 2012).

2.3 Life Cycle of *Plasmodium falciparum*

Malaria is mainly transmitted through the bite of an infected female *Anopheles* mosquito. This species of mosquito needs blood for egg production. During a blood meal, the infected mosquito releases mature forms of the developing parasite, the sporozoites into the capillary bed of the human skin (Figure 2.2). On reaching humans, the sporozoites rapidly move into the parenchymal cells of the liver where they asexually develop and replicate (exoerythrocytic schizogony) into merozoites. Each merozoite when released from the liver can invade a red blood cell. The pathology and symptoms of the disease starts with the invasion and destruction of the red blood cells (Buffet *et al.*, 2011). With respect to *P vivax* and *P ovale* infections, some of the liver forms of the parasite (hypnozoites) could remain dormant and reactivate later to cause the disease, several months or years after an infective bite from a mosquito. In the RBCs, repeats of asexual replication (erythrocytic schizogony; Figure 2.3) occur and yield around 20 merozoites per mature parasite (Guerin *et al.*, 2002).

A small proportion of the merozoites do not undergo this asexual replication. These merozoites differentiate into the sexual forms referred to as macro- (female) and micro-

(male) gametocytes which are enclosed in protective erythrocyte membranes. When drawing blood for egg production, these mature gametocytes can be taken by the mosquito. The protective membranes are shed in the mid-gut of the mosquito and gametogenesis is initiated. The macrogametocyte becomes the macrogamete while the microgametocyte exflagellates into 8 sperm-like microgametes and each of these is capable of fertilizing the macrogamete. When fertilization occurs, a spherical shaped zygote is formed which transforms within 12 to 18 hours into an oval leaf-shaped ookinete. The ookinete crosses the gut wall and settles in mid-gut of the mosquito where it develops into a round oocyst which matures over a period of 10 to12 days to the infective form, (the sporozoites) in an enclosed oocyst capsule. On release from the oocyst, the sporozoites reach and penetrate the salivary gland of the mosquito. The time taken to complete the sexual phase (sporogony; Figure 2.3) is dependent on external environmental factors such as temperature and humidity (Ene *et al.*, 2009).

The factors that determine the extent and intensity of malaria transmission are known to include those that influence the life cycle or development of the parasite in the mosquito vector and also in humans. These include the warm climatic conditions that promote breeding of mosquitoes; the malaria parasite life cycle involves 2 hosts. During a blood meal, a malaria-infected female Anopheles mosquito inoculates sporozoites into the human host. Sporozoites infect liver cells. There, the sporozoites mature into schizonts.

The schizonts rupture and release merozoites. This initial replication in the liver is called the exoerythrocytic cycle. Merozoites infect red blood cells. There, the parasite multiplies asexually. The merozoites develop into ring-stage trophozoites. Some then mature into schizonts. The schizonts rupture, releasing merozoites. Some trophozoites differentiate into gametocytes. During a blood meal, an Anopheles mosquito ingests the male and female gametocytes beginning the sporogonic cycle. In the mosquito's stomach, the microgametes penetrate the macrogametes, producing zygotes. The zygotes become motile and elongated, developing into ookinetes. The ookinetes invade the midgut wall of the mosquito where they develop into oocysts. The oocysts grow, rupture, and release sporozoites, which travel to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle. Immune status of the human host, unavailability of resources to fight the disease and inefficient implementation of proven effective interventions for malaria control (Snow *et al.*, 2005; Wells *et al.*, 2009). The incubation period, which is the time between infective mosquito bites to observation of symptoms in the human host, varies from 7 to 30 days. Shorter incubation periods are observed frequently with *P. falciparum* and longer periods with *P. malariae*. In some rare cases, malaria parasites could also be transmitted from human to human through blood transfusion or from an infected pregnant mother to the unborn child (congenital malaria) (Wells *et al.*, 2009; Dasari *et al.*, 2011).



Source: (Ricardo et al., 2014).

Transmission of malaria occurs through a vector, the mosquito that injected sporozoites into a human during blood meal(s) (1), where they rapidly make their way to the liver and infect hepatocytes and begin asexually (mitotically) replicating (2). After a period of ca. 6–15 days, the liver schizonts rupture, releasing thousands of merozoites into the blood where they invade RBCs (3). Over the next ca. 48 h, the parasite begins replicating mitotically, progressing through a set of stages (ring, trophozoite and schizont), and produces an average of 16 new daughter merozoites per schizont. The schizonts then burst in near synchrony with other parasites, producing the characteristic fever cycle that embodies the clinical manifestations of the disease (4). Some of the merozoites produce gametocytes—the sexual form of the parasite (5), which is ingested by mosquito upon feeding on an infected human (6). Gametocytes, which are both male and female, mate within the gut of the mosquito, undergo meiosis (7), then migrate through the midgut wall of the mosquito, and form an oocyst (8), within which thousands of sporozoites develop (9), that can then infect susceptible mosquitoes and bringing the transmission cycle full circle.

2.4 Plasmodium berghei

It is a protozoan parasite that causes malaria in certain rodents. Originally, isolated from thicket rats in Central Africa, *P. berghei* is one of four Plasmodium species that have been described in African murine rodents, the others being *Plasmodium chabaudi*, *Plasmodium vinckei*, and *Plasmodium yoelii*. Due to its ability to infect rodents and relative ease of genetic engineering, *P. berghei* is a popular model organism for the study of human malaria. Like all malaria parasites of mammals, including the four human malaria parasites, *P. berghei* is transmitted by Anopheles mosquitoes and it infects the liver after being injected into the bloodstream by a bite of an infected female mosquito. After a short period (a few days) of development and multiplication, these parasites leave the liver and

invade erythrocytes (red blood cells). The multiplication of the parasite in the blood causes the pathology such as anaemia and damage of essential organs of the host such as lungs, liver, spleen. *P. berghei* infections may also affect the brain and can be the cause of cerebral complications in laboratory mice. These symptoms are to a certain degree comparable to symptoms of cerebral malaria in patients infected with the human malaria parasite *Plasmodium falciparum* (Franke-Fayard *et al.*, 2010).

Plasmodium berghei is found in the forests of Central Africa, where its natural cyclic hosts are the thicket rat (*Grammomys surdaster*) and the mosquito (*Anopheles dureni*).

2.4.1 Hosts of P. berghei

Plasmodium berghei was first identified in the thicket rat. In research laboratories, various rodents can be infected, such as mice, rats and gerbils (Junaid *et al.*, 2017). The natural insect host of *P. berghei* is likely *Anopheles dureni*, however in laboratory conditions it has also been shown to infect *Anopheles stephensi*.

Plasmodium berghei infections of laboratory mouse strains are frequently used in research as a model for human malaria (Craig *et al.*, 2012). In the laboratory the natural hosts have been replaced by a number of commercially available laboratory mouse strains, and the mosquito *Anopheles stephensi*, which is comparatively easily reared and maintained under defined laboratory conditions.

Plasmodium berghei is used as a model organism for the investigation of human malaria because of its similarity to the Plasmodium species which cause human malaria. *P. berghei* has a very similar life-cycle to the species that infect humans, and it causes disease in mice which has signs similar to those seen in human malaria. Importantly, *P.*

berghei can be genetically manipulated more easily than the species which infect humans, making it a useful model for research into Plasmodium genetics.

