CHAPTER ONE
INTRODUCTION

1.1 Background of the Study

Malaria is a mosquito-borne infectious disease caused by different strains of the protozoan (one-celled microorganism) parasites of the genus *Plasmodium*, transmitted to humans and other animals through the bites of infected female *Anopheles* mosquitoes (WHO, 2015a). Despite increasing research and control efforts, malaria has remained a serious health challenge in Africa and in most of the developing countries of the world (Curtis, 1994). According to the latest estimates released in November, 2017 by World Health Organization, there were about 216 million (range 149–303 million) new cases of malaria, with an estimated 445000 deaths worldwide in 2016 (WHO, 2017). Federal Ministry of health reported that malaria was responsible for nearly 110 million clinical cases and estimated 300,000 deaths per year. It accounts for about 60% of all outpatient attendance, 30% of all hospital admissions, 25% of death in children under one year and 11% of maternal mortality (FMOH, 2005a; FMOH, 2015). It is one of the leading causes of avoidable death in children and pregnant women (Okorosobo *et al*., 2011; WHO, 2015a). One of the major strategies to control malaria is prompt management with effective anti-malaria drugs (WHO, 2015b). The earliest drugs used were the aminoquinolines, among which chloroquine was the mainstay

of nearly 60 years (Obianime and Apirioku, 2011). However, due to the emergence of resistance to chloroquine and other antimalarial drugs, newer antimalarial drugs have been developed including artemisinins. Artemisinin is considered as a perfect replacement for chloroquine because it is a potent and rapidly acting blood schizonticide, eliciting shorter parasite clearance time and rapid symptomatic response than chloroquine and other antimalarial drugs. (Qinghaosu Antimalaria Coordinating Research Group, 1979). Despite its efficacy, artemisinin has pharmacokinetic limitations. Naturally, artemisinin is not soluble in water or oil; it has poor bioavailability, and a short elimination half-life in vivo (~2.5 h), high recrudescence rate of infection (Ashton *et al.*, 1998; Li *et al*., 2007). To overcome some of these problems, semisynthetic derivatives compounds of artemisinin have been developed, to improve the drug's pharmacological properties and antimalarial potency (Klayman, 1985), they include- artesunate, arteether, artemether, artemisone and dihydroartemisinin. These derivatives of artemisinin are more frequently used malaria chemotherapy, because of their effectiveness against *Plasmodium* parasite. The continued use of oral artemisinin-based monotherapies is considered to be a major contributing factor to the development of resistance to artemisinin derivatives. Therefore, the use of the drugs as monotherapy is explicitly discouraged by the World Health Organization (WHO, 2001). This has necessitated the use of combination therapy of artemisinin

with other antimalarial agents known as the artemisinin – based combination therapies. (WHO, 2006; Olliaro and Taylor, 2004). Artemisinin-based combination therapy is the simultaneous use of an artemisinin with other antimalarial agents where both have independent modes of action and thus unrelated biochemical targets on the parasites, artemisinin kills majority of the parasites at the start of the treatment, while the slow-acting partner drug clears the remaining parasites (WHO, 2006). In 2001, the World Health Organization recommended the first-line use of artemisinin-based combination therapy (ACT). The five recommended ACTS are artesunate plus Sulfadoxine plus pyrimethamine (sp), artesunate plus amodiaquine, artemether plus lumefantrine, artesunate plus mefloquine and dihydroartemisinin plus piperaquine (WHO, 2001). In 2005, artemisinin-based combination therapies (ACTs) were adopted as the first-line treatment for uncomplicated malaria in Nigeria, (FMOH, 2005b). This policy change was in line with global trends (WHO, 2001) and was hinged on demonstrated advantages of ACTs over chloroquine and sulfadoxine-pyrimethamine, ACTs are the mainstay of recommended antimalarial treatments today (Olliaro and Wells, 2009). However, due to high intensity of malaria transmission in Africa, these drugs are currently used frequently for presumptive treatment of fever, even in the absence of laboratory confirmed malaria diagnosis. This may increase the risk of incorrect dosing, emergence of resistance, and occurrence of clinical toxicities. Like other

therapeutic agents, ACTs may not be devoid of side effects or toxicities in both human and animal studies. In view of these therefore, the present study is to evaluate the effects of therapeutic doses of Artemisinin – based combination therapies (Artesunate Plus amodiaquine and Dihydroartemisinin plus piperaquine) on Haematological parameters and on histopathology of the liver, kidney, spleen and lungs using *Plasmodium berghei* parasitized mice as a model. Experimental animal model has been paramount towards the understanding of human diseases. (Druilhe *et al*., 2002). They represent the first crucial step in the research of the underlying disease pathogenesis and development of new drug compounds and vaccines (Druilhe *et a*l., 2002), and had paved the way into a modern biomedical research without which we would know very little. The choice of using mice model in this study is base on the fact that mice have similar drug metabolism with man because human and mice are similar physiologically and anatomically (Conn and Parker, 2008). The similarity means that nearly 90% of the Veterinary Medicines that are used to treat animals are the same as or very similar to those developed to treat human patients. Human share approximately 99% of their DNA with mice (Conn and Parker, 2008). The choice of using *Plasmodium berghei* in this study is because the parasite is recognized as valuable model organisms for investigation of human malaria. *P. berghei* have proved to be analogous to

P.falciparum that infect man in most essentials aspects of structure, physiology and life cycle (Frank-Fayand, 2010).

1.2 Statement of the Problem

Malaria has remained one of the leading causes of morbidity and mortality in most developing countries, especially in sub- Sahara region where the disease is endemic. It has remained a serious health challenge in Africa. Despite increasing efforts to reduce malaria infection and transmission, there has been little change in the areas at risk of the disease (WHO, 2015a). In 2016, the World Health Organization recorded 216 million cases of malaria with an estimated 445,000 deaths (WHO, 2017). Since 2000, progress in reducing malaria burden in Africa has lagged behind that of other countries (WHO, 2015a). Individuals are infected repeatedly, this therefore, expose them to repeated treatment with antimalaria drugs. Frequent intake of antimalaria drugs may increase the risk of multidrug resistance and the occurrence of clinical toxicities. The current plans for a wide-scale implementation of artemisinin-based combination therapies across Africa and potentials of the drugs to cause toxicities offer an opportunity to evaluate the effect of these drugs when used in a larger population or community. In view of these therefore, the purpose of this study is to evaluate the effect of artemisinin- based combination therapies on haematological parameters and on the histopathology of the liver, kidney, spleen, and lungs, using an animal model.

1.3 Justification of the Study

As the quest to eradicate malaria continues, there remains a need to gain further understanding on the effect of the disease and its treatment. According to the World Health Organization, an estimated 216 million new cases of malaria and 445,000 deaths were recorded in 2016. To reduce this threat, there is a need to better understand the underlying processes that result in severe disease outcome and mortality. Despite the rapid increase in malaria transmission, there is a little investigation on impact of malaria infection. Information obtained from this study is important to understand the negative impact of malaria and to justify additional efforts, and resources directed towards control and prevention activities. Furthermore, due to high intensity of malaria transmission in Africa, antimalaria drugs are currently used frequently for presumptive treatment of fever, even in the absence of laboratory confirmed malaria diagnosis. The rate of uncontrolled use of drugs has been a challenge in developing countries, especially in Africa. The incidence of drug misuse and drug abuse is on the rise as an increasing number of individuals use drugs arbitrarily without doctor's prescription or for a purpose other than what they are meant for. This may increase the risk of incorrect dosing, emergence of resistance, and occurrence of clinical toxicities. Despite wide-scale implementation of ACTs across Africa, there is a little independent evaluation on the effects of these drugs besides those documented by the manufacturers in its

application for approval. It is however, pertinent for an independent investigation on effects of artemisinin – based combination therapies. Like other therapeutic agents, ACTs may not be devoid of side effects or toxicities in both human and animal studies. Information obtained from this study will also be necessary in malaria endemic countries such as Nigeria where self treatment with anti malaria drugs is pervasive. This is to ensure that the common and rare adverse drug events to ACTs are documented, so as to improve the use of these drugs in the management of malaria infections, to ensure that patients are getting the desired therapeutic benefits rather than negative consequences on account of such adverse drug effects.

1.4 Aim of the Study

The aim of this research is to evaluate the effects of Artemisinin-based combination therapies (ACTs) on the haematological parameters and histopathology of the liver, kidney, spleen and lungs of *Plasmodium berghei* infected mice.

1.5 Objectives of the Study

The objectives of the study were to:

i. determine the effects of therapeutic doses of artesunate/amodiaquine and dihydroartemisinin/piperaquine on the haematological parameters of the *Plasmodium berghei* infected mice,

ii. determine the effects of therapeutic doses of artsunate /amodiaquine and dihydroartemisinin/piperaquine on the liver, kidney, spleen and lungs of the *Plasmodium berghei* infected mice,

iii. compare the effects of artesunate/amodiaquine with ihydroartemisinin /piperaquine in *Plasmodium berghei* infected mice.

iv. ascertain whether the damages (if any) done by malaria infection and its treatment with ACTs are reversible or irreversible.

v. determine the effects of artesunate/amodiaquine and dihydroartemisinin +piperaquine on the body weight of *Plasmodium berghei* infected mice.

CHAPTER TWO

LITERATURE REVIEW

2.1Malaria

The name malaria was derived from two Italian words “mal” and “aria”, which means “bad air”. For many centuries it was believed that certain disease like malaria was caused by poisonous vapour or mist filled with particles from decomposed matter. Prior to the introduction of microscope, everything floating above ground that was invisible to human eye, including dust and bacteria, was called “air”- so in a sense, perhaps this etiological speculations was not far from the truth (Hamelmann and Krafts, 2013).

Malaria is a serious and often fatal disease caused by malaria parasite of the genus *Plasmodium* (WHO, 2015b).

2.2 Causes of Malaria

Malaria is an infectious disease caused by protozoa parasite of the genus *Plasmodium.* The parasites spread through the bites of infected female *Anopheles* mosquitoes called “malaria vectors”. The most dominant species of *Anopheles* mosquito in Nigeria are *Anopheles funestus, Anopheles gambiae complex, Anopheles arabiensis* and *Anopheles melas* (WHO, 2015a; Ayodele *et al*., 2007). There are five species of *Plasmodium* causing human malaria, they are *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium*

malariae and *Plasmodium knowlesi*. However, only *P. falciparum*, *P.ovale,* and *P.malariae* have been reported in the surveys on malaria in Nigeria (FMOH, 2015). *Plasmodium falciparum and Plasmodium vivax* are the most common, but a large majority of the clinical cases and mortalities are caused by *Plasmodium falciparum* (Bozdech *et al.*, 2003; FMOH, 2008; WHO, 2015a). *Plasmodium falciparum* is the commonest specie causing about 97% of uncomplicated malaria and also the specie most responsible for the severe form of the disease that leads to death in Nigeria. *Plasmodium vivax* does not occur in indigenous Nigerians (FMOH, 2015).

2.3 *Plasmodium berghei* Life Cycle

The life cycle of *Plasmodium berghei* follows three stages:

i. **Infection stage**
ii. **Asexual reproduction stage**
iii. **Sexual reproduction stage**

Infection stage: The *Plasmodium berghei* life cycle, illustrated in Fig 2, starts when the haploid sporozoite stage (**1**) is injected into the mammalian host during feeding by an infected *anopheles* mosquito. That is the first stage of *Plasmodium* life cycle (Laurence *et al*., 2002)

Asexual stage: The next stage is the asexual reproduction stage. It is divided into two phases: the pre- erythrocytic or exoerythrocytic) and the erythrocytic phase (Laurence *et al*., 2002).

Pre-erythrocytic or exoerythrocytic phase: Few minutes to several hours after inoculation, the sporozoites circulate in the blood until they reach the liver. Some then make their way into the hepatocytes, to differentiate into exo-erythrocytic or liver stages (**2**), the exo-erythrocytic stages occur in the parenchymal cells of the liver and mature 48 hours after introduction of sporozoites (Smith, 1994), Within the hepatocyte, the sporozoite develop within 47- 52 hours via the trophozoite stage into the mature schizont (3). At maturation the schizont ruptures (4) to release 1500-8000 merozoites into the blood stream. It is now imperative that the merozoites rapidly invade red blood cells if further development is to occur (5). Once this has been achieved the merozoite transforms into the erythrocytic ring form (**6**), which grows to form the trophozoite (7), marking the end of the exoerythrocytic phase of the asexual reproductive stage. At some point during the growth phase, the trophozoite undergoes a selective differentiation that determines whether future development will follow an asexual path or sexual.

The exoerythrocytic phase is not pathogenic and does not produce symptoms or signs of the disease. Its duration is not the same for all parasite species (Ashley *et al*., 2008). *P. vivax and P. ovale*, sporozoites may not follow the reproduction step

and stay dormant (hypnozoites) in the liver; they may be activated after a long time leading to relapses entering the blood stream (as merozoites) after weeks, months or even years. (Ashley *et al*., 2008).

Erythrocytic phase: The haploid merozoites released from the liver schizonts (8), invades red blood cells. As they invade into the cells, they mark the beginning of the erythrocytic phase. The first stage of invasion is a ring stage that evolves into a trophozoite. The trophozoites consume the haemoglobin of the red blood cells, thereby producing crystals of the brown haemozoin that can be observed as the characteristic pigment granules in the cytoplasm. The trophozoites of *P. berghei* have a predilation for reticulocytes up in to 100 percent of which may be infected, whereas only 10 percent of erythrocytes may be infected (Smith, 1994). Towards the end of trophozoite stage, the parasite duplicates its DNA. Replication of DNA is followed by nucler division, leading to binuclear parasite. With this first nuclear division, the parasite enters the schizont stage. Immature and mature *P.berghei* schizonts disappear from the peripheral circulation and sequester in the capillaries of the inner organs, such as the sleen, lungs and adipose tissue. Each mature schizont gives birth to new generation merozoites (erythrocytic schizogony) that, after RBCs rupture, are released into the blood stream in order to invade other RBCs. This is when increase in the parasitemia occurs and clinical manifestations of the disease appear. The liver phase occurs only once while the erythrocytic

phase undergoes multiple cycles (Alan *et al*., 2006). The erythrocytic cycle takes 22-24 hours (Smyth, 1994) in *P. berghei.*

Sexual reproduction stage: In each asexual cycle a small proportion of parasites stop asexual multiplication and differentiate into sexual cells called gamatocytes. The gametocytes, then, mature and become macrogametes (female) (9) and microgametes (male) (10) during a process known as gametogenesis. The microgametes and macrogametes are transferred to the mosquitoes when the mosquito ingests male and female pre-sex cells and blood from an infected intermediate host (Karapelou, 1987). The time needed for the gametocytes to mature differs for each *Plasmodium* species: 3- 4 days for *P. vivax* and *P. ovale*, 6-8 days for *P. malariae* and 8- 10 days for *P. falciparum* (Karapelou, 1987). In *P. berghei* the period is short and takes only 26-30 hours (Smyth, 1994). In the mosquito gut, the microgamete nucleus divides three times producing eight nuclei; each nucleus fertilizes a macrogamete forming a zygote. By contrast microgamete formation is an extremely dramatic event, with gamete formation and escape from the host cell, an event known as exflagellation (12), occurring within approximately 10 min of activation. Zygote formation is the third and final stage of sexual development. It occurs when the microgamete and macrogamete meet and fuse to form the fertilized zygote. The zygote then undergoes a series of changes in shape to form first the retort form ookinete and then the mature banana-shaped

motile ookinete (13). This penetrates the midgut wall to lie under the basal lamina where it rounds up to form an oocyst (14). This matures, undergoing a series of mitotic divisions to form thousands of sporozoites, which are released into the mosquito haemolymph when the oocyst bursts (15). Inside the oocyst, the ookinete nucleus divides to produce thousands of sporozoites (sporogony) (Garnham, 1966). The sporozoites migrate from here to the salivary glands (16), where they wait until they are inoculated into a new host during the next feed, thus completing the cycle (Vickerman and Cox 1972).

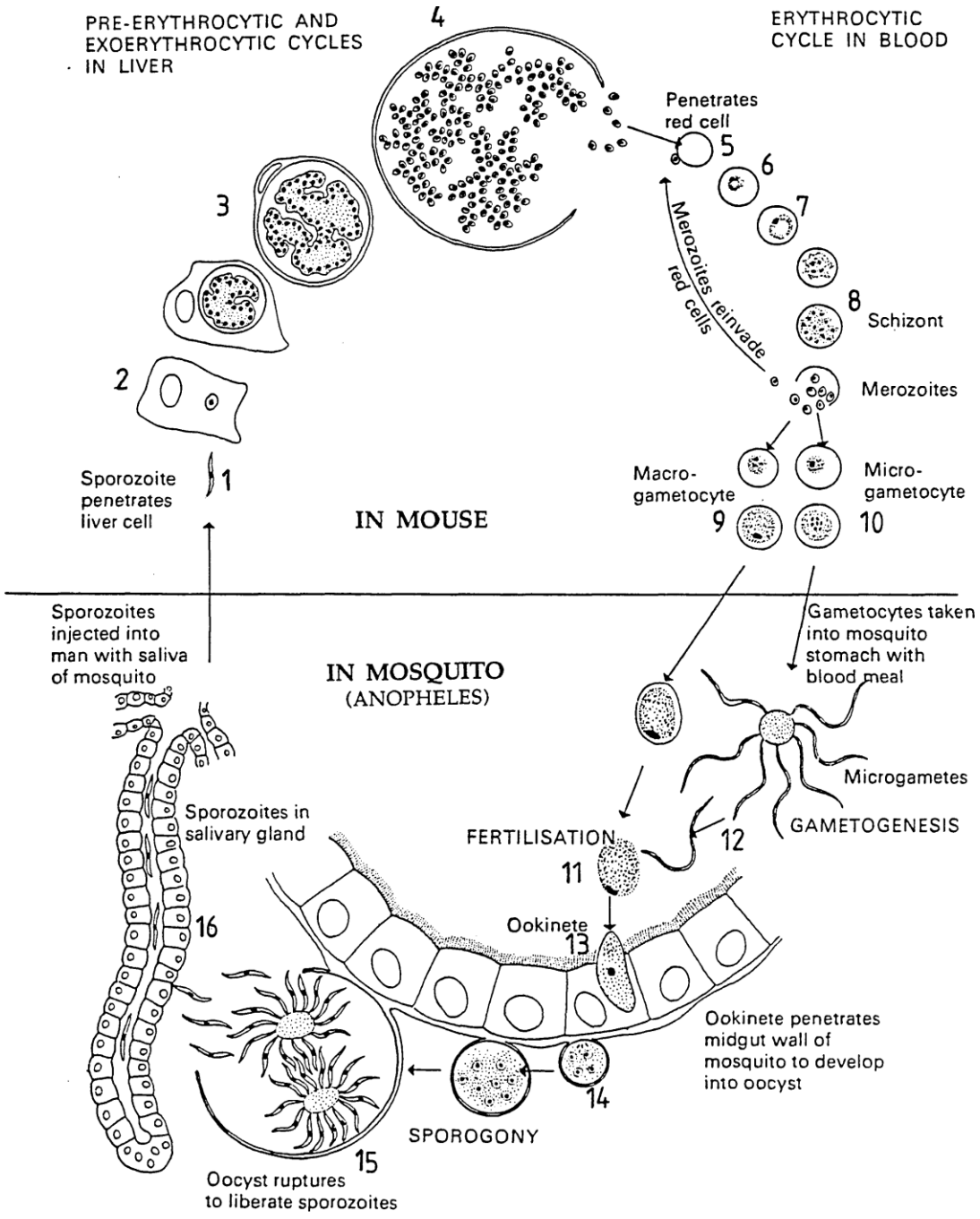

Figure 1. Life cycle of *Plasmodium berghei*

Source: Vickerman and Cox (1972).

2.4 Symptoms of Malaria

In a non-immune individual, symptoms appear seven days or more (usually 10–15 days) after the infective mosquito bite. The first symptoms are nonspecific and similar to systemic viral illness (WHO, 2015b). They are headache, lassitude, fatigue, abdominal discomfort, muscle and joint aches, usually followed by fever, chills, perspiration, anorexia, vomiting and worsening malaise. In young children, malaria may also present with lethargy, poor feeding, and cough. At this early stage of the disease progression, with no evidence of vital organ dysfunction, a rapid, full recovery is expected, provided that prompt, effective antimalarial treatment is given. If ineffective or poor quality medicines are given or if treatment is delayed, particularly in *P. Falciparum,* the parasite often continues to increase and the patient may develop potentially lethal severe malaria (WHO, 2015b).

2.5 Global Malaria Burden

According to the latest estimates released in November, 2017 by World Health Organization, there were about 216 million (range 149–303 million) new cases of malaria, with an estimated 445000 deaths worldwide in 2016 (WHO, 2017). The African Region accounted for most global cases of malaria (88%), followed by the South-East Asia Region (10%) and the Eastern Mediterranean Region (2%). (WHO, 2015a).

In 2015, there were 211 million cases of malaria, with an estimated 438 000 malaria deaths (range 236 000–635 000) worldwide. Most of these deaths occurred in the African Region (90%), followed by the South-East Asia Region (7%) and the Eastern Mediterranean Region (2%) (WHO, 2015a). Children under five are particularly susceptible to malaria illness, infection, and death. In 2015, malaria killed an estimated 306 000 under-fives children globally, including 292 000 children in the African Region (WHO, 2015a). In 2015, the global burden of malaria remained heavily concentrated in 15 countries, mainly in Africa. Since 2000, progress in reducing malaria incidence in these high-burden countries (32%) has lagged behind that of other countries globally (53%) (WHO, 2015a).

2.6 Malaria Burden in Africa

The African region carries a disproportionally high share of global malaria burden, with an estimated 90% of malaria cases and 91% of malaria death in 2016 (WHO, 2017). Nearly half of the people in the world are at risk for malaria infection, but pregnant women and children in Africa are particularly vulnerable. In sub-Saharan Africa, malaria is the leading cause of death for children under five, who account for 78% of all deaths. Infection during pregnancy, particularly among new mothers, increases the risk of maternal mortality, neonatal mortality, and low birth weight (WHO, 2014). In addition to the loss of life, malaria places an economic burden on African nations. It is estimated that malaria costs Africa US$12 billion

per year in direct costs and reduces economic growth by 1.3 percent annually (WHO, 2014). The burden is carried mostly by poor, rural families that have less access to current prevention and treatment services.

2.7 Malaria Burden in Nigeria

Nigeria is known for high prevalence of malaria (Onwujekwe *et al*., 2000; WHO, 2016), and suffers the world's greatest burden, the disease is a leading cause of morbidity and mortality in the country (FMOH, 2005a). Federal Ministry of health reported that malaria was responsible for nearly 110 million clinical cases and estimated 300,000 deaths per year. It accounts for about 60% of all outpatient attendance, 30% of all hospital admissions, 25% of death in children under one year and 11% of maternal mortality (FMOH, 2005a; FMOH, 2015). Monetary loss due to malaria in Nigeria is estimated to be about 132 billion naira in terms of treatment cost and prevention (FMOH, 2005a). Malaria is deemed as not only a public health problem but also a deterrent to the socio-economic growth of the country (Gallup and Sachs 2001; Sachs and Malaney, 2002). Therefore, it imposes a great burden on the country in terms of pains and trauma suffered by its victims as well as loss in outputs and cost of treatments.

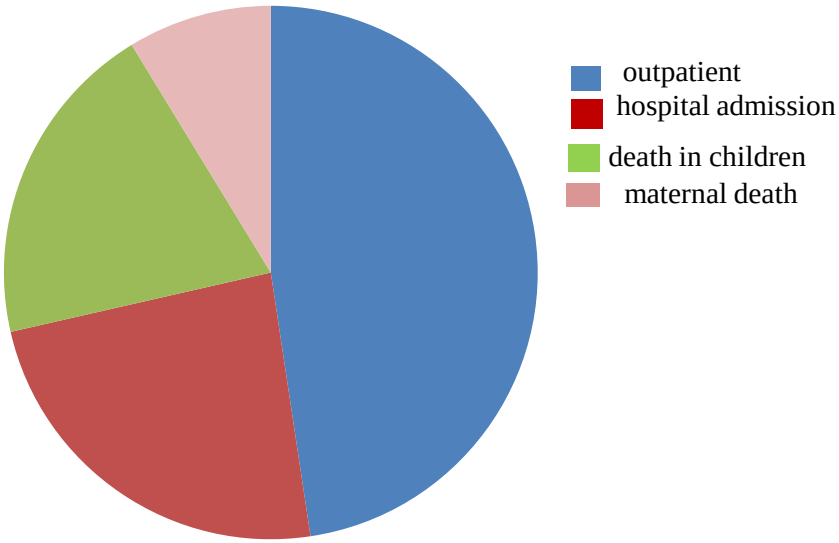

Figure 2: Malaria prevalence in Nigeria.
Source: FMOH, 2015.
(Accessed January, 2017).

2.8 Malaria Transmission

Malaria is transmitted exclusively through the bite of an infected female Anopheles mosquito to man, although infections also occur through transfusion of infected blood (transfusion malaria) and by congenital transmission from mother to her unborn child (congenital malaria) (WHO, 2015a). The mosquito bite introduces the parasite from the mosquito's saliva into a person's blood (WHO, 2014).

About 90% of all malaria deaths in the world today occur in Africa south of the Sahara. This is because the majority of infections in Africa are caused by *Plasmodium falciparum,* the most dangerous of the five human malaria parasites. It is also because the most effective malaria vector – the mosquito *Anopheles gambiae* is the most widespread in Africa and the most difficult to control (WHO, 2002). Malaria affects the lives of almost all people living in the area of Africa. Most people at risk of the disease live in areas of relatively stable malaria transmission; infection is common and occurs with sufficient frequency that some level of immunity develops (WHO, 2002). The intensity of transmission depends on the factors related to the parasite, the vector, the human host, and the environment (WHO, 2015a). About 20 different species of *Anopheles* are locally important around the world (WHO, 2015a). All of the important vector species breed in water and each has its own breeding preferences. They lay their eggs in water, which hatch into larvae, eventually emerging as adult mosquitoes. The

female mosquitoes seek a blood meal to nurture their eggs. Each species of *Anopheles* mosquito has its own preferred aquatic habitat; for example, some prefer small, shallow collections of fresh water, which are abundant during the rainy season in tropical countries (WHO, 2015a). Transmission is more intense in the places where mosquito life span is longer (so that the parasite have time to complete its development inside the mosquito) and where it prefers to bite human rather than other animals. For example, a long life span of and strong human-biting of the African vector species is the main reason why 90% of the world's malaria death are in African (WHO, 2015a). Transmission also depends on climatic conditions that may affect the number and survival of mosquitoes, such as rainfall pattern, temperature, and humidity. In many places, transmission is seasonal, with the peak during and just after the rainy season. Malaria epidemics can occur when climate and other conditions suddenly favour transmission in areas where people have little or no immunity to malaria. They can also occur when people with low immunity move into areas with intense malaria transmission, for instance, to find work, or as refugees. Human immunity is another important factor, especially among adults in areas of moderate or intense transmission conditions. Partial immunity is developed over years of exposure, and while it never provides complete protection, it does reduce the risk that malaria infection will cause severe disease. For this reason, most malaria deaths in Africa occur in young children,

whereas in areas with less transmission and low immunity, all age groups are at risk **(**WHO, 2015a).

2.9 Malaria Prophylaxis

Antimalarial drugs can be used to prevent malaria. For travellers, malaria can be prevented through chemoprophylaxis, which suppresses the blood stage of malaria infections, thereby preventing malaria disease. There are a number of drugs that can help prevent or interrupt malaria in travelers to places where the infection is common. Many of these drugs are also used in treatment. Chloroquine may be used where chloroquine-resistant parasites are not common *(*Jacquerioz and Croft, 2009). In places where *Plasmodium* is resistant to one or more medications, three medications-mefloquine (Lariam), doxycycline (available generically), or the combination of atovaquone and proguanil hydrochloride (Malarone)-are frequently used when prophylaxis is needed. (Jacquerioz and Croft, 2009). Doxycycline and the atovaquone plus proguanil combination are the best tolerated; mefloquine is associated with death, suicide, and neurological and psychiatric symptoms. (Jacquerioz and Croft, 2009). The protective effect does not begin immediately, people visiting areas where malaria exists usually start taking the drugs one to two weeks before arriving and continue taking them for four weeks after leaving (except for atovaquone/proguanil, which only needs to be started two days before and continued for seven days afterward). (Freedman, 2008*).* The use of

preventative drugs is often not practical for those who live in areas where malaria exists, and their use is usually only in pregnant women and short-term visitors. This is due to the cost of the drugs and side effects from long-term use (Fernando *et al.*, 2011). For pregnant women living in moderate-to-high transmission areas, WHO recommends intermittent preventive treatment with sulfadoxine-pyrimethamine, at each scheduled antenatal visit after the first trimester. Similarly, for infants living in high-transmission areas of Africa, 3 doses of intermittent preventive treatment with sulfadoxine-pyrimethamine are recommended, delivered alongside routine vaccinations (WHO, 2006).

