Artesunate-Procyanidin Hybrid Compound: Synthesis, *In vitro* and *In vivo* Antimalarial, Antioxidant and Toxicological Effects

BY

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CERTIFICATION

I certify that this work was carried out by Tijjani Habibu in the Department of Biochemistry, University of Ilorin, under my supervision.

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DEDICATION

This thesis is dedicated to my family; Abdullahi, Amida, Surajudeen, Mudashiru, Muzamilu, Mustapha, Sadiq, Ruqayyah, Muyibat and Qasim.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

ABSTRACT

The goal of global eradication of malaria has remained elusive due to the rapid spread of drug resistant parasites, necessitating urgent development of novel antimalarials. Therefore, the aim of this study was to synthesize a hybrid compound of artesunate and procyanidin and evaluate its antimalarial, antioxidant and toxicological effects using various models. The objectives were to: (i) synthesize and chracterise a hybrid compound from artesunate and procyanidin; (ii) evaluate the *in vitro* antiplasmodial activity and cytotoxicity of the compound; (iii) evaluate its mechanisms of action; (iv) evaluate its suppressive and curative antimalarial activities in *Plasmodium berghei* NK65-infected mice; (v) evaluate the protein-ligand interactions; (vi) evaluate the *in vitro* and *in vivo* antioxidant activities of the compound; and (viii) evaluate its toxic effects.

Artesunate-procyanidin hybrid compound was synthesized using standard chemical protocols and the structure was elucidated using Nuclear Magnetic Resonance and Infra-Red spectroscopy. The compound was evaluated for its activity against *Plasmodium falciparum* W2 strain *in vitro* and *P. berghei* NK65 *in vivo*. Inhibition of β-hematin formation, red blood cell lysis and cytotoxicity against Buffalo Green Monkey (BGM) kidney cell line of the compound were evaluated. The compound was evaluated for antioxidant activity using *in vitro* and *in vivo* models and for toxic effects using selected organ function indices of mice. Protein-ligand interactions were evaluated using molecular docking. Data were analyzed using Analysis of Variance at $P<0.05$.

The findings of this study were that the hybrid compound:

i. was synthesized as a brownish crystal with two pharmacopores linked together by ester bond;
ii. was active against *P. falciparum* W2 (IC_{50}: 0.0335 µg/ml), though its activity was lower than that of artesunate, but higher than that of procyanidin and it was less toxic to BGM cell line (MLD_{50}: 155 µg/ml);
iii. caused higher inhibition of β-haematin formation *in vitro* (IC_{50}: 42.46 µg/ml) compared to chloroquine (IC_{50}: 55.53 µg/ml), though was lower than that of artesunate (IC_{50}: 17.79 µg/ml) and did not cause red blood cell membrane perturbation;
iv. exhibited higher suppressive activity (ED_{50}: <5 mg/kg) than artesunate on day 4 post-inoculation and curative activity (ED_{50}: <5 mg/kg) which favourably compared with artesunate on day 6 post-inoculation against *P. berghei* NK65 in mice;
v. exhibited higher binding affinity for *P. falciparum* Lactate Dehydrogenase (-9.6 kcal/mol) compared to artesunate (-6.2 kcal/mol) and procyanidin (-8.0 kcal/mol);
vi. exhibited higher ferric ion reducing power and DPPH scavenging activity (IC_{50}: 19.68 µg/ml) compared to butylated hydroxytoluene *in vitro* and caused significant increase ($P< 0.05$) in glutathione peroxidase, and glutathione-S-transferase activities in RBC, heart, liver, kidney, and brain of *P. berghei*-infected mice compared to untreated infected controls; and
vii. had no significant effect ($P>0.05$) on atherogenic index and plasma concentrations of sodium, calcium, creatinine, urea and bilirubin but significantly reduced ($P< 0.05$) brain Na^+,K^+Adenosine triphosphatase activity in mice at all doses administered compared to controls.

The study concluded that hybrid compound exhibited antimalarial activity (which favourably compared with artesunate) and antioxidant activity *in vivo*. Artesunate-procyanidin hybrid compound may be explored as an alternative therapy for malaria.

CHAPTER ONE

1.0 Introduction and Literature Review

1.1 Introduction

Malaria is a public health problem posing significant morbidity and mortality, with major economic and developmental challenges in sub-Saharan Africa (Sachs and Malaney, 2002), including Nigeria. Malaria may present itself in a variety of clinical forms, with differing patterns and severity which may be classified into uncomplicated and severe malaria. Globally, there are an estimated 212 million malaria cases and over 428,000 deaths per year (WHO, 2015).

*Plasmodiumfalciparum*infection is responsible for almost all the morbidity and mortality recorded worldwide with about 90% of the reported cases and 85% of the deaths are attributed to malaria in the sub-Saharan Africa (Crawley *et al*., 2010). Nigeria accounts for about a quarter of all malaria cases in Africa (WHO, 2008), mostly caused by *P. falciparum* (Adebayo and Krettli, 2011).

In Africa, a lot of medicinal plants are used in folkmedicine for the treatment of malaria. Some of these plants contain both antimalarial and antioxidant principles which work synergistically for the quick recovery of patients. Antioxidant activity in higher plants has been linked to flavonoids, which are a group of polyphenolic compounds, ubiquitously found in plants and one of the most important classes of phytocompounds with biological activity (Yoshida *et al*., 1989; Zheng and Wang, 2001). Antioxidants play key role in the maintenance of the prooxidant/antioxidant balance by neutralizing the free radicals which are responsible for deleterious process in biological systems (Shahidi, 1997). Their presence in food or body at low concentrations compared with that of an oxidizable substrate markedly delay or prevent the oxidation of that substrate (Robak and Gryglewski, 1988). Flavonoids, tannins, and alkaloids are

some of the active phytoconstitients found in medicinal plants with proven antimalarial and other medicinal properties (Esquenazi *et al*., 2002; Mendonca-Filho *et al*., 2004; Ayoola *et al*., 2008; Adebayo *et al*., 2013). The synthesis of hybrid compounds using potent antimalarials and phytochemicals having antioxidant activity may be of tremendous help in getting novel drugs which may facilitate faster recovery in malaria patients.

1.2 Literature Review

1.2.1 Malaria

Malaria is a disease caused by parasites belonging to the genus *Plasmodium*. *P. falciparum*, *P. Knowlesi, P. vivax*, *P. ovale*, and *P. malariae* are the five species known to infect humans. *P.falciparum* causes the most potentially life threatening infections (Peter and Anatoli, 1998). Malaria remains an important cause of illnessanddeath in children and adults in tropical countries, including Nigeria, despite the advances in the control and treatment of the disease. According to the latest WHO estimates, there were about 216 million cases of malaria in 2016 (with an uncertainty range of 196 to 263 million) and an estimated 445,000 deaths, 91% of which occurs in African region (WHO, 2017).

Malaria infection induces oxidative stress through the increase in the production of reactive oxygen species (ROS) within and outside the infected erythrocyte. This is as a result of a disturbance in the balance of naturally generated oxidants and antioxidants which is upset during infection. The role of antioxidants in malaria treatment is receiving attention because antioxidants have been found to influence host cellular and immunological functions (Spallhoiz *et al.,* 1990; Terahima *et al.,* 2002). There is also increasing indication that natural antioxidants, especially those in some spices, herbs, and medicinal plants may be useful in preventing the deleterious consequences of oxidative stress including that occasioned by malaria (Noda *et*

*al.,*1997). The spread of drug resistance to most currently available antimalarial drugs is a great concern to researchers in the aspect of the treatment of malaria.

P.falciparum remains one of the most infectious parasites causing almost all the malaria morbidity and mortality recorded worldwide (Peter and Anatoli, 1998). Among infected individuals, about 75% rate of infection is acused by *P.falciparum* species followed by *P.* vivax with about 20% rate of infection (Nadjm and Behrens, 2012). *P.falciparum* also represents the most virulent form of human malaria, which cause majority of the deaths (Sarkar *et al*., 2009). Baird (2013) has reported that malaria induced by *P. vivax* may also present a life threatening condition.

Malaria does not only pose a health challenge, it also poses grate economical and developmental challenges to sub-saharan Africa (Sachs and Malaney, 2002). The domestic funding for malaria was estimated to be US$527 million as at 2013, representing 18% of total malaria funding. In addition, the funds required achieving global targets for malaria control and elimination is estimated to about US$5.1 billion is (WHO, 2014). The target research areas include vector control, chemotherapy and vaccine creation for the disease. Insecticide and parasite resistance has hindered research breakthrough in vector control and chemotherapy respectively, while the most advanced malaria vaccine developed (RTS,S/AS01) just completed its 3rd phase trials (Agnandji*et al*., 2015).

1.2.1.1 Lifecycle of Plasmodium species

The life cycle of malaria parasite starts as a no symptom skin stage by the simple bite of a female Anopheles mosquito. The mosquito feeds on human blood and injects the parasites in the form of sporozoites into the skin (Figure 1). Previously before the report by Guilbride *et al.*(2012), the human infection was previously thought to be initiated when sporozoites are

injected by mosquitodirectly into the bloodstream. These skin-infecting sporozoites stage, which last for about 5-8 minutes, initiate rapid suppression of immunity and establishing early tolerance to subsequent lifecycle stages.

The sporozoites find the blood in about 30 minutes, where they migrate to the liver. Liver infections are massively replicative, noninflammatory and asymptomatic. Within few days (5-16 days), the sporozoites grow, divide and produce tens of thousands of blood stage parasite called merozoites which are also highly proliferative (Cox, 2010). Some of the infected malaria species remain dormant for extended periods of time in the liver stage, causing relapses weeks/months later.

Merozoites that have been released from the infected liver cells invade other erythrocytes. They are able to recognize specific proteins on the surface of the erythrocyte, which allows them to invade the cell. After entering the erythrocyte, the parasite undergoes a trophic period and then an asexual replication. Repeatedly for another 1-3 days, the asexual replication occurs and the release of newly formed merozoites from the red bold cell. This results in thousands of parasite-infected red blood cells (RBCs) in host blood stream (Clark *et al*., 2004), leading to illness and other complications of malaria. The young trophozoite is often called a ring form due to its morphology in when stained with Giemsa. As the parasite increases in size, this 'ring' morphology disappears and it is changes to trophozoite. During the trophic period, the parasite ingests the host cell cytoplasm where it breaks down the haemoglobin content into amino acids. The by-product of the parasite haemoglobin digestion is the malaria pigment, haemozoin.