In several aspects the pathology caused by *P. berghei* in mice differs from malaria caused by *P. falciparum* in humans. In particular, while death from *P. falciparum* malaria in humans is most frequently caused by the accumulation of red blood cells in the blood vessels of the brain, it is unclear to what extent this occurs in mice infected with *P. berghei* (Craig *et al.*, 2012). Instead, in *P. berghei* infection, mice are found to have an accumulation of immune cells in brain blood vessels (Craig *et al.*, 2012). This has led some to question the use of *P. berghei* infections in mice as an appropriate model of cerebral malaria in humans (Craig *et al.*, 2012).

Plasmodium berghei can be genetically manipulated in the laboratory using standard genetic engineering technologies. Consequently, this parasite is often used for the analysis of the function of malaria genes using the technology of genetic modification (Janse *et al.*, 2013). Additionally, the genome of *P. berghei* has been sequenced and it shows a high similarity, both in structure and gene content, with the genome of the human malaria parasite Plasmodium falciparum (Hall *et al.*, 2005; Kooij *et al.*, 2007; Otto *et al.*, 2014).

Infected mouse, with *P. berghei* in the lungs, spleen and adipose tissue. Transgenic parasites are visualized by their expression of the bioluminescent reporter protein Luciferase.

A number of genetically modified *P. berghei* lines have been generated which express fluorescent reporter proteins such as Green Fluorescent Protein (GFP) and mCherry (red) or bioluminescent reporters such as Luciferase. These transgenic parasites are important tools to study and visualize the parasites in the living host (Feacham *et al.*, 2010; Franke-Fayard *et al.*, 2010).

A liver cell with *P. berghei* (a schizont with daughter parasites) expressing mCherry (red). Here the parasite membrane is stained green with an antibody, while the nuclei of liver cells and parasites are stained with DAPI (blue).

P. berghei is used in research programs for development and screening of anti-malarial drugs and for the development of an effective vaccine against malaria (Khan *et al.*, 2014).

2.5 Laboratory Diagnosis of Malaria

Laboratory diagnosis of malaria involves identification of malaria parasite or its antigens/products in the blood of infected mice.

2.5.1 Microscopy of malaria parasite

Microscopy is gold standard for laboratory confirmation of malaria. A drop of the infected mice blood is collected by insignificant tail cut, or from a larger venous blood specimen. It is then spread on a glass slide (blood smear), dipped in a reagent that stains the malaria parasites (Giemsa stain), and examined under a microscope at a 1000-fold magnification. Malaria parasites are recognizable by their physical features and by the appearance of the red blood cells that they have infected.

Advantages: Microscopy is an established, relatively simple technique that is familiar to most laboratorians.

Disadvantages: In many developing countries, microscopy is not reliable because the macroscopic are insufficiently trained and supervised and are overworked, the microscopes and reagents are of poor quality, and often the supply of electricity is unreliable. Conversely in nonendemic countries, laboratory technicians are often unfamiliar with malaria and may miss the parasites.

2.5.2 Morphological species and stage diagnosis

Complete knowledge of the morphological features of the different blood stages of the different *Plasmodium* species represents the essential basis of a correct laboratory diagnosis confirmation of malaria infection. It is to be stressed that a correct diagnosis may be done only after attentive and careful observation of a number of microscopic fields (at least 100 microscopic fields should be observed before a thick film may be classed as negative) and of a number of different morphologic characteristics that draw a well-defined picture of the species. A crescent shaped gametocyte in peripheral blood does not obligatorily mean that a *P. falciparum* infection is the cause of the actual clinical complaint and, however, does not rule out the possibility of mixed infection (Aty *et al.*, 2014).

2.5.3 Peripheral blood smears examination for malarial parasite

Peripheral smear examination for malaria parasite is the gold-standard in confirming the diagnosis of malaria. Thin and thick smears prepared from the peripheral blood are used for the purpose. Microscopic examination of the peripheral blood smear provides comprehensive information on the species, the stages and the density of parasitaemia with a sensitivity of 5 to 10 parasites/µl of blood. The efficiency of the test depends on the quality of the equipment and reagents, the type and quality of the smear, skill of the technician, the parasite density and the time spent on reading the smear. The test takes about 20 to 60 minutes depending on the factors mentioned above. It is estimated to cost about 50 to 90 US cents per slide in the endemic countries (Kemgne, 2012).

2.5.4 Preparation of the blood smears

The peripheral blood smears are prepared. Universal precautions are used while preparing the smears for malaria parasites (use of gloves and only disposable needles/lancets; hand washing; careful handling and disposal of the sharp instruments and other materials contaminated with blood to avoid injury) (Khan *et al.*, 2014).

2.5.5 Thin blood smears

The thin smear is air-dried for 10 minutes and then fixed in methanol, by either dipping the thin smear into methanol for 5 seconds or by dabbing the thin smear with a methanol-soaked cotton ball (Khan *et al.*, 2014).

2.5.6 Thick blood smears

A thick blood smear of correct thickness is the one through which newsprint is barely visible. It is dried for 30 minutes and not fixed with methanol. This allows the red blood cells to be haemolysed and leukocytes and any malaria parasites present will be the only detectable elements. However, due to the haemolysis and slow drying, the plasmodia morphology can get distorted, making differentiation of species difficult. Thick smears are therefore used to detect infection and to estimate parasite concentration. Below are the steps involved in smear preparation (Kemgne *et al.*, 2012).

2.5.7 Staining of the blood films

A number of Romanowsky stains like Field's, Giemsa's, Wright's and Leishman's are suitable for staining the smears. Thick films are ideally stained by the rapid Field's technique or Giemsa's stain for screening of parasites. The sensitivity of a thick blood film is 20 parasites (0.0004 %) parasitaemia. Thin blood films stained by Giemsa's or Leishman's stain are useful for specification of parasites and for the stippling of infected

red cells and have a sensitivity of 200 parasites/ μ l (0.004 %). The optimal pH of the stain is 7.2. The slides should be clean and dry and it is better to use neutral distilled water (Nizamuddin, 2009).

2.5.8 Giemsa staining of thin blood films

The fixed thin blood film is covered with Giemsa (diluted 1 in 10 with buffered distilled water pH 7.2) and left for 30 minutes. After that the film is washed with distilled water, drained, dried and examined.

2.5.9 Giemsa staining of thick blood films

The thick film is first dehaemoglobinised in water and then stained with Giemsa.

Rapid giemsa: A 10 % Giemsa is prepared in buffered water at pH 7.1. The slide is then immersed in the stain for 5 minutes. After that, it is rinsed gently for 1 or 2 seconds in a jar of tap water, drained, dried and then examined.

Standard giemsa: A 4 % Giemsa is prepared in buffered solution at pH 7.1. The slide (at least 12 hours old) is immersed in the stain for 30 minutes, rinsed with fresh water, drained, dried and then examined.

2.6 Antimalarials in Clinical Use and Drug Resistance

In all the malaria elimination efforts the world over, antimalarial drugs remain very crucial with the use of insecticide treated nets (ITN) and indoor residual spraying (IRS) of insecticides to strengthen the efforts against combating malaria. The fact of the case is that with all the efforts to eliminate malaria, people in hyper endemic regions such as the sub-Saharan Africa will still contract the disease and without the availability of affordable

and effective drugs, many people will succumb to the devastating effect of malaria (Wells *et al.*, 2009; Aty *et al.*, 2014).