2.10 Classification of Antimalaria Drugs

Anti malarial drugs can be classified according to anti malarial activity or biological activity and according to chemical structure (Seth, 2015; Barar, 2000)

2.10.1 According to antimalarial activity:

i. Tissue schizonticides for causal prophylaxis: These drugs act on the primary tissue forms of the plasmodia which after growth within the liver, initiate the erythrocytic stage. Pyrimethamine and Primaquine have this activity

ii. Tissue schizonticides for preventing relapse: These drugs act on the hypnozoites of *P. vivax* and *P. ovale* in the liver that causes a relapse of symptoms on reactivation. Primaquine is the prototype drug; pyrimethamine also has such activity.

iii. Blood schizonticides: These drugs act on the blood forms of the parasite and thereby terminate clinical attacks of malaria. These are the most important drugs in anti-malarial chemotherapy. These include chloroquine, quinine, mefloquine, halofantrine, pyrimethamine, sulfadoxine, sulfones, tetracyclines etc.

iv. Gametocytocides: These drugs destroy the sexual forms of the parasite in the blood and thereby prevent transmission of the infection to the mosquito. Chloroquine and quinine have gametocytocidal activity against *P. vivax* and *P. malariae*, but not against *P. falciparum*. Primaquine has gametocytocidal activity against all plasmodia, including *P. falciparum*.

iv. Sporontocides: These drugs prevent the development of oocysts in the mosquito and thus ablate the transmission. Primaquine and chloroguanide have this action. Thus in effect, treatment of malaria would include a blood schizonticide, a gametocytocide and a tissue schizonticide (in the case of *P. vivax* and *P. ovale*). A combination of chloroquine and primaquine is thus needed in all cases of malaria (Seth, 2015)

2.10.2 According to chemical structure:

i. Aryl amino alcohols: Quinine, quinidine (cinchona alkaloids), mefloquine, halofantrine.

ii. 3 4-aminoquinolines: Chloroquine, amodiaquine.

iii. **8-aminoquinolines:** Primaquine.

v. **Antimicrobials:**Tetracycline, doxycycline, clindamycin, azithromycin, fluoroquinolones

vi. **Peroxides:** Artemisinin (Qinghaosu) derivatives and analogues- artemether, arteether, artesunate, artelinic acid

vii. **Naphthoquinones:** Atovaquone

viii. **Iron chelating agents:** Desferrioxamine (Barar, 2000).

2.11 Antimalaria Drug Resistance

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 a tolerance of the subject (WHO, 2001). 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 (WHO, 2001). To date, parasite resistance to antimalarials has been documented in three of the five malaria species known to affect humans: *P. falciparum*, *P. vivax*, and *P. malariae*. Parasite resistance results in a delayed or incomplete clearance of parasites from the patient's blood when the person is being treated with an antimalarial (WHO, 2011). The problem of antimalarial drug resistance is compounded by cross-resistance, in which resistance to one drug

confers resistance to other drugs that belong to the same chemical family or which have similar modes of action (WHO, 2011). The resistance of *P. falciparum* to previous generations of medicines, such as chloroquine and sulfadoxine-pyrimethamine (SP), became widespread in the 1970s and 1980s, undermining malaria control efforts and reversing gains in child survival. In an attempt to reduce the incidence of relapse and treatment failure due to large scale resistance to existing drugs, the World Health Organization has advocated the use of Artemisinin-based therapy (ACT) for the treatment of malaria (Olurishe *et al.*, 2007). ACTs are preferred because artemisinin compounds have rapid parasite and fever clearance effects and reduce gametocyte rate with the potential to reduce transmission (Meremikwu *et al.*, 2006).

2.12 Brief Historical Background of Artemisinin

The discovery of artemisinin for malaria therapy by Chinese scientists in the 1970s was one of the greatest discoveries in medicine in the 20th Century **(**Klayman, 1985). Artemisinin originated from northern part of China (Van Agtmael *et al.*, 1999). It is an extraction product of the herb, *Artemisia annua.L*. also known as sweet wormwood (Oreagba, 2010).

Plate 1: *Artemisia annua* .L.
Source: www.//en.wikipedia.org/wiki/Artemisia-annua
(Accessed January, 2018).

The project leading to the discovery of artemisinin was initiated in response to a request from North Vietnamese leaders suffering heavy losses of soldiers due to malaria during the Vietnam War. Chairman Mao and Premier Zhou called for an urgent effort to find solutions. A meeting discussing action plans was held on 23 May 1967 (thus named 'Project 523'), which laid out long- and short-term goals for developing antimalarial therapies. The long-term goal was to discover new effective antimalarial drugs. Because soldiers were dying from malaria, effective antimalarial drugs were also needed in the battlefield immediately. The North Vietnamese, however, lacked a research infrastructure and thus turned to China for help. Under the instructions of Chairman Mao and Premier Zhou, a meeting was held on May 23, 1967, in Beijing to discuss the problem of drug-resistant malaria parasites. This led to a secret nationwide program called project 523, involving over 500 scientists in ~60 different laboratories and institutes (Zhang *et al*., 2006). Although the project's short-term goal was to produce antimalarial drugs that could immediately be used on the battlefield (by 1969 three treatments were established), the project's long-term goal was to search for new antimalarial drugs by screening synthetic chemicals and by searching recipes and practices of traditional Chinese medicine. During their search, Youyou Tu and colleagues investigated more than 2,000 recipes of Chinese traditional herbs, compiling 640 recipes that might have some antimalarial activity. They were tested in a rodent malaria model more than

200 recipes with Chinese traditional herbs and 380 extracts from the herbs. Among the promising results, extracts from *Artemisia annua* L. (Qinghao), a type of wormwood native to Asia, were shown to inhibit parasite growth by 68%. Follow-up studies, however, only achieved 12% to 40% inhibition. Professor Tu reasoned that the low inhibition could be due to a low concentration of the active ingredient in the preparation and began to improve the methods of extraction. After reading the ancient Chinese medical description, "take one bunch of Qinghao, soak in two sheng (~0.4 liters) of water, wring it out to obtain the juice and ingest it in its entirety" in The Handbook of Prescriptions for Emergency Treatments by Ge Hong (283–343 CE) during the Jin Dynasty, she realized that traditional methods of boiling and high-temperature extraction could damage the active ingredient. Indeed, a much better extract was obtained after switching from ethanol to ether extraction at a lower temperature. However, the extract was still toxic. Professor Tu then further removed from the extract an acidic portion that contained no antimalarial activity, leaving a neutral extract with reduced toxicity and improved antimalarial activity. The neutral extract, termed extract number 191, was tested in the mouse malaria, *Plasmodium* berghei, and achieved 100% inhibition in October 1971. She presented her findings at a 523 meeting held in Nanjing on March 8, 1972, providing some critical parameters for other teams to quickly obtain pure artemisinin crystals. Although Tu's team struggled to obtain high-quality crystals

from the plant in the following months, two teams (Zeyuan Luo, Yunnan Institute of Drug Research and the late Zhangxing Wei, Shandong Institute of Chinese Traditional Medicine), using the information and methods she used, soon obtained pure crystals from *A. annua* L. that were highly active against rodent malaria parasites. Tests in humans by Guoqiao Li, Guangzhou University of Chinese Traditional Medicine, using the artemisinin crystals from Yunnan Institute of drug research showed good activity against malaria infection (Miller and Su, 2011). The drug is named Qinghaosu in Chinese. It remained largely unknown to the rest of the world for about seven years until the result of 5,000 traditional Chinese medicines tested by the Chinese scientist for the treatment of malaria were published in the Chinese medical journal in 1979 (Van Agtmael *et al.*, 1999). The news of the discovery thereafter reached scientist outside China. As the worldwide resistance of malaria parasites to existing antimalarial drugs such as chloroquine, quinine and mefloquine quickly emerged in the 1960s, it became urgent to search for more effective antimalaria drugs. In 2006, after artemisinin has become the treatment of choice for malaria, the World Health Organization called for immediate halt to single-drug artemisinin preparation in favour of a combination of artemisinin with another malaria drug, to reduce the risk of a parasite developing resistance (WHO, 2006).

2.13 Mechanism of Action of Artemisinin

Artemisinin is a sesquiterpene lactone endoperoxide, containing a structural feature called peroxide bridge which is believed to be the key to its mode of action **(**Klayman, 1985). Two types of mechanism of action for artemisinin have been proposed, one involving iron (or haem) (Posner 1995; Meshnick *et al.*, 1991)) and the other independent of iron but involving *Plasmodium falciparum* Ca^{2+}-ATPase (PfATP6) (Eckstein-Ludwig *et al*., 2003).

The cleavage of the peroxide bridge in presence of ferrous ion (Fe^{2+}) from haem forms highly reactive free radicals which rapidly rearrange to more stable carbon-centered radicals (Meshnick *et al*. 1996; Posner 1995). It has been suggested that these artemisinin-derived free radicals chemically modify and inhibit a variety of parasite molecules, resulting in parasite's death (Meshnick *et al.*, 1991). A rich source of intracellular Fe^{2+} is haem an essential component of hemoglobin, the malaria parasite is rich in haem iron derived from a breakdown of the host cell hemoglobin. It has long been suspected that Fe^{2+}-haem is responsible for activating artemisinin inside the parasite (Ridley, 2003).

Alternative views propose a direct interaction between artemisinin and *Plasmodium falciparum* Ca^{2+}-ATPase (PfATP6), a calcium pump. PfATP6 is the only sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase gene present in the malaria parasite (Gardner, 2002). It has been suggested as a possible parasite-specific

target. This enzyme is thought to be critical for parasite survival and is damaged by carbon-centered free radicals released during metabolism of artemisinin peroxide. The parasite ingests and degrades up to 80% of the host cell hemoglobin during its growth and replication process, in a compartment called food vacuole. Inside the malaria parasite, artemisinin is activated by free iron which in turn inhibit neighboring PfATP6 in the endoplasmic reticulum by closing the phosphorylation nucleotide binding an actuator domain leading to loss of function of PfATP6 (Ridley, 2003).

Numerous studies have investigated the type of damage oxygen radicals may induce. For example, Pandney *et al* (1999) have observed inhibition of digestive vacuole cysteine proteases activity of malaria parasite by artemisinin. These observations were supported by ex vivo experiment showing accumulation of hemoglobin in the parasite treated with artemisinin and inhibition of hemozoin. Electron microscopic evidence linking artemisinin action to the parasite digestive vacuole has been obtained showing that the digestive vacuole membrane suffers damage soon after the parasite is exposed to artemisinin (Delpilar *et al.*, 2008). This would also be consistent with data showing that the digestive vacuole is already established by the mid-ring stage of the parasite blood cycle (Abubakar *et al.*, 2010) a stage that is sensitive to artemisinin but not other antimalarials.

2.14 Artemisinin and its Derivatives

Artemisinin is a sesquiterpene lactone with a 1, 2, 4-tiroxane ring system. This endoperoxide compound is extracted from the Chinese herb qinghaosu (*Artemisia annua* or annual wormwood) which was used for treating fevers for over two millennia (Qinghaosu Antimalaria Coordinating Research Group, 1979). Artemisinin is a potent and rapidly acting blood schizonticide, eliciting shorter parasite clearance time and rapid symptomatic response than chloroquine and other antimalarial drugs. It acts against the asexual stages and gametocytes and also blocks sporogony, thereby affecting all stages or sites of the parasite life cycle (Obianime and Aprioku, 2011). Despite its efficacy, artemisinin has pharmacokinetic limitations (Qinghaosu Antimalaria Coordinating Research Group, 1979). Naturally, artemisinin is not soluble in water or oil; it has poor bioavailability, and a short elimination half-life in vivo (2.5 h), high recrudescence rate of infection (Ashton *et al.*, 1998; Li *et al.*, 2007). To overcome some of these problems, semisynthetic derivatives compounds of artemisinin have been developed, to improve the drug's pharmacological properties and antimalarial potency (Klayman, 1985), they include- artesunate, arteether, artemether, and artemisone. These Semisynthetic derivatives of artemisinins are obtained by reduction of the carbonyl group of dihydroartemisinin (DHA), the main active metabolite of artemisinin (Haynes *et al.*, 2002; Klayman, 1985). The first

generation of semisynthetic artemisinins includes arteether and artemether, the lipophilic artemisinins, whereas artesunate is the water soluble derivative (Haynes *et al.,* 2002; Klayman, 1985). Artemisone, a second-generation artemisinin, has shown improved pharmacokinetic properties including longer half-life and lower toxicity. These derivatives of artemisinin are more frequently used malaria chemotherapy, because of their effectiveness against *Plasmodium* parasite.

The continued use of oral artemisinin-based monotherapies is considered to be a major contributing factor to the development of resistance to artemisinin derivatives. Therefore, the use of the drugs as monotherapy is explicitly discouraged by the World Health Organization (WHO, 2006). Furthermore, studies have shown that monotherapy with the artemisinin derivatives resulted in some levels of treatment failures (Price *et al.*,1998; Mclintosh and Olliaro, 2000) and that drug combinations markedly delay the emergence of resistance (Chawira *et al.,* 1987). This has necessitated the use of combination therapy of artemisinin with other antimalarial agents known as the artemisinin – based combination therapies as the first line of treatment for uncomplicated *falciparum* malaria in malaria endemic countries (WHO, 2006; Olliaro and Taylor, 2004). Among the drugs used in combination with artemisinin (AS) or its derivatives are lumefantrine (L), mefloquine (MFQ), piperaquine (PQ), sulfadoxine-pyrimethamine (SP) and amodiaquine (AQ), pyronaridine (PYR).

2.15 Artemisinin-based Combination Therapies

Artemisinin-based combination therapy is the simultaneous use of an artemisinin with other antimalarial agents where both have independent modes of action and thus unrelated biochemical targets on the parasites, this has exploited the synergistic and additive potential of the individual drugs, artemisinin kills majority of the parasites at the start of the treatment, while the slow-acting partner drug clears the remaining parasites (WHO, 2006). Artemisinin and its derivatives (Artesunate, Artemether, Arteether, and Dihydro-Artemisinin) produce rapid clearance of parasitemia and rapid resolution of symptoms. Artemisinin compounds are active against all the four species of *Plasmodium* that infect humans (WHO, 2006). A public health advantage of ACTs is that they reduce gametocyte carriage and thus transmissibility of malaria (Snow *et al.,* 2003).

2.16 Pharmacokinetics of artemisinin

Pharmacokinetic properties of artenimol, artesunate, artemether and arteether have been investigated.

2.16.1 Plasma pharmacokinetics

Arteether has been applied by intramuscular injection to Beagle dogs at a dose of 25 mg/kg in an oily solution, and the pharmacokinetic profile in plasma has been determined with electrochemical detection. The rapid absorption with a T_{max} of 45 minutes was followed by a distribution phase with a half-life of 0.84 hours and a

relatively protracted elimination phase with a half-life of 27.95 hours. (Benakis *et al*., 1991). In a large comparative study, the kinetic parameters were determined for artenimol, artesunate, artemether, arteether and artelinate in the plasma of rats. The substances were administered intravenously, intramuscularly and orally at equal doses of 10 mg/kg. At the same time, the conversion of the primary compounds to their common metabolite, artenimol, was also estimated. Large differences in the conversion rates and the calculated "equivalent artenimol dose" were observed between the different primary compounds. For artemether, the conversion rates on the intravenous, intramuscular and oral application, were calculated to be 3.7, 9.0 and 12.4 %, respectively, with "equivalent artenimol doses" of 0.20, 0.26 and 0.61 mg/kg. For arteether the analogous figures were 3.4, 14.2 and 15.9 %, and 0.09, 0.16 and 0.81 mg/kg. Artesunate provided the highest conversion rates and the respective "equivalent doses", with values of 38.2, 25.3 and 72.7 %, and 1.49, 0.87 and 9.67 mg/kg. The differences can be explained by the different chemical and physicochemical properties of the various compounds: Artesunate, is a simple ester of artenimol and undergoes the first-pass metabolism already in the plasma by the action of esterases, whereas the other compounds, on the other hand, have to undergo metabolism by hepatic cytochrome enzymes; additionally, their higher lipophilicity is probably limiting the distribution into the tissues (Li *et al*., 1998).

2.16.2 Absorption

Absorption is fast for all artemisinins. In mice and rats, artemisinin was rapidly absorbed after oral application, with T_{max} of 0.5 - 1 hour (China Cooperative Research Group, 1982a). Furthermore, the amount remaining in the muscle after intramuscular administration of arteether in an oily preparation has been determined after single and multiple doses (Li *et al*., 1999).

In humans, the relative oral bioavailability of artemisinins decreases after multiple doses. A study with an *in situ* intestinal perfusion model in rats was therefore conducted in order to investigate the possibility that this decreased bioavailability might be the result of an increased efflux mediated by P-glycoprotein. In this model, pretreatment of the animals for 5 days with an oral artemisinin dose of 60 mg/kg did not influence the jejunal permeability of artemisinin, and artemisinin was shown not to be a substrate for P-glycoprotein. The observed decrease in relative oral bioavailability of artemisinin in humans should, therefore, be related rather to an induction of metabolism than to any effect on absorption (Svensson *et al*., 1999).

2.16.3 Distribution

In the early Chinese studies, organ distribution of artemisinin has been estimated by radioactivity determination or by thin-layer chromatography. The resulting pattern 1 hour after oral administration of 900 mg/kg artemisinin to rats showed

highest levels in liver, followed by brain, plasma, and lung, with kidneys, muscle, heart and spleen at somewhat lower levels of exposure. The tissue content of radioactivity in mice after intramuscular administration of ^{3}H-artemether decreased considerably within 24 hours; again, the liver was the most exposed organ, but brain levels were very low in this experiment. In pregnant mice treated with ^{3}H-artemisinin, radioactivity could also be detected by autoradiography in the foetuses (China Cooperative Research Group, 1982a). In another early study, tissue distribution of artesunate has been investigated in rats after an intravenous injection of 50 mg/kg. Rats were killed either 10 or 60 minute after drug administration, and tissue concentrations were measured by a radioimmunoassay. Shortly after drug administration, the levels in the intestine were markedly higher than in any other organs; brain levels were second, followed by the concentrations in kidney and liver. One hour after the injection, the levels were markedly reduced and were more similar between organs; artesunate concentrations in brain and fat seemed to be declining at a lower rate than in other organs (Zhao and Song, 1989). A prominent property of artemisinins is their propensity for selective distribution into infected erythrocytes. Erythrocytes infected with *P. falciparum* have been shown to take up artemisinin derivatives to concentrations greater than 100-fold of those which were found in uninfected erythrocytes under the same treatment conditions (Meshnick *et al*., 1996). This selective uptake has been demonstrated to

involve a carrier-mediated mechanism, in contrast to the simple, passive diffusion of artemisinins into non-infected red blood cells (Vyas *et al*., 2002).

2.16.4 Metabolism

The first and most important step in the biotransformation of artemisinin and of all its derivatives consists in the formation of the dihydro-form of artemisinin, artenimol. The further metabolic breakdown has also been investigated and described in some publications. The hepatic metabolism and biliary excretion of the formed metabolites have been investigated in rats for artenimol and for β-artemether. Both compounds were given as intravenous injections at a dose of 35 μm/kg; after application of artenimol, 34.8 % of the radioactivity was recovered from the bile within the first hour after application, and 48.4 % were recovered between 0 and 5 hours postdose. The principal metabolite was found to be the glucuronide of artenimol, and minor metabolites were demonstrated to be products of reductive cleavage and rearrangement of the endoperoxide bridge (Maggs *et al*., 1997). With artemether, 38.6 % of the radiolabel was recovered from bile within the first 3 hours postdose, and 42.3 % were found within the first 5 hours. The major metabolites in bile for the time interval of 0 -3 hours were artenimol (22.6 % of the dose), the glucuronide of 9α-hydroxy-artemether (33.4 %) and glucuronides of various other hydroxylated metabolites. Glucuronidation products were also found to be present in the urine of malaria patients who had received a dose of 120

mg artesunate (Ilett *et al*., 2002).The further metabolism of artemisinin has been investigated with human and rat liver microsomes *in vitro*. In this study, CYP 2A6 and CYP 2B6 were identified as the isoenzymes mainly responsible for the formation of four distinct metabolites. When the rats used for the preparation of the liver microsomes were pretreated with artemisinin, an induction of metabolic activity ranging from 2.5- to 7.2-fold was observed. CYP 3A4 did not seem to play a major role in these biotransformations (Svensson *et al*., 2003). Three of the four metabolites formed by rat liver microsomes were also found in human microsomal incubations; they have not been characterized, but it has to be assumed that the main metabolite should correspond to artenimol. While for the metabolism of artemisinin, artemether and arteether the cytochrome P450 enzymes play the major role in the transformation into the primary metabolite artenimol, common esterases perform the hydrolytic cleavage of the ester bond in artesunate. This difference has most probably to be regarded as the cause for the more rapid transformation of artesunate into the active metabolite, artenimol than can be observed with the other artemisinins.

2.16.5 Excretion

After oral administration of ^{3}H-artemisinin or ^{3}H-artenimol to mice, 80% of the radioactivity was excreted in the urine during 24 hours. After an intravenous injection of ^{3}H-artemether, 68% of the radioactivity was recovered in urine and

faeces during the first 24 hours, while 95% were excreted within 72 hours (China Cooperative Research Group, 1982a). Only very little artesunate was found to be present in urine, faeces and bile (0.3, 0.01, and 0.3 % of the applied dose, respectively). (Zhao and Song, 1989).

2.17 Amodiaquine

Amodiaquine, a 4-aminoquinoline with a mode of action similar to that of chloroquine. Amodiaquine was first introduced as an alternative to chloroquine since it appeared to have activity against chloroquine- resistant *Plasmodium falciparum* parasites. The drug was actively used for malaria prophylaxis but consequently removed from the Essential Drugs List due to rare but serious adverse effects of hepatitis and agranulocytosis associated with its long-term use. The drug is being re-introduced as a component of the artesunate-amodiaquine combination therapy for the treatment of uncomplicated malaria, particularly in Africa (Merck, 1983).

2.17.1 Mechanism of action

In vitro studies suggest that amodiaquine inhibits the digestion of hemoglobin as the antimalarial mode of action (Famin and Ginsburg, 2002). The drug also inhibits the glutathione-dependent destruction of ferriprotoporphyrin IX in the malaria parasite, resulting in the accumulation of this peptide, which is toxic for the parasite (Polina and Aweeka, 2008).

2.18 Pharmacokinetics of Amodiaquine

2.18.1 Absorption

After oral administration, amodiaquine is rapidly absorbed from the gastrointestinal tract. In the liver, it undergoes rapid and extensive metabolism to N-diethyl-amodiaquine (DEAQ) which concentrates in blood cells. Peak plasma concentrations are reached after a mean of 30 minutes (healthy volunteers), and 1.75 hours in malaria patients. It is likely that N-desethyl-amodiaquine, not amodiaquine, is responsible for most of the observed antimalarial activity, and that the toxic effects of amodiaquine after oral administration may in part be due to desethylamodiaquine (Winstanley *et al*., 1987).

2.18.2 Distribution

After oral administration of amodiaquine (600 mg) to 7 healthy adult males, amodiaquine underwent rapid conversion to desethylamodiaquine.The Peak plasma concentrations (Cmax) of these metabolites are reached after a mean of 3.4 hours (healthy volunteers). The mean plasma concentration of desethylAQ is 6-7 times greater than amodiaquine (healthy volunteers). DesethylAQ accumulates in red cells to give a red cell. Plasma ratio of 3:1.Thereafter the concentration of amodiaquine declined rapidly and was detectable for no more than 8h. (Winstanley *et al*., 1987). The distribution half times observed after intravenous injection (3 mg base per kg over 10 minutes) to seven healthy adult male volunteers (geometric mean 1.7; range 0.4 to 55 minutes) were significantly faster than those observed

after intravenous infusion (10 mg base per kg over 4 hours) to 10 adult patients with *falciparum* malaria (geometric mean 22.2; range 5 to 126 minutes). The plasma concentration-time profiles were biphasic. After bolus injection, the apparent volume of the central compartment (1.1; range 0.3 to 3.6 l/kg) was one-quarter of estimated after the infusion (4.6; range 0.5 to 29.3 l/kg). The study suggested that there was probably an additional distribution phase in the malaria patients obscured by the slower rate of infusion. (White *et al*., 1987).

2.18.3 Biological half-life

The mean terminal half-life of AQ is 5.2 hours. DesethylAQ has a longer terminal half-life than AQ with widely reported values ranging from 9 - 31 days. Amodiaquine 600 mg was given by mouth the apparent terminal half-life of amodiaquine was 5.2 + 1.7 (range 0.4 to 5.5) minutes and the geometric mean of the estimated elimination phase half-lives was 2.1 (range 0.5 to 5.7) hours (White *et al*., 1987).

2.18.4 Metabolism

In the liver, amodiaquine undergoes rapid and extensive metabolism to N-desethyl-amodiaquine (DEAQ) which concentrates in blood cells. When amodiaquine is given orally relatively little of the parent compound is present in the blood. Hepatic biotransformation to desmethyl amodiaquine (the principal biologically active metabolite) is the predominant route of amodiaquine clearance

with such a considerable first pass effect that very little orally administered amodiaquine escapes untransformed into the systemic circulation (Winstanley *et al*., 1987).

2.18.5 Excretion

AQ and its metabolites are >90% protein bound and are eliminated by renal excretion. Amodiaquine and desmethyl amodiaquine have been detected in the urine several months after administration (Winstanley *et al*., 1987).

2.19 Piperaquine

Piperaquine a synthetic bisquinoline compound belonging to a 4-amioquinoline group of antimalarials. (Davis *et al*., 2005). Piperaquine has a slow and longer schizontocidal activity against erythrocytic stages of both *P. vivax* and *P. falciparum* and chloroquine-resistant plasmodium strains. (Raynes,1999; Raynes, et al., 1996; Vennerstrom et al., 1992).

2.19.1 Mechanism of action

Most evidences conclusively propose inhibition of parasite heme-digestion pathway, similar to action of chloroquine (O'Neill et al., 1998; Ahmed et al., 2008) Failure to inactivate heme and toxicity of drug-heme complexes is thought to kill the parasites via oxidative damage to membranes, digestive proteases or other critical biomolecules such as nucleic acid, phospholipids etc. A good activity against chloroquine resistant strains may be attributed to bulky bisquinoline

structure itself, which possibly also contributes to inhibition of chloroquine efflux transporters from the parasite food vacuole (Raynes, 1999; Raynes *et al*., 1996)

2.20 Pharmacokinetics of Piperaquine

2.20.1 Absorption

Piperaquine, a highly lipophilic compound, is slowly absorbed. In humans, piperaquine has a T_{max} of approximately 5 hours following a single and repeated dose (Liu *et al*., 2007). The presence of food doubles the systemic exposure of piperaquine compared with when administered alone.

2.20.2 Metabolism

In vitro metabolism studies demonstrated that piperaquine is metabolized by human hepatocytes (approximately 85 % of piperaquine remained after 2 hours incubation at 37°C). Piperaquine was mainly metabolized by CYP3A4 and to a lesser extent by CYP2C9 and CYP2C19. Five human urinary metabolites of piperaquine were identified in two healthy male volunteers after an oral single dose of the dihydroartemisinin-piperaquine combination. The major metabolites are a carboxyl acid cleavage product and an N-oxidated piperaquine product. The carboxylic acid metabolite thus appears to be the major piperaquine metabolite in both the rat and human beings (Tarning *et al*., 2008).

2.20.3 Distribution

Piperaquine is highly bound to human plasma proteins, the protein binding observed in *in vitro* studies was >99 %. Moreover, from *in vitro* and *in vivo* data in animals, piperaquine tend to accumulate in RBC. Pharmacokinetic parameters observed for piperaquine in humans indicate that this active substance has a large volume of distribution (730 l/kg; CV % 37.5 %).

2.20.4 Biological half-life

Piperaquine has long elimination half life relative to the dosing interval with mean terminal half lives ranging from 11-18 days and 17-23 days following a single dose and multiple dose administration respectively (Ahmed *et al*., 2008).

2.20.5 Elimination

Studies in rats suggest that renal and biliary clearance of piperaquine is negligible, and it is therefore assumed that piperaquine is eliminated primarily through hepatic metabolism (Tarning et al., 2008).

2.21 Efficacy of Artemisinin- Based Combination Therapies

Artesunate + amodiaquine combination had been reported to be safe and effective in the treatment of highly drug-resistant *falciparum* malaria in African children (Sinou, *et al*., 2009). Oguche *et al* (2014), evaluated the efficacy of 3-day regimens of artemether-lumefantrine and artesunate-amodiaquine in 747 children < 5 years of age with uncomplicated malaria from six geographical areas of Nigeria

and discovered that both treatments were well tolerated safe and are efficacious treatments for uncomplicated *falciparum* malaria in young Nigerian children. Study on efficacy of non-artemisinin- and artemisinin-based combination therapies for uncomplicated falciparum malaria in Cameroon was carried out by Whegang *et al*, (2010), it was discovered that artesunate-amodiaquine (AS-AQ) is as effective as other drugs (artesunate-sulphadoxine-pyrimethamine [AS-SP], artesunate-chlorproguanil-dapsone[AS-CD],artesunate-mefloquine[AS-MF],dihydroartemisinin-piperaquine [DH-PP], artemether-lumefantrine [AM-LM], amodiaquine, and amodiaquine-sulphadoxine-pyrimethamine [AQ-SP]). AM-LM appeared to be the most effective with no treatment failure due to recrudescence, closely followed by DH-PP. Sowunmi *et al*., (2009), reported that artesunate+mefloquine clears parasitaemia and reduces gametocyte carriage more rapidly and causes lesser fall in hematocrit than mefloquine alone, but both regimes are an effective treatment for uncomplicated *P.falciparum* in Nigerian children. Onasanya and Ademowo (2013), discovered that taking Artemisinin-naphthoquinone (ANQ) for three consecutive days at 7.3mg/kg gave a significant difference in parasitaemia clearance in mice on day 2 compared to ANQ single dose, restored PCV faster than ANQ single dose, confers a higher mean survival rate compared to ANQ taken for a single day. Ewenighi *et al* (2013) also discovered that Artesunate + amodiaquine therapy have higher parasitic clearance

rate compared to the amodiaquine therapy in *P.berghei* infected mice and concluded that Artemisinin-based Combination Therapy (ACT) is more effective than monotherapy in the treatment of malaria. In several studies the fixed combination dihydroartemisinin-piperaquine has resulted in high cure rates (>95%) with excellent tolerability in the treatment of adults and children with *P. falciparum* malaria (Denis *et al*., 2002; Ashley *et al*., 2004; Karunajeewa *et al*., 2004; Tran *et al*., 2004). The fixed dihydroartemisinin-piperaquine combination has been shown to be more effective than the commonly used antimalarial combinations of artesunate-amodiaquine and artemether-lumefantrine in patients with multidrug-resistant *P*.falciparum and *P. vivax* (Hasugian *et al*., 2007; Ratcliff *et al*., 2007). Overall risk of treatment failure at day 42 was 45% (n=375; 95%CI, 36%-53%) for artesunate-amodiaquine and 43% (n=166; 95%CI, 38%-48%) for artemether-lumefantrine compared with 19% (n=379; 95%CI, 14%-23%) and 13% (n=168; 95%CI, 7.2%-19%), respectively for the dihydroartemisinin-piperaquine comparative arm when investigated in two large patient studies in Southern Papua, Indonesia (Hasugian *et al*., 2007; Ratcliff *et al*., 2007). No significant difference in true recrudescence could be detected between dihydroartemisinin-piperaquine and artemether-lumefantrine with a mean value of 4.7% and 4.1%, respectively. Both treatments were well tolerated with mild and transient adverse advents. The combination dihydroartemisinin-piperaquine reduced true recrudescence

significantly (4.8%) compared with artesunate-amodiaquine (16%) and was better tolerated. Almost all patients (97%) were parasite free within 48 hours by dihydroartemisinin-piperaquine treatment demonstrating the rapid parasite clearance of dihydroartemisinin. The increased duration of post-treatment prophylactic effect of the piperaquine combination displays the benefit of piperaquine as a partner drug in ACTs.