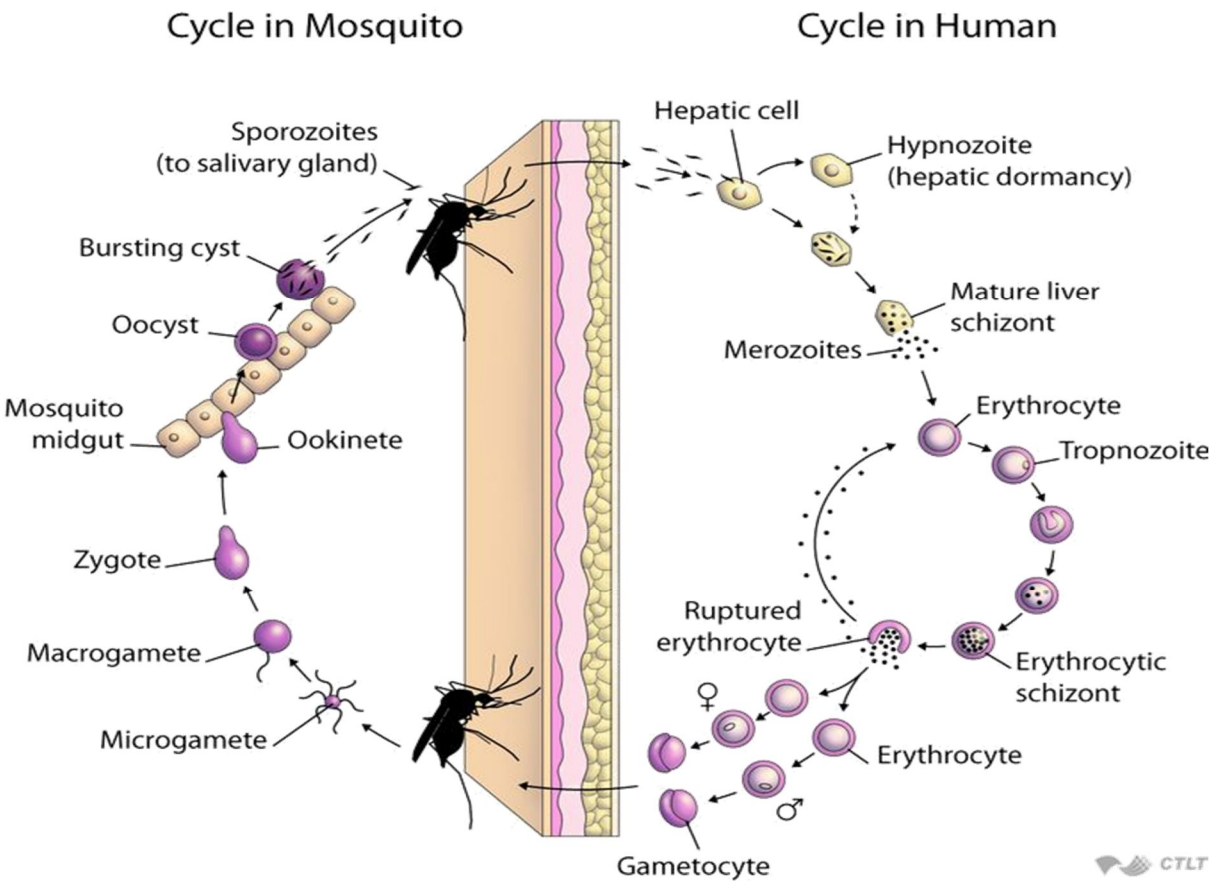


Figure 1: Life Cycle of Malaria Parasites

Source: Richard (2007)

The trophozoite stage ends by nuclear division, which thus initiate the beginning of the schizont stage of the life cycle. These stages consist of 3 – 5 rounds of nuclear replication followed by a budding process. The host erythrocyte ruptures and releases the merozoites. Thereleasedmerozoites invade new erythrocytes and initiate another round of schizogony. The blood-stage parasites in a host usually undergo a synchronous schizogony. The simultaneous rupture of the infected blood cells (iRBC) and the concomitant release of antigens and waste products accounts for the intermittent fever paroxysms seen in malaria infection (Talman *et al.,* 2005). Blood stage schizogony in *P.falciparum* differs from the other human species of malaria parasites in that the trophozoite- and the schizont-infected erythrocytes adhere to capillary endothelial cells and are not seen in the peripheral circulation.

Some of the merozoitesinfect blood cells and leave the cycle of asexual multiplication. Instead of replication, the merozoites in these cells develop into sexual forms of the parasite, called the male and female gametocytes, which circulate in the blood stream. When a mosquito bites an infected human, it ingests the gamatocytes. Gametocytes do not cause pathology in the human host by themselves except when taken upand will disappear from the circulation if not taken up by a mosquito. In the mosquito gut, the infected human blood cells burst, thereby releasing the gametocytes, which develops further into mature sex cells called gametes. Male and female gametes fuse to form zygotes, which develop into actively moving ookinetes within 12-24 hours, the ookinetes burrow into the mosquito's midgut wall and form oocyst.

Growth and division of each oocyst produces several thousands of active sporozoites. After 8-15 days, the oocyst burst, releasing sporozoites into the body cavity of the mosquitoes,

from which they travel to the mosquites salivary gland. Salivary gland sporozoites efficiently invade liver cells, but cannot re-invade the salivary glands (Talman *et al.,* 2005). The cycle of human infection is re-starts when a mosquito takes a blood meal, injecting the sporozoites from its salivary glands directlyinto the human bloodstream.

1.2.1.2 Parasite haemoglobin detoxification

Haemoglobin is a protein with 3452 amino acid sequence, which is found in the RBC. It is an important transport protein which is also very important in oxygen transport. Haemoglobin is degraded by a number of metabolic enzymes, which include the aspartic proteases (plasmepsin I, II, III, IV), cysteine proteases (falcipain 1, 2, 3) and metalloproteases (falcilysin) (Omara-Opyene *et al.,* 2004; Rosenthal *et al.,* 2002; Barnerjee and Goldberg, 2001). The food vacuole is the site for the catabolism. Its acidic nature (a pH ranging between 5.0 - 5.4) allows for optimal haemoglobin catabolism (Barnerjee and Goldberg, 2001). It is also a site for parasite proteolysis, peptide transport and haemozoin formation.

P. falciparum solidly depends on available haemoglobin for survival and development inside the RBC, digesting about 60 – 80% of available haemoglobin in the food vacuole (Krogstad *et al.,* 1985; Egan *et al.,* 2002;Hayward *et al.,* 2006). Their action of haemoglobin degredation release peptide chains and free haem (Fe^{2+} protoporphyrin IX) which needs to be detoxified. The toxicity results from the irreversible oxidation of the metal ion from Fe^{2+} protoporphyrin IX by molecular oxygen (de Villiers and Egan, 2009) to give a redox pair of hydroxo/aqua ferritoprotoporphyrin IX (HO^{-}/H_2O^{-} Fe^{3+} protoporphyrin IX) (Chou *et al*., 1980).

The mechanism of detoxification is different in vertebrate and protozoan parasite. Vertebrates detoxify haematin by catabolism using the haem oxygenase, which *P. falciparum* lacks (Barnerjee and Goldberg, 2001), while parasite detoxifies haematin by sequestering the

free haematin into a microcrystalline form known as haemozoin which usually appears as a dark malaria pigment under the microscope. While haematin is soluble in a lipid environment, and possess lipid peroxidation ability to compromise membrane integrity, sequestered haemozoin released after detoxification is insoluble in lipid environment, lacks lipid peroxidation ability due to its decreased pro-oxidant nature and as such safe and non-toxic to the parasite (Oliveira *et al.,* 2002).

The variation in haem detoxification pathway of *P. falciparum* with respect to human pathway serve as anattractive drug target (Figure 2). Drugs are targeted at the parasitic stage where the multiplying protozoans cause clinical pathologies. Inhibition of the haem detoxification pathway results in a buildup of high concentration of the toxic free haem inside the parasite, which leads to the death of the parasite (Choi *et al,* 2002). Humans detoxify free haem through oxygenase/biliverdin reductase pathway while *Plasmodium* speciesdetoxifies through biomineralization, this difference allow for drug target where only the parasite detoxification pathway is inhibited.

The knowledge of parasite's mechanism of haemozoin formation is critical to successfully tackling malaria burden; it also helps in elucidating the mechanism of action of antimalarial drugs that act by interfering with haemozoin formation. Quinoline antimalarials are known to act by binding to haemozoin crystals and inhibiting their growth, thereby causing an increase in toxic haematin concentration and consequently leading to the death of the parasite (Weissbuch and Leiserowitz, 2008).

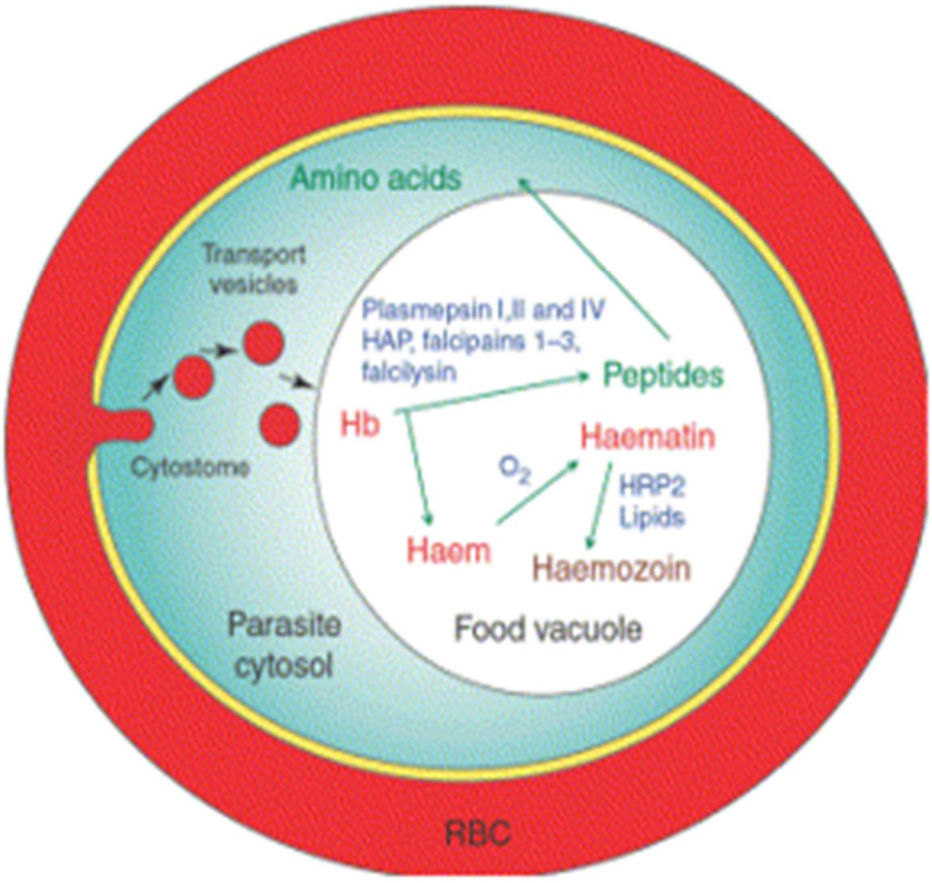


Figure 2: Haemoglobin uptake and degradation by *Plasmodium* in host RBC

Source: Egan(2008)

The existence and resemblance of β-haematin (a synthetic haematin product) and Haemozoin (the detoxified end product of haem) has been reported by Bohle *et al.* (1997) and Slater and Cerami (1992). β-haematin is a cyclic dimer of Fe^{2+} protoporphyrin IX (Pagola *et al*., 2000), consisting of haem linked by a propionate side chain (Figure 3). It has provided an alternative *in vitro* method for the assessment of β-haematin inhibition assay for testing of drugs and other compounds (Ncokazi and Egan, 2005).