The problem is further aggravated by the rampant resistance development of P. *falciparum* to the antimalarials in current use. Antimalarial drug resistance can be defined as the ability of the malaria parasite 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 (WHO, 2012; Bruce-Chwatt et al., 2016). A modification of this definition 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 has been reported (Aty et al., 2014). 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 (Olasehinde et al., 2012). Resistance generally appears to occur through spontaneous mutations that confer reduced sensitivity to a given drug or class of drugs. For some drugs, just a single point mutation is required to confer resistance while for others multiple mutations appear to be required. Provided the mutations are not deleterious to the survival or reproduction of the parasite, drug pressure will remove susceptible parasites while resistant parasites survive (Mayxay et al., 2007).

The spread of drug and insecticide resistance is on the increase due to the evolutionary pressure being put on both the *Plasmodium* parasite and the mosquito. The Thai-Cambodia border continues to be the historical site for emergence of resistance to antimalarial drugs as the initial signs of resistance to both the older drugs such as chloroquine and the newer artemisinins have all been first reported from this area (Noedl *et al.*, 2010; Satimai *et al.*, 2012). With the exception of the antifolates, the modes of action and resistance mechanisms of available antimalarials are not clearly understood.

However, the resistance mechanisms appear to involve among others increased elaboration of inhibited (or targeted) enzymes, modification of drug transport systems, increase production of enzymes that inactivate the drugs as well as alternative metabolic pathways (Petersen *et al.*, 2011).

Currently, the available antimalarial drugs are basically derivatives of certain core structures which can be grouped into three main classes; quinolones (comprising the aryl amino alcohols, amino quinolones and the bisquinolone), anti-folates and the artemisinins (Achan *et al.*, 2011). Some antibiotics have also exhibited antimalarial properties and these include the tetracyclines and clindamycin.

2.7 Antibiotics Used Against Plasmodium Parasites

Some antibiotics have either shown potent activity against the *Plasmodium* parasites or synergize the activity of other antimalarials. Tetracycline and its derivatives such as doxycycline are very potent antimalarials and are used for both treatment and prophylaxis (Kremsner *et al.*, 1994). In regions where response to quinine has deteriorated, tetracyclines are often used in combination with quinine to improve cure rates. Clindamycin has been used but offers only limited advantage when compared to other available antimalarial drugs as parasitological response is slow and recrudescence rates are high (Kremsner *et al.*, 1989).

Doxycycline is a tetracycline compound derived from oxytetracycline. The tetracyclines are still used widely in many types of infection even though they were one of the earliest groups of antibiotics to be developed. Doxycycline is a bacteriostatic agent that interferes with protein synthesis process by binding to the 30_s ribosomal subunit thus preventing the 50s and 30s units from bonding (Schwartz *et al.*, 2012). Doxycycline is primarily employed for chemoprophylaxis in regions where quinine resistance exists. It is also used in resistant cases of uncomplicated *P. falciparum* but has a very slow action in acute malaria. The common side effects include permanent enamel hypoplasia, transient depression of bone growth, gastrointestinal disturbances and some increased levels of photosensitivity. Due to its effect on bone and tooth growth it is not used in children under 8, pregnant or lactating women and those with a known hepatic dysfunction (Schwartz *et al.*, 2012). Tetracycline is only used in combination for the treatment of acute cases of *P. falciparum* infections due to its slow onset. Unlike doxycycline, tetracycline is not used in chemoprophylaxis. Oesophageal ulceration, gastrointestinal upset and interferences with the process of ossification and depression of bone growth are known to occur. The majority of side effects associated with doxycycline are also experienced.

Clindamycin, a derivative of Lincomycin, has a slow action against blood schizonticides. It is only used in combination with Quinine in the treatment of acute cases of resistant *P*. *falciparum* infections and not as a prophylactic (Schwartz *et al.*, 2012).

2.8 Natural Products Used Against *Plasmodium* Parasites

Throughout the ages, natural products have been the most consistently successful source of antimalarial drugs and lead compounds for antimalarial drug development. As previously stated, the *Cinchona* bark extract was the first malaria treatment option discovered and quinine was one of the alkaloids isolated from this extract. Despite the adverse effects and the resistance developed by some of the malaria parasites to it, quinine is still a very important chemotherapeutic agent and is used even in severe and complicated conditions whenever all the other drugs fail to provide therapeutic response. The development of artemisinin forms the Chinese herb *Artemisia annua* is another typical example of natural product source of potent antimalarial (Dasari and Bhakdi, 2012).

Natural products have also provided structural pharmacophores for the development of effective antimalarials. The synthetic antimalarials such as chloroquine, amodiaquine and primaquine, are but structural analogues of quinine. The prenylated naphthoquinone lapachol from *Tabebuia* species (Bignoniaceae) provided the pharmacophores that led ultimately to the development of the antimalarial atovaquone, a synthetic 2-alkyl-3-hydroxy-1, 4-naphthoquinone (Vianal *et al.*, 2003; Wells & Gutteridge, 2011). Further discussion on plant natural products as drugs and or lead compounds for the anti-infective drug development is in section 1.6 of this thesis.

2.9 Antiplasmodial Assays Methods

The traditional antiplasmodial bioassay tests are all based on the measurement of the effect of the compound or extract on the growth and development of malaria parasites. The antiplasmodial effect (endpoint) is generally characterized by inhibition of parasite growth and, consequently, their multiplication; these are parameters that can be measured *in vitro* in several different ways. The parasites are mixed with medium (usually RPMI 1640 culture medium) and the growth in test cultures is measured relative to a drug-free control. Variations in parasite density, haematocrit and the stage of the malaria parasite in its life cycle may have a significant impact on the outcome of these tests (Bankole *et al.,* 2016). It is therefore necessary to control the culture for parasite density, hematocrit and developmental stage of the parasites as far as possible. The assays usually result in sigmoid dose–response curves when performed with serial dilutions of the compound or extract. For the *in vivo* methods, the malaria infection is induced in laboratory animals which are then treated with varying concentrations or preparations of the compound. In
the course of treatment, parasitaemia levels are determined by analysing blood samples taken from the animals. The antiplasmodial effect in both cases is usually measured as the lowest concentration of the compound that produces 50% inhibition of the parasite growth. The most commonly used methods for determining the antiplasmodial effect of compounds and extracts are the WHO micro-test, the isotopic test and measurement of pLDH or HRP2 (Desjardins *et al.*, 2009).

In the WHO micro-test, thin blood smears of the incubated cultures are prepared, fixed and stained and then observed under a light microscope. Growth inhibition due to the compound or extract defined as the difference between the percentage parasitaemia of test culture and the corresponding positive control (culture without test compound or extract) is calculated as follows (Ene *et al.*, 2009).

% Growth inhibition =
$$\frac{(\% \text{ Parasitemia CIRBC} - \% \text{ parasitemia DIRBC}) \times 100\%}{\% \text{ parasitemia CIRBC}}$$

Where CIRBC is the percentage parasitaemia of infected RBC without extracts (i.e. control); DIRBC is the percentage parasitaemia of infected RBCs incubated with the test compound. The Percentage growth inhibition is plotted against the log concentration of the test compound to get the sigmoid curve.