2.22 Effects of Artemisinin-Based Combination Therapies (ACTs).

Nosten and White (2007) reported that artemisinin and its derivatives on their own have low toxicological effects and that any toxicity observed in artemisinin combination treatment may be due to the partner drug agents such as amodiaquine, lumefantrine, mefloquine, and piperaquine.

2.22.1 Effects on haematological parameters

The haematological abnormalities that have been reported in malaria treatment include anaemia, thrombocytopenia, lymphocytosis and rarely disseminated intravascular coagulation (Facer, 1994), leukopenia, leukocytosis, neutropenia, neutrophilia, eosinophilia, and monocytosis also have been reported (Murphy and Oldfield, 1996). A variety of hematological alterations like progressively increasing anemia, thrombocytopenia, leukocytosis or leukopenia has been reported in cases of malaria (Koltas *et al*., 2007). Francis *et al* (2014) reported that the haematological parameters of malaria infected patients and the controls, the

total WBC, the absolute lymphocytes, monocytes and ESR were significantly higher in malaria infected patients than in the controls. However, the PCV, absolute neutrophils, eosinophil, and platelets count were lower significantly in the malaria patients than in the controls. There was no significant difference in the basophil count between the patients and the controls. The Packed Cell Volume and platelets count were significantly higher in the male patients than in the females. Total white blood cell and erythrocytes Sedimentation Rate were higher significantly in the females than in the males. Sowunmi *et al* (2009) discovered significant fall in haematocrit after treatment with artesunate and amodiaquine in children. Artemether caused significant reduction of the hematological profile of rats in a dose-dependent manner. Discontinuation of the drug use, however, showed gradual recovery of the depressed indices of the blood parameters (Osonuga *et al*., 2012). Intravenous application of artesunate for three consecutive days to rats in doses of up to 240 mg/kg induced a dose-dependent decrease in reticulocyte numbers, as well as decreases in erythrocytes, haematocrit and haemoglobin; the decrease in reticulocyte numbers was generally the most sensitive measure of artesunate effects (Xie *et al*., 2005). Intramuscular administration of 6 mg kg^{-1} artemether over a 3 months period induced a decreased red blood cell (RBC) count (anemia) in dogs (Yin *et al*., 2014). A study by Kareem *et al* (2014) indicates that administration of a therapeutic dose of palaxin

(dihydroartemisinin) to the healthy individual will not significantly alter the Packed Cell Volume (PCV), Red Blood Cell Count (RBC) and White Blood Cell (WBC). Their results also suggest that variation in these parameters is not sex dependent. Whereas haemoglobin level increased significantly in male rats treated with the drug, the level of haemoglobin was not altered in the female rats. Both were reduced to normal in the process of recovery from the effect of the drug. Utoh-Nedosa *et al* (2009) discovered that dihydroartemisinin significantly elevated the Packed Cell Volume (PCV), the total White Cell Count (WBC), the percentage neutrophil count (NC) and the percentage Lymphocyte Count (LC) in rats. Aprioku and Obianime (2011a) reported that artesunate-, dihydroartemisinin-, and artemether significantly and dose-dependently increased white blood cell count and lymphocyte count while the neutrophil count was decreased by artemether at double therapeutic dose in rats. Agomo *et al* (2008) investigated the efficacy, safety, and tolerability of artesunate/mefloquine in the treatment of uncomplicated *Falciparum* malaria and reported that the mean of all the parameters was within the normal limits, though white blood cell counts showed a slight increase. The increase was more prominent in the lymphocyte count with a decrease in neutrophils as treatment progressed. There was a slight increase in haemoglobin on day 7 before returning to normal on day 28. Haematology findings of Omotosho *et al* (2014) reveal that there was intravascular haemolysis, leucocytosis with a

significant decrease in neutrophils in the group of rats treated with artesunate at 4mg/kg body weight on the first day followed by 2mg/kg body weight for six consecutive days. Bigoniyaa *et al* (2015) discovered that artesunate significantly increases total WBC, neutrophil, eosinophil, packed cell volume and mean cell hemoglobin at 8 mg/kg/day dose in rats while haemoglobin, total red blood cell, platelet, lymphocytes, basophil, mean cell volume and mean corpuscular hemoglobin concentration have not changed.

2.22.2 Effects on biochemical parameters

Studies with animals had shown that artemisinin and its analogs have adverse effects on biochemical parameters in rabbits (Ngokere *et al.,* 2004). Olayinka and Ore (2013) reported that activities of plasma ALP, ALT, AST, and GGT were significantly higher in the animals treated with dihydroartemisinin. Plasma Total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides in the two treated groups were significantly increased. These were accompanied by a significant increase in the MDA level in the two treated groups. Furthermore, the two doses of P-ALAXIN® (dihydroartemisinin) significantly reduced hepatic ascorbic acid and GSH with a concomitant reduction in activities of hepatic GST. Similarly, there was a significant reduction in the activities of hepatic Catalase, and SOD. Farombi *et al* (2000) reported significant decrease in the activities of aniline hydroxylase, p-nitroanisole, O-demethylase and pentoxyresorufin O-dealkylase, significant

increased in the cholesterol per phospholipid ratio, significant decreased in the activities of microsomal lipids, hepatic glutathione (GSH) level, glutathione S-transferase (GST), significant decrease in malondialdehyde (MDA) formation following administration of rats with therapeutic doses of three structurally related antimalarial drugs, amodiaquine, mefloquine) and halofantrine. Lower level of brain cholesterol (CH), triacylglycerol (TAG) and total proteins (TBP) in the group treated with 8.75±2.86mg/kg of amodiaquine plus artesunate for six days, and a higher level of these bio-molecules in the group treated with 8.75±2.86mg/kg of amodiaquine plus artesunate for three days has been reported (Ekong *et al.*, 2007). Utoh-Nedusa *et al* (2009) also reported that dihydroartemisinin has no effect on serum level of Alanine aminotransferase, serum alkaline phosphatase, and serum aspartate aminoTransferase activities. Obianime and Aprioku (2009) reported increase in basal serum prostate acid phosphatase by amodiaquine and sulfadoxine/primethane, significant increase in serum urea by Artesunate/sulfadoxine/primethane in doses tested, increased in Basal serum creatinine level by sulfadoxine/pyrimethamine and decrease by artesunate/amodiaquine at their subclinical doses, significant decrease in control serum uric acid level by artesunate/amodiaquine after administration of half their clinical doses, and also significant reduction in total blood cholesterol after administration of clinical doses of artesunate and amodiaquine. Aprioku and

Obianime (2011b) also reported a dose-dependent increase in serum prostate acid phosphatase and glutamate oxaloeacetic transaminase in artesunate and dihydroartemisinin exposed animals, dihydroartemisinin also causes a dose-dependent increase in serum glutamate pyruvate transaminase and triglyceride level. Adekunle *et al* (2009) reported a transient decrease in serum concentrations of total protein and albumin in malaria patients treated with artemether™. Oluwatosin *et al* (2008) reported that artemether, artemether-lumefantrine, and halofantrine caused a significant increase in reduced glutathione level in the liver of rats, there is no significant difference in kidney glutathione level; furthermore, the drugs increased the liver and kidney enzymatic antioxidant status in the animals. Aniefiok *et al* (2009) reported that artemisinin only elevated the activities of serum aspartate aminotransferase, Alanine amino transferase, and serum alkaline phosphatase significantly at four dose levels. When 1.50mg/kg^{-1} of folic acid was concurrently administered with artemisinin, the elevated serum level of ASAT, ALAT, and ALP was significantly reversed almost completely. Omotosho *et al* (2014) revealed elevated levels of serum Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) in the group of rats treated with artesunate at 4mg/kg body weight on the first day followed by 2mg/kg body weight for six consecutive days. Jahas *et al* (2014) disclosed that artesunate administered at a dose of 110mg/kg body weight for 14 days caused a significant increase in the

levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and gamma-glutamyltransferase in the serum of Wistar rats. The levels of superoxide dismutase and catalase in liver homogenate were also decreased. Bigoniya *et al* (2015) discovered that artesunate treatment for 45 days significantly increased Serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), serum alkaline phosphate (ALP), cholesterol (TC), triglyceride (TG), total protein, bilirubin, and glucose level at 8 mg/kg/day dose. It has a non-significant effect on serum total protein, albumin, creatinine, and urea. Al-Ani *et al* (2013) indicated that artemisinin (8mg/ Kg/day) causes a significant increase in the activity of GOT; GPT; ACP; ACP; ALP, LDA activities with a corresponding decrease in cholesterol level, total protein and blood glucose in rats.

2.22.3 Neurotoxicity of ACTs

Artemisinins, under specific circumstances, exhibit neurotoxicological effects in nonclinical animal models. In a pharmacokinetic study with repeated intramuscular injection of arteether at a dose of 20 mg/kg/day for 8 days to dogs, a syndrome of progressive clinical neurological defects in conjunction with cardio-respiratory collapse and death was noted, while no such effects were seen to occur at the lower dose of 10 mg/kg. These findings were subsequently investigated further in dogs given intramuscular doses of 5, 10, 15and 20 mg/kg/day for 28 days, and in rats

given arteether or artemether in intramuscular doses of 12.5, 25 and 50 mg/kg/day for 28 days. Lesions were observed in the brains of both rats and dogs, and consisted of scattered neuronal degeneration and necrosis, characterized by swelling and rounding of nerve cell bodies, increased eosinophilia, vacuolization of cytoplasm with loss of Nissl substance (central chromatolysis), swelling and fading of nuclei, and separation and clumping of fibrillar and granular components of nucleoli; similar effects were seen in the rat brains (Brewer *et al*., 1994). These findings have been reproduced and confirmed in a number of other studies in mice, rats, and monkeys, and the histopathological lesions in the brainstem have been observed either in the presence or the absence of clinically manifest neurotoxicity (Genovese *et al*., 1995; Petras *et al.,* 1997; Kamchonwongpaisan *et al*., 1997; Genovese *et al*., 1998a; Genovese *et al*., 1998b; Genovese *et al*., 1999; Genovese *et al*., (2000). Petras *et al*., 2000; Nontprasert *et al*., 2002). It was recognized that this neurotoxicity, although probably a class effect of artemisinins, was dependent not only on the dose applied but also on the mode of application and the physical-chemical properties of the derivative tested. Two studies in mice investigated the aspects of compound differences as well as differences due to the application route. Artemether, in doses of 30, 50, 75 and 100 mg/kg, and artesunate, in doses of 30, 50 and 100 mg/kg, were given daily as intramuscular injections for 28 days. Mortality was higher for artemether in the high dose group compared with

artesunate at the same dose. Neurological observations at the high dose showed a clear distinction between artesunate, which had practically no effect, and artemether, where only about 10% of the animals remained neurologically normal. Also, olfactory responses, estimates as the time needed to find food, were significantly impaired in the artemether but not in the artesunate-treated groups (Nontprasert *et al*., 1998). In an analogous setting with treatment for 28 days, mice received intramuscular or oral doses of artemether and artesunate. Artemether was administered intramuscularly at doses of 150 and 200 mg/kg; artesunate was given either in aqueous solution at doses of 150, 200 and 250 mg/kg, as well as in an oil preparation at 100 mg/kg. Oral doses were also given as different formulations, either as an aqueous solution or in the form of oil-coated food pellets; doses ranged from 100 to 300 mg/kg. Artemether again proved to affect neurological parameters more than artesunate, and the intramuscular injection was more toxic than the oral application (Nontprasert *et al*., 2000). Several studies have shown that high doses of artemisinin can produce neurotoxicity such as selective damage to brainstem centres, gait disturbances in mice and rats (Nontprasert *et al*., 1998; Nontprasert *et al*., 2002), loss of spinal cord and pain response mechanisms in animals (Genovese *et al*., 1995). Others showed some varying degree of cell clustering, cellular hypertrophy, degenerative and necrotic changes, and increase intercellular vacuolations in the stroma of the stomach of rats of artesunate-treated animals

(Eweka and Adjene, 2008). Loss of brainstem, eye reflexes, prominent neuropathic lesion sharply limited to the brain stem have also been reported by Brewer *et al* (1994). Ekong *et al* (2009a) reported that amodiaquine plus artesunate cause the destruction of Purkinje cortical layers, alteration in the cerebellum which may result in cerebellar dysfunction manifesting in some motor problems like dizziness, gait disturbances, and convulsion. Ekanem *et al* (2009) reported that artequine (combination therapy comprising of artesunate and mefloquine) induced dose-dependent reactive astrocytes formation in the hippocampus which may impair uptake of neurotransmitters and alter neuronal environment thus altering hippocampus function such as learning and memory. Adebayo *et al* (2010) reported that sole administration of artesunate and co-administration of artesunate and amodiaquine for malaria may increase the risk of coronary heart diseases and that co-administration of artesunate and amodiaquine for malaria treatment in a patient with heart-related diseases should be done with caution. Yin *et al* (2014) observed a prolonged QT interval and neuropathic changes in the central nervous system, which demonstrated the cortex and motor neuron vulnerability in dogs after intramuscular administration of 6 mg kg^{-1} artemether over a 3 months period. Ajibade *et al* (2012b) reported degenerative changes and loss of cellular components with reduced population of Purkinje cells in the group of rats treated with 4mg/kg/body weight and 2mg/kg/body weight of Artesunate.

2.22.4 Embryotoxicity of ACTs

Preclinical studies in rodents have demonstrated that artemisinins can induce fetal death at high dose levels but that at lower doses congenital malformations may be produced. The malformations can be induced in rodents only within a narrow window in early embryogenesis (WHO, 2003). Artemisinins had been demonstrated to be embryo lethal and teratogenic in rats and rabbits (WHO, 2003). Developmental toxicity has been observed in rats following treatment on single days between days 10 and 14 postcoitum (PC) when artesunate was administered orally at 17 mg/kg (White, 2006). Day 11 pc was the most sensitive day for the induction of embryo lethality; day 10 pc was the most sensitive day for the induction of malformations (Cardiovascular defects and shortened and/or bent long bones). No developmental toxicity was seen following administration of the same dose administered on day 9 pc or following 30 mg/kg on day 16 or 17 pc (White, 2006). Clark, (2009) discovered that Single oral doses of artesunate, dihydroartemisinin, arteether and artemether administered to rats during a sensitive period of organogenesis caused embryo deaths and malformations (malformed long bones and ventricular septal defects). Extended oral dosing (12 days or more) of monkeys once daily with 12 mg/kg-d artesunate also caused embryo deaths. The initial embryotoxic effect in both species was to kill primitive erythroblasts which

are present in the embryo for a few days of gestation in rats and several weeks in primates. The malformations that occurred in rats are attributed to a transient depletion of the primitive erythroblasts. In monkeys, when treatment at 12 mg/kg-d was shortened to 3 or 7 days, the embryos survived but likely suffered a transient loss of primitive erythroblasts. The Chinese investigations for the registration documentation have been conducted on artemisinin and artemether in rats with relatively high doses. Doses are presented as fractions of the LD_{50}, i.e., 1/400, 1/200 and 1/25 corresponding to 14, 28 and 223 mg/kg (LD_{50} equal to 5576 mg/kg), and application was either in the first 6 days of pregnancy, or on days 7 to 12, or 13 to 19, respectively. Dosing in the very early stage had no effect on the development of the foetuses, while treatment at the later stages resulted in a complete embryonic loss. When treatment was administered between pregnancy days 6 and 15 (comparable to current protocols), about 50% loss of embryos was observed at the lowest dose, while at the higher doses total loss was observed again. A umbilical hernia was the only malformation detected in 6.1% of the foetuses when 14 mg/kg was administered on days 6 to 8 of pregnancy. Results in mice were described as comparable (China Cooperative Research Group, 1982b). Artemether has also been tested in mice and rats by intramuscular injection, and analogous results are reported. In no instances, teratogenic effects were reported (China Cooperative Research Group, 1982b). Further studies conducted with

artemether in mice, rats, and rabbits, and of artesunate in rats have been summarized by Longo *et al* (2006); they have generally provided an analogous picture, with large embryonic losses at relatively modest doses. In mice, 100% resorptions were observed at a dose of 21.4 mg/kg, while in rats and rabbits >90% resorptions were seen at 10.7 and 2.7 mg/kg, respectively. Further investigations have been conducted with rat embryos in culture by Longo *et al* (2006). They studied the effects of dihydroartemisinin (DHA) in rat whole embryo cultures. DHA was added to the culture medium for the entire 48-h culture, 1.5 h at the beginning or at the end of the culture at 0.01-2 microg/mL. DHA affected primarily red blood cells during yolk sac hematopoiesis. Higher concentrations and longer exposure inhibited angiogenesis. Tissue damage (cell deaths) and effects on embryo morphology (neural tube, branchial arches, somites and caudal region defects) were attributed to these events. The primary mechanism of artemisinin-induced embryotoxicity in the rat is a sustained depletion of primitive erythrocytes (embryonic erythroblasts). There was a marked reduction in primitive erythrocytes within 24 hours following a single dose of 17 mg/kg on day 10 or 11 pc. Embryos were viable until day 13 pc (postcoitum), but the majority had died by day 14 pc (White, 2006). These results suggest an inhibitory action on the development of early erythroid precursor cells, leading to anaemia and hypoxia, including

cardiovascular defects and resulting finally in damage to growing tissues and embryonic death (White, 2006).

2.22.5 Effects of ACTs on body organs

Artemisinins are selectively distributed into P. *falciparum* infected erythrocytes, where they cause malaria parasite's death through the generation of free radicals (Vyas *et al*., 2002; Little *et al*., 2009). However, these drugs are also distributed in other organs including the liver, CNS, lungs, kidney, spleen, etc (Zhao and song, 1989; Vyas *et al*., 2002) making such organs possible targets of toxicity. Studies with animal had shown that artemisinin and its analogues cause acute hepatotoxicity in guinea-pigs (Nwanjo and Oze, 2007) acute morphological changes in visceral organs of rabbits (Ngokere *et al*., 2004) and liver inflammation associated with prolonged use of relatively high doses of artemisinin (Leonardi et al., 2001). Prophylactic use of amodiaquine has been associated with fatal cases of agranulocytosis and hepatitis, mainly in non-immune adults (Hatton, *et al*., 1986; Neftel *et al*., 1986) Although it is considered that agranulocytosis and hepatitis are unlikely to occur when amodiaquine is used for treatment (Olliaro *et al*., 1996) neutropenia (Adjuik *et al*., 2002) have been reported after administration of treatment doses of amodiaquine, alone or in combination with artesunate. Histological studies by Utoh-Nedusa *et al* (2009) with dihydroartemisinin on vital organs of rats reveal no evidence of toxicity in the

heart, liver, lungs, and intestine. Onyije and Hart (2012) discovered sinusoidal congestion, infiltration of inflammatory cells and loss of tissue architecture in the rats administered with 6mg/kg of oral artesunate, there was no form of distortion in the tissue architecture of the liver in the group administered with 2mg/kg of oral artesunate. Soniran *et al* (2012) discovered that physical examination of the internal organs of mice showed mild enlargement of the spleen (splenomegaly) in the group treated with artesunate while the liver, lungs, and kidneys of the two groups treated with chloroquine and artesunate, respectively, were without macroscopic changes. A study by Ekong *et al (*2009b) on the effect of amodiaquine, on the histomorphology of the spleen of wister rats, showed that administration of overdose and chronic doses of amodiaquine is detrimental to the spleen; therefore excess doses should not be taken especially as a prophylactic. Olurishe *et al* (2007) discovered the presence of regions of focal necrosis, perivascular cuffing and fatty degeneration in the liver of mice that was immunosuppressed with cyclophosphamide, infected with *Plasmodium berghei*, and then treated with lamivudine-artesunate co-administration. Shahbazfar *et al* (2011) reported that broiler treated with artemisinin at doses of 17, 34, 68, and 136 ppm for 36 days showed various lesions in the liver, kidney, and brain while heart, lung, and spleen had no lesion. Degenerative lesions like intracytoplasmic eosinophilic inclusions were seen in both kidney and liver but the fatty change was

seen only in the liver. There was no relationship between severity of the liver lesions and drug dosage. A study by Ejiofor *et al* (2009) on histopathological effects of artemether on selected organs in rats revealed no histopathological effect in the organs of rats treated with 1.5 mg kg^{-1}. However, rats treated with 7.5 and 15 mg kg^{-1} revealed necrositic lesions with mononuclear cellular infiltration in the liver and brain. The liver had focal area necrosis, while the brain had liquefactive necrosis, neuronal degeneration, congested blood vessels, hemorrhage, and vacuolations. The interstitial spaces of the glomerulus and renal tubules of one kidney from rats that received 15 mg kg^{-1} had focal area fibrositis-necrosis. Izunya *et al* (2010) observed sinusoidal congestion with cytoplasmic vacuolation (hepatocyte oedema) and mild inflammation of the portal tracts in the liver of rats treated with 4 mg/kg b. w of artesunate daily for 3 days followed by 2 mg/kg b. w daily for 4 for next days, and in rats treated with 8 mg/kg^2 b. w of artesunate daily for 3 days followed by 4 mg/kg b. w daily for next 4 days. Their study suggests that artesunate at normal dose has a toxic effect on the liver cells and could be a potential hepatotoxic drug. Intramuscular administration of 6 mg kg^{-} artemether over a 3 months period induced concurrent extramedullary hematopoiesis in the spleen and inhibition of erythropoiesis in the bone marrow of dogs (Yin *et al*., 2014). Peys *et al* (2006) discovered high liver weight and slight diffuse hepatocellular hypertrophy and distal tubular dilatation, together with

flattened epithelium, in the kidneys of eight beagle dogs treated with 45 mg/kg/dosing of *β*-artemether for 2 weeks by oral gavage. Bigoniya *et al* (2015) reported that artesunate treatment for 45 days at 4 and 8 mg/kg/day showed sinusoidal dilation, cytoplasmic vacuolation, focal necrosis, sinusoidal congestion and extensive inflammatory changes in the liver of rats, whereas kidney was free of any deleterious effect. Jahas *et al* (2014) disclosed that artesunate administration at a dose of 110mg/kg body weight for 14 days caused a pattern of hepatocellular necrosis in the liver of rats. Danladi *et al* (2013) discovered severe glomerular degeneration, focal haemorrhage, oedema, congested blood vessels, cloudy swelling of the tubules and necrosis of the tubule both in cortex and medulla of the kidney in the rat treated with artesunate. These pathological effects were observed to be dose and duration dependent. In other words, the higher the dose given, the more severe the damage observed and the longer the duration of the dose the more severe the damage observed. Izunya *et al* (2011) reported that artesunate at normal and double normal dose has no effects on the histology of the heart in Wistar rats. Study of Ajibade *et al* (2012a) revealed distortions, disaggregation, vacuolation and degenerative changes in cardiac tissue of rats treated with 4 mg kg^{-1} body weight and 2 mg kg^{-1} body weight of artesunate orally.

2.22.6 Effects of ACTs on Reproductive organs and parameters

Most antimalarial agents have been associated with male reproductive dysfunction in laboratory animals (Joshi *et al*., 1996; Adeeko and Dada, 1998; Parveen *et al*., 2003). Studies with mice/albino rats had shown that chloroquine, quinine (Adeeko and Dada, 1998), halofantrine (Orisakwe *et al*., 2003) and artemisinins (Nwanjo and Oze, 2007) adversely affect sperm parameters. Obianime and Aprioku (2009) discovered significant decreases in total sperm count and sperm motility, with increases in abnormal sperm cells (morphology), debris and premature sperm cells in male guinea pigs treated with half, normal and double clinical doses of artesunate; artesunate/sulfadoxine/pyrimethamine; artesunate/amodiaquine and their combinants (sulfadoxine/pyrimethamine and amodiaquine). The effects were maximal at the subclinical doses and synergistic in the ACTs, compared to those of the individual partner agent. Okey and Olorunshola (2012) reported that Halofantrine Hydrochloride and Artesunate given at normal therapeutic doses, have no adverse effect on sperm concentration and testicular weight, but have the potential to cause oligospermia following prolong use. Rajput *et al* (2012)disclosed that the testis of mice treated with oral doses of 150mg/kg.b.wt and 300mg/kg.b.wt of artesunate for a period of 14, 21 and 45 days showed some varying degree of cell clustering, cellular hypertrophy and intercellular vacuolations specifically in the germinal cell layer, resulting in a decline in sperm production. The germinal

cell nuclei were highly pyknotic. Vacuolization within the interstitium was also observed particularly in the Leydig cell cytoplasm. Histopathological studies of Ajah and Eteng (2010) on the effect of single acute dose of *Artemisia annua .L*. on testes and ovaries of Wistar rats showed adverse lesion on the ovary ranging from atretic-degenerating corpus luiteum, with the loss of connective substances, the arrest of ovarian follicle maturation to complete absence of ovarian follicle. No adverse histopathological changes were observed in the testes of the rats (Adebayo *et al.,* 2010). Similarly, Aprioku and Obianime (2011) reported that artemether caused no significant changes in the semen parameters but there was significant reduction in the epidermal sperm concentration and mortality in the artesunate and dihydroartemisinin treated animals. Further study by Obianime and Aprioku (2009) revealed that artesunate, ACTs, and their recombinants significantly decreased serum testosterone level in the male guinea pig. In addition, artesunate and amodiaquine caused a moderate decrease in the serum luteinizing, follicle stimulating hormone without effect on prolactin. Izunya *et al* (2010) reported that oral administration of artesunate at normal and double normal doses may be toxic to the testes at higher doses and that it is uncertain if these changes are reversible. Adebayo *et al* (2010) reported that sole administration of artesunate and co-administration of artesunate/amodiaquine causes no change in the sperm count, sperm abnormality, and serum testosterone level. A study by Raji *et al* (2005)

showed that oral administration of artemisinin derivatives, artemether caused a significant reduction in the progressive sperm motility, viability, sperm count and serum testosterone levels in a dose-dependent fashion during an acute administration of the drugs in male rats. Olumide and Raji (2011) reported that artesunate did not cause significant effect during short- term administration but significantly reduce aforesaid parameters in long-term administration, visible lesion in the testicular and epididymal structures were observed during histological studies, although infertility was not significantly reduced. Furthermore, In-vitro studies showed dose and duration dependent changes in Sertoli cell viability and ds-DNA integrity. Tijani *et al* (2010) reported that graded doses of co-artesiane (combination of artemether-lumefantrine) elicit depletion of the antioxidant defense system of adult wister rats. The mean sperm count, motility, and viability were reduced in treated animals when compare with the control group. A study by Nwanjo and Oze (2007) on the antifertility activity of dihydroartemisinin in male albino rats showed a decrease in sperm count, sperm motility, and sperm viability which were also both dose and duration dependent. Abnormalities noticed in sperms were "curved mind piece", small and pyriform heads observed only in some groups. A significant decrease in serum testosterone level was also reported. The study of Gbotolorun *et al* (2011) showed that Amodiaquine Hydrochloride is deleterious to the ovary by prolonging the length of the oestrous cycle, producing

widespread follicular atresia and depletion of the enzymatic antioxidant status of SOD and CAT and consequently resulting in a state of oxidative stress in the ovary.

2.22.7 Effects on body and organ weight

Tijani *et al* (2010) reported that acute administration of co-artesiane (combination of artemether and lumefantrine) caused an increase in body weight while testicular weight was lower in all the treated groups though not significant. Ayodele *et al* (2007) reported no significant change in weight of testis and other accessory reproductive organs in artemether-lumefantrine exposed animals. Utoh-Nedosa *et al* (2009) reported an increase in mean body weight on the administration of artemisinin in rats. Olumide *et al* (2011) reported a significant change in body and relative organ weight of rats administered artesunate (2.9 mg/kg B.W) for 5 days while there were significant reductions in these parameters in the rats treated for 6weeks when compared with their control. Izunya *et al* (2010) reported that artesunate at their normal and double normal dose has no effect on body weight of rats but may be toxic to testis at higher doses.

2.22.8 Effects on animal behaviour

Ekong *et al* (2010) reported that artesunate may not be harmful at its recommended dose and may not affect behaviour unlike when taken in excess of dose and or

time. Even at this doses/time, there may have been no behavioural manifestation. Furthermore, artesunate/ amodiaquine combination also showed no significant change in the behaviour of rats, an indication that this drug may not have altered locomotion and may neither be anxiologic nor anxiogenic at its recommended doses or time (Ekong *et al.*, 2010).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental Site

The study was carried out in the animal house of the Zoology Department, Nnamdi Azikiwe University, Awka. Strategically, Awka is located midway between two major cities - Onitsha and Enugu, with 6.21 latitude and 7.07 longitudes in south-eastern Nigeria (Ezenwaji *et al.*, 2013).