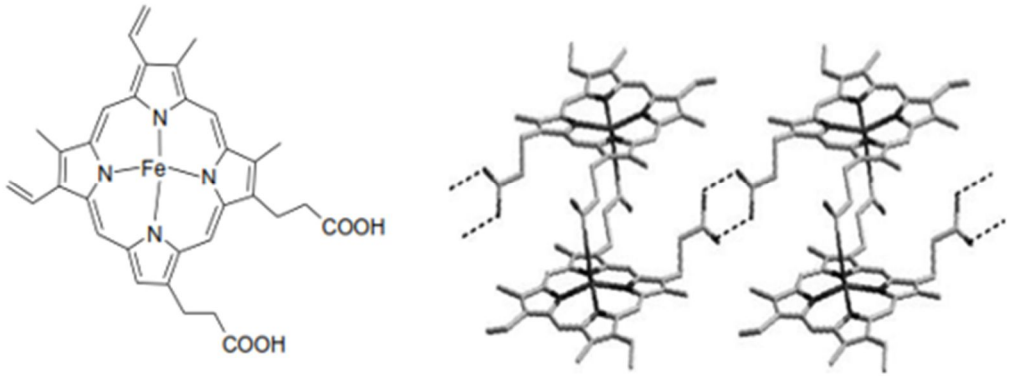


Figure 3: Structures of Fe protoporphyrin IX (Haem) and β-haematin dimer

Source: Egan (2008)

1.2.1.3 Oxidative Stress in Malaria

Oxidative stress is a phenomenon, which occurs, normally in the human system, kept in check by various enzyme systems to maintain the*in vivo* redox homeostasis (Rahal *et al*., 2014). The imbalance between the prooxidant and antioxidant components is what is termed "Oxidative stress" (Rahal *et al*., 2014). Oxidative stress develops in malaria during the development of the parasite in the host cells. About 80% of circulating hemoglobin is consumed by the parasite leading to changes in structure, stiffness, viscosity and volume of erythrocytes (Esposito *et al.,* 2008). Iron containing ferriprotoporphyrin IX is release by the digestion, which is toxic to the parasite, being a pro-oxidant and catalyzes the production of reactive oxygen species. Oxidative stress contributes to many pathological conditions which include heart disease (Elahi and Matata, 2006), atherosclerosis (Galle *et al*., 2006), cancer (Klaunig and Kamendulis, 2004) stroke (Alexandrova *et al*., 2004) and several neurodegenerative diseases (Emerit *et al*., 2004).

1.2.1.3.1 Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS)

Reactive oxygen species (ROS) are activated forms of oxygen, which present damaging effect to a biological system. They include free radicals such as hydroxyl radicals (OH^-), perhydroxyl radical (HO_2^-), superoxide anion radicals (O^{2-}), and the free radical nitric oxide (NO^-) as well as non-free radicals such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Halliwell, 1995). Other specific examples are presented in Table 1. The constant oxidative stress observed in infected erythrocytes (iRBC) during malaria is caused by ROS or reactive nitrogen species (RNS) generated from endogenous source during the digestion of host cell haemoglobin and associated biochemical reactions and exogenous sources such as the immune system of the host. Activated molecular oxygen, neutrophils and macrophages generate large

amounts of ROS and RNS, leading to an imbalance in the formation of oxidizing species and the activity of antioxidants. This imbalance is reported to trigger alterations in redox status, which is an important mechanism of human host response to malaria infection (Percário *et al.*, 2012). Smoking (Cho *et al.*, 2005), exposure to pollutants (Church and Pryor, 1985), hyperoxia (Comhair *et al.*, 2000) or radiation (Chiu *et al.*, 1993) as well as heavy metals (Stohs and Bagchi, 1995) are other exogenous sources of ROS.

During metabolism, respiration consumes molecular oxygen (O_2) converting it to water with release of energy. The cytochrome c oxidase-catalyzed reaction transfers of four electrons to oxygen, in principle without intermediates, producing partially reducing oxygen species. About 1–3% of electrons passing through the chain cause oxygen to be prematurely and incompletely reduced (Esra *et al.*, 2012), resulting in the generation of superoxide radical ($^{\cdot}O_2^-$) which leads to a cascade of other ROS as illustrated in Figure 4.O_2^- can react with H_2O_2 and generate OH^-, even though it's not particularly reactive (Liochev and Fridovich, 2002). It can inactivate specific enzymes or initiate lipid peroxidation in its protonated form, hydroperoxyl $HO_2^{\cdot}$ (Van Raamsdonk and Hekimi, 2009). Superoxide anions are converted to hydrogen peroxide (H_2O_2) through dismutation by superoxide dismutase (SOD) in Haber–Weiss reaction, and then catalase act on hydrogen peroxide to form water. Metal catalyst such as Fe^{2+}, can react with hydrogen peroxide (H_2O_2) in the Fenton reaction to generate hydroxyl radical (Flora, 2009). Hydroxyl radicals are the most reactive among all the ROS, it can damage carbohydrates, lipids and proteins.

Table 1: Specific Examples of Reactive Oxygen and Nitrogen Species

Reactive Oxygen Species	
Radicals	**Non-radicals**
Superoxide O_2^-	Hydrogen peroxide H_2O_2
Hydroxyl OH^-	Hypochlorous acid HOCl
Peroxyl RO_2^-	Hypobromine acid HOBr
Alkoxyl RO^-	Ozone O_2
ɔperoxyl HO_2^-	Singlet oxygen
Reactive Nitrogen Species	
Radicals	**Non-radicals**
Nitric oxide NO^-	Nitrogen dioxide NO_2
Nitrous acid HNO^2	Nitroxyl cation NO
	Nitroxyl anion NO^-

Source: Caimi *et al*. (2004)

$$Fe^{3+} + O_2 \rightarrow Fe^{2+} + O_2 \text{Haber} - \text{Weiss}$$

$$\boldsymbol{Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot \text{Fenton reaction}}$$

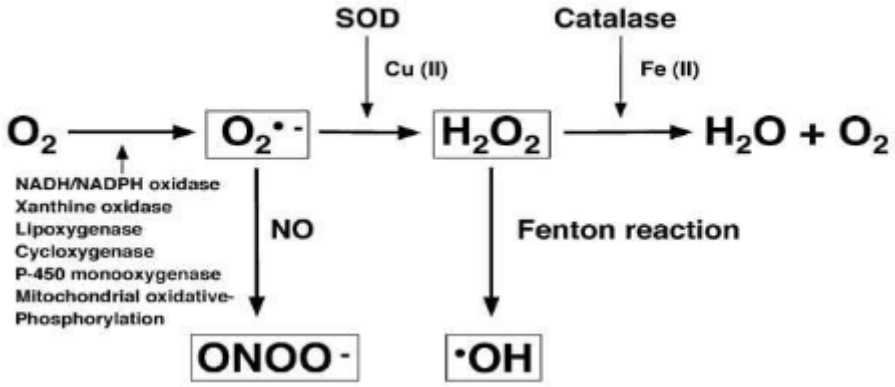


Figure 4: Fenton reaction

Source: Fenton (1984)

1.2.1.4 Immune Response in Malaria

The immune system plays several vital role in fighting the invasion of foreign agents in the body. Inmate immunity as well as adaptive immunity plays vital roles in protection of the body. Such immunological responses against malaria parasite are complex and stage-specific (Holder, 1999). Upon invasion of the body, the malaria parasite induces a specific immune response, as mechanism to stimulate the release of cytokines from human peripheral blood mononuclear cells (PBMC) (Doolan *et al*., 1994). These cytokines play an important function in activating a cascade of host immunological responses such as the host's monocytic cells (Esparza *et al*., 1987) neutrophils (Djeu 1990), T cells (Yokota *et al*., 1988) and natural killer (NK) cells (Østensen*et al*., 1987).

The hepatocytes are major sites of symptomatic and asymptomatic stage of malaria parasite development. Thus, the *Plasmodium* parasite development at the hepatocytic stage is the major target of protective immune response (Kwiatkowski, 1995). Antigens specific to the liver stage of parasite development and antigens brought along with invading sporozoites are processed by the invaded host cell and presented on the surface of infected hepatocytes in combination with MHC I (Weiss*et al*., 1990) (Figure 5). Cytotoxic T lymphocytes recognize the presented cells, Natural killer cells kills them, and CD4+ T cells are stimulated to produce interferon γ triggering a cascade of immune reactions, which arrest the proliferation of intracellular parasite (Weiss*et al*., 1990; Wang *et al*., 1996).

Altered immune response in human malaria occurs in the late phase of acute malaria and last for a long period even after the clearance of the parasite from circulation (Hviid *et al*., 1991). Pro-inflammatory and anti-inflammatory cytokines such as interferon γ, interleukin 4,

interleukin 10, and interleukin 12 among other cytokines mediate inflammatory response to malaria infection.

1.2.1.4.1 Interferon γ

Interferon γ isa macrophage-activating factor that is involved in the innate immune response to malaria infection. It mainly initiatesthe production of the CD4+ and CD8+T lymphocytes in a specific immune-response or Natural Killer cells in a non-specific response (Weiss *et al*., 1993). Plasma concentrations of interferon γ are reported to be higher in clinical cases of malaria than in asymptomatic cases, and there seem to be a relationship between interferon γ secretion and occurrence of fever (Malaguarnera and Musumeci, 2002).

1.2.1.4.2 Interleukin 4

Interleukin 4 is produced by Th2 and activated by basophil or mast cells (Figure 5). It is involved in the activation of cytotoxic T lymphocytes, Natural killer cells and macrophages. It is also a mediator of CD4+/CD8+ crosstalk, which is a necessary step for the development of immunity against malaria (Kumaratilake and Ferrante, 1992).

1.2.1.4.3 Interleukin 10

Interleukin 10 is also an important cytokine. Its concentration is found to be high in plasma of acute malaria patients (Wenish *et al*., 1995). Interleukin 10 inhibits cytokine production in CD8+ and Th 1 cells, but not in Th2 cells counterpart. Many interleukin 10 producing CD4+ and CD8+ T cells co-express interferon γ in malaria infected children and adults (Winkler *et al*., 1999).

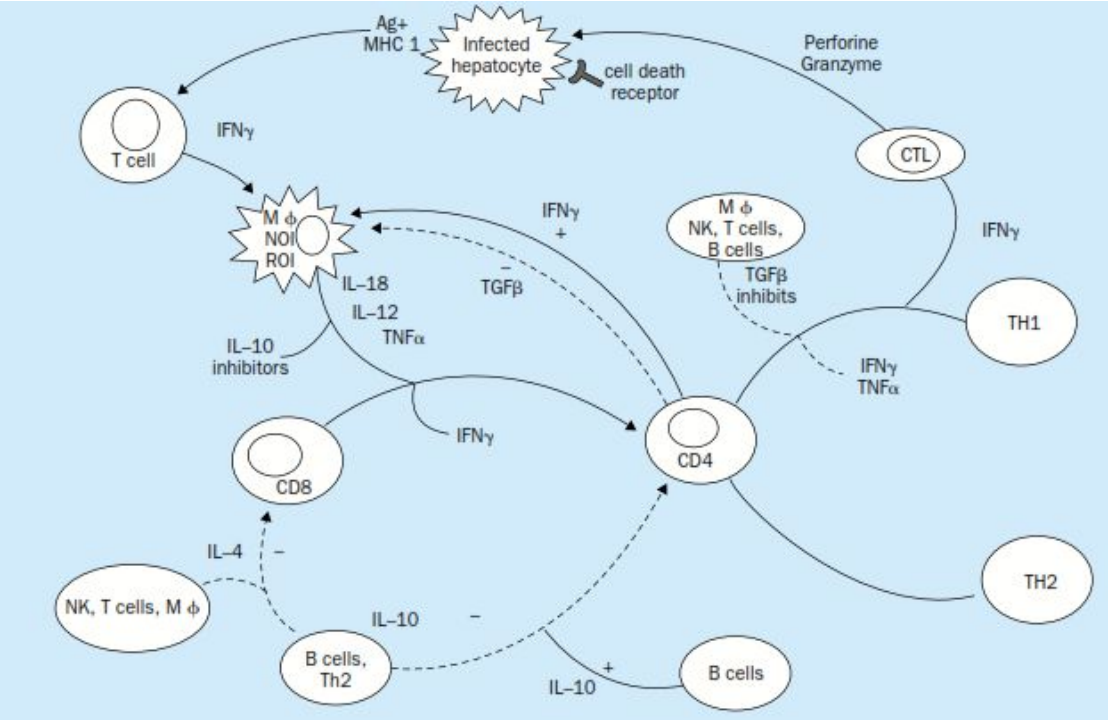


Figure 5: Immunological response during malaria infection

Source: Malaguarnera and Musumeci (2002)

1.2.1.4.4 Interleukin 12

Interleukin 12 is a potent immunomodulatory cytokine and has been established to be effective in conferring protection against viral, intracellular parasitic and bacterial infections (Malaguarnera and Musumeci, 2002). The induction of interleukin 12 is a direct consequence of CD8+ and CD4+ T cell activation: interferon γ production precedes and initiates production of interleukin 12, which in turn induces interferon γ production by Natural Killer (NK) cells in a positive feedback loop that represents an important amplifying mechanism (O'Garra and Arai, 2000).