2.10 Selected Plant Species for Antiplasmodial Activity Screening

Polyalthia longifolia popularly called the "Mast Tree" of the family Annonaceae is native to the drier regions of India and Sri Lanka. It is commonly cultivated in Pakistan (Verma *et al.*, 2008; Katkar *et al.*, 2010) and many other tropical countries around the world. The Annonaceae, on the basis of source of edible fruits and oils, is of very significant economic importance. *Polyalthia* is a large genus of shrubs and trees consisting of about 120 species which are distributed in Asia and tropical Africa. Few of these species are *P. cerasoides*, *P. suberosa*, *P. nemoralis*, *P. barnesii*, *P. viridis*, *P. acuminata*, *P. macropoda* and *P. oliveri*.

In Nigeria and other West African countries *P. longifolia* is usually cultivated in residential areas, especially along streets and around houses as an ornamental plant and a wind-break. *P. longifolia* is an evergreen, tall and slender tree (up to12 meter) that grows symmetrically and produces fresh and shining green foliage. The entire length of the plant is covered by long and wavy leaves. The beautiful contrast of new golden and coppery brown leaves against old dark-green leaves make a spectacular show.

There are two varieties of *P. longifolia* and both are very commonly cultivated in Nigeria; *P. longifolia* Thwaites and *P longifolia* var. pendula. The Thwaites variety is also a tall handsome evergreen tree with straight bole and all other feature and uses just like the pendula except that Thwaites has horizontal branches while pendula is of droppingbranching style, a habit that gives it a very beautiful narrow columnar shape. Almost all parts of the plant are used in many traditional systems of medicine all over the world for the treatment of various ailments in humans and animals. These include treatment of skin diseases, cancer, fever, diabetes, helminthiasis and hypertension (Ichino *et al.*, 2006; Verma *et al.*, 2008). Aside the medicinal uses, *P. longifolia* serve as an ornamental plant and a wind-break.



Figure 2.4 Polyalthia longifolia Variety Pendula

(Source: A picture of the plant on F.U.T MINNA BOSSO campus)

Reports indicate the presence of phytochemicals such as aporphine, azafluorene, and protoberberine alkaloids, clerodane diterpenes, ent-halimanes, altholactone, proanthocyanidin, quercetin and its glycosides and steroids *in Polyalthia longifolia*. Other constituents present include saponins, flavanoids, and terpenoids in addition to various amino acids such as proline, glutamic acid and methionine. All parts of the plant have been reported to exhibit various bioactivities including antibacterial, antifungal, antitumour, antiulcer, antioxidant, antiparasitic, antifeedant, antipyretic, hypoglycaemic, antidyslipidemic, and antihypertensive properties (Nair & Chanda, 2006; Chang *et al.*, 2006; Faizi *et al.*, 2008; Katkar *et al.*, 2010; Bhatta *et al.*, 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 The Plant Species Used in the Study

The plant selected and investigated for antiplasmodial activity was *Polyalthia longifolia* var. pendula. This plant was selected after a thorough search through ethno botanical survey reports by several researchers including Addae-Kyereme *et al.* (2001), Asase and Oppong-Mensah (2009), Batista *et al.* (2009), Asase *et al.* (2010), and personal communications with Traditional healers and herbalists in Nigeria.

3.2 Collection and Identification of Experimental Plant Species

Leaves of matured *Polyalthia longifolia* plant were collected from Bosso Local Government area of Niger state. The plant material was identified by a Botanist of the Plant Biology Department of Federal University of Technology, Minna.

3.3 Experimental Animals

Swiss male albino mice weighing 28-35 g were obtained from the animal facility of Federal University of Technology, Minna. The mice were housed in plastic cages and divided into seven groups (3 mice per group) in a controlled environment. They were allowed to acclimatize for 2 weeks. During this period, the mice were fed on standard mice-chew diets *ad libitum* (Vianal *et al.*, 2003).

3.4 Preparation of Sample

Leaves of matured *P. longifolia* plant were air dried under room temperature of 26-28 ^oc and made into powder using mortar and pestle (Mabeku *et al.*, 2011). The extract was obtained from the powder (25 g) by cold maceration in 70 % methanol for 7

2 hours at room temperature (Abdullahi et al., 2015). The extract was filtered and the

excess methanol was evaporated under a reduced pressure in a water bath. The dried extract was put in a clean sterile container for further use.

3.5 Extraction of Crude Extract

One hundred grams of the powdered sample was poured into 500 mL flask and 500 mL of methanol poured into the flask and extracted using reflux extractor for 2 hours. The extract was filtered and another 500 mL of methanol was added to the residue and allowed to extract for another 2 hours and then filtered. The extract was concentrated by placing in water bath. A total of 300 g of the plant sample was extracted following same procedure.

3.6 Partitioning of Crude Extract

Extraction of the plant material was carried out according to the method of Amita and Shalini (2014). Briefly, one hundred grams (100 g) of powdered plant extract was extracted with 400 cm³ of methanol for 6 hours. The resulting mixtures were filtered and concentrated with a rotary evaporator at 50 °c to obtain a residue labeled methanol extract. The Marc was removed, air-dried repacked and partitioned with N-hexane (400 cm³) in increasing order of polarity. The entire resulting fraction were transferred into sterile universal containers and stored at 4 °c until required for use.

3.7 Acute Toxicity Study

The safety of the extract were evaluated by determining its median LD_{50} using the Locke's (1983) method with slight modifications. The experiments were in one phase, mice were randomly divided into five groups consisting of three mice each. The five groups were administered orally with crude plant extracts grated concentrations of 10,

100, 400, 800 and 1600 mg/kg body weight respectively. The animals were all kept under the same condition and observed for toxicity signs and mortality for 24 h. Median LD_{50} values were calculated as geometric mean of the dose that resulted in 100 % lethality and that which caused no lethality at all.

3.8 Quantitative Determination of Phytochemical Components.

3.8.1 Total phenol determination

Ziegler *et al.* (2008) method was used to determine total phenol content of aqueous extract of *Datura metel* seed. 0.01 g of the crude extract was dissolved in 10 mL of distilled water, and 0.5 mL was oxidized by 2.5 mL of 10 % Folin-Ciocalteu's reagent which was then neutralized by 2 mL of 7.5 % sodium carbonate. The reaction mixture was incubated at 45 ^oC for 40 minutes. Absorbance was read at 765 nm using double beam Shimadzu UV spectrophotometer, UV-1800. Standard garlic acid was used to prepare the calibration curve.

3.8.2 Total flavonoids determination

Total flavonoid content of the aqueous extract of *Polyalthia longifolia* leaf was determined using the method of (Chang *et al.*, 2006). 0.5 mL of the extract was added to a test tube containing 1.5 mL of absolute methanol, 0.1 mL of 10 % aluminum chloride, 0.1 mL of 1M sodium acetate and 2.8 mL of distilled water and incubated at ampient temperature for 30 minutes. The absorbance was read at 415 nm with double beam shimadzu UV-spectrophotometer, UV-1800. Standard quercetin was used to prepare the calibration curve.