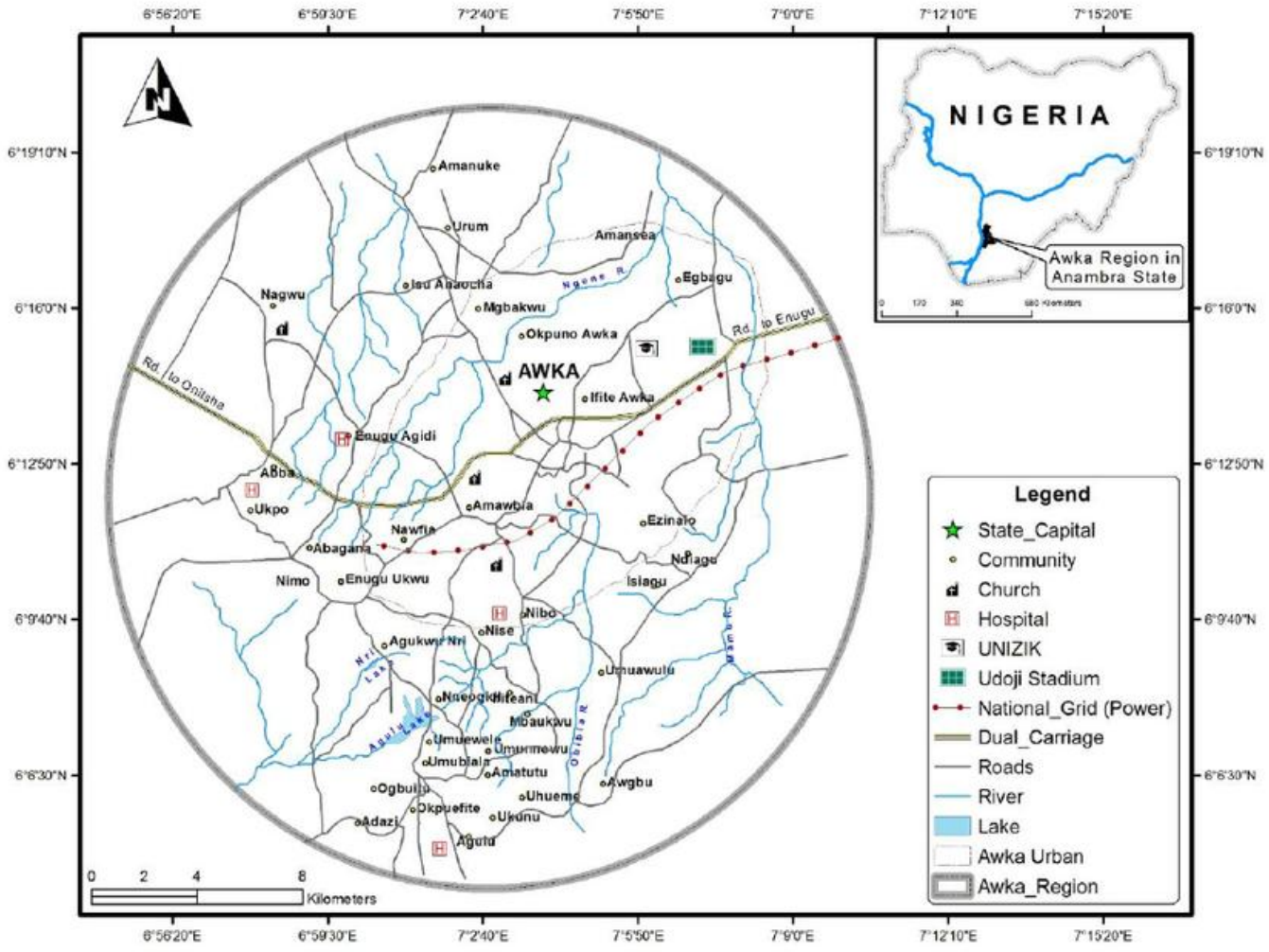

Figure 3: Map of Awka Showing Experimental Site.

Source: Ezenwaji *et al.*, (2013).

3.2 Study duration

The study was carried out from April 2018 to August 2018.

3.3 Experimental Animals

Adult albino mice of 8 weeks old with average weight between 24 - 27g were used for this study. The animals were obtained from the animal house of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka.

Plate 2: Some Albino Mice Used for the Study

3.4 Procurement of Parasites

The *Plasmodium berghei (*NK 65 strain) used for this study was obtained from Rresearch Laboratory Unit, Biochemistry, and Nutrition Division, Nigeria Institute of Medical Research, Yaba, Lagos, Nigeria.

3.5. Procurement of Drugs

Camosunate® (Artesunate100mg plus Amodiaquine 300mg, manufactured by Adams Pharmaceutical, China). P-Alaxin® (Dihydroartemisinin 40mg plus Piperaquine 320mg, manufactured by Blis gvs Pharmaceutical Ltd, India) were used for the study.

3.6. Experimental Design

One hundred and eighty (180) albino mice weighing between 24 - 27g were used for this study. The experiment was divided into three replicates, each replicate contains sixty (60) animals of both sexes which were randomly distributed into six (6) groups of ten (10) animals each (n=10). Each group contains five (5) males and five (5) females in separate cages with sawdust as beddings. The beddings were replaced regularly for prevention of infection. The animals were fed with grower's marsh (Vital feed Limited, Sapele) and were given access to water from public supply *ad libitum* till the end of the experiment. They were allowed to acclimatize

for a period of one week prior to the commencement of the study. The animals were identified using concentrated picric acid solution, base on their weight and drug dose received. The picric acid was applied to some body parts (head, limb, trunk, stomach, and tail) of the animals. The animals were identified based on the body part where the picric acid was applied.

3.7. Experimental Treatment

3.7.1. Inoculation of mice with *Plasmodium berghei*

The infection of the recipient mice was initiated by injection of the parasite preparation from the donor mice to healthy test mice via an intraperitoneal route as described by David *et al* (2004) and Peter and Anatoli (1998). Briefly, 1ml of *Plasmodium berghei* – infected red blood cells obtained from the retro – orbital sinus of the infected mice was diluted with 5ml of phosphate buffered saline (PBS) so that each 0.2ml of the blood that was subsequently injected contained approximately 1×10^7 *Plasmodium berghei* parasitized red cells. Animals from Groups A, B, C, D, and E were inoculated with the *Plasmodium berghei*.

3.7.2. Determination of Degree of Parasitaemia

Degree of parasitaemia was determined using the method of Warhurst and Williams (1996). Peripheral blood smears were prepared by using blood obtained from the tail veins of infected mice. A drop of blood was taken from the tail of the infected mice and placed at a distance of about an inch from one end of the

microscopic slide and dispersed along the length of the slide width with another slide. The thin films were fixed in methanol for 3 minutes and then stained with Giemsa stain for 30 minutes. The stain was diluted with the sodium phosphate buffer at pH 7.2 in the ratio of 1:10 (1 ml of the Giemsa stain to 9 ml of buffer solution). After 30 minutes, the slides were rinsed with tap water and then air dried. Blood smears were examined at a magnification of x100 by oil immersion light microscopy (Binocular light microscope; Olympus; XS2-107BN) and scanned with Moticam Images plus 2.0 digital camera (Motic China Group Ltd. 1999-2004). In a particular field, the total number of red blood cells (TRBC), as well as the total number of parasitized RBC (PRBC), was counted. Parasitemia in each field was calculated with this formular-

$$\text{Parasiteamia (\%)} = \frac{\text{No of PRBC}}{\text{No of´ RBC}} \times \frac{100}{1}$$

Ten fields were counted on each slide and the mean percentage parasitaemia was recorded for each mice. Parasite count in each of the groups of animals was determined on day 0, 3, 5, and 7. The mice were not treated until the parasitemia was established on day 7. After the last day of drug administration, animals in groups D and E were followed up to 28 days, during which parasitemia was also determined.

3.7.3 Drug Administration

Drug administration commenced on day 8 after inoculation. Two Camosunate tablets® (100mg of Artesunate + 300mg of Amodiaquine) and one P-Alaxin tablet® (40mg of Dihydroartemisinin + 320mg of Piperaquine) were powdered separately in a mortar, mixed with 20ml of distilled water and administered in mg/kg body weight as recommended by the World Health Organization (2015b). Drugs were administered as aqueous suspensions with oral gavage using the formula;

$$\frac{\text{Weight of animal(kg)} \quad X \quad \text{Dosage (mg/kg)}}{\text{Concentration of drug (mg/ml)}}$$

The drug suspension was continuously agitated in order to deliver the drugs homogeneously to the animals. This experiment was to mimic the dose and duration of administration of the drugs in human as recommended by the World Health Organization (WHO, 2015b). Drug administration started at day 8 after the establishment of parasitemia, as follows:

Table1: Experimental design for drug administration in the *Plasmodium* parasitized mice

GROUPS	TREATMENT	DOSAGE (mg/kg).
Group A	Normal saline	Infected control
Group B	Artesunate+Amodiaquine	4/10mg/kg for 3 days
Group C	Dihydroartemisinin+Piperaquine	4/18mg/kg for 3days
Group D	Artesunate+Amodiaquine	4/10mg/kg for 3days, allowed to recover for 28 days
Group E	Dihydroartemisinin+Piperaquine	4/18mg/kg for 3days, allowed to recover for 28 days
Group F	Normal Saline	Uninfected control

Animals in groups D and E were used to study recrudescence and reversibility of antimalaria drug-induced toxicity in *P. berghei* infected mice.

3. 8. Data Collection

3. 8.1. Weight Assessment

As an index of the physical status of the animals, the weight of each animal was monitored over the period of the study. The animals were weighed before and after drug administration using Electronic weighing balance (Labtech; model; BL7501; Range: 750g - 0.1g)

3. 8.2 Collection of blood samples

After 24 hours, blood samples were collected from all the experimental groups After 28 days post - recovery, blood samples were also collected from animals in groups, D and E for haematological investigation. Blood samples were collected through retro – orbital sinus with a capillary tube into ethylenediaminetetraacetic acid (EDTA) tubes.

3. 9. Estimation of haematological parameters:

Blood was collected from the animals in all the groups and assayed for White blood cell count, White blood cell differential count, Red blood cell count and Haemoglobin concentration using standard methods.

3. 9.1 White blood cell count

White blood cell count was estimated with haematocytometer using the principle of a calibrated capillary tube for blood sampling described by Baker *et al* (1998). Briefly, 950µl of diluting fluid was dispensed into a small clean dry test tube using micropipette, then, 50µl of well mixed EDTA anticoagulated blood was added to the diluting fluid in the test tube. The diluted sample was then mixed and loaded

into the improve Neubauer counting chamber. The white cells present in the 4 corners of the chamber were counted and recorded.

3. 9.2 White blood cell differential count

A drop of blood was taken from the tail of the infected mice and placed at a distance of about an inch from one end of the microscopic slide and dispersed along the length of the slide width with another slide, the thin film was air-dried and fixed with methanol for 3 seconds, air-dried and then stained with field stain A and B for 6 seconds. The slides were washed, drained and air-dried. Lymphocytes, neutrophils, eosinophils, basophils and monocytes were identified and counted under the 100x objective lens.

3. 9.3 Estimation of Packed Cell Volume (PCV)

Packed cell volume was estimated using centrifugation method of Dacie and Lewis (1984). A small volume of blood was collected from the tip of the animal tail (tail tip amputation) into a heparinized capillary tube, about three-quarters of the tube. The capillary tubes were sealed with sealant and placed in the numbered slots of the haematocrit centrifuged with the sealed end against the rim, then, spun at 10,000rpm for 5 minutes to separate the blood into plasma and packed cells. Immediately after centrifuging, the packed cell volume was read using the microhaematocrit reader.

3. 9.4 Red Blood Cell count

Red blood cell count was estimated with haematocytometer using the principle of a calibrated capillary tube for blood sampling described by Baker *et al* (1998). Briefly, 4ml of red blood cell diluting fluid was drawn with a Pasteur pipette and then transferred into a small, clean and dried test tube. Then, 20µl of blood was drawn with a micropipette and then added to the tube, the diluted blood was mixed gently, 10µl was withdrawn from the diluted blood and then loaded into the counting chamber. When the cells have settled out of suspension, the number of red blood cells lying in the 5 of the 0.04 mm^2 areas were counted and recorded.

3. 9.5 Estimation of Haemoglobin concentration

Haemoglobin concentration was estimated by Sahli's haemoglobinometric method. Hydrogen chloride was placed in diluting tube up to the lowest mark; 0.02ml of well mixed whole blood was dispensed with micropipette into diluting tube and mixed. The diluted haemoglobin was allowed to sit at room temperature for 10 minutes. After 10 minutes, distilled water was added in drops and mixed until it has exactly the same colour with comparism standards.

3.10. Collection of Tissue for Histopathological Studies

After collection of blood, animals were collected from each group, anesthetized in chloroform vapour and dissected. The liver, kidney, lungs and spleen were collected from all the groups.

3.11 Histopathological Analysis

This was carried out as described by Bancroft and Gamble (2002).

3.11.1 Procedure:

Histopthological analysis was carried out using the following steps-

1. Fixation

After collection, the liver, spleen, lung and kidney from the various groups of mice were, immediately fixed in 10% formol saline. The organ samples were plunged in the fixative solution. The fixative container was stirred gently for a few seconds to make sure the organ samples do not stick to the container surface.

2. Dehydration

The organs were dehydrated in ascending grades of ethanol (70%, 80%, 90%, 100%, Absolute I, Absolute II), within 1 hour intervals.

3. Clearing

The organs were cleared using xylene to remove opacity from the dehydrating tissue.

4. Infiltration

The tissue block was infiltrated with paraffin that acts as a support during the sectioning. Alcohol was discarded from the last dehydration step; the vial was filled with melted paraffin and allowed to equilibrate for 1 hour in an incubator set at $58^{O}C$.

5. Embedding

Once tissue samples are infiltrated by paraffin, they are removed from the cassettes and carefully positioned inside a metal base mold. The mold was filled with molten paraffin wax with a melting point of 50 -52^{o} c and then immediately placed on a cooling surface in other to solidify.

6. Sectioning

The edges of the block were trimmed with a sharp razor blade so that the upper and lower edges of the block are parallel to the edges of the knife. Sectioning was accomplished by using a cutting apparatus called a microtome. The blocks were sectioned at 5– 6 microns. The paraffin block was mounted on the microtome holder. Sections are cut as a ribbon and are floated on a water bath maintained at 45°C to stretch the paraffin section. A standard microscope glass slide was placed under the selected tissue section and removed from the water bath. Tissue sections were then allowed to dry.

9. Staining

The paraffin sections were dewaxed in xylene, rehydrate in 100% and 95% ethanol, washed in running tap water for 3 min. Stained for 3–5 minutes in hematoxylin, then, washed in running tap water for 3 minutes, decolorized briefly in acid alcohol for 2 seconds,washed and blue in running tap water for 3 minutes, staineded for 2–5 minutes in 0.1% aqueous eosin, rinsed in tap water for 30

seconds. Dehydrated in 95% ethanol two times for 2 minutes, dehydrate again in 100% ethanol two times for 2 minutes, then Cleared in clearing agent two times for 2 minutes.

10. Microscopic Observation of the slide

The sections were examined under Olympus/3H light microscope-Japan (x400) for histopathological changes. Photomicrographs were captured using a Moticam Images plus 2.0 digital camera (Motic China Group Ltd. 1999-2004) fixed onto the light microscope (Olympus/3H light microscope).

3.12 Statistical Analysis

Statistical Analysis was carried out using statistical package for social sciences (SPSS) version 22. Comparisons between values obtained in treated groups and control groups were performed with one-way analysis of variance (ANOVA). Thereafter, post – hoc test was carried out using Least Significant Difference (LSD) to find out the level of significance at $\alpha = 0.05$. Paired Sample T – test was used for finding significant difference between initial and final body weight of the mice.

CHAPTER FOUR

RESULTS

4.1. Observation of *Plasmodium berghei* Erythrocytic Stage (Asexual Forms)

In the present study, *P. berghei* erythrocytic stage was observed inside the red blood cells of mice after being inoculated intraperitonealy with 0.2 ml of *P.berghei* infected red blood cells. Ring stage of the parasite was seen in the blood films stained with Giemsa solutions (Plate 3).

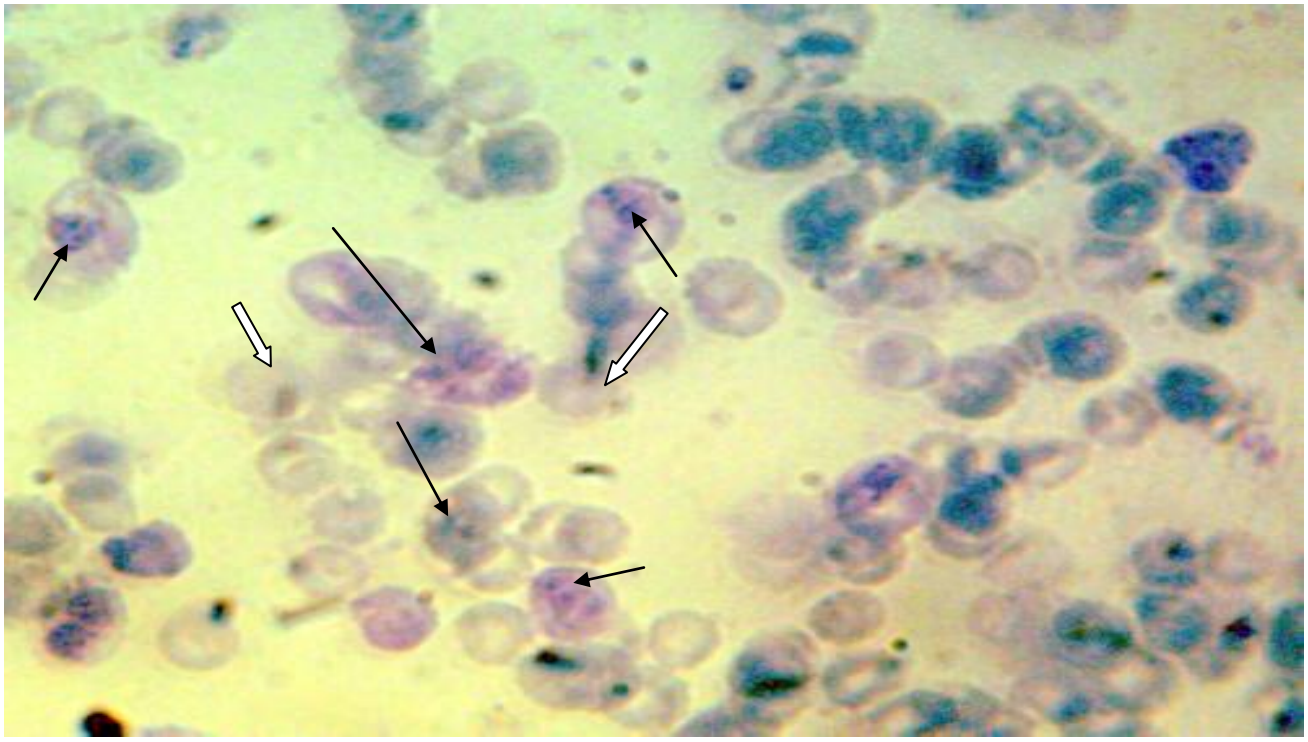

Plate 3: Blood film of *P. berghei* infected mice showing parasitized red blood cells (dark arrows) and unparasitized cells (white arrows). Giemsa stain, x100.

4.2. Levels of parasitaemia of *P. berghei* infected mice before treatment

Figure 4: showed an increase in parasitaemia with days of infection. Percentage parasitaemia was lowest (3%) on day 3 post infections and increased progressively, peak parasitaemia (24%) was observed on day 7 post infection. This model of malaria is lethal, death follows the peak parasitaemia. 30% mortality was recorded on day 7, followed by 50% on day 14, by day 21 post infection, 100% mortality were observed in untreated group (Figure 4).

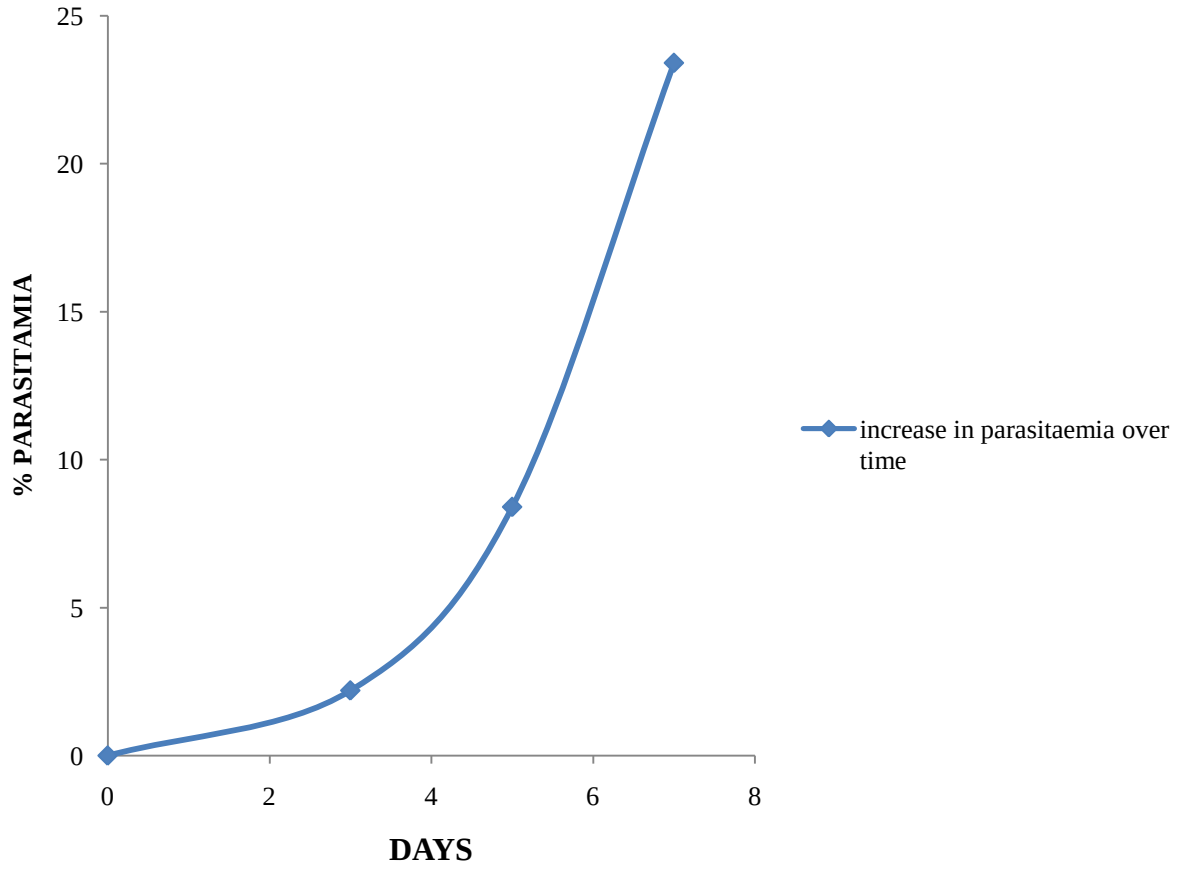

FIGURE 4: Parasitaemia Levels of *P. berghei* Infected Mice before treatment

4.3 Parasitaemia Levels of *P. berghei* Infected Mice after treatment with Artemisinin-Based Combination Therapy

There was a gradual decrease in parasitaemia during treatment with Artesunate/amodiaquine (A/A) and dihydroartemisin/piperaquine (D/P).The drugs remarkably decreased parasitaemia in all the treated groups. Complete clearance of parasites was achieved before day 28 in all the treated groups, while all the infected untreated mice died before day 21 post infection. However, parasite clearance rate was faster in A/A treated groups than in D/P treated groups. No recrudescence was observed in all the treated groups (Figure 5).

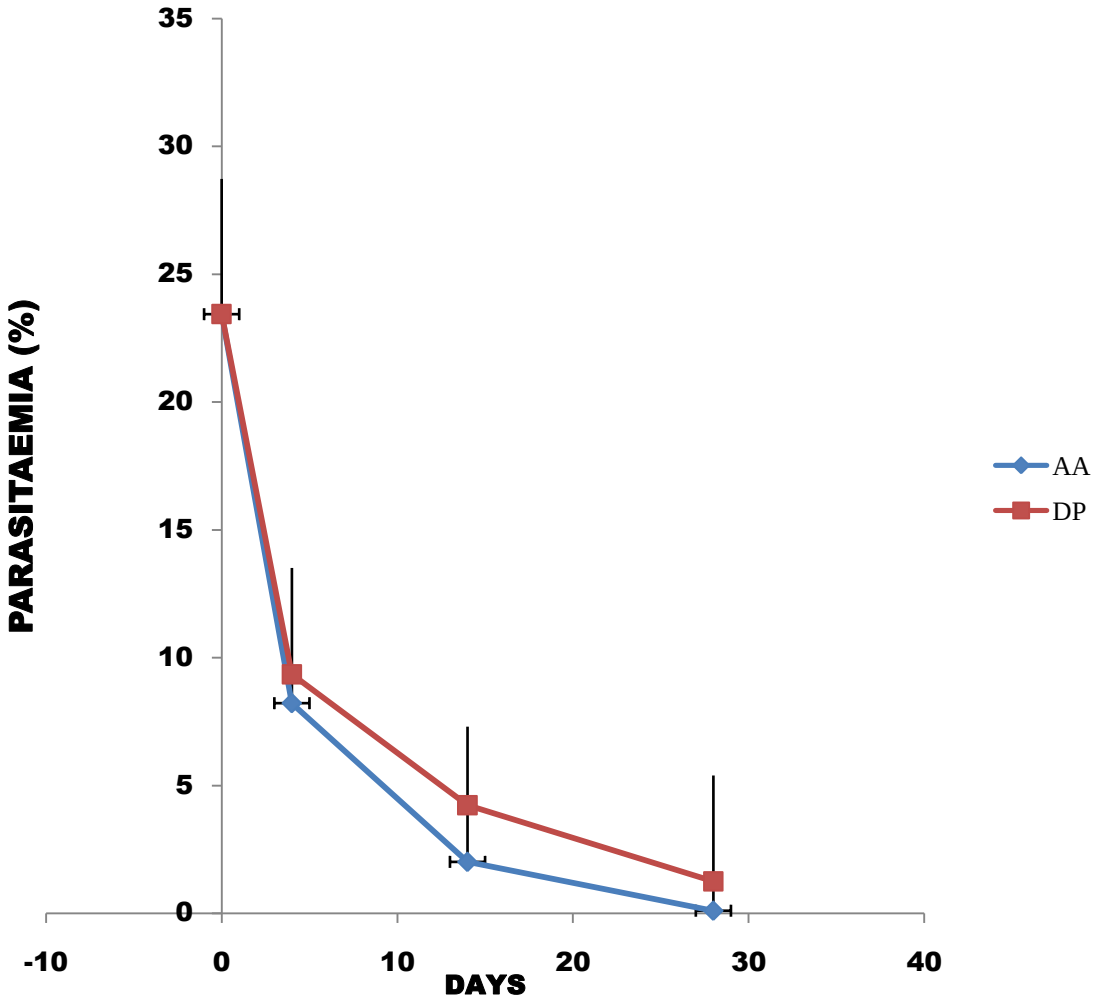

FIGURE 5: Parasitaemia level after treatment with Artemisinin- Based Combination Therapy

AA = Artesunate + Amodiaquine

DP = Dihydroartemisinin + piperaquine

4.4. Effects of Artesunate+Amodiaquine(AA) and Dihydroartemisinin +Piperaquine (DP) on white blood cell (mm^3 x 10^3) count in *P. berghei* infected mice.

With reference to Figure 6 and Appendix 3, there was a significant difference ($P < 0.05$) in white blood cell count in the infected untreated group (16.54± 0.546 mm^3 x 10^3), the group treated with artesunate+amodiaquine (12.87± 0.38 mm^3 x 10^3) and the group treated with dihydroartemisinin+piperaqune (10.65±0.07 mm^3 x 10^3) compared to the uninfected untreated group (7.42 ± 0.23 mm^3 x 10^3). Significant decrease in WBC was observed in the group treated with Artesunate +amodiaquine(12.87±0.38mm^3x10^3), group treated with dihydroartemisinin +piperaquine (10.65±0.07 mm^3 x 10^3) and recovery group treated with artesunate+amodiaquine (7.78±0.59 mm^3 x 10^3) compared to the infected untreated group (16.54± 0.54 mm^3 x 10^3). A significant decrease was also observed in the group treated with dihydroartemisinin+piperaqune(10.65±0.07mm^3 x 10^3) compared to the group treated with artesunate+amodiaquine (12.87± 0.38 mm^3 x 10^3). However, white blood cell count was restored during the recovery period in the group treated with dihydroartemisinin+piperaquine (7.65 ± 0.17mm^3 x 10^3) to a level that was not significantly different ($P > 0.05$) from the uninfected untreated group (7.42 ± 0.23 mm^3 x 10^3), white blood cell count was not completely restored in the artesunate+amodiaquine treated group (8.89 ± 0.59 mm^3 x 10^3) since the

value obtained was significantly higher ($P < 0.05$) than that of the uninfected untreated group ($7.42 \pm 0.23\ mm^3 \times 10^3$).