1.2.1.5 Clinical Symptoms of Malaria

Malaria is associated with clinical symptoms that include intermittent fever, which occurs due to the rapture of RBC and release of merozoites and toxic substances into the blood system. This is followed by respites and another section of intermittent fever,triggering a chain of immune system response to combat the causative agent. The protozans feed on the RBC of their host, this also leads to anaemia if repeated malaria infection occurs.Other symptom include high temperature, glomerulonephritis (kidney inflammation),hypoglycaemia (low glucose levels),accumulation of fluids in the lungs (pulmonary oedema),acute renal failure and metabolic acidosis (Winstanley, 2001; Wilairatana *et al.,* 2002).

1.2.1.6 Diagnosis of malaria

Malaria is diagnosed mainly by microscopic examination of prepared blood smears, polymerase chain reaction (PCR) base testing and immunosorbent based rapid diagnostic test (RTD). These methods are well documented by the WHO bench aids for the diagnosis of malaria (WHO, 2000); however, the methods all have their advantages and disadvantages. Microscopic examination requires no use of sophisticatedequipment to carry and requires no advance training

skills. While ELISA base testing and PCR are very accurate and specific, they are expensive and require advance training. PCR is also usedin the differentiation of recrudescence and re-infection of parasite in host.

1.2.1.7 Treatment of malaria

Currently Artemisinin-based Combination Therapy (ACT) is recommended for the treatment of *P. falciparum* malaria. Fast acting artemisinin-based compounds (artemether dihydroartemisinin and artesunate) are combined with other slow acting drug from a different class to avoid resistance of the parasite to any of the antimalarials when administered alone. Other drugs used in ACT combination include amodiaquine, mefloquine, lumefantrine,sulfadoxine/pyrimethamine and piperaquine. Implementation of the recommendation to use ACTs is limited by the small number of affordable co-formulated and available antimalarial drugs. The co-formulated drug is one, in which two different drugs are combined in one tablet, and it ensures both drugs are used. ACTs also ensure fast action, high efficacy and the reduced likelihood of resistance development.

Artemether/lumefantrine (AL) was the first fixed-dose artemisinin-based combination therapy (ACT) recommended and pre-qualified by World Health Organisation for the treatment of uncomplicated malaria caused by *P.falciparum*. It has been shown to be effective both in sub-Saharan Africa and in areas with multi-drug resistant *P.falciparum*. Artemether/lumefantrine is currently recommended as a first - line treatment for uncomplicated malaria in several countries. However, its complex treatment regimen of two (2) doses daily for three (3) days could affect adherence.

1.3 Artesunate

Artesunate, also known as dihydroartemisinin-12-α-succinate, is a potent, semisynthetic antimalarial compound derived from its parent compound Artemisinin in a two-step reaction (Chekem and Wierucki, 2006). In search for a better and more soluble antimalarial compound, Artesunate, artemether, and arteether were synthesised from artemisinin. These analogs have improved solubility, absorption and pharmacokinetics (Li *et al*., 1998).

Artemisinin is effective against blood and early gametocyte stages of *Plasmodiumfalciparum* (Piyaphanee *et al*., 2006; White, 2008). Due to resistance by malaria parasite, Oral artemisinin monotheray (oAMT) was replaced by the use of Artemisinin Combination Therapies (ACT), where a fast acting artemisinin derivative is administered together with a slow acting antimalarial compound usually from another class. oAMT is believed to contribute to the development and spread of resistance to artesmisinin and its derivatives, thus a need for its withdrawal.

Resistance by malaria parasite remains the main challenge to malaria chemotherapy. Chloroquine resistance was reported in 1980's, in parts of Southeast Asia and South America, as well as regions along the east coast of Africa. Chloroquine was replaced as a first line drug for treatment of uncomplicated *P. falciparum* malaria by ACT (WHO, 2001). Regions with the common usage of Artemisinin as monotherapy, such as Western Cambodia, have shown drug resistance in clinical studies to Artemisinin (White, 2008). Oduola *et al*. (1992) and Randrianarivelojosia *et al*. (2001) have also reported reduced sensitivities by clinical parasite isolates from Nigeria and Madagascar to artemisinins derivatives respectively. Decreased responses to treatments specifically to artesunate and artemether have also been reported in western Thailand (Luxemburger *et al*., 1998), India (Gogtay *et al*., 2000) and Sierra Leone (Sahr

et al., 2001). Preserving the activities of the Artemisinin derivatives is now a challenge to malaria researchers.

Artemisinin-based combination therapy (ACT) is highly effective and still remains the currently recommended first-line therapy for treatment of uncomplicated malaria (WHO, 2010; 2015). The World Health Organization have recommended four different forms of ACT, which are artemether/ lumefantrine, artesunate + mefloquine, artesunate+Sulfadoxine / pyrimethamine and artesunate+amodiaquine. Other combinations are also recommended based on region and resistance expressions in the regions. It also recommended close monitoring of resistance and change of drug combinations due to pattern changes overtime (WHO, 2015).

Artemisinin and artesunate, since their discoveries, have undergone several modification and drug combinations in order to improve their activities or prevent the development of resistance against them. The synthesis of artesunate was a form of modification of its parent compound, artemisinin (Figure 6). The two-step reaction involve reduction and esterification using diisobutylaluminium hydride (DIBAL) and succinic anhydride respectively (Chekem and Wierucki, 2006). This modification has increased the solubility of artesunate, thereby solving one of the problems hindering the activity of artemisinin. This also allows artesunate to be recommended as a rectal, intramuscular as well as intravenous medication in severe malaria **(**Barnes *et al*., 2004;Harin *et al*., 2006).

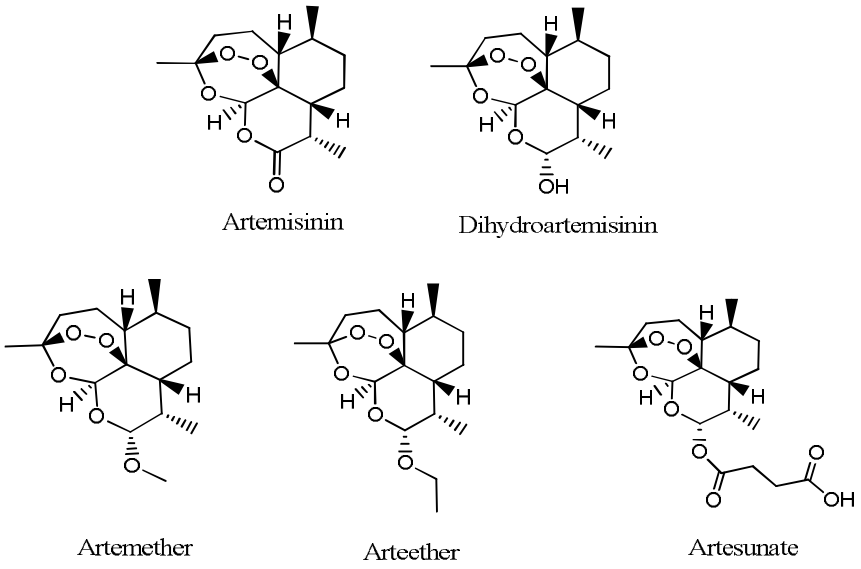


Figure 6: Artemisinin and it derivatives

Source: Francoise *et al*. (2000); Meena and Sandhya (2012)

1.3.1 Mechanism of action of Artesunate

Several mechanisms have been proposed for the antimalarial activities of the artemisinins and their bioactivation pathways including the generation of an activated intermediate reactive metabolite, production of free radicals and alteration of membrane transport properties of malaria parasite which inhibits the nutrient flow in parasite (Eckstein-Ludwig *et al*., 2003; Krishna *et al*., 2004; German and Aweeka, 2008). The artemisinin binding site is not clearly understood and has been proposed to inhibit the sarcoplasmic reticulum Ca^{2+}-transporting ATPases (SERCAs), specifically within the parasites PfATP6 (Afonso *etal*, 2006; Golenser *etal*., 2006; Beez*etal*., 2011). The endoperoxide bridge found within the artesmisinins mediate the end products giving rise to a carbon-centred radical which alkylates various proteins in the parasite; it also increases oxidative stress and inhibits angiogenesis which leads to cytotoxicity, which has found application in anti-cancer therapy (Meshnick, 2002; Haynes *et al*., 2002).

Artesunate has a short half-life of between 20-45min by oral route (OS) (Morris *et al*., 2011) and is metabolised through esterase-catalyzed hydrolysis, within this short time, to dihydroartemisinin which is the active metabolite responsible for the antimalarial activity (Teja-isavadharm *et al*., 2001; Gautam *et al*., 2009). Dihydroartemisinin (DHA) is converted to its inactive form through glucuronidation via UDP-glucuronosyltransferases (Ilett *et al*., 2002). A little amount of DHA is also eliminated via the bile as minor glucuronides (Teja-isavadharm *et al*., 2001). Artesunate inhibits DNA synthesis which reduces the growth of the parasite; it also reduces the energy production in the parasite by inhibiting respiratory process of the parasite (Yi *et al*., 1987).

1.3.2 Improving the activities of Artesunate

Since the discovery of artemisinin, several modifications have been done to the ACT parent compound including synthesis of dihydroartemisinin, artesunate and artemether (Brossi*et al*., 1988; Yu-Ming and Herman, 1993; Chekem and Wierucki, 2006), in an attempt to improve its effectiveness and delay/avoid the occurrence of resistance to the drug (Figure 7). The most widely accepted method, approved as WHO recommended method is the combination of the fast acting artemisinins with another slow acting drug usually from another class of antimalarial. Other, attempts to improve the activity have also been documented. These include synthesis of hybrid compounds including nanohybrids and embedding the artemisinin derivative in a synthetic matrix.