3.8.3 Determination of tannins

Tannin content of the crude extract was determined using the method of (Thaithong, 1983; Bankole *et al.*, 2016). 0.2 g of the crude extract was weighed into a 50 mL beaker and 20 mL of 50 % methanol was added to it and covered with Para film and heated in water bath at 80 ^oC for 1hour. The reaction mixture was shaken thoroughly to ensure uniformity. The extract was then filtered into a 100 mL volumetric flask, and 20 mL of distilled water, 2.5 mL of Folin-Denis' reagent, and 10 mL of sodium carbonate were added and mixed properly. The reaction mixture was then allowed to stand for 20 minutes at room temperature for the development of bluish-green coloration. The absorbance was taken at 760 nm using double beam shimadzu UV-spectrophotometer, UV-1800. Standard tannic acid was used to prepare the calibration curve.

3.8.4 Determination of saponins

Saponins content of the crude extract was determined using the method of (Oloyed, 2005; Ogungbamigbe *et al.*, 2005). 0.5 g of the crude extract was weighed and dissolved in 20 mL of 1NHCl and boiled in water bath at 80 ^oC for 4 hours. The reaction mixture was cooled and filtered. 50 mL of petroleum ether was added and the ether layer was collected and evaporated to dryness. Thereafter, 5 ml of acetone-ethanol (1:1), 6 mL of ferrous sulphate and 2 mL of concentrated sulphuric acid were added and allowed to stand for 10 minutes. The absorbance was taken at 490 nm. Standard saponins were used to prepare the calibration curve.

3.8.5 Total alkaloid determination

Total alkaloid of the crude extract was determined using method of Oloyed, (2005). In this method, 0.5 g of the crude extract was weighed and dissolved in 5 mL of mixture of 96 % ethanol:20 % H₂SO₄ (1:1) and then filtered. 1 mL of the filtrate was then added to a test tube containing 5 mL of 60 % H₂SO₄ and allowed to stand for 5 minutes. Thereafter, 5 mL of 0.5 % formaldehyde was added and allowed to stand at room temperature for 3 hours. The absorbance was read at wavelength of 565 nm. Vincristine extinction coefficient (E₂₉₆, ethanol {ETOH} = 15136M⁻¹cm⁻¹) was used as reference alkaloid.

3.9 Preliminary Test for the Crude Extract

Preliminary test of the crude extract was performed using nine mice. Animals were grouped into three groups and inoculated with 0.2 mL of blood infected by *P. berghei* intraperitoneally. In such a way, group I-III treated with extract at doses of 150, 300 and 600 mg/kg. Based on the test conducted, the curative effect of the crude extract on parasitaemia was confirmed using 4-day curative test.

3.10 Grouping and Dosing of Animals

The studies of the fractions were performed using twelve mice for each solvent fraction. Mice were randomly assigned into four extract treated groups and two controls three mice per group for each fraction and inoculated. Group IV, V, VI, VII were treated with solvent fractions of the plant extract at doses of 150, and 300 mg/kg, respectively. The doses were selected based on preliminary test. Group IX was treated with chloroquine 5 mg/kg. Administration was performed via the oral route using gavage. Volume administered was calculated based on individual mouse body weight and 0.45 mL is the maximum volume administered.

3.11 Inoculation of Parasite

To infect the mice, blood sample was collected from a donor mouse with a rising parasitaemia of about 30-37 % (Jigam *et al.*, 2011a; Adedeji *et al.*, 2012). After determination of the percentage parasitaemia and erythrocytes count, the donor mouse was sacrificed by decapitation and blood was collected into a petridish containing 0.5 % trisodium citrate. The collected blood was then diluted with isotonic saline in proportions indicated by both determinations (Okokon *et al.*, 2011). The inoculum consisted of 5×10^7 *P. berghei* parasitized erythrocytes per mL. Each mouse used in the experiment was infected intraperitoneally with 0.2 mL of infected blood containing about 1 x 10^7 *P. berghei* parasitized erythrocytes.

3.12 Determination of Haematological Parameters

The haematological components including haemoglobin (Hb), red blood cell (RBC) mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), white blood cell (WBC), platelet count (PLC) were determined using the automated haematological analyzer SYSMEX KX 21, a product of SYSMEX corporation, Japan employing the method described by Dacie and Lewis

3.13 Determination of Body Weight

The body weight of each mouse in all groups was measured (day 0) before inoculating parasite and on day 3 (i.e. 72 h) after infection and after treatment.

3.14 Packed Cell Volume Measurement

Blood was collected from tail of each mouse in heparinized microhaematocrit capillary tubes. The capillary tubes were filled to $2/3^{rd}$ of their height with blood and sealed at one end with sealing clay. It was then placed in a microhaematocrit centrifuge (Hettich

haematokrit, Germany) with the sealed ends outwards. The blood was centrifuged at 11,000 rpm (relative centrifugal force (RCF) =11498.63xg) for 5 min. The tubes were then taken out of the centrifuge and PCV was determined using the standard Microhaematocrit Reader (Hawksley and Sons, England). It was measured before inoculating the parasite and after treatment in prophylactic tests. PCV is a measure of the proportion of RBCs to plasma in the whole blood and determined using the relation shown below (Dikasso *et al.*, 2006).

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

3.15 Determination of Parasitaemia

Thin blood smears were prepared from tail snip of each mouse 72 h after infection in prophylactic test on microscopic slides. The slides were dried and fixed with absolute methanol. The slides were stained with 10 % Giemsa at pH 7.2 for 10 min and then washed gently using distilled water and air dried at room temperature. Two stained slides were prepared for each mouse and examined under microscope with an oil immersion nosepiece of 100x magnification power. Five different fields on each slide were examined to calculate the average parasitaemia (Jigam *et al.*, 2011b). Percentage parasitaemia was calculated by counting infected RBC and total RBC from Giemsa stained thin blood films of the blood that was collected from the tail snip of each mouse in all groups using the formula shown below (Oyewole *et al.*, 2008).

% Parasitaemia =
$$\frac{\text{Number of parasitized RBC X 100.}}{\text{Total number of RBC.}}$$

The mean % parasitaemia was recorded for each animal and for each group; it was used to determine variations in parasitaemia level with time of infection. The average percentage

suppression of parasitaemia was calculated for each dose level by comparing the parasitaemia in infected controls with those of treated mice with the following formula (Aarthi and Murugan, 2011).

3.16 Determination of Mean Survival Time

Mortality was monitored daily and the number of days from the time of inoculation of the parasite up to death was recorded for each mouse in the treatment and control groups throughout the follow up period of 14 days (D0-D13) for all the models. The mean survival time (MST) for each group was then calculated using the following formula (Mengiste *et al.*, 2012).

3.17 Data Analysis

All the results are expressed as mean \pm SEM for each group. All the grouped data were statistically evaluated and the significance of various treatments was calculated using oneway ANOVA followed by Turkey's HSD post hoc test. A p-value of < 0.05 was considered significant. All data processing was done using Microsoft excel 2010 and Statistical package for social sciences (SPSS) data analysis software version 16.