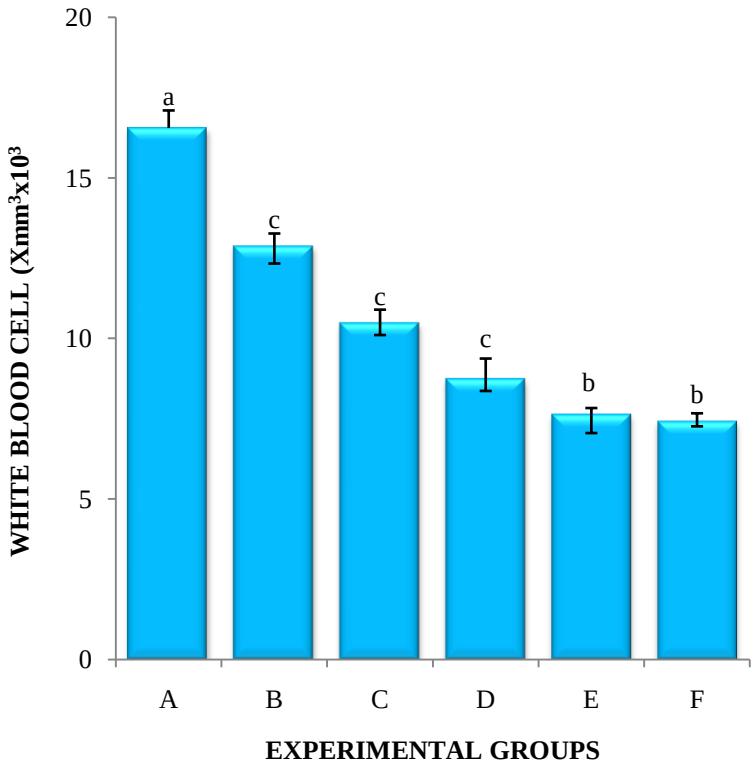

Figure 6: Effects of Artesunate+ Amodiaquine and Dihydrodroartemisinin+Piperaquine on WBC count in *p. Berghei* Infected Mice.

Bars represent mean ± standard error of means.

a = significant difference from normal control at $p < 0.05$ using ANOVA and LSD post Hoc test.
b = significant difference from parasitized control at $p < 0.05$ using ANOVA and LSD post Hoc test
c = significant difference from parasitized and normal control at $p<0.05$

A= infected and untreated (parasitized control)
B = infected and treated with artesunate+amodiaquine (mg/kg) for 3 days
C= infected and treated with dihydroartemisinin+ piperaquine (mg/kg) for 3 days
D= infected and treated with artesunate+amodiaquine (mg/kg) in for 3 days, then allowed to recover for 28 days
E= infected and treated with dihydroartemisinin+piperaquine (mg/kg) for 3 days, then allowed to recover for 28 days
F= uninfected and untreated (Normal saline)

4.5 Effects of artesnate+amodiaquine and dihydroartemisinin+piperaquine on Lymphocyte count in (%) *P. berghei* infected mice.

From Figure 7 and Appendix 6, there was a significant difference ($P < 0.05$) in lymphocyte count in the infected untreated group (77.13±1.02 %) compared to the uninfected untreated group (65.47± 1.83 %). No significant difference ($P > 0.05$) in lymphocyte count was observed in the group treated with artesunate + amodiaquine (77.53±0.55 %) and the infected untreated group (77.13±1.02 %), more so, significant increase ($P < 0.05$) in the lymphocyte count was observed in the group treated with dihydroartemisinin+piperaquine (82.47±1.11 %) compared to the infected untreated group (77.13±1.02 %). A significant increase ($P < 0.05$) in lymphocyte was also observed in all the treated groups except in the recovery group treated with artesunate+amodiaquine compared to the uninfected untreated group (65.47± 7.09 %).

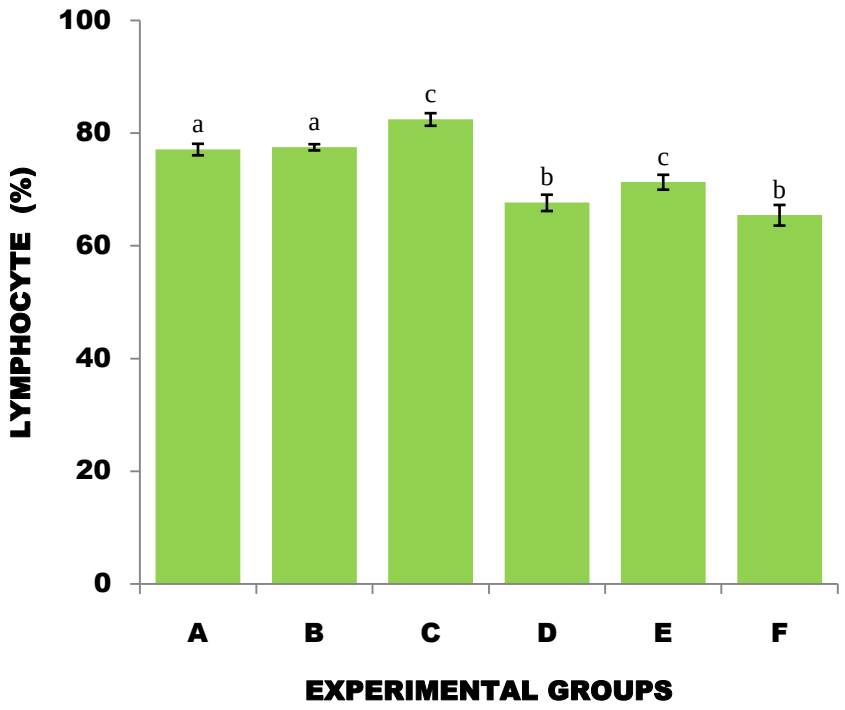

Figure 7: Effects of Artesunate +Amodiaqune and Dihydroartemisinin+Piperaquine on Lymphocyte Count in *P. Berghei* Infected Mice.

Bars represent mean ± standard error of means.

a = significant difference from normal control at $p < 0.05$ using ANOVA and LSD post Hoc test.

b = significant difference from parasitized control at $p < 0.05$ using ANOVA and LSD post Hoc test

c = significant from parasitized and normal control at $p<0.05$

A= infected and untreated (parasitized control)
B = infected and treated with artesunate+amodiaquine in (mg/kg) for 3 days
C= infected and treated with dihydroartemisinin+ piperaquine (mg/kg) for 3 days
D= infected and treated with artesunate+amodiaquine in (mg/kg) for 3 days, then allowed to recover for 28 days
E= infected and treated with dihydroartemisinin+piperaquine in (mg/kg) for 3 days, then allowed to recover for 28 days
F= uninfected and untreated (normal saline).

4.6 Effects of artesunate+amodiaquine and dihydroartemisinin+ piperaquine on neutrophil (%) count in *P. berghei* infected mice

Figure 8 and Appendix 9 showed a significant difference ($P < 0.05$) in neutrophil count between the infected untreated group (16.13± 0.88 %) and the uninfected untreated group (33.33±1.739 %). No significant difference($P > 0.05$) in neutrophil count was observed in the groups treated with artesunate+amodiaquine for 3 days (17.27±0.65%) and dihydroartemisinin

+piperaquine for 3 days (15.93±1.12 %) compared to the infected untreated group (16.13± 0.88%). Neutrophil count was significantly decreased ($P > 0.05$) in groups treated with artesunate+amodiaquine for 3 days (17.27±0.65%), dihydroartemisinin+piperaquine for 3 days (15.93±1.12) and in the recovery group of dihydroartemisinin+piperaquine (7.53 ±1.89) when compared to the uninfected untreated group (33.33 ± 1.73%). After the recovery period, neutrophil count was restored in the group treated with artesunate+amodiaquine to a level (31.27± 1.64%) that was not significantly different when compared to the uninfected untreated group (33.33±1.73%)

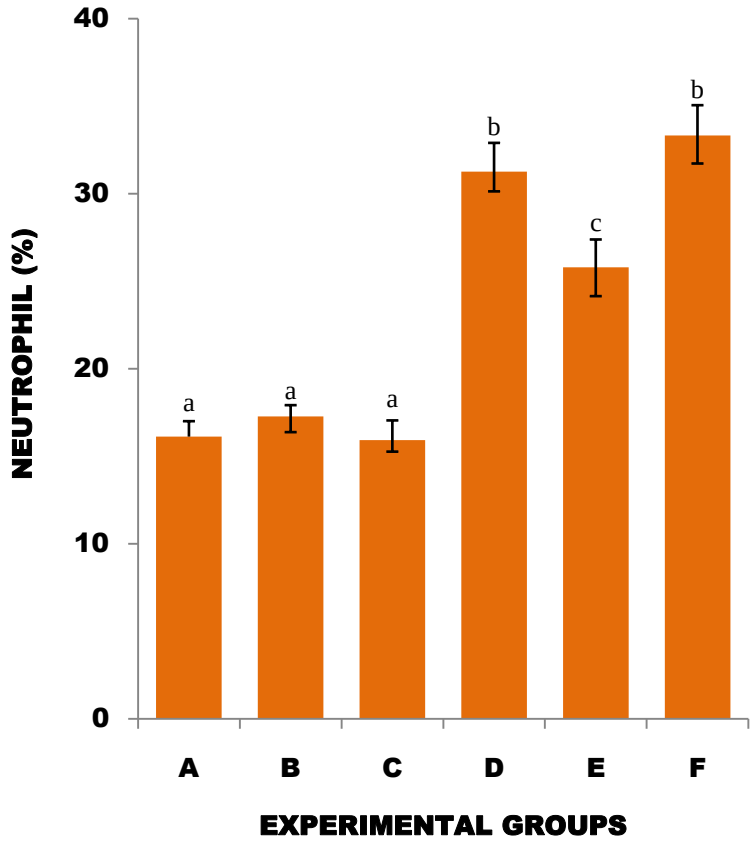

Figure 8: Effects of Artesunate+ Amodiaquine and Dihydrodroartemisinin +Piperaquine on Neutrophil Count in *P. Berghei* Infected Mice.

Bars represent mean ± SEM.
a = significant difference from normal control at $p < 0.05$ using ANOVA and Least LSD post Hoc test
b = significant difference from parasitized control at $p < 0.05$ using ANOVA and LSD post Hoc
c= significant from parasitized and normal control at $p<0.05$

A= infected and untreated (parasitized control)
B = infected and treated with artesunate+amodiaquine (mg/kg) for 3 days
C= infected and treated with dihydroartemisinin+ piperaquine (mg/kg) for 3 days
D= infected and treated with artesunate+amodiaquine (mg/kg) for 3 days, then allowed to recover for 28 days
E= infected and treated with dihydroartemisinin+piperaquine (mg/kg) for 3 days, then allowed to recover for 28 days
F= uninfected and untreated (Normal control)

4.7 Effects of artesnate+amodiaquine and dihydroartemisinin+piperaquine on basophil count (%) in *P. berghei* mice.

Figure 10 and Appendix 12 showed a significant difference ($P < 0.05$) in basophil count of infected untreated group (1.07± 0.18%) compared to the uninfected untreated group (0.60 ± 0.13 %). The groups treated with artesunate+amodiaquine for 3 days (1.00±0.16 %) showed no significant difference ($P > 0.05$) when compared to the infected untreated group (1.07±0.18 %). A significant decrease ($P < 0.05$) was observed in the group treated with dihydroartemisinin+piperaquine for 3 days (0.53±0.13 %) compared to the infected untreated group (1.07± 0.18 %). After recovery period, a significant decrease was observed in the recovery groups when compared to the infected untreated group. There was no significant difference ($P > 0.05$) in all the treated groups compared to the uninfected untreated group.

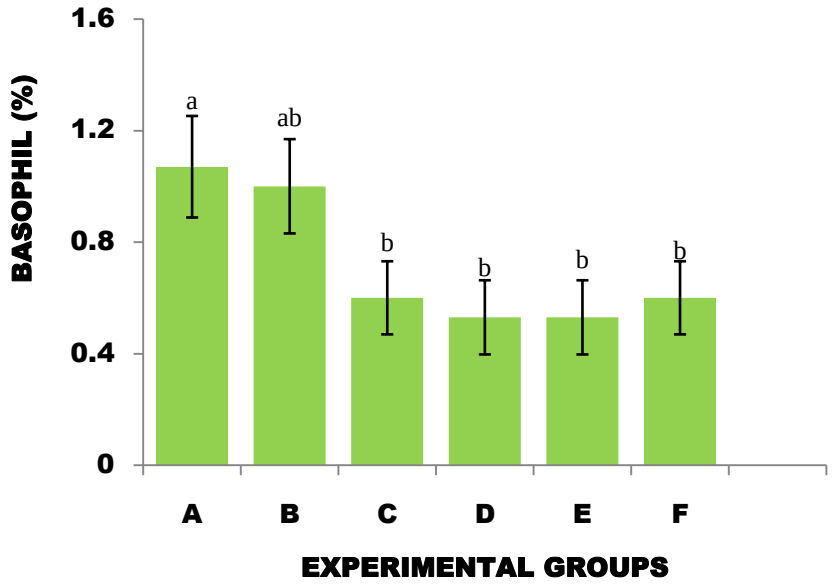

Figure 9: Effects of Artesunate+ Amodiaquine and Dihydroartemisinin+Piperaquine on Basophil Count in *P.berghei* Infected Mice.

Bars represent mean ± Standard error of means

a = significant difference from normal control at $p < 0.05$ using ANOVA and LSD post Hoc test.
b = significant difference from parasitized control at $p < 0.05$ using ANOVA and LSD post Hoc
ab = not significantly difference from parasitized and normal control

A= infected and untreated (parasitized control)
B = infected and treated with artesunate+amodiaquine (mg/kg) for 3 days
C= infected and treated with dihydroartemisinin+ piperaquine (mg/kg) for 3 days
D= infected and treated with artesunate+amodiaquine (mg/kg) for 3 days, then allowed to recover for 28 days
E= infected and treated with dihydroartemisinin+piperaquine (mg/kg) for 3 days, then allowed to recover for 28 days
F= uninfected and untreated (Normal saline)

4.8 Effects of artesunate+ amodiaquine and dihydroartemisinin+piperaquine on eosinophil count (%) in *P. berghei* infected mice.

Figure 10 and Appendix 15 showed a significant difference ($P < 0.05$) in eosinophil count in the infected untreated group (1.40 ± 0.13 %), the group treated with artesunate+amodiaquine (1.33 ± 0.15 %) and the group treated with dihydroartemisinin+piperaquine(0.73 ± 0.15%) compared to the uninfected untreated group (0.40 ± 0.13%). No significant difference ($P > 0.05$)was observed in the group treated with artesunate+amodiaquine (1.33 ±0.15%) and the infected untreated group (1.40 ± 0.13%). Significant decrease in eosinophil count was observed in the group treated with dihydroartemisinin+pipiraquine (0.73 ± 0.15) compared to the group treated with artesunate+amodiaquine for 3 days (1.33 ±0.15). A significant decrease ($P < 0.05$) was also observed in the group treated with dihydroartemisinin+pipiraquine (0.73 ± 0.15%), the recovery group treated with artesunate+amodiaquine (0.53±0.13%) and the recovery group treated with dihydroartemisinin+pipiraquine (0.67±0.12%) compared to the infected untreated group (1.17 ± 0.17%).

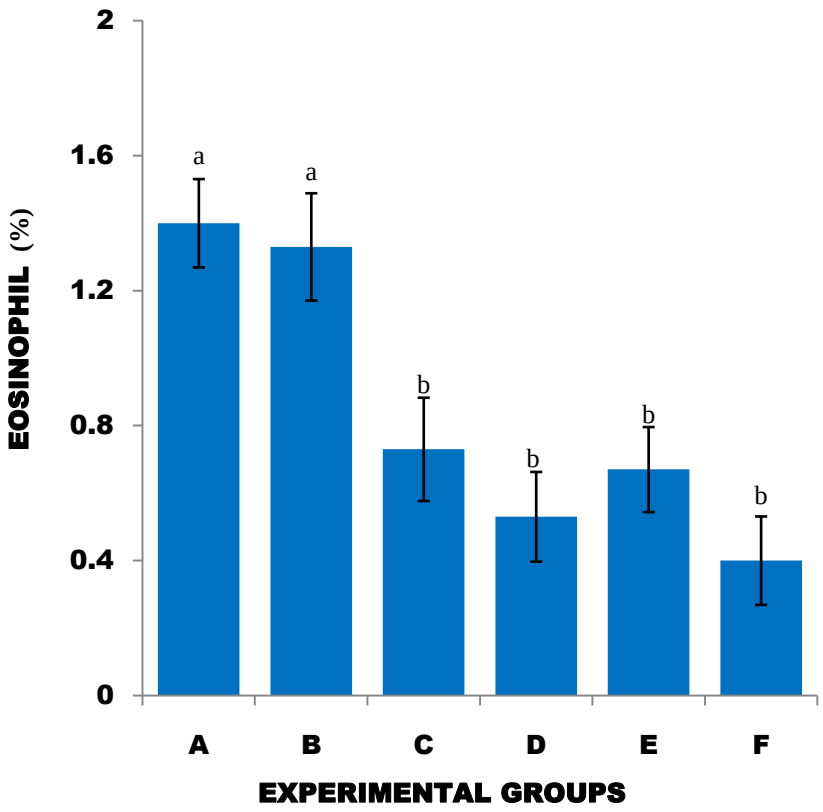

Figure10: Effects of Artesunate+Amodiaquine and Dihydroartemisinin+Piperaquine on Eosinophil Count in *P. Berghei* Infected Mice.

Bars represent mean ± Standard Error of Mean

a = significant difference from normal control at $p < 0.05$ using ANOVA and LSD post Hoc test.
b= significant difference from parasitized control at $p < 0.05$ using ANOVA and LSD post Hoc

A= infected and untreated (parasitized control)
B = infected and treated with artesunate+amodiaquine (mg/kg) for 3 days
C= infected and treated with dihydroartemisinin+ piperaquine(mg/kg) for 3 days
D= infected and treated with artesunate+amodiaquine (mg/kg) for 3 days, then allowed to recover for 28 days
E= infected and treated with dihydroartemisinin+piperaquine(mg/kg) for 3 days, then allowed to recover for 28 days
F= uninfected and untreated (Normal saline)

4.9 Effects of artesunate+ amodiaquine and dihydroartemisinin+piperaquine on monocyte count (%) in *P. berghei* infected mice

Figure 11 and Appendix 18 showed a significant difference ($P < 0.05$) in monocyte count in the infected untreated group (4.40±0.21%) and the group treated with artesunate+amodiaquine (2.27±0.22%) compared to the uninfected untreated (0.73±0.11%). Significant decrease ($P < 0.05$) was observed in the group treated with dihydroartemisinin+ piperaquine (0.47±0.21%) compared to the group treated with artesunate+amodiaquine (2.27±0.22%). A significant decrease ($P < 0.05$) in monocyte count was also observed in the group treated with artesunate+amodiaquine (2.27±0.22%), the group treated with dihydroartemisinin+piperaquine (0.47±0.21%), recovery group treated with arteunate+amodiaquine (0.53±0.13%) and the recovery group treated dihydroartemisinin+piperaquine (0.60±0.23%) compared to the infected untreated group (4.40±0. 21%). No significant difference was observed in the group treated with dihydroartemisinin+ piperaquine (0.47±0.21%), recovery group treated with arteunate+amodiaquine(0.53±0.13%) and the recovery group treated dihydroartemisinin+piperaquine for 3 days (0.60±0.23%) compared to the uninfected untreated group (0.73±0.11%).

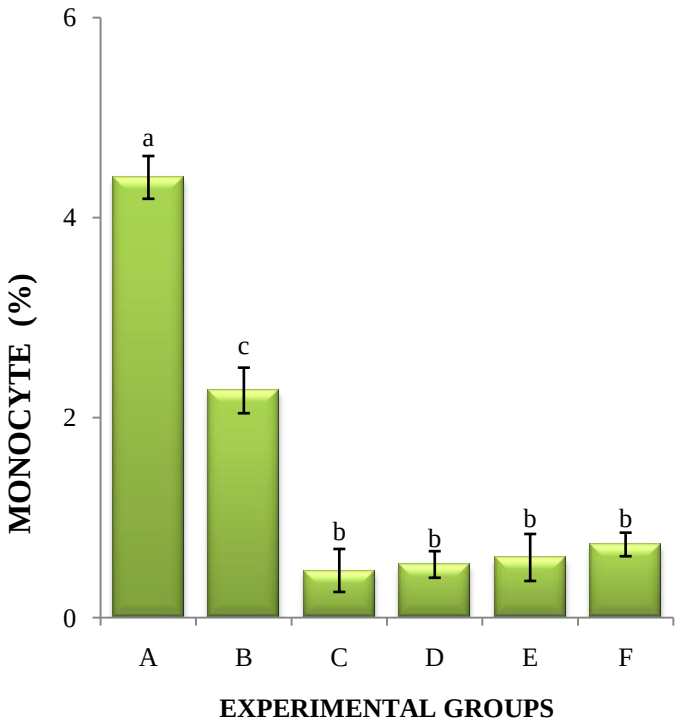

Figure 11: Effects of Artesunate+ Amodiaquine and Dihydroartemisinin+Piperaquine on Monocyte Count in *P. Berghei* Infected Mice.

Bars represent mean ± Standard Error of Mean.

a = significant from normal control at $p < 0.05$ using ANOVA and Tukey post Hoc test.
b = significant difference from parasitized control at $p < 0.05$ using ANOVA and LSD post Hoc
c = significant from parasitized and normal control at $p<0.05$

A= infected and untreated (parasitized control)
B = infected and treated with artesunate+amodiaquine (mg/kg) for 3 days
C= infected and treated with dihydroartemisinin+ piperaquine (mg/kg) for 3 days
D= infected and treated with artesunate+amodiaquine (mg/kg) for 3 days, then allowed to recover for 28 days
E= infected and treated with dihydroartemisinin+piperaquine (mg/kg) for 3 days, then allowed to recover for 28 days
F= uninfected and untreated (Normal saline)

4.10 Effects of artesunate+ amodiaquine and dihydroartemisinin+piperaquine on red blood cell count (x10^{12}/ml) in *P. berghei* infected mice

Figure 12 and Appendix 21 showed a significant difference ($P < 0.05$) in Red blood cell count in the infected untreated group (4.60 ± 0.48x10^{12}/ml) compared to the uninfected untreated group (7.82±0.84). A significant decrease was also observed in the group treated with artesunate+amodiaquine for (6.10±0.15 x10^{12}/ml) and the group treated with dihydroartemisinin+ pipiraquine (6.16±0.09 x10^{12}/ml) compared to the uninfected untreated group (7.82±0.84 x10^{12}/ml). No significant difference ($P > 0.05$) was observed between the group treated with artesunate+amodiaquine (6.10± 0.15 x10^{12}/ml) and the group treated with dihydroartemisinin+pipiraquine (6.16 ± 0.09 x10^{12}/ml). Insignificant difference ($P > 0.05$) was also observed in the recovery group treated with artesunate+amodiaquine (7.64±0.26 x10^{12}/ml) and the recovery group treated with dihydroartemisinin+piperaquine (8.15 ± 0.25 x10^{12}/ml) compared to the uninfected untreated group (7.82±0.84 x10^{12}/ml). A significant ($P < 0.05$) increase in RBC was observed in all the treated groups and their recovery groups compare to the infected untreated group.

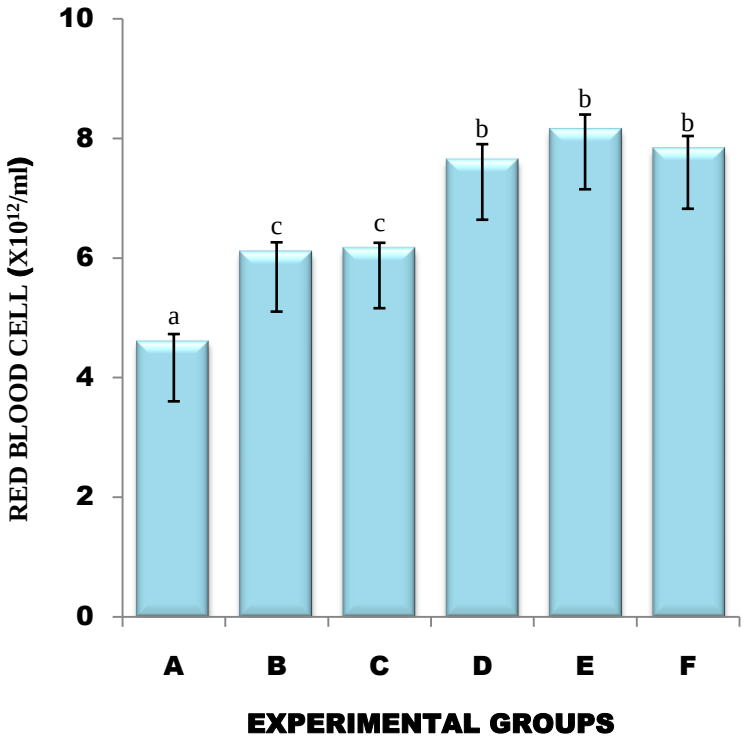

Figure: 12: Effects of Artesunate+ Amodiaquine and Dihydroartemisinin+Piperaquine on Red Blood Cell Count (RBC) in *P. berghei* Infected Mice.

Bars represent mean ± Standared Error of Mean.

a = significant difference from normal control at $p < 0.05$ using ANOVA and LSD post Hoc test.
b = significant difference from parasitized control at $p < 0.05$ using ANOVA and LSD post Hoc test
c = significant from parasitized and normal control at p<0.05

A= infected and untreated (parasitized control)
B = infected and treated with artesunate+amodiaquine (mg/kg) for 3 days
C= infected and treated with dihydroartemisinin+ piperaquine (mg/kg) for 3 days
D= infected and treated with artesunate+amodiaquine (mg/kg) for 3 days, then allowed to recover for 28 days
E= infected and treated with dihydroartemisinin+piperaquine (mg/kg) for 3 days, then allowed to recover for 28 days
F= uninfected and untreated (normal saline)

4.11 Effects of artesunate+ amodiaquine and dihydroartemisinin+piperaquine on packed cell volume (PCV) (%) in *P. berghei* infected mice.

Figure 13 and Appendix 24 showed a significant difference ($P < 0.05$) in PCV in the infected untreated (21.27 ± 1.22%) compared to the uninfected untreated group (41.33±0.61%). A significant decrease ($P < 0.05$) was also observed in the group treated with artesunate+amodiaquine for (34.47±1.00%) and the group treated with dihydroartemisinin+piperaquine (37.00±0.62%) compared to the uninfected untreated group (41.33 ± 0 .61%). No significant difference ($P > 0.05$) was observed between the group treated with artesunate+amodiaquine (34.47±1.00) and the group treated with dihydroartemisinin+piperaquine for 3 days (37.00±0.62). Packed cell volume was significantly ($p>0.05$) elevated in all the infected treated groups, and in all the recovery groups when compare to the infected untreated group. However, after recovery period, PCV level was restored in the groups treated with artesunate+amodiaquine (41.53±0.45%) and dihydroartemisinin+piperaquine (39.40 ± 0.89) to the levels that were not significantly ($P > 0.05$) different when compared to the uninfected untreated group (41.33 ± 0 .61).

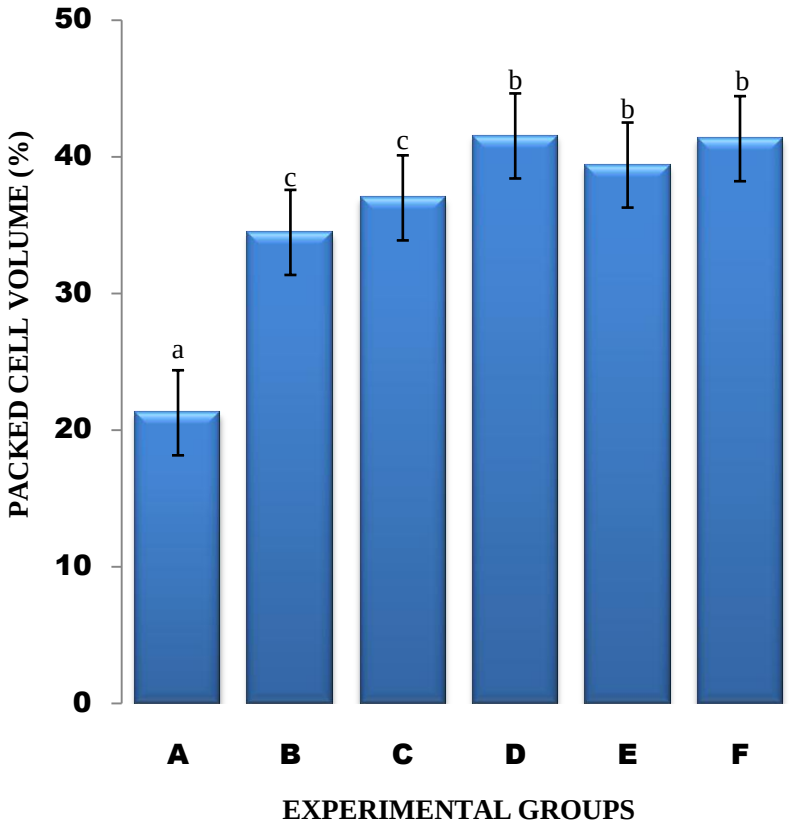

Figure: 13: Effects of Artesunate+Amodiaquine and Dihydroartemisinin+Piperaquine on Packed Cell Volume (PCV) in *P. berghei* Infected Mice.

Bars represent mean ± Standard Error of Means.

a = significantly different from normal control at $p < 0.05$ using ANOVA and LSD post Hoc test.

b = significantly different from parasitized control at $p < 0.05$ using ANOVA and LSD post Hoc test
c = significant from parasitized and normal control at p<0.05

A= infected and untreated (parasitized control)
B = infected and treated with artesunate+amodiaquine (mg/kg) for 3 days
C= infected and treated with dihydroartemisinin+ piperaquine (mg/kg) for 3 days
D= infected and treated with artesunate+amodiaquine (mg/kg) for 3 days, then allowed to recover for 28 days
E= infected and treated with dihydroartemisinin+piperaquine(mg/kg) for 3 days, then allowed to recover for 28 days
F= uninfected and untreated (normal saline)

4.12 Effects of artesunate+ amodiaquine and dihydroartemisinin+piperaquine on haemoglobin concentration (Hb) (g/dl) in *P. berghei* infected mice.