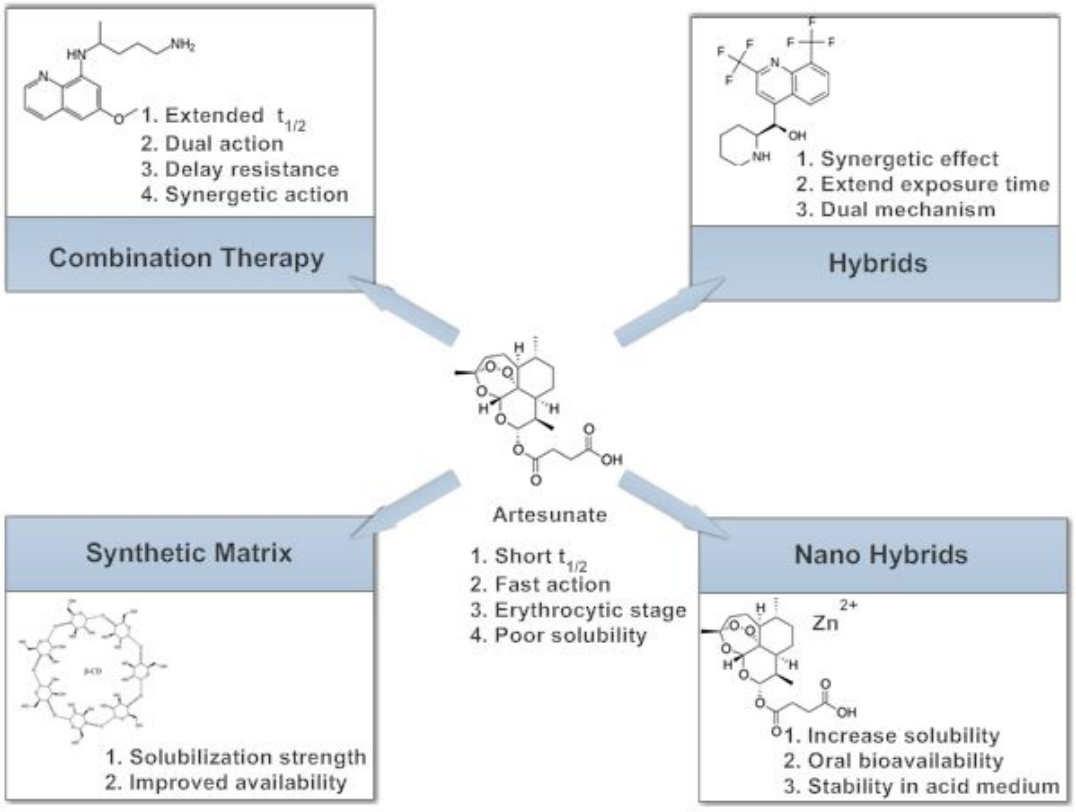


Figure 7: Methods employed in improving the antimalarial activity of Artesunate

Adapted from: White and Olliaro (1996); White (1998); Majori (2004); Kremsner and Krishna (2004); Douroumis and Fahr (2013)

1.3.2.1 Combination Therapy

The resistance of malaria parasite to chloroquine in regions of Southeast Asia, South America and along the east coast of Africa led to the withdrawal of chlorquine for ACT as first-line treatment for uncomplicated *Plasmodiumfalciparum* malaria (WHO, 2001). ACT was introduced due to emergence of parasite resistance to artemisinin monotherapy. Different ACT combination have being in use since then with continued monitoring (Figure 8). The principle behind the drug of choice for combinations is that the drugs should act synergistically (Majori, 2004) or exhibit their mechanisms of action through different modes such that the evolution of drug resistance is prevented (White, 1998). When the sensitivity of one combination decreases the partner drug is replaced by another with higher sensitivity. Artesunate combinations have been done with Primaquine, Mefloquine, Pyronaridine, Amodiaquine, and Piperaquine as single formulated dose. Others are combinations of Chloroproguanil-Dapsone, Sulfadoxine-Pyrimethamine, Atovaquone-proguanil, and some antibiotic combinations which include clindamycin, tetracycline, and doxycycline.

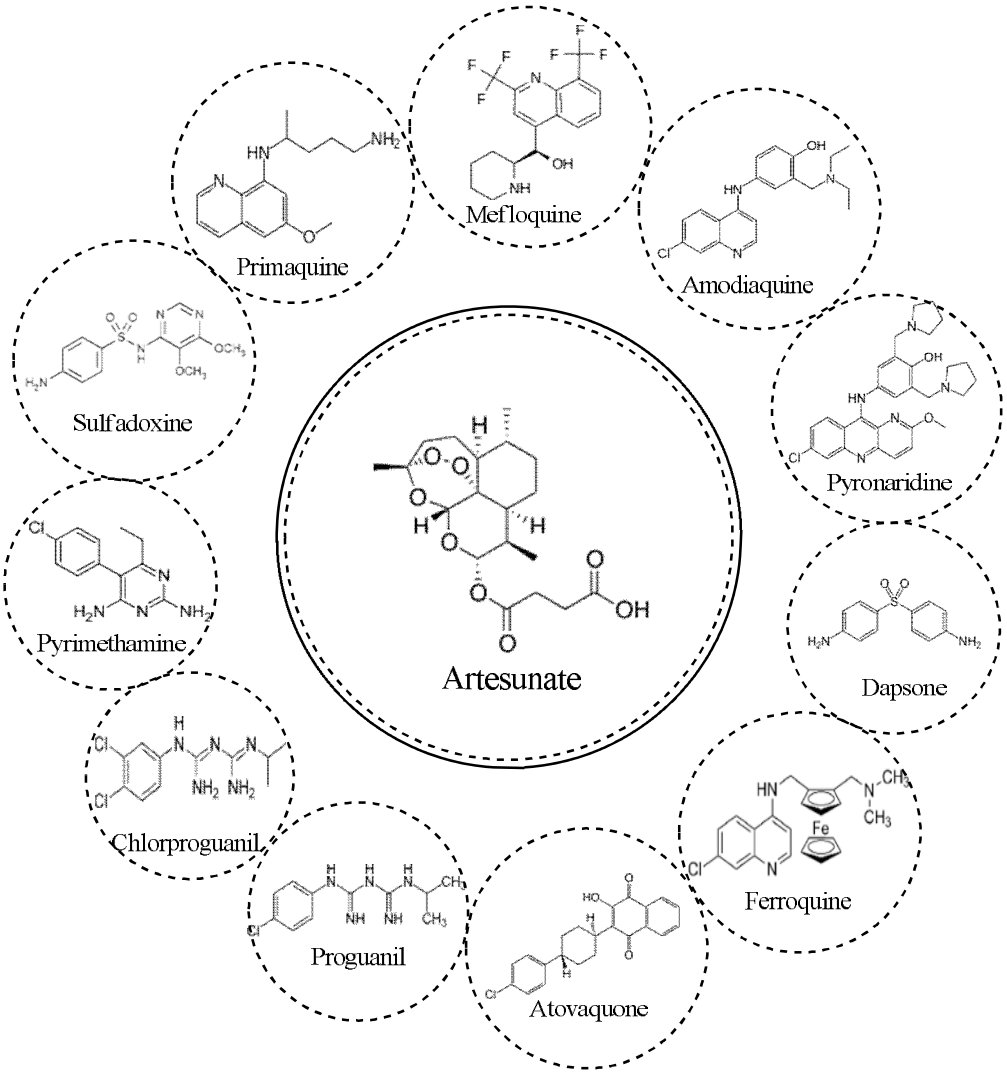


Figure 8: Chemical Structures of Artesunate and its partner drugs

Adapted from: de Pilla Varotti *et al*. (2008); Chadha *et al*. (2011); Wani *et al*. (2015)

1.3.1.1 Primaquine-artesunate

Primaquine (PQ) is highly effective against the gametocyte of *P. falciparum*, and equally effective as a hypnozoitocide drug for the treatment of *P. vivax* and *P. ovale* infections (Galappaththy *et al*., 2013; Graves *et al*., 2012; Baird and Hoffman, 2004; WHO, 2012). The drug is well absorbed and with a longer half-life (3.7 to 9.6 h) when compared to artesunate, its ACT partner (Edwards *et al*., 1993; Cuong *et al*., 2006; Elmes *et al*., 2006).

1.3.1.2 Mefloquine-artesunate

Mefloquine (MQ) was initially used in combination therapy with Sulfadoxine/pyrimethamine, and was successfully used in the treatment of resistant *P.falciparum* (Nosten *et al*., 1987), until the resistance developed few years later (Nosten *et al*., 1991). The new formulation of artesunate-mefloquine was suggested by the WHO technical consultation team on antimalarial drug combination therapy in 2001, the fixed dose contain ASMQ which is to be taken daily over a period of three days (WHO, 2001; ter Kuile *et al*., 1995). MQ provides a longer half-life of about 2 weeks and rapid clearance of asexual blood-stage of parasite and gametocyte (White, 1998).

1.3.1.3 Amodiaquine-artesunate

Amodiaquine belongs to the class 4-aminoquinoline. It has both schizonticidal and gametocytocidal activities against *Plasmodium* species. Amodiaquine and its active metabolite desethylamodiaquine have a longer half-life (5 hours and above 6 days respectively) (Krishna and White, 1996) when compared to artesunate. The combination of artesunate and amodiaquine as ACT demonstrated its superiority over the use of amodiaquine alone in African children (Adjuik *et al*., 2002).

1.3.1.4 Pyronaridine-artesunate

Pyronaridine is another drug developed in combination with artesunate for the treatment of uncomplicated *P. vivax* and *P. falciparum* malaria (Poravuth *et al*., 2011; Rueangweerayut *et al*., 2012). Their combination is in the ratio of 3:1 respectively. Pyronaridine has been reported to be active against erythrocytic stages of *Plasmodium* infection in mouse animal models (Ye and Shao, 1990; Looareesuwan *et al*., 1996). Its *in vitro* activities against multi-drug resistant *P. falciparum* isolates have also been documented (Institute of Parasitic Diseases Chinese Academy of Medical Sciences, 1980). Resistance to Pyronaridine has been developed by some *Plasmodium* strains (Wu, 1988; Li *et al*., 1995).

1.3.1.5 Sulfadoxine-Pyrimethamine-artesunate

The combinations of artesunate with mefloquine, amodiaquine or Sulfadoxine - Pyrimethamine are among the strongly recommended antimalarial drugs by WHO to be used for the treatment of uncomplicated malaria in children and as well as adult except in first trimester of pregnancy (WHO, 2015). Sulfadoxine /pyrimethamine combination with MQ was also previously used in the treatment of resistant *P.falciparum* before the development of resistance to their combination (Nosten *et al*., 1987; Nosten *et al*., 1991). Sulfadoxine-Pyrimethamine-artesunate combination has been used in different countries for the treatment of uncomplicated malaria (Obonyo *et al*., 2003; Marquiño *et al*., 2005), and it is reportedly well-tolerated, with no severe adverse drug reactions (Obonyo *et al*., 2003; Marquiño *et al*., 2005). The drug combination ensured a rapid decrease in fever and asexual parasite density; presence of gametocytemia was also significantly lowered in the combination therapy compared with SP alone treatment (Obonyo *et al*., 2003; Marquiño *et al*., 2005).

1.3.1.6 Chlorproguanil-Dapsone-artesunate

Artesunate, Dapsone and Chlorproguanil were developed into an affordable, fixed dose ACT for use in Africa, in tackling the emergence of resistance of parasites to Chlorproguanil – Dapsone combination. They are metabolised after oral administration into the active metabolites dihydroartemisinin, monoacetyl dapsone and chlorcycloguanil respectively. The latter provide a longer half-life to the ACT (Zuidema *et al*., 1986; Edstein and Veenendaal, 1987). The antifolates combination is similar to the SP, but with a rapid elimination from the body (Winstanley*et al*.1997).

1.3.1.7 Atovaquone-proguanil-artesunate

Artesunate-atovaquone-proguanil combination is highly effective and well-tolerated in the treatment of multidrug-resistant *P. falciparum* malaria (van Vugt *et al*., 1999; van Vugt *et al*., 2002). In the right combinations as single formulated drug, they provide alternative treatment for pregnant women infected with multidrug- resistant *falciparum* malaria (McGready*et al*., 2003). Although it may be expensive in its formulation, it provides a 3 day rescue for pregnant women (McGready*et al*., 2003). The inclusion of artesunate does not influence the pharmacokinetics of other components (van Vugt *et al*., 1999).