CHAPTER FOUR

4.0 **RESULTS AND DISCUSSION**

4.1 RESULTS

4.1.1 Qualitative phytochemical constituent of the crude methanol extract of *P*. *longifolia*

The result of the qualitative phytochemical constituent of *P. longifolia* is contained in Table 4.1. The result showed that the crude methanol extract of *P. longifolia* revealed the presence of metabolites of medicinal importance. These include Phenol, Flavonoids, Saponins, Alkaloids, and Tannins.

Table 4.1 Qualitative Phytochemical Constituent of Crude Methanol Extract of P. Image: Image of the second sec

Phytochemica	Inference
Phenol	+
Flavonoid	+
Tannin	+
Saponin	+
Alkaloid	+

Keys; + = Present

- = Absent

4. 1.2 Quantitative phytochemical constituents of the crude and methanol extract of *P. longifolia*

The result of the qualitative phytochemical constituent of *P. longifolia* is contained in (Table 4.2). The result showed that Saponins was significantly highest (500.78 ± 2.37 mg/100g) compared to other phytochemicals detected. Alkaloid was found to be the least (30.99 ± 3.05 mg/100g).

 Table 4.2 Quantitative Phytochemical Constituent of the Crude and Methanol

 Extract of P. longifolia

Phytochemical	Concentration (mg/100g)	
Phenol	322.85±1.02	
Flavonoid	240.04±0.41	
Tannins	97.03±2.10	
Saponins	500.78±2.37	
Alkaloid	30.99±3.05	

Values followed by different superscript alphabets on the same row are significantly different at p>0.05

Values are presented in mean \pm standard error of two determinations.

4.1.3 Acute oral toxicity of the crude and methanol extract of P. longifolia

The result of the acute oral toxicity of the crude and methanol extract of *P. longifolia* as detailed in Table 4.3. Throughout the toxicity bio-assay, no gross physical or behavioral change such as lacrimation, sleep, reduction in their motor and feeding activities, depression, hair erection or abnormal secretions were recorded for the treatments. All the mice survived up to the end of the experimental period. The LD₅₀ of each of the extract can thus be extrapolated to be more than 1600 mg/kg body weight.

 Table 4.3 Acute Oral Toxicity of the Crude Methanol Extract of Polyalthia

 longifolia.

Extract	Dose mg/kg b.wt	Mortality
Crude	10	0/3
	100	0/3
	400	0/3
	800	0/3
	1600	0/3

Locke's (1983) with slight modification.

No visible sign of the lethality for toxicity like distress were discovered after the administration of the extract. This implies that the median and maximum lethal dose of the crude extract is beyond the maximum test dose 1600 mg/kg body weight of the mice.

4.1.4 Parasitaemia of *P. berghei*-infected mice treated with crude and solvent fraction of *P. longifolia*

The mean parasitaemia level of *P. berghei* in infected mice treated with the crude methanolic extract and solvent fraction of *P. longifolia* is contained in Table 4.5. On the general note, where a parasite reduction was recorded, in both crude and fraction, it was a dose-dependent reduction. For the crude extract, the mean parasite count of the mice treated with 150 and 300 mg/kg b.wt decreased throughout the experimental period. However, the mice group treated with 600 mg/kg b.wt of the crude extract showed a daily parasitaemia reduction till day 7 of the experiment. The parasitaemia count $(0.00\pm0.00\%)$

of mice treated with 600 mg/kg b.wt, on day 7 and was significantly (p<0.05) lower than the negative control group (19.67 \pm 1.45 %), but not significantly different from the parasitaemia count of the positive control group (0.00 \pm 0.00 %).

 Table 4.4 Parasitaemia of P. berghei-Infected Mice Treated with Crude and Solvent

 Fraction of P. longifolia

Extract	Dose (Mg/kg b.wt	Day 3	Day 4	Day 5	Day 6	Day 7
Crude	150	13.33±3.84 ^b	$7.33{\pm}2.03^{a}$	4.33±.03 ^a	5.00 ± 1.00^{b}	2.00±0.58 ^c
	300	9.33±0.88 ^a	5.67±0.67 ^a	3.67±1.86 ^a	3.67±0.67 ^a	1.67±0.67 ^b
	600	8.00±2.00 ^a	6.00±0.58 ^a	2.33±0.33 ^a	2.00±1.15 ^a	0.00±0.00 ^a
Ethylacetate	150	12.33±7.88 ^b	7.33±2.40 ^a	2.67±0.88ª	1.00±0.08ª	0.33±0.33 ^b
	300	14.67±1.86 ^b	6.00±2.00 ^a	1.67±1.20 ^a	2.00±1.15 ^a	0.33±0.33 ^b
N-Hexane	150	15.33±5.24 ^c	9.67±3.48ª	3.33±1.20 ^a	2.00±1.15 ^a	$1.00{\pm}1.00^{b}$
	300	19.00±3.21°	7.67±2.60 ^a	3.33±1.67 ^a	2.33±0.33 ^a	0.00±0.00 ^a
Untreated	-Ve	12.00±0.58 ^b	13.67±0.67 ^b	15.67±1.45°	17.67±0.88°	19.67±1.45°
Standard	+Ve	19.67±2.19	12.33±1.20 ^b	9.00±2.00 ^b	2.00±1.53 ^a	0.00±0.00 ^a

Note: Value are expressed as mean \pm Standard error of three replicates

Values followed with the same superscript alphabet on the same column are not significantly different at p>0.05, n=3unit=%.

4.1.5 Weight of *P. berghei* infected mice treated with crude and solvent fraction of *P. longifolia*.

The effect of crude and solvent fraction of *P. longifolia* on body weight in *P. berghei* infected mice is detailed in Table 4.5. Although there were no significant differences (p0>0.05) in the mean weight change of the treatments and controlled group on day zero (before infection), the mean weight change varied significantly among the crude extract treated and control group on day 5 (72 hours after infection). For instance, among the crude extract treated group, the weight reduced slightly from day zero to day 5, where most of the extract treated group and normal control was recorded an increase in weight, compared to their weight on day 5. Generally, there was a progressive decrease in weight of the negative control (untreated group) throughout the experimental period.

Extract	Dose (Mg/Kg b.wt)	Before	72hr After	After
		Infection	Infection	Treatment
Crude	150	34.06±1.65 ^b	27.35±2.93 ^a	22.37±1.03 ^a
	300	33.00±1.50 ^b	28.23±4.35ª	27.07±3.67 ^b
	600	28.79±4.59ª	25.25 ± 1.87^{a}	21.73±3.56 ^a
Ethylacetate	150	32.49±3.16 ^b	26.64±1.10 ^a	21.17±2.77 ^a
	300	26.05±0.61ª	31.89±3.70 ^b	26.67±3.43 ^a
N-Hexane	150	26.62±2.60 ^a	34.47±2.18 ^b	21.33±2.95ª
	300	26.35±2.25ª	30.17±3.41 ^b	28.87±3.98 ^b
Untreated	-VE	33.59±1.22 ^b	34.46±2.18 ^b	32.39±0.88°
Standard	+VE	$34.24{\pm}1.04^{b}$	36.63±0.32 ^b	34.13±0.70°

Table 4.5 Body Weight of P. berghei-Infected Mice Treated with Crude and Solvent Fraction of P. longifolia

Note: Value are expressed as mean \pm Standard error of three replicates

Values followed with the same superscript alphabet on the same column are not significantly different at p>0.05, n=3.