From Figure 13 and Appendix 27, there was a significant difference ($P < 0.05$) in Hb in the infected untreated group (4.56 ± 0.20g/dl) compared to the uninfected untreated group (10.93 ±0.13g/dl). No significant difference ($P > 0.05$) in Hb was observed between the group treated with artesunate+amodiaquine (7.72 ±0.14g/dl) and the group treated with Dihydroartemisinin + piperaquine (7.80±0.25g/dl), Although treatment elevated the heamoglobin level in the groups treated with artesunate+amodiaquine for 3 days (7.72±0.14g/dl), Dihydroartemisinin+ piperaquine (7.80±0.25g/dl), artesunate+amodiaquine recovery group (10.18±0.11g/dl) and Dihydroartemisinin+ piperaquine recovery group (10.42±0.12g/dl), their Hb concentration were still significantly ($P < 0.05$) lower when compare to the uninfected untreated group (10.93±0.13g/dl). However, haemoglobin level was significantly ($P > 0.05$) elevated in all the infected and treated groups and their recovery groups compared to the infected untreated group.

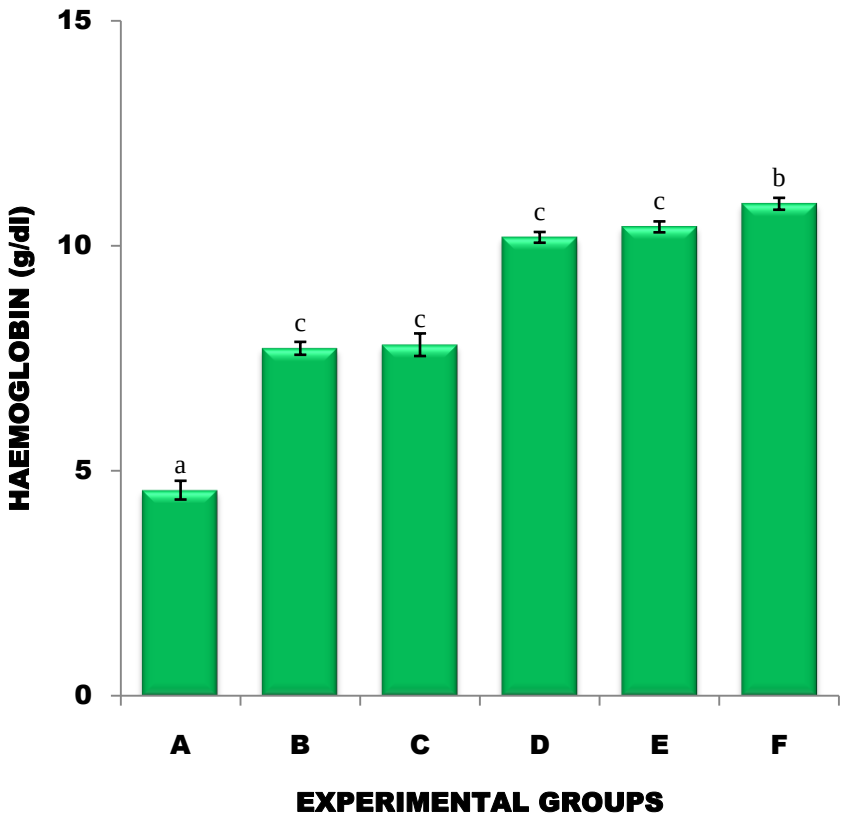

Figure 14: Effects of Artesunate+ Amodiaquine and Dihydroartemisinin+Piperaquine Haemoglobin Concentration (Hb) in *P. berghei* Infected Mice.

Bars represents mean ± Standard Error of Mean

a = significantly different from normal control at $p < 0.05$ using ANOVA and LSD post Hoc test.

b = significantly different from parasitized control at $p < 0.05$ using ANOVA and LSD post Hoc
c = significant from parasitized and normal control at p<0.05

A= infected and untreated (parasitized control)
B = infected and treated with artesunate+amodiaquine (mg/kg) for 3 days
C= infected and treated with dihydroartemisinin+ piperaquine (mg/kg) for 3 days
D= infected and treated with artesunate+amodiaquine (mg/kg) for 3 days, then allowed to recover for 28 days

E= infected and treated with dihydroartemisinin+piperaquine (mg/kg) for 3 days, then allowed to recover for 28 days F= uninfected and untreated (normal Saline)

4.13 Effects of artesunate+amodiquine and dihydroartemisin+piperaquine on body weight (g) in *P. berghei* infected mice.

Figure 15 showed a significant difference ($P < 0.05$) in initial body weight (27.87±1.82g) of the infected untreated group compare to their final body weight (24.00 ± 1.59g). No significant difference ($P > 0.05$) was observed between initial body weight (24.53 ± 0.89) and final body weight (25.00 ± 3.29g) of the group treated with artesunate + amodiaquine. A significant increase ($P > 0.05$) was observed in the initial weight (27.33 ± 1.27g) compared to final weight (30.13 ± 6.08g) in the group treated with dihydroartemisinin + piperaquine. There was also a significant increase ($P > 0.05$) in final body weight (28.80 ± 0.43g) compare to the initial body weight (24.93 ± 0.98g) in the recovery group treated with artesunate + amodiaquine . There was also a significant increase($P > 0.05$) in final body weight (30.33 ± 3.24g) compared to the initial body weight (25.87 ± 3.58g) in the recovery group treated with dihydroartemisinin + piperaquine for. There was also a significant increase ($P > 0.05$) in the final body weight (31.27 ± 1.73g) compared to the initial body weight (25.93 ± 4.38) in the uninfected untreated group.

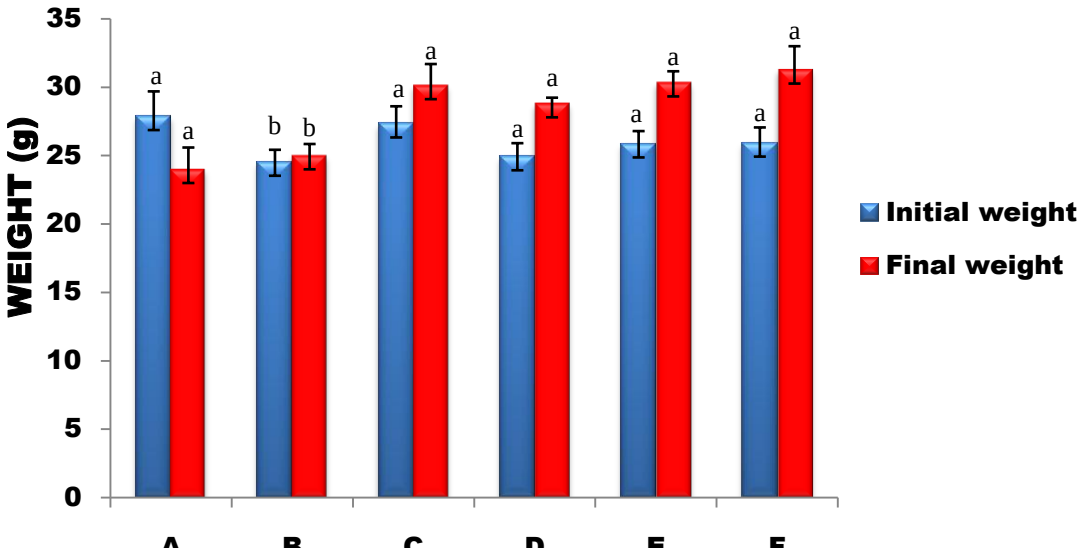

Figure 15: Effects of Artesunate+Amodiquine and Dihydroartemisin+Piperaquine on Body Weight in *P. berghei* Infected Mice.

Bars represent mean ± Standard error of mean

a = significant at $p < 0.05$ using paired sample t test

b = Not significant at $p > 0.05$ using paired sample t test

A= infected and untreated (parasitized control)
B = infected and treated with artesunate+amodiaquine (mg/kg) for 3 days
C= infected and treated with dihydroartemisinin+ piperaquine(mg/kg) for 3 days
D= infected and treated with artesunate+amodiaquine(mg/kg) for 3 days, then allowed to recover for 28 days
E= infected and treated with dihydroartemisinin+piperaquine (mg/kg) for 3 days, then allowed to recover for 28 days
F= uninfected and untreated (normal Saline)

4.14 Effects of artesunate+amodiaquine and dihydroartemisinin+piperaquine on histology of the liver in *P. berghei* infected albino mice.

Histological studies revealed some damages caused to the liver by malaria parasite infection and treatment. The liver histopathology of groups infected and untreated (plate 4) and the group infected and treated with Artesunate+amodiaquine for 3 days (plate 6) showed remarkable periportal inflammatory cells infiltration which was mixed with the presence of precursor cells showing evidence of hepatic haematopoiesis (blood cell formation) as shown in plate 30 The liver sections also had pigmentations. These lesions were minimal in the liver of the group treated with Dihydroartemisinin+piperaquine for 3 days which had widespread vacuolar degeneration of hepatocytes (plate 7). However, no remarkable changes were seen in their recovery groups except cytoplasim vacuolation which may be a fatty change in the liver as a result of increased feeding and minimal inflammatory cells infiltration (plates 8 and 9)

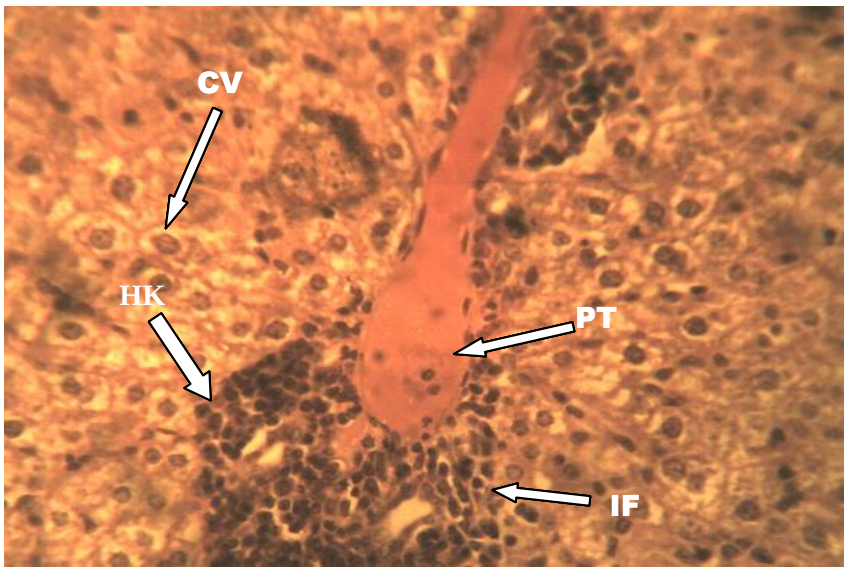

Plate 4: A section of liver from the group infected and untreated showing remarkable periportal inflammatory cells infiltration (IF), hypatrophy of the kuffer cell (HK), Haemozoin pigmentations, cytoplasmic vacuolation (CV), Portal tract (PV). H and E x400

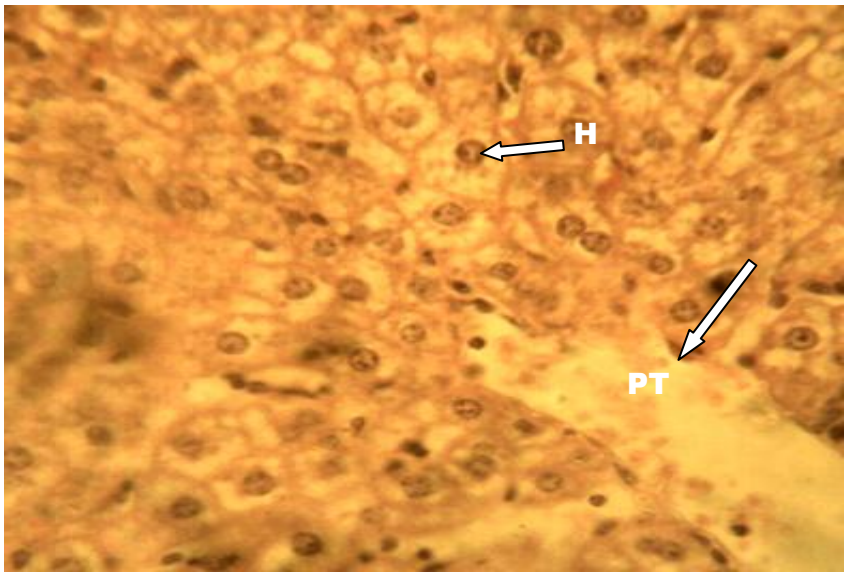

Plate 5: A section of liver from uninfected untreated group (normal liver) showing normal portal tracts (PT) and normal hepatocyte (H). H and E x400.

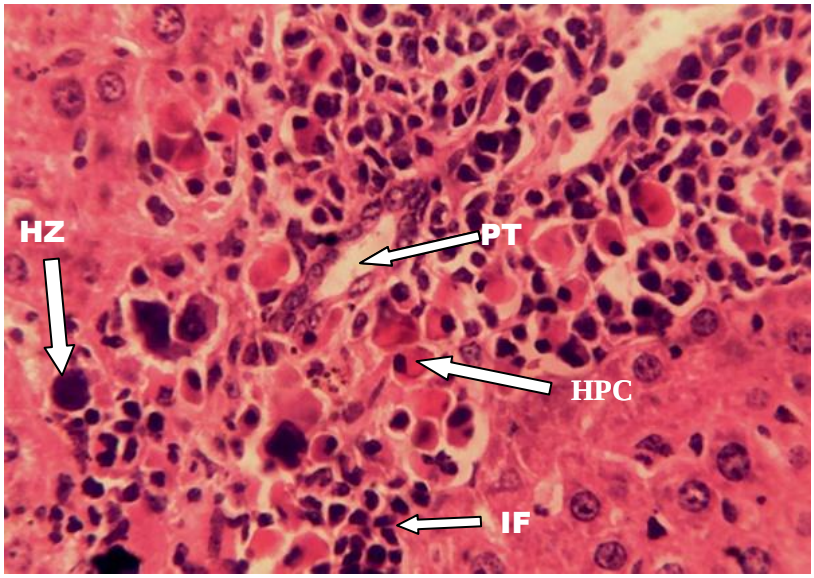

Plate 6: A section of liver from mice infected and treated with artesunate+amodiaquine for 3 days showing remarkable periportal inflammatory cells infiltration (IF), which was mixed with presence of haemopoietic precursor cells (HPC), Haemozoin pigmentations (HZ), Portal Tract (PT). H and E x400

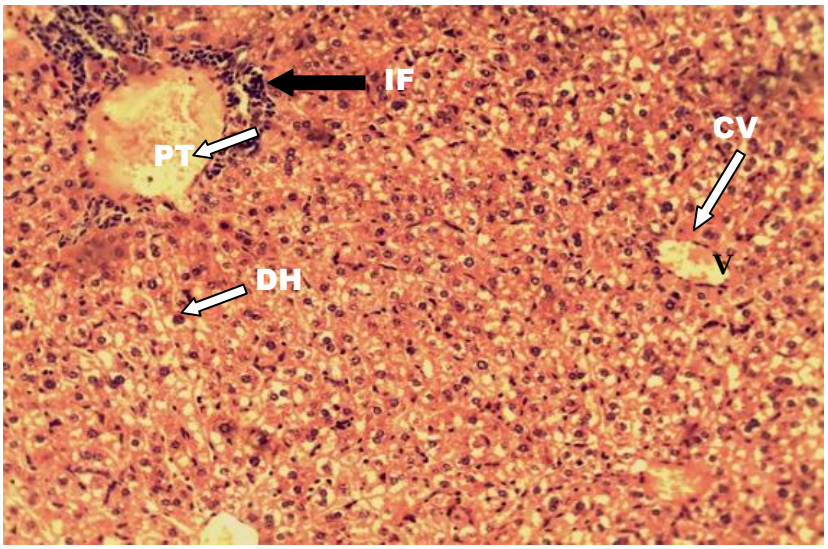

Plate 7: A section of liver from mice infected and treated with dihydroartemisinin+piperaquine for 3 days showing normal central vein (CV), minimal periportal inflammatory cells infiltration (IF) and widespread vacuolar degeneration of hepatocytes (DH), Portal Vein (PT), H and E x100.

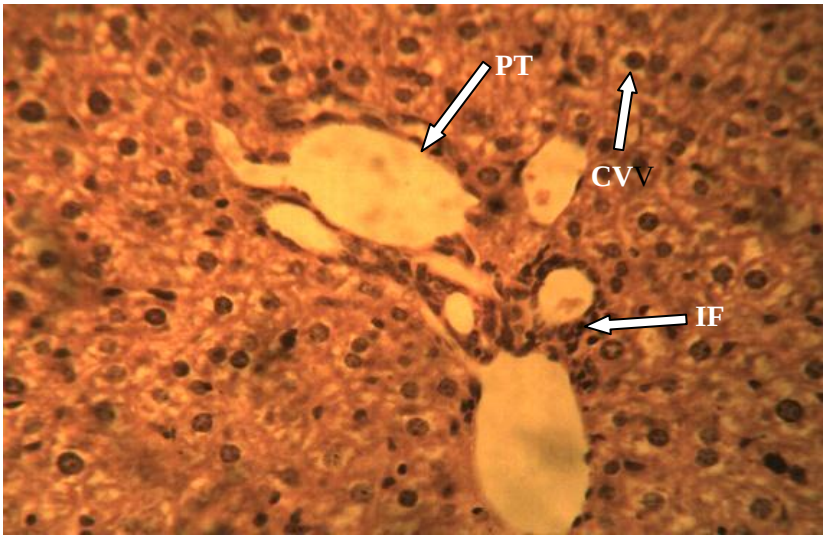

Figure 5: A section of liver from mice infected and treated with artesunate+amodiaquine for 3 days and allowed to recover for 28 days, showing Portal tract (PT), mild inflammation (IF), and Cytoplasmic Vacuolation (CV), H and E x400

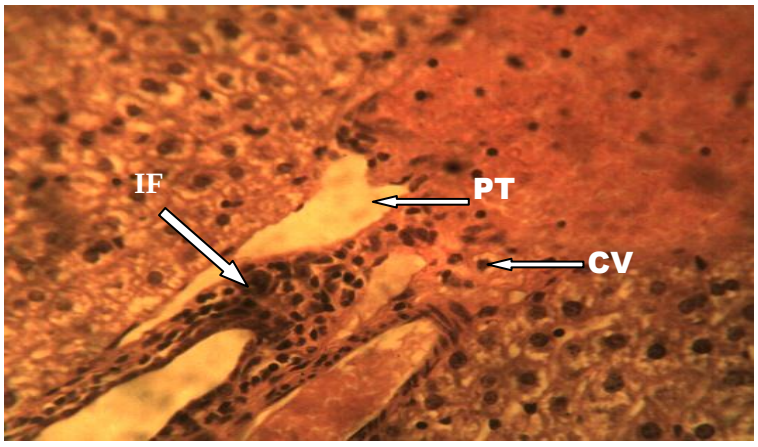

Plate 9: A section of liver from mice infected and treated with dihydroartemisinin+piperaquine for 3 days and allowed to recover for 28 days, showing cytoplasmic vacuolation (CV), minimal inflammatory cells (IF), and normal portal tract (PT). H and E x400.

4.15. Effects of Artesunate+amodiaquine and Dihydroartemisinin+piperaquine on the histology of the kidney in *P.berghei* infected albino mice.

There was inflammation of the renal pelvis in the infected untreated group **(**Plate 10**)**, and the group treated with dihydroartemisinin+piperaquine (Plate 13). The kidney tissues of all the recovery groups showed no histopathological change (Plates 14 and 15).

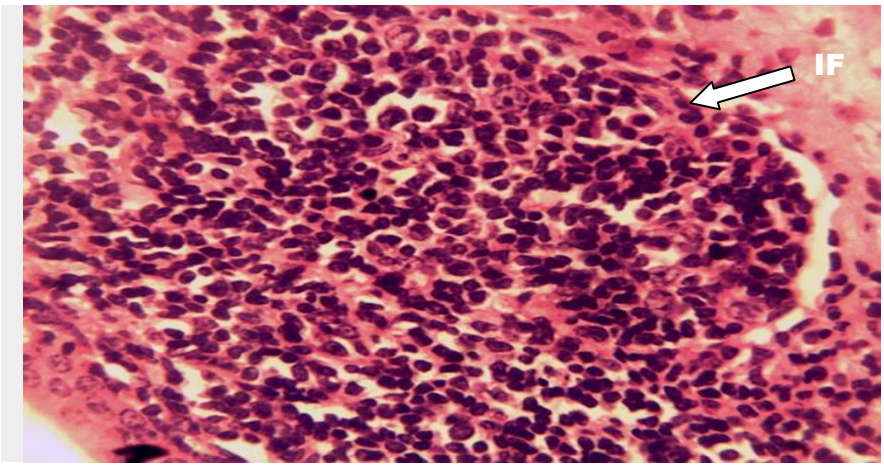

Plate 10: A section of kidney from mice infected and untreated showing severe inflammation of the renal medulla/pelvis (IF). H and E x400.

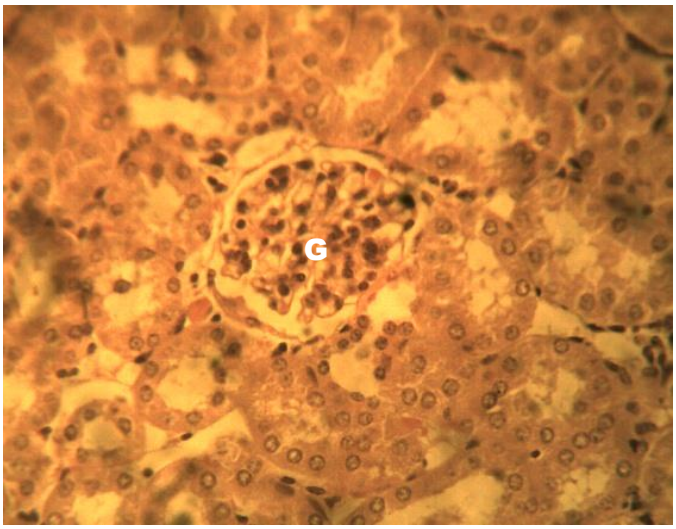

Plate 11: A section of kidney from the uninfected untreated group (normal control), showing normal Glomerulus (G) and tubules. H and E x400

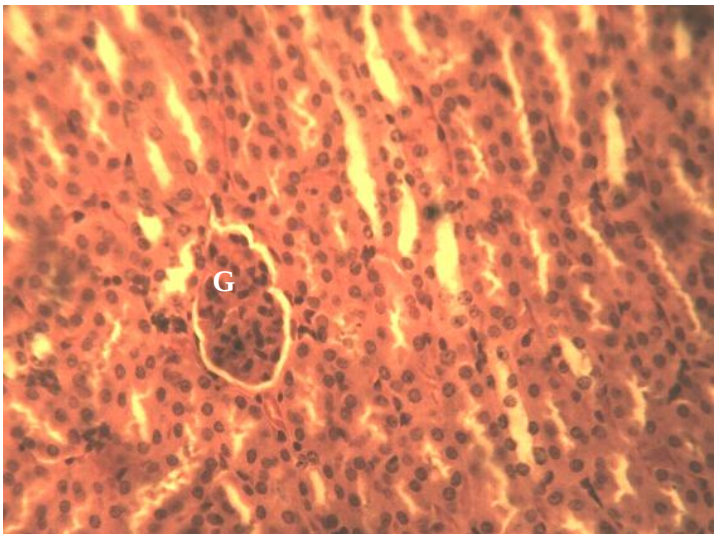

Plate 12: A section of kidney from mice infected and treated with artesunate+amodiaquine for 3 days showing normal Glomerulus (G) and tubules. H and E x400

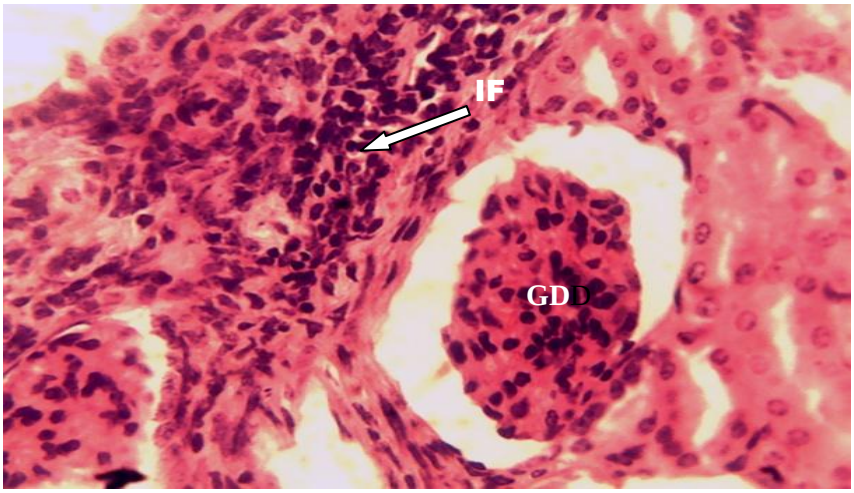

Plate 13: A section of the kidney from the group infected and treated with dihydroartemisinin+piperaquine for 3 days showing inflammation of the renal medulla/pelvis (IF) and glomerular degeneration (GD) H and E x400

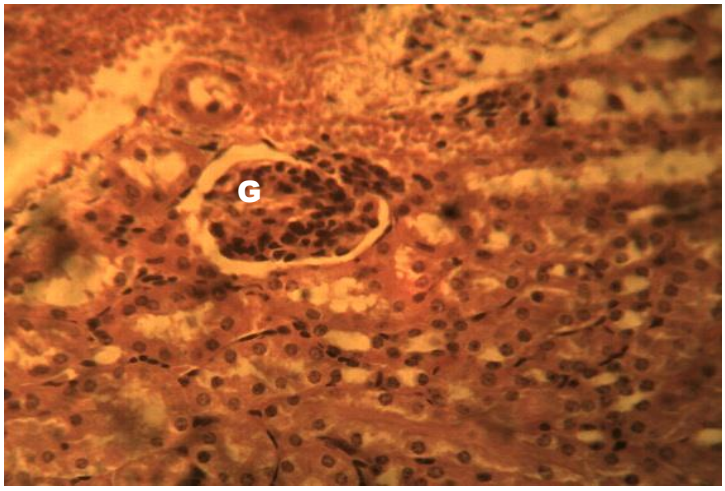

Plate 14: A section of the kidney tissues from the group infected and treated with artesunate+amodiaquine for 3 days and allowed to recover for 28 days, showing normal glomerulus (G) and tubules. H and E x400.

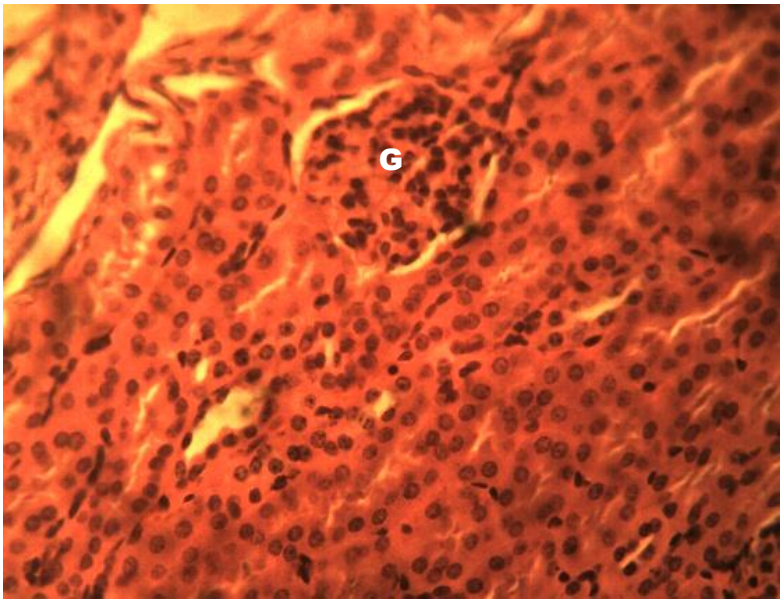

Plate 15:A section of liver from the group infected and treated with and dihydroartemisinin +piperaquine and allowed to recover for 28 days showing normal glomerulus (G) and tubules. H and E x400.

4.16. Effects of Artesunate+amodiaquine and Dihydroartemisinin+piperaquine on the histology of the spleen in *P.berghei* infected albino mice.

In the spleen, hemozoin pigments and macrophages were widely seen in the splenic sinusoids, in the infected untreated group, groups treated with artesunate+amodiaquine

and the group treated with dihydroartemisinin+piperaquine. There was a loss of the typical structure of the germinal center which was in these groups had apoptotic lymphocytes with tinged macrophage. Widespread megakaryocytes and other haemopoietic precursor cells within the red pulp where also discovered in these groups (Plates 16, 18 and 19). However, their recovery groups showed traces of hemozoin pigments in the splenic sinusoids, apoptotic lymphocytes with tinged macrophages in the germinal center and presence of megakaryocyets. (Plate 20 and 21).

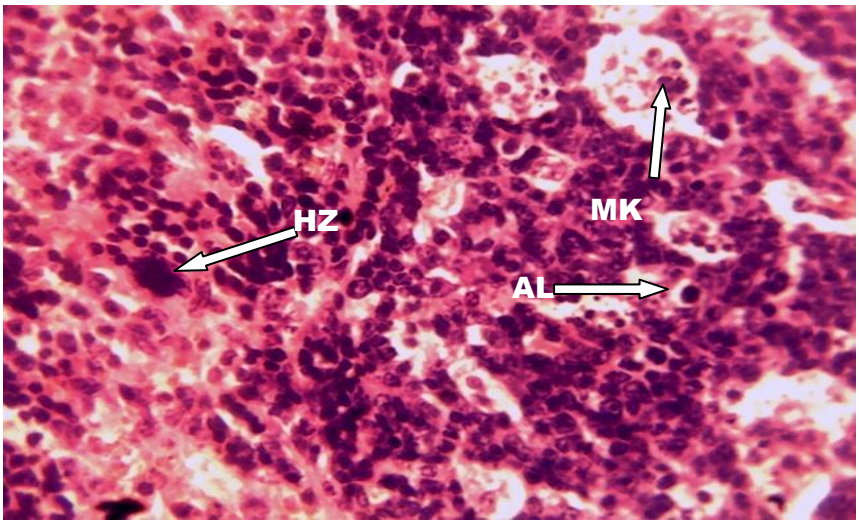

Plate 16: A section of the spleen from the infected untreated group, showing haemozoin pigments (HZ), apoptotic lymphocytes with tinged macrophages in the germinal centres (AL), Widespread megakaryocytes (MK), and other haemopoietic precursor cells within the red pulp H and E x400.