1.3.1.8 Ferroquine-artesunate

Ferroquine, a derivative of chloroquine, possesses great antimalarial properties. Ferroquine and its other derivatives such as hydroxyferroquines, trioxaferroquines, chloroquine-bridged ferrocenophanes have been extensively studied due to their promising antimalarial properties (Wani *et al*., 2015). Ferroquine is a 4-aminoquinoline analogue and the most successful of the chloroquine derivatives and has been used in combination with artesunate for a dual mode of action, better activities and possible means for delay of emergence of resistance to

both compounds. Ferroquine alone is effective against resistance chloroquine strain of *P. falciparum* (Biot, 2004). One of its mechanisms of action is by the inhibition of the formation of hemozoin in parasite (Biot *et al*., 2005).

1.3.1.9 Antibiotics

Antibiotics are used in the prevention and treatment of a vast range of diseases caused by microorganisms. Just like malaria, the treatment of bacterial infection does not only pose health challenge but also economic burdens due to decline in its effectiveness as the organisms develop resistance to the drugs (Gandra *et al*., 2014). The combination of an effective antibiotic with an antimalarial in areas of malaria endemic region is recommended where this combination is effective and also safe (White and Olliaro. 1996; WHO, 2006). Doxycycline, clindamycin and tetracycline have been used specifically in combination with artesunate in treatment of malaria.

1.3.2.2 Hybrids

Hybrids are chemical compounds with two or more different structures, having different biological functions and dual activity (Meunier, 2007). Hybrid molecules are synthesized in order to bring a new pharmacophore; this pharmacophore moiety is expected to bring a fresh/new property to the hybrid. Hybrid molecules with higher antimalarial effect or with synergistic effects are reported to be good candidates for new antimalarial development. According to Morphy and Rankovic (2005), hybrid molecules can be classified into Conjugates, Cleavage conjugates, Fused hybrids and Merged hybrids. A hybrid can be termed conjugate when a stable linker exists between them, which was not found initially in any of the individual component. When the linker is metabolised and the individual entities are release, the hybrid is termed cleavage conjugate. In a fused hybrid, a small linker reduces the distance between the different entities, such that the different pharmacophores are close to each other. Merged hybrid

possessestwo pharmacophores that are bound together such that the hybrid appearssmaller. Examples of synthesized artesunate hybrid molecules include artesunate and mefloquine hybrid (de Pilla Varotti *et al*., 2008) and indoloquinoline and artesunate hybrids (Wang *et al*., 2014).

1.3.2.2.1 Mefloquine and Artesunate hybrid molecule

Artesunate and mefloquinehave been used in combination therapy since 2001 (WHO, 2001), as well as with Sulfadoxine/pyrimethamine in treatment of *P.falciparum* (Nosten *et al*., 1987). The choice of Artesunate and Mefloquine as ACT partners is ideal, combining fast acting and slow eliminating drug respectively (Wiseman *et al.,* 2006). The endoperoxide ring of artesunate and the quinolinic ring of mefloquine, where combined in a new MEFAS hybrid molecule (de Pilla Varotti *et al*., 2008). MEFAS had new features different from the parent compounds, combining antimalarial compounds from two different classes, and was more potent than the parent compounds when administered alone and in different mass proportions. The hybrid compound was effective against both chloroquine-sensitive (3D7, IC_{50} 1.1 ng/ml) and chloroquine resistant (W2, IC_{50} 1.0 ng/ml) strains of *P. falciparum* parasites (de Pilla Varotti *et al*., 2008). MEFAS was active *in vivo* against *P. berghei*, with recrudescence after 30 days of parasite monitoring (de Pilla Varotti *et al*., 2008).

1.3.2.2.2 Indoloquinoline and artesunate

Wang *et al*. (2014) synthesized series of indoloquinoline hybrids, and evaluated their synergistic antimalarial properties. Indoloquinoline was linked with artesunate through an amide bond. The hybrids expressed low cytotoxicity, increased antimalarial activity and inhibition of β-haematin formation. They were active against chloroquine - sensitive (NF54) and -resistant (K1) strains of *P.falciparum*. In *in vivo* studies, the hybrids showed good antiplasmodial activities,

reducing parasitaemia by 89.6% on the 4th day and expressing a mean survival time of 7.7 days (Wang *et al*., 2014).

1.3.2.3 Nanohybrid

Artesunate nanohybrids were prepared by intercalating it into a zinc salt (Kim *et al*., 2015), in attempt to increase its solubility, oral bioavailability and prevent its degradation in acidic medium (Davis *et al*., 2001; Gabriëls and Plaizier-Vercammen, 2003; Chadha *et al*., 2012). Kim *et al*. (2015) also encapsulated the artesunate-Zinc-Basic Salt (ZBS) in an enteric coating agent to further decrease the release of artesunate and prevent its decomposition in intestinal pHs. The synthesised nanohybrid significantly improved the aqueous solubility and chemical stability of artesunate in acidic conditions.

1.3.2.4 Synthetic matrix

Drug solubility is very important for delivery to target regions/organs. Drug candidates from biological base screening or combination chemistry are mostly lipophilic and are expected to exert their therapeutic action across biological membranes or membrane associated proteins (Fahr and Liu, 2007). Artesunate is a water-soluble antimalarial drug; however, improving its solubility could increase it delivery and therapeutic effects. The use of a carbohydrate and non-carbohydrate matrices has been employed in increasing solubility of drugs and enhancing their delivery to target areas (Fahr and Liu, 2007; Douroumis and Fahr, 2013). This process transforms hydrophobic drugs to more water-soluble ones thereby improving their delivery systems.

Chadha *et al*. (2011) investigated the inclusion of artesunate in a carbohydrate matrix. β-cyclodextrin (β-CD) along with other matrix where used in this study. The artesunate-cyclodextrin molecules increased the solubilization strength of artesunate which was highest in

Me-β-CD (Chadha *et al*., 2011). More so, the binary Me-β -CD lyophilized suspension was effective *in vivo* against *P. berghei* infected mice with no mortality recorded for the duration of the study (Chadha *et al*., 2011).

1.3.2.5 Slight Modifications

Sodium artesunate is a basic salt as opposed to the acidic form, a recommended form of artesunate for administration in severe malaria due to the solubility of the earlier. It's a water soluble compound which can be administered either as an intravenous or intramuscular drug (Batty *et al*., 1996). Artesunate and sodium artesunate are more active in parasite clearance when compared with quinine and some other antimalarials because of their fast action, better tolerance and less pain; however, sodium artesunate is less potent when compared to its acid form (Batty *et al*., 1996). Its activities in resistant malaria parasite strains are also well-documented (Hien and White, 1993; Batty *et al*., 1996). Sodium artesunate was found to increase urinary excretion of sodium, chloride and potassium ions following administration in wistar rats (Campos *et al*., 2001). Campos *et al*. (2001) also reported increase in metabolites of nitric oxide (nitrite and nitrates) which were linked with protective effects in malaria (Taylor-Robinson and Looker, 1998).

1.3.2.6 Co-Crystal

Co-crystals of artesunate and nicotinamide prepared using two separate methods of solvent evaporation increased the solubility and dissolution rate of artesunate but no significant increase in the *in vivo* antimalarial activity of artesunate alone was observed (Setyawan *et al*., 2015). Co-crystal formation has been used to design crystals with improved properties such as solubility, bioavailability, and stabilityamong other properties without altering the effectiveness of the parent compound (Chadha *et al*., 2012). The effect of nicotinamide in the crystal formed

with artesunate, seem to improve the dissolution of artesunate in water but not its antiparasitic activities *in vivo* (Setyawan *et al.,* 2015).

1.4 Ethnomedicinal treatment of malaria

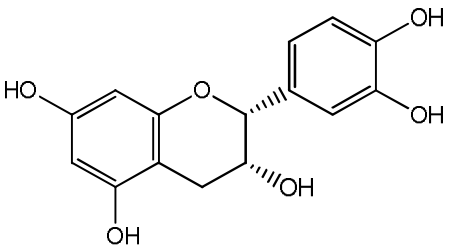
Several species of plant are used by some indigenous people in Nigeria for medicinal purposes which include conventional treatment of malaria. *Cocos nucifera*, *Fagara zanthoxyloides* and *Boswella dalzielli* are examples of Nigerian plants with antimalarial activities (Kassim *et al*., 2005; Shuaibu *et al*., 2008; Adebayo *et al*., 2012). The active antimalarial principles of some of these plant species used in Nigerian folk medicine have been reported for malaria treatment and have been isolated (Adebayo and Krettli, 2011). Among the active compounds isolated majority are alkaloids (Adebayo and Krettli, 2011).

Flavonoids also play important roles as active compounds in many African traditional malaria therapies.They have been shown to grately affect the human endothelial system at normal dietary concentrations, suggesting the potential of reducing the severity of malaria infections and facilitating parasite clearance via immune response (Maranz and Deitsch, 2010). Polyphenolic compounds such as flavonoids, of which catechins (a monomer of procyanidin) are the most prominent, have been reported to be present in abundance in the coconut husk fiber and are responsible for its antinociceptive, antibacterial, antileishmanial, antiviral and free radical scavenging activities (Esquenazi *et al*., 2002; Mendonca-Filho *et al*., 2004).*In vitro*anticytoadhesion activity has been documented for epigallocatechin gallate, a flavonoid that is commonly synthesized in plant leaves and is especially abundant in green tea (Dormeyer *et al*., 2006).

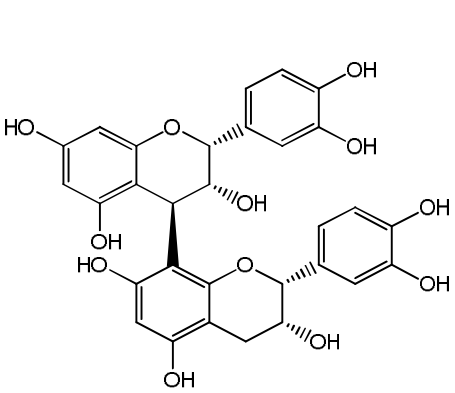
1.5 Procyanidins

Procyanidin (PC) is a class of polyphenolic polymer composed of flavan-3-ol ((+)-catechin) and (-)-epicathechin as monomers, linked mainly through C4–C8 bonds, and C4–C6 linkages in other polymers (Figure 9). Flavonoids are polyphenolic compounds that are ubiquitous in nature and are categorized according to their chemical structures into flavornols, catechins, flavanones, isoflavones, flavones, anthocyanidins and chalcones. Over 4,000 flavonoids have been identified, many of which occur in vegetables, fruits and beverages; beer, coffee, tea, wine and fruit drinks (Tanwar and Modgil, 2012). Cocoa and Coconut husk fibre have been reported to be high in flavonoids and polyphenolic content (Richelle *et al*., 2001; Esquenazi *et al*., 2002; Mendonca-Filho *et al*., 2004; Adebayo *et al*., 2013) which are responsible for their therapeutic importance.