4.1.6 Packed cell volume of *P. berghei* infected mice treated with crude and solvent fraction of *P. longifolia*.

The mean PVC of *Plasmodium berghei* infected mice treated with crude and solvent extract of *P. longifolia* was represented in Table 4.6. The PCV recorded for all the extract treated groups and the controls were not significantly higher than one another. Three days after the establishment of the parasite have been confirmed, the PCV of the mice recorded varied significantly. Thereafter, the PCV dropped except for the group treated with standard drugs. At 3 days post parasite infection, the highest PCV was recorded for the standard group (39.13 ± 1.58), although this was not significantly different from (P>0.05). The lowest PCV was recorded for untreated group after treatment from (42.67 ± 2.03 %) to (29.67 ± 1.76 %).

Extract	Dose (mg/kg b.wt))	Before	72hrs After Infection	After Treatment
		Infection		
Crude	150	38.0±2.31ª	31.00±1.53 ^b	28.20±0.12ª
	300	35.67±6.17 ^a	34.33±3.93 ^b	35.67±1.20 ^a
	600	36.00±5.86 ^a	34.83±1.74 ^b	26.53±1.53ª
Ethylacetate	150	38.00±3.06 ^a	32.33±2.96 ^b	27.33±5.90 ^a
	300	35.67±0.67 ^a	25.67±2.19 ^a	33.00±3.79°
N-Hexane	150	39.00±4.93 ^a	27.17±0.93 ^a	35.47±1.27°
	300	39.00±3.61 ^a	24.33±2.73 ^a	34.47 ± 2.60^{b}
Untreated	-VE	42.67±2.03 ^b	34.00±4.36 ^b	29.67±1.76 ^c
Standard	+VE	40.67±1.45 ^b	39.13±1.58°	32.0.3±1.55 ^c

Table 4.6 Packed Cell Volume of P. berghei-Infected Mice Treated with Crude and Solvent Fraction of P. longifolia

Note: Value are expressed as mean \pm Standard error of three replicates

Values followed with the same superscript alphabet on the same column are not significantly different at p>0.05, n=3.

4.1.7 Haematology of *P.berghei*-infected mice treated with crude and fraction of *Polyalthia longifolia*.

The result of the effect of methanol extracts and solvent fractions of *Polyalthia longifolia* on haematological parameter of *P.berghei* mice is obtained in Table 4.7. At the end of the experiment the lowest (9.00 ± 1.02) HB was recorded for the group treated with 150 mg/kg b.wt of the crude fraction of *Polyalthia longifolia*, it was not significantly different from the mean HB recorded for the mice treated with 300 mg/kg b.wt crude fraction. Similarly, the MCV of the negative control was significantly (P>0.05) lowered than the MCV of the crude and fractionated extract of the treated group. The mean RBC, WBC, and PLC recorded for the crude and fractional extract, mostly at the high doses was significantly P>0.05 higher than those for the infected untreated (negative control) group.

	HB (g/dl)	PCV (%)	MCV(Fi)	MCH (pg)	MCHC (g/dl)	RBC (%)	PLC (%)	TWBC (%)
Crude 150	9.00±1.02 ^b	22.00±0.03°	51.00±0.07 ^e	21.00±0.01 °	37.00±0.13 ^d	5.00±0.31 ^a	249.00±0.23 ^f	4.20±0.45 ^a
300	9.30±0.12 ^b	28.00±0.23 ^d	54.00 ± 0.12^{f}	24.00±0.10 ^c	37.00±0.01 ^e	5.00±0.06 ^a	249±0.67 ^g	4.50±0.34 ^a
600	13.20±0.51 ^b	37.00±0.01 ^d	47.00±0.21 ^e	22.00±0.10 ^c	39.00±0.13 ^d	5.70±0.11 ^a	162.00 ± 0.34^{f}	5.50±0.11 ^a
Ethyl acetate 150	14.20±0.30 ^b	43.00±0.76 ^c	40.00±0.91°	18.00±0.21 ^b	41.00±0.54 ^c	7.30±0.03 ^a	194.00±0.01 ^d	6.60±0.64 ^a
300	12.30±0.23 ^b	44.00±0.01 ^d	49.00±0.34 ^e	19.00±0.03 ^c	42.00±0.21 ^d	7.40±0.03 ^a	164.00 ± 0.31^{f}	6.00±0.30 ^a
N-Hexane 150	10.90±0.01 ^b	32.00±0.05 ^e	50.00±0.22 ^g	20.00±0.06 ^c	40.00 ± 0.07^{f}	5.50±0.12 ^a	170.00 ± 0.05^{h}	24.00±0.03 ^d
300	10.20±0.45 ^b	34.00±0.12 ^d	45.00 ± 0.23^{f}	18.00±0.12 ^c	40.00±0.03 ^e	6.40±0.01 ^a	143.00±0.09 ^g	12.70±0.34 ^b
Untreated	9.00±0.12 ^b	34.00±1.02 ^e	38.00 ± 0.12^{f}	13.00±0.46 ^c	31.00±0.21 ^d	2.60±0.22 ^a	260.00±0.17 ^g	2.00±0.12 ^a
Standard	12.00±0.45°	37.00±0.01 ^e	50.00±0.13 ^g	20.00 ± 0.12^d	39.00 ± 0.03^{f}	10.50±0.18 ^b	300.00 ± 0.21^{h}	4.50±0.01 ^a

Table 4.7	Haematology of	f P. berghei-Ir	fected Mice	Treated with	Crude and	Fraction of	of Polyalthia	longifolia.
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Note: Effect of crude and fraction of *Polyalthia longifolia* on haematological parameters of *Plasmodium berghei* infected mice Value are expressed as mean \pm Standard error of three replicates.

Values followed with the same superscript alphabet on the same column are not significantly different at Keys: HB = Haemoglobin Count; PCV = Parked Cell Volume; MCV = Mean Corpuscular Volume; MCH = Mean Corpuscular Haemoglobin; MCHC = Mean Corpuscular Haemoglobin Concentration; RBC = Red blood cell count; WBC = White blood cell count; PLC = Platelet Count

4.1.8 Mean survival time of *P. berghei* infected mice treated with crude and fraction of *Polyalthia longifolia*.

The mean survival time of *Plasmodium berghei* infected mice treated with crude and fraction *of Polyalthia longifolia* is contained in table 4.8. The highest survival time (days) was recorded for the group treated with the standard drug (29.14±0.14); although this is not significantly different from the mean survival time recorded for the group treated 300 mg/kg b.wt crude and fraction(ethylacetate and n-hexane). The mean survival time was significantly lowest in the lowest in the negative controlled group; it was observed that their mean survival time is not up to 14 day of the experiment.







Figure 4.2 Mean Survival Time of *P. berghei* Infected Mice Treated with Fraction of *Polyalthia longifolia*.

4.2 DISCUSSION

Malaria is one of the world's most deadly infectious diseases. Spread and emergence of resistance to the front-line antimalarial drugs including artemisinin is the major challenge that jeopardize all recent gains in malaria control and has major implications for public health (Omotayo, 2003; Dondorp *et al.*, 2010; Ashley *et al.*, 2014). The scientific community is now underway to combat this problem by searching for new, affordable and effective antimalarial agents from medicinal plants and other sources (Omotayo, 2003; Gamo, 2014). The present study was aimed to determine the *in vivo* antiplasmodial activity of crude extract and fraction of *P. longifolia* in *P. berghei* infected mice using 4-day suppressive tests.