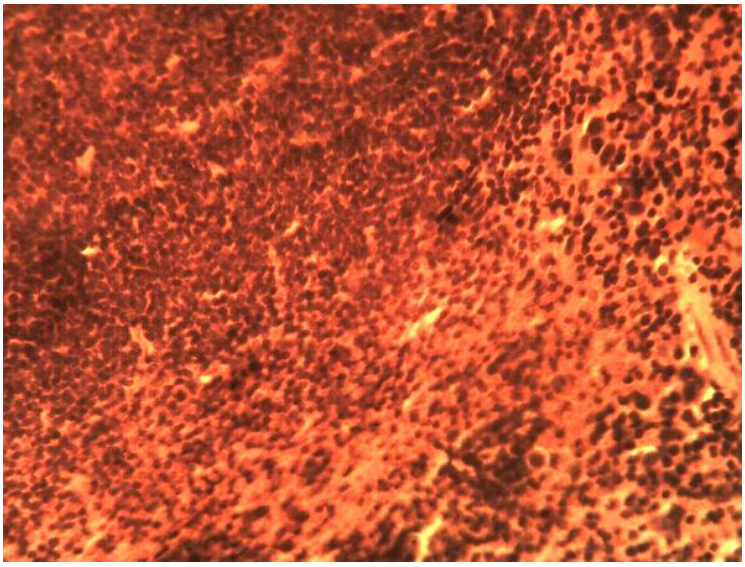

Plate 17: A section of the spleen from the infected untreated group (normal control) showing normal spleen architecture.

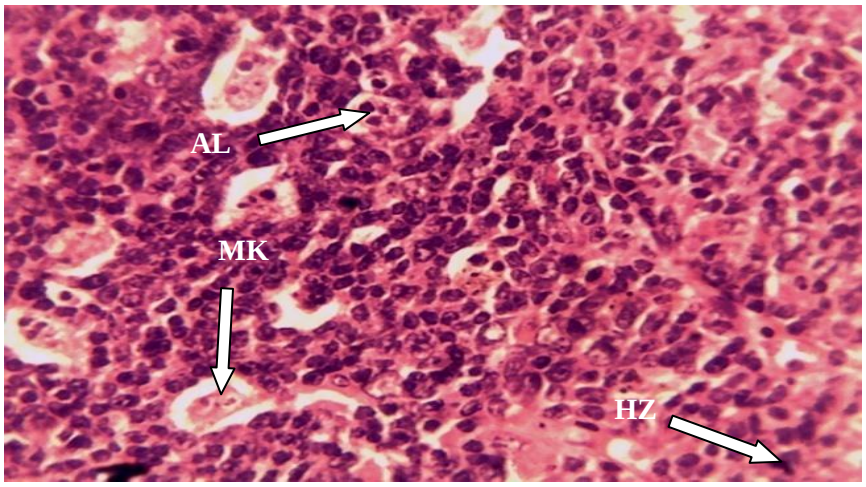

Plate 18: A section of the spleen from the group infected and treated with artesunate+amodiaquine for 3 days, showing haemozoin pigments in the splenic sinusoids within the red pulp (HZ) and apoptotic lymphocytes with tinged macrophages in the germinal centers (AL). There were also widespread megakaryocytes (MK) and other haemopoietic precursor cells within the red pulp. H and E. X400.

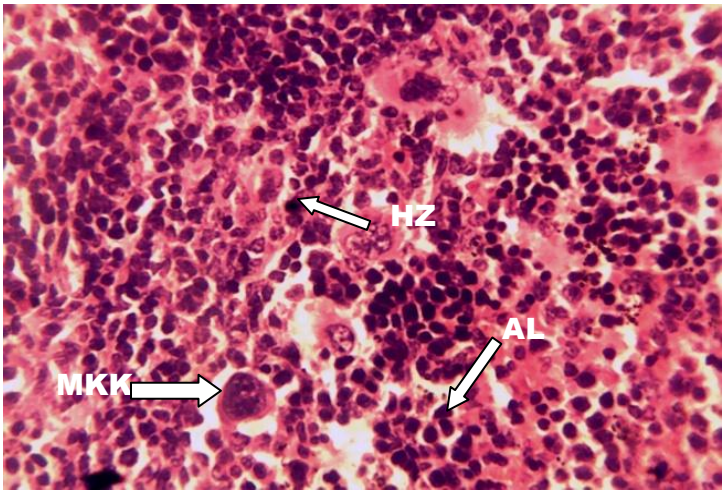

Plate 19: A section of the spleen from the group infected and treated with Dihydroartemisinin+ piperaquine for 3 days, showing haemozoin pigments in the splenic sinusoids within the red pulp (HZ) and apoptotic lymphocytes with tinged macrophages in the germinal centers (AL). There were also widespread megakaryocytes (MK) and other haemopoietic precursor cells within the red pulp. H and E. x400

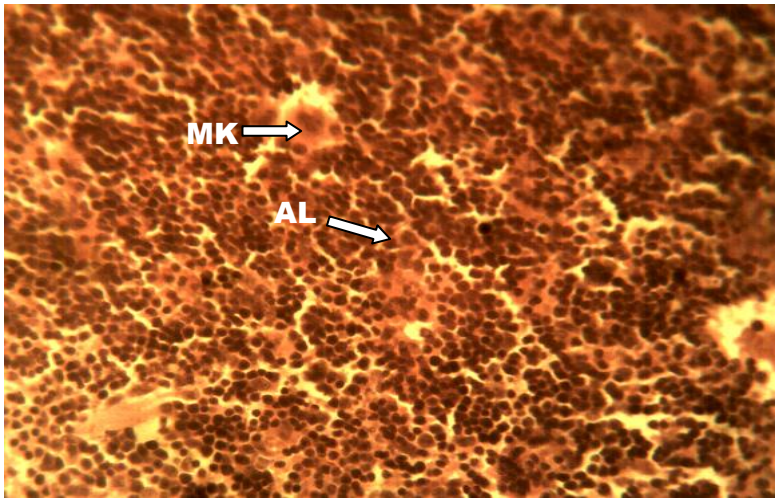

Plate 20: A section of the spleen from the group infected and treated with artesunte+amodiaquine for 3 days, then allowed to recover for 28 days, showing traces of haemozoin pigments in the splenic sinusoids, apoptotic lymphocytes with tinged macrophages in the germinal centres (AL), and presence of megakaryocytes (MK). H and E x 400

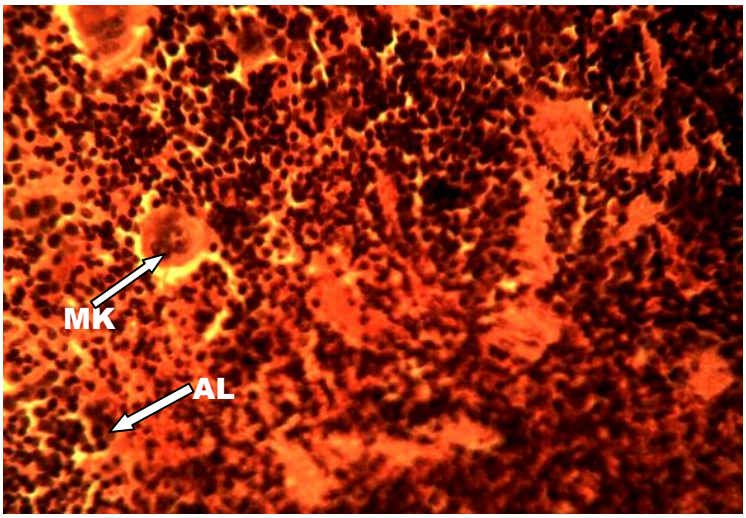

Plate 21: A section of the spleen from the group infected and treated with dihydroartemisinin+piperaquine and allowed recovering for 28 days showing traces of haemozoin pigments in the splenic sinusoids and apoptotic lymphocytes with tinged macrophages in the germinal centers (AL) and presence of megakaryocytes (MK). H and E. x400

4.17. Effects of Artesunate+amodiaquine and Dihydroartemisinin+piperaquine on the histology of the lungs in *P.berghei* infected Swiss albino mice.

Pigment accumulations occurred in the parenchymal of the lungs and pulmonary emphysema was seen in the lungs of the group infected and untreated, group treated with artesunate+amodiaquine and the group treated with dihydroretamisinin+piperaquine (plates 22, 24 and 25). No remarkable histopathological change was observed in the recovery groups (plates 26 and 27).

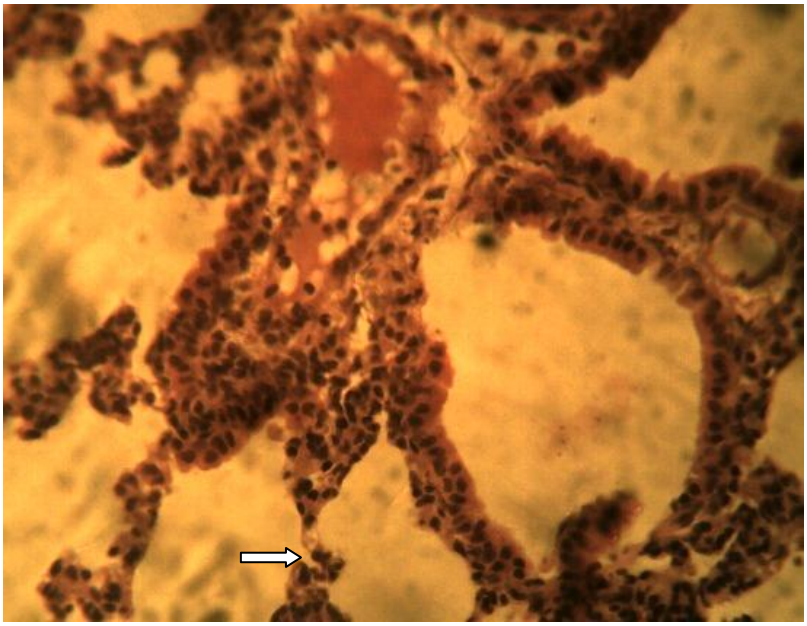

Plate 22: A section of the lung from the infected untreated group, showing Pigment accumulations in the parenchymal of the lungs and pulmonary emphysema (arrow). H and E. x400

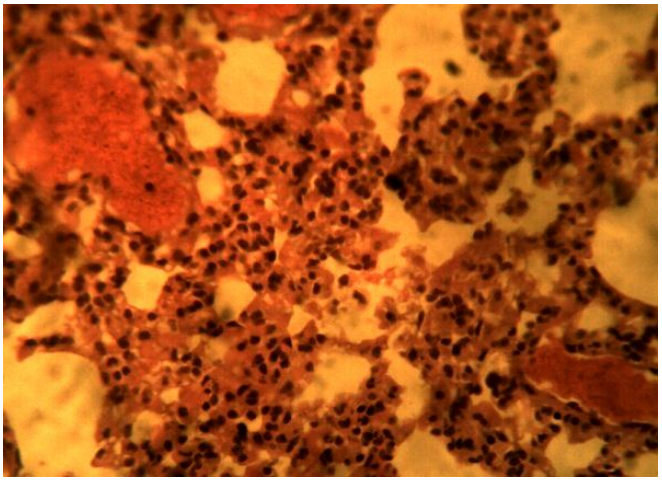

Plate 23: A section of lung from mice uninfected and untreated (normal control). No histopathological change was observed. H and E x400

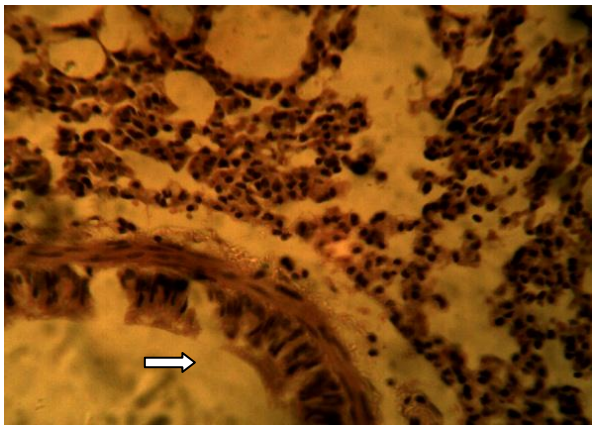

Plate 24: A section of the lung from the group infected and treated with artesunate+amodiaquine for 3 days showing pigment accumulations in the parenchymal of the lungs and pulmonary emphysema (arrow).

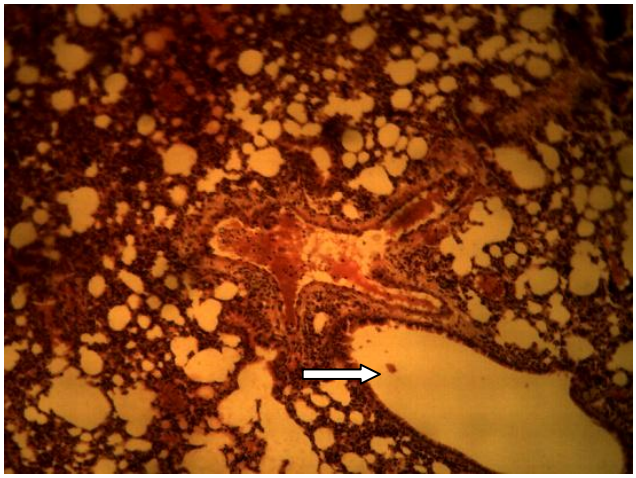

Plate 25: A section of the lung from the group infected and treated with dihydroartemisinin+piperaquine for 3 days, showing pigment accumulations in the parenchymal of the lungs and pulmonary emphysema (arrow).

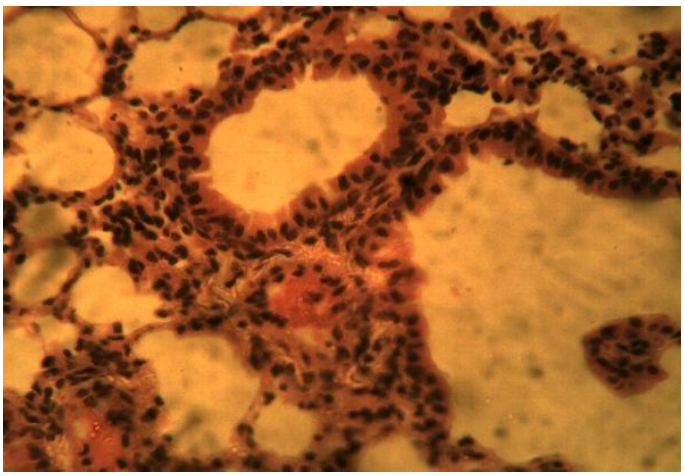

Plate 26: A section of the lung from the group infected and treated with artesunte+amodiaquine for 3 days, then allowed to recover for 28 days. No remarkable histopathological change was observed.

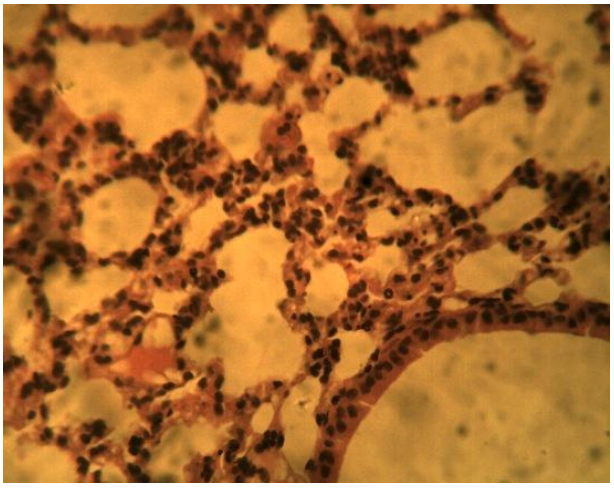

Plate 27: A section of lung from mice infected and treated with dihydroartemisinin+piperaquine and allowed to recover for 28 days. No remarkable histolopathological change was observed.

CHAPTER FIVE

DISCUSSION

From the result of this study, rapid onset of malaria occurred within 3 days following inoculation with the *Plasmodium* parasite. The parasitaemia level increased on day 8 postinfection which is in line with the view that parasitaemia increases progressively after inoculation or infection until the point of death in the absence of suitable treatment (Trampuz *et al.*, 2003; Breman and Egan, 2001). A high degree of parasitaemia with ultimate death in the infected untreated group suggested severe degree of infection in this model. Death is also the ultimate complication of many *P. falciparum* cases in human. The total clearance of the malaria parasite and non-recrudescence observed in this study after treatment with artesunate+amodiaquine and dihydroartemisinin + piperaquine showed that the drugs are still effective against malaria parasites that have not developed resistance yet. This agreed with the report of Whegang *et al* (2010) who reported that Artesunate+amodiaquine is as effective as other ACTs. Ewenighi *et al* (2013) also discovered that Artesunate + amodiaquine therapy have higher parasitic clearance rate compared to the amodiaquine therapy in *P. berghei* infected mice.

Changes in total white blood cell count and differential white blood cells count observed in this present study reflected the response of the mice to *P. berghei*

infection. White blood cells (WBC) are important part of body's defense which made up the body immune system. The elevated total white blood cell count, lymphocytes and monocyte counts in the infected untreated and infected treated groups are as a result of the body's attempt to fight the infection. The observed increase was due to abnormalities introduced by the malaria parasite, following an infection; the body triggers an alarm to activate body's natural defense system. Upon identification of the threat, white blood cells start replication and production of more number of defense cells to counter and fight off the infection. So, eventually number of white blood cells grows by many folds in the body, which can be found in a blood test. The results showed a higher number of leukocytes, predominantly of the lymphocytes and monocytes, being a major feature in malaria infected individuals. Lymphocytes, particularly T- cells, and monocytes play a major role in immunity to *Plasmodium* malaria. Treatment of the infected groups with Artesunate+amodiaquine and dihydroartemisinin+piperaquine restored the indices altered during the infection to a normal level. However, total white blood cell count was not completely restored during the period of recovery. This indicated that restoration of the total white blood cell count to a normal level after malaria infection and treatment with artesunate+amodiaquine and dihydroartemisinin+piperaquine for three days may take longer than 28 days. Lymphocyte, neutrophils and monocytes level was restored though not completely

during recovery. This result sowed that *Plasmodium* infection and subsequent treatment with ACTs have a reversible alteration in the white blood cell count and white blood cell differential. This is in line with the study of Kareem *et al* (2014). In this study, massive lost in the total number of red blood cells (RBC) and rapid decrease in the haemoglobin (Hb) and packed cell volume (PCV), in *P. berghei* infected mice showed a characteristic sign of severe anaemia which is one of the major clinical manifestations of severe malaria in human. Reductions in these parameters were more evident in the infected untreated group than in the infected treated groups. This is in line with the reports of Koltas et al (2007) and Sowunmi et al (2009). Ovuakporage (2011) also reported that malaria parasite reduced Red blood cell count, Packed cell volume (PCV) and Haemoglobin concentration (Hb). Liver and spleen were pigmented, congested and swollen from the accumulation of the malaria pigment, haemozoin, which led to discolouration. This finding is in line with that of Soniran et al 2012 and Basir et al 2012. Haemozoin (malaria pigment) is a disposal product formed from the digestion of red blood cells by malaria parasites, it is released into the blood stream after rupture of the infected red blood cells at the end of each parasite replication cycle and it is observed in either the cytoplasm or outside hepatocyte and kupffer cells of the liver as black or brownish granules. It is also found in the spleen especially in the red pulp (Bates and Bedu-Addo, 1997). However, this molecule has been shown to be actively engulfed by

phagocytes, and to modulate microphage functions, indicating that hemozoin potentially contributed to the development of malaria immunopathogenesis (Francis et al., 1997; Sherry et al., 1995). In this study, it was discovered that the liver and the spleen were still dark after 28 days recovery period. It has been reported that haemozoin remains in the body for long period and appeared to be actively redistributed (Frita et al., 2012). It disappeared from the liver over 270 days after parasite clearance, while the amount of haemozoin in the spleen increased 8- fold (Frita et al., 2012). If haemozoin accumulated in the tissues as shown in this study and continues to be biologically active, it may have tremendous implications for the host in terms of host immunity to secondary infections, which are very common in malaria-endemic areas (Millington et al., 2006).

Histopathology of the liver revealed remarkable periportal inflammatory cells infiltration including the presence of haemopoietic precursor cells, deposition of malaria pigment (haemozoin) and hypertrophy of the kupffer cells in the sinusoids in both infected untreated and infected treated groups. These findings are in line with the reports of Onyije and Hart (2012), Al-Ani et al (2013) and Basir et al (2012). Inflammation cells infiltration is the term used to denote single or multiple, focal, randomly distributed aggregates of inflammatory cells that are seen in the liver as a background lesion. The presence of haemopoietic precursor cells

observed in the liver is an indication of extramedullary haemopoiesis (blood cell formation in the liver). Extramedullary hematopoiesis is the proliferation of haematopoietic cells (Those cells responsible for producing red blood cells, white blood cells, and platelets) outside the bone marrow in response to the production of too few blood cells to satisfy the body's demand (Choi et al., 2004).This insufficient production is caused by either bone marrow replacement disease (myelofibrosis or chronic myelogenous leukemia) or hemolytic anemia. (Choi et al., 2004), where extramedullary haematopoiesis involves an organ, there is usually radiographic evidence of its enlargement (Choi et al., 2004). This may contribute to liver and spleen enlargement observed in this study. This study also documented hypertrophy of the kupffer cells in the sinusoids. Kupffer cells are macrophages that are attached to the luminal surface or inserted in the endothelial lining of hepatic sinusoids. Kupffer cells play a key role in host defense by removing foreign, toxic and infective substances from the portal blood and by releasing beneficial mediators (Chua et al., 2013). The fatty change was observed in the group treated with dihydroartemisinin+piperaquine for 3 days and in all the recovery groups. Fatty change implies the presence of multiple small droplets of fat within the cytoplasm of cells without nuclear displacement. Fat accumulates due to the inability of the cell to metabolize fat. When the process of fat metabolism is disrupted, the fat can accumulate in the liver in excessive amounts,

thus resulting in a fatty liver (Reddy and Rao, 2006). Fatty change is often associated with metabolic disturbances, excess consumption of a diet containing foods with a high proportion of calories coming from lipids (Chalasani, 2012) and or more likely a reflection of toxicity, possibly involving mitochondrial disturbances. The minimal fatty change noted in this study was similar to a previous finding (Whitten et al., 2011). However, all the hepatotoxicities observed in the infected treated groups were not observed in their recovery groups except presence of fats which may be indication of increased feeding. Therefore, malaria infection and its treatment with artesunate+amodiaquine and dihydroartemisinin +piperaquine induced reversible effects in the liver of mice. Moreover, the recovery group of dihydroartemisun+piperaquine showed focal areas of inflammatory reaction, this was regarded as a delayed toxicity effect of the drug. The liver is susceptible to these toxicities because all the foreign substances and drugs are metabolized and inactivated in the liver. The organ is also involved during the hepatic stage of malaria where malaria sporozoites developed into merozoites (Adachi et al., 2001).

In the spleen, haemozion (malaria pigments) was also observed in the infected groups. This organ is the site for the breakdown and removal of abnormal or worn-out red blood cells. Therefore, the spleen also contributed to the accumulation of hemozion pigments molecules which was also noticed in the liver of the infected

groups. The widespread of malarial pigments was found to be consistent with the elevated parasitaemia level in the infected mice; higher pigmentation could further impair the macrophage function (Helegbe et al., 2011) and trigger the host immune system to release more cytokines (Turrini et al., 1993). The release of pro-inflammatory cytokines may have caused splenic tissue abnormalities as observed in the treated mice. Loss of the typical structure of the germinal center was also observed in the spleen of all the infected groups. This finding is in line with the report of Basir et al (2012). Apoptotic lymphocytes with tinged macrophages in the germinal centers were observed in the spleen. Apoptosis is characterized by shrinkage of individual lymphocytes, condensation of nuclear chromatin, and fragmentation of apoptotic cells into membrane-bound bodies (apoptotic bodies, which are subsequently phagocytized by macrophages (tingible body macrophages). There were also widespread megakaryocytes and other hamopoeitic precursor cells within the red pulp in all the infected groups. Megakaryoblast is a precursor cell to a megakaryocyte during haematopoiesis (the process involved in the formation of blood cellular components). The presence of haemopoietic precursor cells observed in the spleen is an indication of extramedullary haemopoiesis as found in the liver. Extramedullary hematopoiesis is the formation and development of blood cells outside the medullary spaces of the bone marrow (Johns and Christopher, 2012). It occurs most often in the spleen in association

with degenerative and inflammatory conditions, including lymphoid hyperplasia, hematomas, and thrombosis. (Ballegeer et al., 2007). This is in line with the report of Soniran et al (2012). However, traces of haemozoin pigments in the splenic sinusoids were observed after the recovery period.

In the kidney, inflammation of the renal pelvis was observed in the infected untreated group and the group treated with dihydroartemisinin+piperaquine. Inflammation is a vital part of the body's immune response. Inflammation of the renal pelvis is most commonly associated with an infection. It was hypothesized that the release of malaria antigens activates monocyte cells, to release proinflammatory cytokines and activate cell-mediated response, causing renal problems (Barsoun, 1998). Glomerular degeneration observed in this study had been reported by Danladi et al (2013) during artesunate treatment in albino rat. The kidneys provide the common pathway for the excretion of many drugs and their metabolites, and hence are frequently subjected to high concentrations of potentially toxic substances. Drugs and their metabolites are taken up selectively and concentrated by the renal tubular cells before excretion into urine, so high intracellular concentrations are attained, particularly in the renal medulla, which has relatively little vasculature compared with the cortex. As a result, direct toxic damage occurs, generally affecting the renal, tubular cells and renal papillae.

In the lungs, accumulation of malaria pigment (haemozoin) in the parenchymal and pulmonary emphysema was observed. No remarkable histological alteration was observed in all the recovery groups.

In this study, the body weights of the infected untreated and infected treated mice were monitored in other to know the effects of malaria infection and treatment on factors such as food and water intake, metabolism and gut function. The infected treated groups and their recovery groups progressively gained weight until the end of the experiment whereas a progressive decrease in body weight was observed in the infected untreated group. The decrease in body weight of the infected untreated mice was clearly evident from the third day post infection, and presumably due in part to the decrease in food intake. Decrease in body weight may also be the consequences of disturbed metabolic function and hypoglycaemia that has been reported to be associated with malaria infection (WHO, 2011).

5.1 CONCLUSION

Malaria is one of the causes of death and illness in children and adults, mainly in tropical countries. Increasing parasite resistance and failure of single drug treatment of malaria in many endemic countries has led to a widespread promotion of Artemisinin-based combination therapy. Artemisinin derivatives are effective against Plasmodium parasite. Combination therapies consisting of

artesunate+amodiaquine and dihydroartemisinin+piperaquine have better parasite clearance and efficacies as have been revealed by this study. Moreover, it was observed that anaemia is one of the most common complications of malaria infection and treatment. Plasmodium infection and bone marrow dysfunction contributed to anemia during malarial infection. However, treatment with artesunate+amodiaquine and dihydroartemisinin+piperaquine ameliorated anaemia in the treated groups and restored blood parameters to a normal level within 28 days after treatment. This study also demonstrated that malaria infection and treatment with artesunate+amodiaquine and dihydroartemisinin+piperaquine was toxic to the liver, kidney, and spleen even at normal doses, although there were signs of recovery after 28 days, new toxicities were observed during recovery period which may be regarded as delayed toxicity. Finally, liver, kidney and spleen were more affected in this study than the lungs. Dihydroartemisinin/piperaquine was more toxic to the kidney than Artesunate/amodiaquine which was more toxic to the liver.

5.2 RECOMMENDATIONS

To avoid the damages done by malaria parasite and its treatement with artemisinin –based combination therapies, it is therefore recommended that-

i. prompt use of ACTs will reduce the burden of malaria-associated anaemia and organ toxicities initiated by Plasmodium parasite.

ii. treatment of malaria with Artesunate+amodiaquine and dihydroartemisinin+ piperaquine may contribute to malaria associated anaemia, care should therefore be taken to follow up the treatment with blood building agent.

iii. drug users should be educated with emphasis on the adverse effects when drugs are abused which will help to reduce the abuse of drugs in developing countries.

iv. antimalarial drugs artesunate+amodiaquine and dihydroartemisinin+ piperaquine should be cautiously used in malaria patients who have the previous history of liver and kidney diseases.

v. Care should be taken when the drugs are used as curative agent or as prophylaxis.

vi. more efforts should be directed towards improvement in the use of ACTs for management of malaria.

vii. high level of personal malaria awareness and individual application of protective measures like the use of treated mosquito net are critical for reducing malaria infection rates.