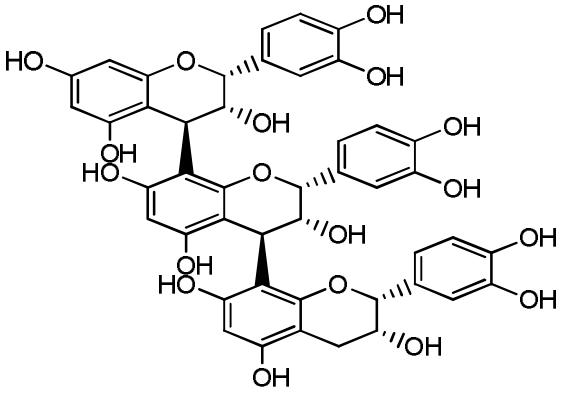
The flavonoids have gained considerable interest recently because of their potential benefits to human health. Experimental evidences have demonstrated that flavonoid compounds have several biological activities including anti-allergic, antimutagenic, anti-cancer, anti-HIV, anti-oxidant, anti-platelet, anti-inflammatory and radical scavenging activities (Harborne, 1994). Murakami (2003) has shown that diglycosides of flavanols (a specific type of flavonoids) impede life cycle of malaria parasites, whereas monoglycosides completely inhibited proliferation of trophozoite stage of parasites. Procyanidin B_2 extracted from the leaves of *Anogeissus leiocarpus* has been reported to be active against multidrug pyrimethamine/chloroquine-resistant strain with an IC_{50} of 5.3 µM *in vitro* (Attioua *et al*., 2011). Procyanidins are also abundant in grape seeds and have been reported to have health benefits that include antioxidant, anticarcinogenic, and anti-inflammatory effects (Kondo *et al*., 2000).



Epicatechin



Procyanidin B_2



Procyanidin C_1

Figure 9: Structures of oligomeric and polymeric Procyanidins

Source: Qin et al., 2005; Silva *et al*., 2013

1.5.1 Classification of Procyanidin

The building blocks of Procyanidin are flavonoids, which are characterized by a 15-carbon (C6–C3–C6) backbone consisting of one oxygenated heterocyclic and two aromatic rings (Figure 10). Procyanidin exist as monomers to polymers, with more B-type than the A-type structures. B-type are ploymerised by a carbon-carbon bond between the position 8 of the terminal units and the other preceding unit while the A-type are linked using the carbon-carbon bond on position 2 and 7 or positions 2 and 5. Other forms of linkages also exist. Procyanidin C1, is a C-type structure linked in B-type trimer.

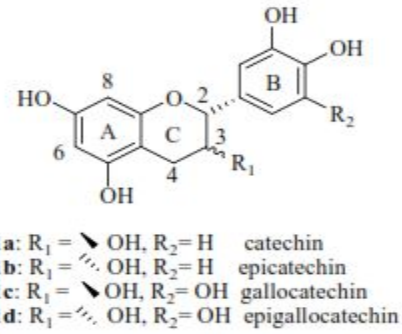


Figure 10: The structure of monomeric procyanidins

Source: Hashida *et al*. (2006)

1.6 Sites and Mechanismsof action of antimalarial drugs

Antimalarial drugs have been deployed to target different stages in the life cycle of malaria parasite. The replicative, noninflammatory and asymptomatic liver schizonts are acted upon by tissue schizontocidal drugs such as primaquine, proguanil, tetracycline, which act on early stages of parasite development in the liver, before the release of merozoites into the blood. Hypnozoitocidal drugs also kill dormant hypnozoites in the liver, preventing relapses of infection while sporontocidal drugs inhibit the development of oocysts in mosquito, decreasing malaria transmission (Pukrittayakamee *et al*., 2004).

The symptomatic erythrocyte stages are treated using blood schizontocidal drugs such as quinine, mefloquine, chloroquine, atovaquone and artemisinins which clears the parasite before clinical signs emerge.The site of action of these antimalarial drugs is summarized as follows:

1. Blocking Parasite Detoxification of Heme

Mechanism of action of the class of antimalarial drugs known as the Quinolines and related compounds is through the blocking of parasite detoxification of Heme, a product of their catabolism of haemoglobin from which they derive their source of amino acid. Quinine, chloroquine, amodiaquine, primaquine, piperraquine and mefloquine are all members of this family.

They act by interfering with the sequestration of ferriprotoporphyrin IX moieties, the toxic hemeproduced when hemoglobin is digested by the intra-erythrocytic parasite to obtain a major portion of its amino acid requirements. The heme, in the form of β-hematin dimers, is normally complexed by a process of biomineralization into hemozoin (the parasite-specific substance), which is deposited in a crystalline form in the lysosome-like acidic food (or

digestive) vacuole, into which the hemoglobin is initially imported by endocytosis (Kuhn *et al*., 2007; Klonis *et al*., 2010).

2. Competitive inhibitors of natural substrates

The treatment of malaria also explore the principle of antifolate drugs. Examples of such drugs are pyrimethamine, proguanil and the sulfa drugs (sulfadoxine and the sulfone). These drugs target key metabolites in the bacteria, acting as competitive inhibitors to dihydrofolate reductase and other key enzymes.

3. Alteration Ca^{2+} homeostasis

An initially promising study revealed that artemisinin inhibits PfATP6, the only sarco/endoplasmic reticulum calcium-dependent ATPase (SERCA) ortholog in *P.falciparum*, which consequently impedes the crucial Ca^{2+} homeostasis of the parasite (Eckstein-Ludwig *et al*., 2003).

4. Collapse of Mitochondrial membrane potential

Atovaquone is one of the potent antimalarial drugs introduced as a chemotherapeutic agent against malaria. Atovaquone is a substituted hydroxynaphthoquinone that acts as a structural analogue of coenzyme Q, it is described as a broad spectrum antiparasitic drug that collapses the mitochondrial membrane potential (Srivastava *et al*., 1997).

Its mechanism of action is by the inhibition of electron transfer in the mitochondria and its action does not only result in a loss of the membrane potential, but also impedes pyrimidine biosynthesis, two essential processes whose inhibition leads to parasite death. The fact that the human respiratory chain is not a target is based on the structural differences between the human and plasmodial coenzyme (Kessl *et al*., 2005).

1.7 Resistance to antimalarial drugs

The widespread availability of cheap and effective antimalarial drugs has kept the morbidity and mortality of malaria in check (White *et al*., 1999). The loss of these drugs to resistance may represent the single most important threat to the health of people in tropical countries (White *et al.,* 1999). Resistance to chloroquine is widespread across Africa and resistance to Pyrimethamine-sulphadoxine, a drug introduced to replace chloroquine is increasing (Ronn *et al*., 1996). As treatments lose their effectiveness, morbidity and mortality from malaria will inevitably continue to rise (White *et al.,* 1999).

Artemisinin and its derivatives (artemether, artesunate, dihydroartemisinin) are the most potent and rapidly acting of the antimalarial drugs (White, 1997). Artemisinin derivatives are stiil very effective, but reports of an incremental creep in resistance are emerging in many places (Dondorp *et al*., 2009; Sene *et al*., 2010). Resistance to artemisinin by *P.falciparum* has been reported in 5 countries; Cambodia, Leo Peoples Democratic, Myanmar, Thailand and Viet Nam (Noedi *et al*., 2008; WHO, 2014). Parasite isolates from regions inNigeria and Madagascar have been reported to exhibit reduced sensitivity to artemisinins (Oduola *et al*., 1992; Randrianarivelojosia *et al*., 2001). Maintaining the therapeutic efficacy of existing antimalarial drugs is therefore necessary. This has been done over the years through different means.

Malaria parasites, just like bacteria, have developed over the years several mechanisms of drug resistance. These mechanisms include their ability to mutate, prevent the permeability of drugs and rapid efflux of chemical substances before they could elicit their therapeutic effects. The genetic basis of the mechanism of chloroquine resistance was better understood by the discovery of the key gene, P*fCRT* ('chloroquine resistance transporter' [CRT]) in 2000 (Fidock *et al*., 2000).Also, the *P.falciparum*multidrug resistance (PfMDR) 1 protein encoding gene has

been implicated in the drug resistance mechanism (Henry *et al*., 2008; 2009).The treatment failure of atovaquone, resulting in increased mortality, has been linked to the appearance of mutations in the *cytochrome b*gene of the parasite (Korsinczky *et al*., 2000; Berry *et al*., 2006).

Chloroquine is a low cost, safe and effective drug which was used for treatment and prevention of malaria infection. Development of resistance to it was of great concern to researchers and a wakeup call for the development of new antimalarials that will be effective against the chloroquine-resistant strains. Chloroquine resistance by *P.falciparum* and *P. vivax* has rendered the drug inappropriate for the treatment of malaria in many areas (Whitby, 1997; Barat *et al*., 1998). The resistance by *P.falciparum* to chloroquine has been narrowed down to the massive efflux of the drug outof the digestive food vacuole (the site chloroquine action in inhibiting β-hematin formation) through the membrane drug pump/drug channel. Presence of P*fCRT* mutated gene was associated with marked reduction in the accumulation of CQ by the parasite (Martin *et al.,* 2010). This has been found to be the major difference between CQ-resistant parasites and CQ-sensitive parasites (Dorsey *et al.,* 2001).

1.8 Antioxidant Enzymes

Antioxidant enzymes protect the biological system from the toxic consequences of free radicals and reactive oxygen species by catalyzing various reactions to neutralize them. The major enzymatic antioxidants found in the cells include glutathione-S-transferase (GSTs, EC 2.5.1.18), superoxide dismutases (SODs, EC 1.15.1.11), catalase (CAT, EC 1.11.1.6) and glutathione peroxidases (GSH-Pxs, EC 1.11.1.9) (Halliwell and Gutteridge, 2007). Reduction in antioxidant enzymes such as catalase, glutathione peroxidase, superoxide dismutase has been observed in plasma of malaria-infected individuals (Steinbrenne and Sies, 2009).

1.8.1 Superoxide Dismutase

Superoxide dismutase (SOD, EC 1.15.1.1) catalyzes the dismutationof superoxide (O_2^-) radical into either hydrogen peroxide (H_2O_2) or ordinary molecular oxygen(O_2).It is the major antioxidant defense system protecting against superoxide radical. SOD exists in three isoforms in mammals, which are cytoplasmic Cu/ZnSOD, mitochondrial Mn/SOD and extracellular Cu/ZnSOD (Fukai and Ushio-Fukai, 2011). They require catalytic Cu or Mn for their activation and play critical roles in redox signaling, vascular function and diseases (Fukai and Ushio-Fukai, 2011).

1.8.2 Glutathione peroxidase

Glutathione peroxidases (GSH-Pxs, EC 1.11.1.9) are family of tetrameric enzymes containing selenocysteine, a unique amino acid within its active site. GSH-Pxs reduce H_2O_2 and lipid peroxides to alcohols using glutathione as substrate. There are at least four different GSH-Pxs encoding genes in mammals, giving rise toselenocysteine containing GPx1 to GPx4 (Ursini *et al.,* 1995). GPx1 and GPx4 (or phospholipid hydroperoxide GPx) are both cytosolic enzymes abundant in most tissues. GSHPx-1, the cellular GSH-Px is most ubiquitous among all of them and has been implicated in the development and prevention of many diseases such as cancer and cardiovascular diseases (Lubos *et al*., 2011).

1.8.3 Glutathione-S-transferase

Glutathione-S-transferase (GST, EC 2.5.1.18) is among the antioxidant enzymes of important biochemical activities. GST catalyses the inactivation of secondary metabolites, such as unsaturated aldehydes and hydroperoxides. Similar to SOD, GST exists in three major forms: cytosolic, mitochondrial and membrane-associated microsomal GSTs (Jakobsson *et al*., 1999;

Ladner *et al*., 2004; Robinson *et al*., 2004). Decrease in the activities of GST has been reported in of *P. berghei* infected mice (Okeola *et al*., 2011).

1.8.4 Catalase

Catalase (CAT, EC 1.11.1.6) is an enzyme which catalyze the decomposition of hydrogen peroxide produced by the action of SOD or oxidase, such as xanthine oxidase to molecular oxygen and water. Catalase is tetrameric with four identical mononers known to contain a heme group at the active site (Aebi, 1974). Catalase is described to have one of the highest turnover rates of all enzymes, with the ability to convert approximately 6 million molecules of hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2) within a minute (Mates *et al*., 1999). Catalase hasbeen implicated during *Plasmodium* infections with significant decrease in activities of the enzyme (Mathews and Selvam, 1991; George *et al.,* 2012).