The 4-day curative test is a standard test commonly used for antimalarial screening (Akele, 2013) and the determination of percent inhibition of parasitaemia is the most reliable parameter. A mean group parasitaemia level of less than or equal to 90% that of mock-treated control animals usually indicates that the test compound is active in standard screening studies (Peter and Anatoli, 1998). Therefore, it is clear from the result indicated in (table 2) that in *P. berghei* infected mice treated with the extracts of *P. longifolia*, the percentage of parasitaemia measured changed significantly from those in the control animals.

Plasmodium berghei (ANKA strain) was used in the prediction of treatment outcome (Bantie *et al.*, 2014) and hence it was an appropriate parasite for the study. Even though the rodent malaria model, *P. berghei*, is not exactly similar to that of the human *Plasmodium* parasites, it is the first step to screen most of the *in vivo* antimalarial activities of test compounds (Fidock *et al.*, 2004). Moreover, several conventional antimalarial agents, such as chloroquine, halofantrine, mefloquine and more recently

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

Acute toxicity test has been identified generally as the first preliminary test in any toxic study; they provide data on the relative toxicity that is likely to rise from a single or brief exposure to any substance. The assay is used to estimate LD_{50} of natural products, different extract from natural products, have been reported to possess different level of toxicity which majorly depend on the level of toxico-metabolite inherent in them (Sofowora, 1993). In the present study the *in vivo* profile assay of the crude extract revealed that Flavonoids exert antiplasmodial activity by chelating with the nucleic acid base pairing of the parasite (Okokon *et al.*, 2016), there by producing antiplasmodial effect, other mode of action include modulation of the host immunity to tackle disease and inhibition of plasmodia enoyl-ACP reductase (FABI enzyme), a key regulator of type ii fatty synthase (FAS II) in *P. falciparum* (Mustofa *et al.*, 2000; Idih *et al.*, 2017), flavonoid may also bind parasite serine threonine kinase, with high affinity and affect its development (Ferreira *et al.*, 2010; Mills-Robertson *et al.*, 2012). Some other metabolite may exact their antiplasmodial efficacy either by causing elevation of red blood cell oxidation by inhibiting protein synthesis (Miller *et al.*, 1994; Al-Adhroey *et al.*, 2011).

The observation that no death with an oral dose of 1600mg/kg body weight of the extracts could indicate that the methanolic extract of *P. longifolia* may imply that this plant could safely be used to treat malaria. This was consistent with the results of the previous study on toxicity of *Gardenia lutea* by Akele (2013) and Murithi *et al.* (2014).

In vivo antiplasmodial activity can be classified as moderate, good and very good if an extract displayed a percent parasite suppression equal to or greater than 50% at a dose of 600, 300 and 150mg/kg body weight per day, respectively (Muñoz *et al.*, 2000; Deharo *et al.*, 2001; Aty *et al.*, 2014). Based on this classification, crude extracts of *P. longifolia*,

exhibited a good antiplasmodial activity, with a dose dependent inhibition against *P*. *berghei* infection in mice

Among the fractions, the Ethylacetate and N-hexane fractions were found to possess higher percentage suppression than crude fraction. This was evident from the chemo suppression obtained during the four-day curative test, suggesting the possible localization of the active ingredients in these two fractions (Mabeku *et al.*, 2011; Abdullahi *et al.*, 2015).

All crude extracts and fractions prolonged the mean survival time of the study mice indicating that the plant suppressed *P. berghei* and reduced the overall pathologic effect of the parasite on the study mice (Munoz *et al.*, 2000; Idih *et al.*, 2017).

Anaemia, body weight loss and body temperature reduction are the general features of malaria infected mice (Asase *et al.*, 2005; Idih *et al.*, 2017). So, ideal antimalarial agents obtained from plants are expected to prevent body weight loss in infected mice due to the rise in parasitaemia. The crude extracts (methanol) of *Polyalthia longifolia* leaves significantly prevented weight loss at higher two doses in a dose dependent manner. Whereas Ethylacetate and N-hexane fraction of *Polyalthia longifolia* leaves significantly prevented weight loss at all dose in a dose independent manner. This suggests the possibility of the localization of appetite suppressing components even at lower dose in this fraction and nutrients and other immunomodulatory substances in the N-hexane fraction). This result agrees with that of the previous studies on hydro alcoholic extract of *Asparagus africanus* (Dikasso *et al.*, 2006; Bantie *et al.*, 2014).

A decrease in the metabolic rate of infected mice occurred before death and was accompanied by a corresponding decrease in packed cell volume (Dondorp, 2005; Mengiste *et al.*, 2012). Active compounds could prevent the rapid dropping of packed cell volume. All doses of methanol and the highest dose of crude extract did have protective

effect against reduction, which reflects constituents responsible for this effect were likely found in these extracts. Unlike the crude extracts, all fractions failed to prevent parasite packed cell volume reduction compared to negative control unlike the standard drug. This could be attributed to the effect of the extract as it may have hypothermic effect on the treated mice (Bantie *et al.*, 2014).

After malaria infection, the host (human or mouse) suffer from anaemia (Chinchilla *et al.*, 1998; Otto *et al.*, 2014). The underlying cause of anaemia includes; loss of infected erythrocytes through parasite maturation, destruction of uninfected red cells in the spleen and liver by macrophages activation and/or enhanced phagocytosis, reduced erythropoiesis and dyserythropoiesis (Lamikanra *et al.*, 2007). This necessitates haematocrit (packed cell volume) analysis that evaluates the effectiveness of the extract in preventing haemolysis.

The effect of crude extracts and fractions of *P. longifolia* leaves extract on the packed cell volume (PCV) was also evaluated. Both the crude extracts and fractions significantly prevented PCV reduction when compared to negative control in a dose dependent manner.

CHAPTER FIVE

5.0 CONLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

The present study indicates that *in vivo* antiplasmodial effect of crude extract and solvent fractions of *Polyalthia longifolia* have significant antimalarial activity. This plant extracts also exhibited safety profile at the maximum dose of 1600 mg/kg. The antimalarial effect of solvent fractions of methanol extract demonstrated less as compared to the crude extracts, although the N-hexane fraction had protected body weight loss at all dose level and displayed greater parasite suppression among the fractions. Therefore, the crude extracts and solvent fractions could represent a new source for the development of new plant based antimalarial agent. Moreover, the data would provide evidence to uphold the claims made by the African traditional medicine practitioners.

5.2 RECOMMENDATIONS

From the present study, the following works are suggested for further investigation on the plant.

- i. Mechanism of antimalarial action of *P. longifolia* should be further purified and standardized to be used as drug for anti-malaria.
- ii. .Further quantitative phytochemical investigation to clearly identify and quantify the antiplasmodial bioactive components from the plant should be investigated.
- iii. Endemic regions should consider the using of extracts of *Polyalthia longifolia* as alternative remedies for the management of malaria.

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APPENDICES

Appendix A



Plate i Crude Methanol, Ethylacetate and N-hexane fractions of the plant extract

Appendix B



Plate ii The Mice in cages