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APPENDIX

APPENDIX 1

WHITE BLOOD CELL COUNT

	REPLICATES			
GROUPS		I	II	III
A	1	14. 8	19.3	17.1
	2	14.7	20.4	17. 6
	3	14.5	16. 6	15. 6
	4	14. 9	20.4	17.7
	5	14. 8	14. 9	15. 6
B	1	13.2	11. 8	12.5
	2	13.0	16. 8	14. 9
	3	13.1	10.1	11. 6
	4	12. 8	13.3	13.0
	5	12.7	12.0	12.3
C	1	10. 6	12.0	11.3
	2	10.5	12. 9	11.7
	3	10.4	10.0	10.2
	4	10. 9	6.1	8.5
	5	10. 8	10.7	10.7
D	1	12.4	11.3	11. 8
	2	12.5	5.4	6. 9
	3	7.0	9.7	8.3
	4	7.7	7. 8	7.7
	5	9.2	5.4	6. 6
E	1	9.1	7.0	8.0
	2	7.4	6. 5	6. 9

	3	7.5	7.2	7.3
	4	7.7	7. 8	7.7
	5	9.2	5.4	6. 6
F	1	7. 9	5.2	6.5
	2	8.5	6. 8	7. 6
	3	8.1	8. 6	8.3
	4	6. 8	7. 9	7.3
	5	7. 9	6.7	7.3

APPENDIX 2

Descriptives statistics and Anova of white blood cell count

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
A	15	16.547	2.1172	.5467	15.374	17.719	14.5	20.4
B	15	12.873	1.5031	.3881	12.041	13.706	10.1	16.8
C	15	10.487	1.5615	.4032	9.622	11.351	6.1	12.9
D	15	8.760	2.3494	.6066	7.459	10.061	5.4	12.5
E	15	7.653	.6675	.1723	7.284	8.023	6.5	9.1
F	15	7.427	.9106	.2351	6.922	7.931	5.2	8.6
Total	90	10.624	3.6162	.3812	9.867	11.382	5.2	20.4

ANOVA of white blood cell count

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	940.180	5	188.036	70.625	.000
Within Groups	223.647	84	2.662		
Total	1163.826	89			

APPENDIX 3

Post Hoc test for white blood cell count

Least significant difference(LSD)

(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	3.6733*	.5958	.000	2.488	4.858
	C	6.0600*	.5958	.000	4.875	7.245
	D	7.7867*	.5958	.000	6.602	8.972
	E	8.8933*	.5958	.000	7.708	10.078
	F	9.1200*	.5958	.000	7.935	10.305
B	A	-3.6733*	.5958	.000	-4.858	-2.488
	C	2.3867*	.5958	.000	1.202	3.572
	D	4.1133*	.5958	.000	2.928	5.298
	E	5.2200*	.5958	.000	4.035	6.405
	F	5.4467*	.5958	.000	4.262	6.632
C	A	-6.0600*	.5958	.000	-7.245	-4.875
	B	-2.3867*	.5958	.000	-3.572	-1.202
	D	1.7267*	.5958	.005	.542	2.912
	E	2.8333*	.5958	.000	1.648	4.018
	F	3.0600*	.5958	.000	1.875	4.245
D	A	-7.7867*	.5958	.000	-8.972	-6.602
	B	-4.1133*	.5958	.000	-5.298	-2.928
	C	-1.7267*	.5958	.005	-2.912	-.542
	E	1.1067	.5958	.067	-.078	2.292
	F	1.3333*	.5958	.028	.148	2.518
E	A	-8.8933*	.5958	.000	-10.078	-7.708
	B	-5.2200*	.5958	.000	-6.405	-4.035
	C	-2.8333*	.5958	.000	-4.018	-1.648
	D	-1.1067	.5958	.067	-2.292	.078
	F	.2267	.5958	.705	-.958	1.412
F	A	-9.1200*	.5958	.000	-10.305	-7.935
	B	-5.4467*	.5958	.000	-6.632	-4.262
	C	-3.0600*	.5958	.000	-4.245	-1.875
	D	-1.3333*	.5958	.028	-2.518	-.148
	E	-.2267	.5958	.705	-1.412	.958

*. The mean difference is significant at the 0.05 level.

APPENDIX 4
PERCENTAGE LYMPHOCYTE

GROUPS	REPLICATES		
	I	II	III
A	83 80 84 75 79	74 70 75 72 78	79 75 80 74 79
B	80 80 79 75 74	74 79 80 77 78	77 79 79 76 76
C	80 80 79 88 86	80 75 90 81 87	80 77 84 84 86
D	67 74 74 72 59	74 64 63 69 59	70 73 68 70 59
E	71 70 56 78 71	71 70 86 75 77	71 70 70 77 74
F	64 72 60 54 54	60 75 73 68 74	62 74 67 61 64

APPENDIX 5

DESCRIPTIVE STATISTICS AND ANOVA FOR LYMPHOCYTE

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
A	15	77.13	3.962	1.023	74.94	79.33	70	84
B	15	77.53	2.134	.551	76.35	78.71	74	80
C	15	82.47	4.324	1.116	80.07	84.86	75	90
D	15	67.67	5.615	1.450	64.56	70.78	59	74
E	15	71.33	5.122	1.323	68.50	74.17	56	78
F	15	65.47	7.090	1.831	61.54	69.39	54	75
Total	90	73.60	7.683	.810	71.99	75.21	54	90

ANOVA

LYMPHOCYTE

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3196.000	5	639.200	26.095	.000
Within Groups	2057.600	84	24.495		
Total	5253.600	89			

APPENDIX 6

POST HOC TEST FOR LYMPHOCYTE COUNT

LEAST SIGNIFICANT DIFFERENCE (LSD)

(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	-.400	1.807	.825	-3.99	3.19
	C	-5.333*	1.807	.004	-8.93	-1.74
	D	9.467*	1.807	.000	5.87	13.06
	E	5.800*	1.807	.002	2.21	9.39
	F	11.667*	1.807	.000	8.07	15.26
B	A	.400	1.807	.825	-3.19	3.99
	C	-4.933*	1.807	.008	-8.53	-1.34
	D	9.867*	1.807	.000	6.27	13.46
	E	6.200*	1.807	.001	2.61	9.79
	F	12.067*	1.807	.000	8.47	15.66
C	A	5.333*	1.807	.004	1.74	8.93
	B	4.933*	1.807	.008	1.34	8.53
	D	14.800*	1.807	.000	11.21	18.39
	E	11.133*	1.807	.000	7.54	14.73
	F	17.000*	1.807	.000	13.41	20.59
D	A	-9.467*	1.807	.000	-13.06	-5.87
	B	-9.867*	1.807	.000	-13.46	-6.27
	C	-14.800*	1.807	.000	-18.39	-11.21
	E	-3.667*	1.807	.046	-7.26	-.07
	F	2.200	1.807	.227	-1.39	5.79
E	A	-5.800*	1.807	.002	-9.39	-2.21
	B	-6.200*	1.807	.001	-9.79	-2.61
	C	-11.133*	1.807	.000	-14.73	-7.54
	D	3.667*	1.807	.046	.07	7.26
	F	5.867*	1.807	.002	2.27	9.46
F	A	-11.667*	1.807	.000	-15.26	-8.07
	B	-12.067*	1.807	.000	-15.66	-8.47
	C	-17.000*	1.807	.000	-20.59	-13.41
	D	-2.200	1.807	.227	-5.79	1.39
	E	-5.867*	1.807	.002	-9.46	-2.27

*. The mean difference is significant at the 0.05 level.

APPENDIX 7

NEUTROPHIL COUNT

GROUPS	REPLICATES		
	I	II	III
A	10 12 11 18 17	19 22 16 20 16	15 17 13 19 17
B	16 15 14 20 22	19 15 15 18 19	18 15 14 19 20
C	17 19 19 10 12	19 24 10 19 11	18 21 14 14 12
D	30 24 25 25 40	24 35 40 30 41	27 29 32 27 40
E	27 29 42 19 28	24 29 14 25 21	26 29 28 22 24
F	34 26 38 43 45	38 23 25 30 35	36 24 31 37 35

APPENDIX 8

DESCRIPTIVE STATISTICS AND ANOVA FOR NEUTROPHIL COUNT

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
A	15	16.13	3.420	.883	14.24	18.03	10	22
B	15	17.27	2.549	.658	15.86	18.68	14	22
C	15	15.93	4.367	1.127	13.52	18.35	10	24
D	15	31.27	6.364	1.643	27.74	34.79	24	41
E	15	25.80	6.178	1.595	22.38	29.22	14	42
F	15	33.33	6.726	1.737	29.61	37.06	23	45
Total	90	23.29	8.832	.931	21.44	25.14	10	45

ANOVA

NEUTROPHIL

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4686.222	5	937.244	34.893	.000
Within Groups	2256.267	84	26.860		
Total	6942.489	89			

APPENDIX 9

POST HOC TEST FOR NEUTROPHIL COUNT

LEAST SIGNIFICANT DIFFERENCE(LSD)

(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	-1.133	1.892	.551	-4.90	2.63
	C	.200	1.892	.916	-3.56	3.96
	D	-15.133*	1.892	.000	-18.90	-11.37
	E	-9.667*	1.892	.000	-13.43	-5.90
	F	-17.200*	1.892	.000	-20.96	-13.44
B	A	1.133	1.892	.551	-2.63	4.90
	C	1.333	1.892	.483	-2.43	5.10
	D	-14.000*	1.892	.000	-17.76	-10.24
	E	-8.533*	1.892	.000	-12.30	-4.77
	F	-16.067*	1.892	.000	-19.83	-12.30
C	A	-.200	1.892	.916	-3.96	3.56
	B	-1.333	1.892	.483	-5.10	2.43
	D	-15.333*	1.892	.000	-19.10	-11.57
	E	-9.867*	1.892	.000	-13.63	-6.10
	F	-17.400*	1.892	.000	-21.16	-13.64
D	A	15.133*	1.892	.000	11.37	18.90
	B	14.000*	1.892	.000	10.24	17.76
	C	15.333*	1.892	.000	11.57	19.10
	E	5.467*	1.892	.005	1.70	9.23
E	F	-2.067	1.892	.278	-5.83	1.70
	A	9.667*	1.892	.000	5.90	13.43
	B	8.533*	1.892	.000	4.77	12.30
	C	9.867*	1.892	.000	6.10	13.63
	D	-5.467*	1.892	.005	-9.23	-1.70
	F	-7.533*	1.892	.000	-11.30	-3.77
F	A	17.200*	1.892	.000	13.44	20.96
	B	16.067*	1.892	.000	12.30	19.83
	C	17.400*	1.892	.000	13.64	21.16
	D	2.067	1.892	.278	-1.70	5.83
	E	7.533*	1.892	.000	3.77	11.30

*. The mean difference is significant at the 0.05 level.

APPENDIX 10
BASOPHIL COUNT

GROUPS	REPLICATES		
	I	II	III
A	0 1 0 1 0	1 1 2 2 1	2 2 1 1 1
B	0 0 1 1 0	2 2 1 1 1	2 1 1 1 1
C	0 0 1 0 1	1 1 0 0 1	1 0 1 1 1
D	1 1 0 1 0	1 0 0 0 0	1 0 1 1 1
E	0 0 1 1 0	1 0 0 0 0	1 1 0 1 1
F	0 1 1 0 1	1 1 1 0 1	1 0 0 1 0

APPENDIX 11

DESCRIPTIVE STATISTICS AND ANOVA FOR BASOPHIL COUNT

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
A	15	1.07	.704	.182	.68	1.46	0	2
B	15	1.00	.655	.169	.64	1.36	0	2
C	15	.60	.507	.131	.32	.88	0	1
D	15	.53	.516	.133	.25	.82	0	1
E	15	.53	.516	.133	.25	.82	0	1
F	15	.60	.507	.131	.32	.88	0	1
Total	90	.72	.600	.063	.60	.85	0	2

ANOVA

BASOPHIL

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.456	5	.891	2.712	.025
Within Groups	27.600	84	.329		
Total	32.056	89			

APPENDIX 12

POST HOC TEST FOR BASOPHIL COUNT

LEAST SIGNIFICANT DIFFERENCE (LSD)

(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	.067	.209	.751	-.35	.48
	C	.467*	.209	.028	.05	.88
	D	.533*	.209	.013	.12	.95
	E	.533*	.209	.013	.12	.95
	F	.467*	.209	.028	.05	.88
B	A	-.067	.209	.751	-.48	.35
	C	.400	.209	.059	-.02	.82
	D	.467*	.209	.028	.05	.88
	E	.467*	.209	.028	.05	.88
	F	.400	.209	.059	-.02	.82
C	A	-.467*	.209	.028	-.88	-.05
	B	-.400	.209	.059	-.82	.02
	D	.067	.209	.751	-.35	.48
	E	.067	.209	.751	-.35	.48
	F	.000	.209	1.000	-.42	.42
D	A	-.533*	.209	.013	-.95	-.12
	B	-.467*	.209	.028	-.88	-.05
	C	-.067	.209	.751	-.48	.35
	E	.000	.209	1.000	-.42	.42
	F	-.067	.209	.751	-.48	.35
E	A	-.533*	.209	.013	-.95	-.12
	B	-.467*	.209	.028	-.88	-.05
	C	-.067	.209	.751	-.48	.35
	D	.000	.209	1.000	-.42	.42
	F	-.067	.209	.751	-.48	.35
F	A	-.467*	.209	.028	-.88	-.05
	B	-.400	.209	.059	-.82	.02
	C	.000	.209	1.000	-.42	.42
	D	.067	.209	.751	-.35	.48
	E	.067	.209	.751	-.35	.48

*. The mean difference is significant at the 0.05 level.

APPENDIX 13

EOSINOPHILCOUNT

GROUPS	REPLICATES		
	I	II	III
A	2	2	2
	2	2	2
	1	1	1
	1	1	1
	1	1	1
B	2	2	2
	2	0	1
	1	2	1
	1	2	1
	1	1	1
C	2	0	1
	1	0	0
	1	0	1
	1	0	1
	1	1	1
D	1	0	1
	1	0	0
	0	1	1
	1	0	1
	0	0	1
E	0	1	1
	0	1	1
	1	0	0
	1	1	1
	0	1	1
F	0	1	1
	1	0	0
	0	0	0
	0	1	1
	1	0	0

APPENDIX 14

DESCRIPTIVE STATISTICS AND ANOVA FOR EOSINOPHIL COUNT

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
A	15	1.40	.507	.131	1.12	1.68	1	2
B	15	1.33	.617	.159	.99	1.68	0	2
C	15	.73	.594	.153	.40	1.06	0	2
D	15	.53	.516	.133	.25	.82	0	1
E	15	.67	.488	.126	.40	.94	0	1
F	15	.40	.507	.131	.12	.68	0	1
Total	90	.84	.652	.069	.71	.98	0	2

ANOVA

EOSINOPHIL

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13.289	5	2.658	9.100	.000
Within Groups	24.533	84	.292		
Total	37.822	89			

APPENDIX 15

POST HOC TEST FOR EOSINOPHIL COUNT

LEAST SIGNIFICANT DIFFERENCE (LSD)

(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	.067	.197	.736	-.33	.46
	C	.667*	.197	.001	.27	1.06
	D	.867*	.197	.000	.47	1.26
	E	.733*	.197	.000	.34	1.13
	F	1.000*	.197	.000	.61	1.39
B	A	-.067	.197	.736	-.46	.33
	C	.600*	.197	.003	.21	.99
	D	.800*	.197	.000	.41	1.19
	E	.667*	.197	.001	.27	1.06
	F	.933*	.197	.000	.54	1.33
C	A	-.667*	.197	.001	-1.06	-.27
	B	-.600*	.197	.003	-.99	-.21
	D	.200	.197	.314	-.19	.59
	E	.067	.197	.736	-.33	.46
	F	.333	.197	.095	-.06	.73
D	A	-.867*	.197	.000	-1.26	-.47
	B	-.800*	.197	.000	-1.19	-.41
	C	-.200	.197	.314	-.59	.19
	E	-.133	.197	.501	-.53	.26
	F	.133	.197	.501	-.26	.53
E	A	-.733*	.197	.000	-1.13	-.34
	B	-.667*	.197	.001	-1.06	-.27
	C	-.067	.197	.736	-.46	.33
	D	.133	.197	.501	-.26	.53
	F	.267	.197	.180	-.13	.66
F	A	-1.000*	.197	.000	-1.39	-.61
	B	-.933*	.1 7	.000	-1.33	-.54
	C	-.333	.197	.095	-.73	.06
	D	-.133	.197	.501	-.53	.26
	E	-.267	.197	.180	-.66	.13

*. The mean difference is significant at the 0.05 level.

APPENDIX 16
MONOCYTE COUNT

GROUPS	REPLICATES		
	I	II	III
A	5 5 4 4 3	4 5 6 5 4	4 5 5 4 3
B	2 3 1 3 3	3 4 2 2 1	2 3 1 2 2
C	1 0 0 1 0	0 3 0 0 0	0 1 0 1 0
D	1 0 0 1 0	1 1 0 1 0	1 1 0 1 0
E	1 0 0 1 0	3 0 1 0 0	2 0 1 0 0
F	1 1 1 1 0	0 1 1 1 0	1 1 1 1 0

APPENDIX 17
DESCRIPTIVE STATISTICS AND ANOVA FOR MONOCYTE COUNT

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
A	15	4.40	.828	.214	3.94	4.86	3	6
B	15	2.27	.884	.228	1.78	2.76	1	4
C	15	.47	.834	.215	.00	.93	0	3
D	15	.53	.516	.133	.25	.82	0	1
E	15	.60	.910	.235	.10	1.10	0	3
F	15	.73	.458	.118	.48	.99	0	1
Total	90	1.50	1.623	.171	1.16	1.84	0	6

ANOVA

MONOCYTE

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	185.967	5	37.193	64.373	.000
Within Groups	48.533	84	.578		
Total	234.500	89			

APPENDIX 18
POST HOC TEST FOR MONOCYTE COUNT
LEAST SINIFICANT DIFFERENCE (LSD)

(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	2.133*	.278	.000	1.58	2.69
	C	3.933*	.278	.000	3.38	4.49
	D	3.867*	.278	.000	3.31	4.42
	E	3.800*	.278	.000	3.25	4.35
	F	3.667*	.278	.000	3.11	4.22
B	A	-2.133*	.278	.000	-2.69	-1.58
	C	1.800*	.278	.000	1.25	2.35
	D	1.733*	.278	.000	1.18	2.29
	E	1.667*	.278	.000	1.11	2.22
	F	1.533*	.278	.000	.98	2.09
C	A	-3.933*	.278	.000	-4.49	-3.38
	B	-1.800*	.278	.000	-2.35	-1.25
	D	-.067	.278	.811	-.62	.49
	E	-.133	.278	.632	-.69	.42
	F	-.267	.278	.339	-.82	.29
D	A	-3.867*	.278	.000	-4.42	-3.31
	B	-1.733*	.278	.000	-2.29	-1.18
	C	.067	.278	.811	-.49	.62
	E	-.067	.278	.811	-.62	.49
	F	-.200	.278	.473	-.75	.35
E	A	-3.800*	.278	.000	-4.35	-3.25
	B	-1.667*	.278	.000	-2.22	-1.11
	C	.133	.278	.632	-.42	.69
	D	.067	.278	.811	-.49	.62
	F	-.133	.278	.632	-.69	.42
F	A	-3.667*	.278	.000	-4.22	-3.11
	B	-1.533*	.278	.000	-2.09	-.98
	C	.267	.278	.339	-.29	.82
	D	.200	.278	.473	-.35	.75
	E	.133	.278	.632	-.42	.69

*. The mean difference is significant at the 0.05 level.

APPENDIX 19

RED BLOOD CELL COUNT

GROUPS	REPLICATES		
	I	II	III
A	4.2 5.2 4.2 4.8 4.8	3.6 4.9 4.0 4.3 5.1	4.8 5.1 4.1 4.6 5.0
B	6.5 6.5 6.6 6.9 6.9	5.9 4.5 5.3 5.7 6.8	6.2 6.5 5.5 6.3 6.8
C	6.6 6.6 6.7 6.0 6.0	5.5 5.7 6.4 6.0 6.1	6.1 6.1 6.5 6.0 6.0
D	8.0 8.0 8.0 7.0 6.5	6.1 8.6 9.9 8.0 7.0	7.0 8.3 8.9 7.5 6.7
E	8.0 6.4 8.0 8.9 9.4	7.8 7.2 9.7 7.3 8.9	7.9 6.8 8.8 8.1 9.1
F	8.6 8.9 8.8 7.9 6.4	8.5 7.5 7.7 6.2 7.9	8.5 8.2 8.2 7.0 7.1

APPENDIX 20
DESCRIPTIVE STATISTICS AND ANOVA
OF RED BLOOD CELL COUNT

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
A	15	4.607	.4847	.1252	4.338	4.875	3.6	5.3
B	15	6.107	.6170	.1593	5.765	6.448	4.5	6.8
C	14	6.164	.3522	.0941	5.961	6.368	5.5	6.7
D	16	7.644	1.0545	.2636	7.082	8.206	6.0	9.9
E	15	8.153	.9694	.2503	7.616	8.690	6.4	9.7
F	15	7.827	.8413	.2172	7.361	8.293	6.2	8.9
Total	90	6.767	1.4595	.1538	6.461	7.072	3.6	9.9

ANOVA

RBC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	139.603	5	27.921	46.928	.000
Within Groups	49.977	84	.595		
Total	189.580	89			

APPENDIX 21
POST HOC TEST FOR RED BLOOD CELL COUNT
LEAST SIGNIFICANT DIFFERENCE (LSD)

(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	-1.5000*	.2817	.000	-2.060	-.940
	C	-1.5576*	.2866	.000	-2.128	-.988
	D	-3.0371*	.2772	.000	-3.588	-2.486
	E	-3.5467*	.2817	.000	-4.107	-2.987
	F	-3.2200*	.2817	.000	-3.780	-2.660
B	A	1.5000*	.2817	.000	.940	2.060
	C	-.0576	.2866	.841	-.628	.512
	D	-1.5371*	.2772	.000	-2.088	-.986
	E	-2.0467*	.2817	.000	-2.607	-1.487
	F	-1.7200*	.2817	.000	-2.280	-1.160
C	A	1.5576*	.2866	.000	.988	2.128
	B	.0576	.2866	.841	-.512	.628
	D	-1.4795*	.2823	.000	-2.041	-.918
	E	-1.9890*	.2866	.000	-2.559	-1.419
	F	-1.6624*	.2866	.000	-2.232	-1.092
D	A	3.0371*	.2772	.000	2.486	3.588
	B	1.5371*	.2772	.000	.986	2.088
	C	1.4795*	.2823	.000	.918	2.041
	E	-.5096	.2772	.070	-1.061	.042
	F	-.1829	.2772	.511	-.734	.368
E	A	3.5467*	.2817	.000	2.987	4.107
	B	2.0467*	.2817	.000	1.487	2.607
	C	1.9890*	.2866	.000	1.419	2.559
	D	.5096	.2772	.070	-.042	1.061
	F	.3267	.2817	.249	-.233	.887
F	A	3.2200*	.2817	.000	2.660	3.780
	B	1.7200*	.2817	.000	1.160	2.280
	C	1.6624*	.2866	.000	1.092	2.232
	D	.1829	.2772	.511	-.368	.734
	E	-.3267	.2817	.249	-.887	.233

*. The mean difference is significant at the 0.05 level.

APPENDIX 22

PACKED CELL VOLUME

	REPLICATES		
	I	**II**	**III**
A	**17** **19** **23** **35** **18**	**25** **22** **16** **17** **20**	**21** **21** **20** **26** **19**
B	**33** **45** **34** **35** **34**	**30** **35** **37** **30** **33**	**31** **40** **35** **32** **33**
C	**40** **39** **38** **36** **35**	**35** **40** **40** **35** **33**	**37** **39** **39** **35** **34**
D	**41** **40** **42** **40** **42**	**45** **42** **40** **45** **39**	**43** **41** **41** **42** **40**
E	**40** **31** **39** **40** **35**	**45** **45** **40** **40** **39**	**42** **38** **40** **40** **37**
F	**40** **40** **40** **45** **39**	**40** **40** **45** **45** **40**	**40** **40** **42** **45** **39**

APPENDIX 23
DESCRIPTIVE STATISTICS AND ANOVA
OF PACKED CELL VOLUME

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
A	15	21.27	4.758	1.228	18.63	23.90	16	35
B	15	34.47	3.907	1.009	32.30	36.63	30	45
C	15	37.00	2.420	.625	35.66	38.34	33	40
D	15	41.53	1.767	.456	40.55	42.51	39	45
E	15	39.40	3.481	.899	37.47	41.33	31	45
F	15	41.33	2.380	.615	40.02	42.65	39	45
Total	90	35.83	7.694	.811	34.22	37.44	16	45

ANOVA

PCV

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4363.167	5	872.633	80.966	.000
Within Groups	905.333	84	10.778		
Total	5268.500	89			

APPENDIX 24
POST HOC TEST FOR PACKED CELL VOLUME
LEAST SIGNIFICANT DIFFERENCE (LSD)

(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	-13.200*	1.199	.000	-15.58	-10.82
	C	-15.733*	1.199	.000	-18.12	-13.35
	D	-20.267*	1.199	.000	-22.65	-17.88
	E	-18.133*	1.199	.000	-20.52	-15.75
	F	-20.067*	1.199	.000	-22.45	-17.68
B	A	13.200*	1.199	.000	10.82	15.58
	C	-2.533*	1.199	.038	-4.92	-.15
	D	-7.067*	1.199	.000	-9.45	-4.68
	E	-4.933*	1.199	.000	-7.32	-2.55
	F	-6.867*	1.199	.000	-9.25	-4.48
C	A	15.733*	1.199	.000	13.35	18.12
	B	2.533*	1.199	.038	.15	4.92
	D	-4.533*	1.199	.000	-6.92	-2.15
	E	-2.400*	1.199	.049	-4.78	-.02
	F	-4.333*	1.199	.001	-6.72	-1.95
D	A	20.267*	1.199	.000	17.88	22.65
	B	7.067*	1.199	.000	4.68	9.45
	C	4.533*	1.199	.000	2.15	6.92
	E	2.133	1.199	.079	-.25	4.52
	F	.200	1.199	.868	-2.18	2.58
E	A	18.133*	1.199	.000	15.75	20.52
	B	4.933*	1.199	.000	2.55	7.32
	C	2.400*	1.199	.049	.02	4.78
	D	-2.133	1.199	.079	-4.52	.25
	F	-1.933	1.199	.111	-4.32	.45
F	A	20.067*	1.199	.000	17.68	22.45
	B	6.867*	1.199	.000	4.48	9.25
	C	4.333*	1.199	.001	1.95	6.72
	D	-.200	1.199	.868	-2.58	2.18
	E	1.933	1.199	.111	-.45	4.32

*. The mean difference is significant at the 0.05 level.

APPENDIX 25

HEAMOGLOBIN CONCENTRATION

	REPLICATES		
	I	II	III
A	3.9 4.0 4.0 6.1 4.4	4.2 4.3 5.9 5.2 3.5	4.1 4.2 5.0 5.7 4.0
B	8.5 8.0 8.3 8.2 8.0	7.0 8.0 6.5 7.0 8.1	7.7 7.5 7.4 7.6 8.0
C	7.4 8.2 8.4 8.0 8.1	8.0 8.3 8.0 8.4 7.9	7.7 8.2 8.2 8.2 8.0
D	10.1 10.0 9.2 10.0 10.0	10.0 11.0 10.6 11.0 10.1	10.1 10.5 9.9 10.5 10.0
E	10.4 10.2 10.3 9.8 10.6	11.6 10.0 11.0 10.0 10.5	10.8 10.1 10.6 9.9 10.5
F	11.0 11.2 11.0 12.0 11.0	11.2 10.0 10.0 11.0 11.0	11.0 10.0 10.5 11.5 11.0

APPENDIX 26
DESCRIPTIVE STATISTICS AND ANOVA FOR HAEMOGLOBIN CONCENTRATION

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
A	3	4.567	.8077	.2085	4.119	5.014	3.5	6.1
B	3	7.720	.5557	.1435	7.412	8.028	6.5	8.5
C	3	7.800	.9725	.2511	7.261	8.339	4.4	8.4
D	3	10.187	.4627	.1195	9.930	10.443	9.2	11.0
E	3	10.420	.4724	.1220	10.158	10.682	9.8	11.6
F	3	10.933	.5122	.1323	10.650	11.217	10.0	12.0
Total	18	8.604	2.3001	.2424	8.123	9.086	3.5	12.0

ANOVA

HB

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	434.346	5	86.869	199.962	.000
Within Groups	36.492	84	.434		
Total	470.838	89			

APPENDIX 27
POST HOC TEST FOR HAEMOGLOBIN CONCENTRATION LEAST SIGNIFICANT DIFFERENCE (LSD)

(I)	(J)	Mean Difference (I-	Std. Error	Sig.	95% Confidence Interval

GROUPS	GROUPS	J)			Lower Bound	Upper Bound
A	B	-3.1533*	.2407	.000	-3.632	-2.675
	C	-3.2333*	.2407	.000	-3.712	-2.755
	D	-5.6200*	.2407	.000	-6.099	-5.141
	E	-5.8533*	.2407	.000	-6.332	-5.375
	F	-6.3667*	.2407	.000	-6.845	-5.888
B	A	3.1533*	.2407	.000	2.675	3.632
	C	-.0800	.2407	.740	-.559	.399
	D	-2.4667*	.2407	.000	-2.945	-1.988
	E	-2.7000*	.2407	.000	-3.179	-2.221
	F	-3.2133*	.2407	.000	-3.692	-2.735
C	A	3.2333*	.2407	.000	2.755	3.712
	B	.0800	.2407	.740	-.399	.559
	D	-2.3867*	.2407	.000	-2.865	-1.908
	E	-2.6200*	.2407	.000	-3.099	-2.141
	F	-3.1333*	.2407	.000	-3.612	-2.655
D	A	5.6200*	.2407	.000	5.141	6.099
	B	2.4667*	.2407	.000	1.988	2.945
	C	2.3867*	.2407	.000	1.908	2.865
	E	-.2333	.2407	.335	-.712	.245
	F	-.7467*	.2407	.003	-1.225	-.268
E	A	5.8533*	.2407	.000	5.375	6.332
	B	2.7000*	.2407	.000	2.221	3.179
	C	2.6200*	.2407	.000	2.141	3.099
	D	.2333	.2407	.335	-.245	.712
	F	-.5133*	.2407	.036	-.992	-.035
F	A	6.3667*	.2407	.000	5.888	6.845
	B	3.2133*	.2407	.000	2.735	3.692
	C	3.1333*	.2407	.000	2.655	3.612
	D	.7467*	.2407	.003	.268	1.225
	E	.5133*	.2407	.036	.035	.992

*. The mean difference is significant at the 0.05 level.