1.9 Organ Function Indices

1.9.1 Liver Function Indices

The liver is a vital organ of the body and a vital organ for the survival of malaria parasite during infection and development. The liver is affected in the early stage of malaria infection (Miller *et al.,* 2002) which leads to significant alteration in the host hepatocytes and its morphology (White and Ho, 1992). As such, assessment of the liver is vital in monitoring the level of infection or treatment of the disease. Liver function tests include a list of biochemical indices such as concentrations of total protein, albumin, globulin and bilirubin in the plasma and other biochemical marker enzymes.

1.9.1.1 Albumin

Albumin is the most abundant plasma protein, where it accounts for about 55-60% serum protein concentration (Gosling, 1995). It is produced in the liver as non-glycosylated, negatively

charged single polypeptide, which are not stored in the liver but secreted into the portal circulation. Albumin functions in the transportation of many substances such as bile acid, copper, folate, zinc and several drugs. Albumin is also involved in nutrient transport, waste removal and osmotic pressure regulation. Albumin can modulate oxidant effects through scavenging radicals, modifying redox balance and regulating cell-signaling (Peters, 1996; Quinlan *et al*., 2005).

1.9.1.2 Globulin

Globulins are an important class of protein. They are manufactured in the liver, play important roles in blood clotting, and respond to foreign body invasion. Globulins are classified into alpha, beta and gamma globulins. Their functions include the inhibition of an enzyme that digests protein, inhibition of compounds vital in blood coagulation and a protein that can transport the element copper.

1.9.1.3 Bilirubin

Bilirubin is the main product of haemoglobin catabolism in the red blood cell. The liver is responsible for the conversion of the unconjugated bilirubin released to a conjugated bilirubin. They are then conjugated to mono and diglucuronides by the UDP- glucuronyltransferase (Mauro *et al*., 2006). Bilirubin could be toxic especially in newborn leading to a condition known as neonatal jaundice. Bilirubin concentration is therefore measured as marker liver function.

1.9.1.4 Marker enzymes

Activities of various enzymes are used as markers for liver function indices. Examples are glutamate dehydrogenase, malate dehydrogenase, gamma glutamyl transferase alanine and

aspartate aminotransferases and alkaline phosphatase. The activities of these enzymes are increased in the blood when there is liver damage.

1.9.2 Kidney Function Indices

The kidney is an organ responsible for filtration of waste products from the blood. In addition, they maintain electrolyte balance in the body and control blood pressure. Kidney function test are done to assess the ability of the kidney to perform its normal excretory functions or otherwise. Acute renal failure has been reported in malaria, mostly from the *P.falciparum* infections (Das, 2008). This occurs with no clearly explained mechanism (Das, 2008). However, it can be measured as abnormal values in electrolytes, urinary sediments and increase urinary protein excretion among other signs (Maheshwari *et al*., 2004). Plasma concentrations of electrolytes, urea, uric acid and creatinine are among the kidney function indices assayed for during the study.

1.10 Plasma Electrolytes

1.10.1 Calcium Ion

Calcium (Ca^{2+}) ion constitute a major part the teeth and bone. It also participates in the activations of enzymes such as ATPases, lipases, succinic dehydrogenase and transmission of nerve impulses. Its absorption is regulated by the presence of calcium-binding proteins and vitamin D. Excess calcium are eliminated by the kidneys or they can cause depression of cardiac activities which leads to respiratory and cardiac failure (Soetan *et al*., 2010).

1.10.2 Chloride Ion

Chloride (Cl^-) ionis the principal anion in extracellular fluid (Soetan *et al*., 2010). It is involved in fluid and electrolyte balances as well as in chloride shift in HCO^{3-} transport in

erythrocytes. Increase in chloride ion concentration is related to acidosis, while their decrease with serum albumin may indicate dehydration (Guyton ad Hall, 2006).

1.10.3 Bicarbonate Ion

Bicarbonate (HCO_3^-) ion playsan important role in maintaining the body buffering system. It is a byproduct of the body's metabolism which is excreted as carbon dioxide by the lungs. High serum bicarbonate concentration may result from metabolic alkalosis while low serum bicarbonate may result from renal dysfunction (Guyton and Hall, 2006).

1.10.4 Potassium Ion

Potassium (K^+) ionis the principal cation in intracellular fluid (Soetan *et al*., 2010). It is important in maintenance of body acid/base balance, muscle contraction, cardiac function, cell membrane and Na^+/K^+-ATPase activities (Soetan *et al*., 2010). Increased levelof serum potassium (Hyperkalaemia) is seen in shock, dehydration, chronic renal failure and Addison's disease (Soetan *et al*., 2010).

1.10.5 Sodium Ion

Sodium (Na^+) ionis the principal cation in extracellular fluid (Soetan *et al*., 2010). It plays a significant role in the regulation of plasma volume and acid/base balance. Na^+ is involved in the maintenance of osmotic pressure of the body fluid, preserve muscle irritability, cell permeability and Na^+/K^+-ATPase activities (Soetan *et al*., 2010). Its extracellular concentration also allows for the absorption of monosaccharides, bile salts, pyrimidines and amino acids and (Soetan *et al*., 2010). Its plasma concentration also affects cell volume: increased concentration (hypertonicity), shrinks cells and decreased concentration (hypotonicity) swells cells (Sterns, 2015).

1.10.6 Phosphate Ion

Phosphate (PO_4^{3-}) ion is among the major intracellular anions in mammals. It is essential in cellular structure, cytoplasm and mitochondria, and contributes to the structure of DNA, phospholipids and is present in high-energy compound such as ATP (Bazydlo *et al*., 2014).

1.10.7 Urea

Urea is the principal nitrogenous waste product of body metabolism. It is generated from amino acid and protein catabolism and eliminated from the body by the kidney through the formation of urine. Urea concentration in the plasma reflects the balance between its production in the liver and elimination by the kidney. Defect in urea elimination by the kidney, allows its accumulation in the blood which subsequently damages the nephrons and results in reduced glomerular filtration and presence of urea in the blood (ureamia) (Guyton and Hall, 2006).

1.10.8 Uric acid

Uric acid isa product of endogenous purine metabolism. It is an organic compound that is produced in the liver and excreted by the kidney and intestine at different ratios (de Oliveira and Burini, 2012). Uric acid circulates in the plasma as urate, a monovalent sodium salt because it exists as weak acid in nature with high dissociation constant (Liebman *et al*., 2007). It possessesan excellent antioxidant capacity, a function attributed to their double bond nature (Sautin and Johnson, 2008). Elevated uric acid is seen in renal failure, chronic disease and other diseases associated with nucleic acid metabolism.

1.10.9 Creatinine

Creatinine is the final product of muscle creatine metabolism (Gao *et al*., 2010). Creatinine is thus excreted as a waste product by the kidney at a relatively low rate (Gao *et al*., 2010). Serum creatinine level is an indicator of glomerular filtration rate, which gives useful

information about the kidney. Abnormally, high value of creatinine implies malfunction or failure of the kidneys (Guyton and Hall, 2006).

1.11 Cardiovascular disease Indices

Circulatory collapse and thesymptoms associated with impaired hemodynamic function are characteristics of complicated *Plasmodiumfalciparum* malaria (WHO, 2000). Ehrhardt *et al*. (2004, 2005) have shown that cardiac enzymes are elevated in complicated malaria. Similarly, ejection fraction assessed by ultrasound in children with severe malaria was significantly reduced when on admission compared with whendischarge (Yacoub *et al.,* 2010). Hypercholesterolemia and hypertriglyceridemia are both observable features of uncomplicated and complicated malaria (Mohanty *et al*., 1992; Davis *et al*., 1993; Das *et al.,* 1996). However, Imrie *et al*. (2004) reported no correlation between severity of malaria infection and extent of HDL – cholesterol decrease. Human serum HDL– cholesterolis necessary for *P.falciparum*in *in vitro*culture. However, it has been reported that HDL– cholesterol can be toxic for the parasite at high concentrations (Grellier *et al*., 1991). Imrie *et al.* (2004) have also reported that in the absence of serum, HDL– cholesterol in low concentration (0.75 mg/ ml) supported growth of *P.falciparumin vitro*, whereas itshigh concentration (3 mg/ml), it was toxic to the parasite. Recent findings, however, suggest that the *Plasmodium* genome contains genes encoding enzymes of phospholipids metabolism, allowing *de novo* synthesis of phosphatidyl choline via the kneddy pathway and necessitating only the uptake of the small choline molecule (Vial *et al.,* 2003).

1.11.1 Total Cholesterol

Cholesterol is an amphipathic lipid and thus an essential structural component of all cell membranes and of the outer layer of plasma lipoproteins. Cholesterol is present in tissues and in

plasma lipoprotein combined with a long-chain fatty acid, as cholesteryl ester or as free cholesterol. It is synthesized in many tissues from acetyl-CoA precursor and is ultimately eliminated from the body through the liver as cholesterol or bile salts in the bile (Fredrickson and Levy, 1972). Lipoprotein transports free cholesterol in the circulation, where it readily equilibrates cholesterol in other lipoproteins and in membranes. Cholesteryl ester is a stored form of cholesterol found in most tissues. It is transported as cargo in the hydrophobic core of lipoproteins (Tietz, 1994).

1.11.2 Triglycerides

Triglycerides are esters derived from glycerol and three fatty acids. It is the main constituent of vegetable oil and animal fats (Nelson and Cox, 2000). Triglycerides are major component of very low density lipoprotein (VLDL) and chylomicrons which play important role as energy source and transporters of dietary fat. They contain more than twice as much energy (9 kcals/g or 38 kj/g) as carbohydrates and proteins (Nelson and Cox, 2000). In the human body, high levels of triglycerides in the blood stream have been linked to atherosclerosis and by extension, the risk of heart disease and stroke (Murray *et al*., 2000).

1.11.3 High Density Lipoprotein

High Density Lipoprotein (HDL) is also known as alpha lipoprotein. It is composed of 13% triglyceride, 46% phospholipid, 29% cholesterol ester, 6% cholesterol and 6% fatty acids (Nelson and Cox, 2000). HDL-C mediates the removal of cellular cholesterol by carrying cholesterol away from the body cells and tissues to the liver for excretion (Murray *et al.,* 2000). Increase in the concentration of HDL-C correlates inversely with coronary heart disease (Philip, 1995).

1.11.4 Low Density Lipoprotein

Low Density Lipoprotein (LDL) is also known as beta lipoprotein. LDLis a primary carrier of cholesterol. Increase in its concentration in blood can lead to atherosclerosis and eventually stroke (Stryer, 1995). LDL-Cholesterol along with total cholesterol and triglycerides are reportedly related to prevalence of Coronary Heart Disease (Alixandra *et al*., 2010).

1.11.5 Atherogenic index

Atherogenic index (AI) refers to the ratio of LDL-cholesterol to HDL-cholesterol or the ratio of total cholesterol concentration to HDL-cholesterol concentration (Balogun and Adebayo, 2007). It has been used as an indicator of cardiovascular disease (Panagiatakos *et al*., 2003). According to Ng *et al*. (1997), atherogenic index greater than 5 indicates ahigh risk of atherosclerosis.

1.12 Enzymes studied

1.12.1 Aspartate Aminotransferase

Aspartate aminotransferase (AST, E.C. 2.6.1.1) catalyses the transamination reaction involving the transfer of an amino group from a 2-amino acid to a 2-oxo acid, requiring pyridoxal-5-phosphate as cofactor. AST exists in two different isoenzyme forms known as the cytoplasmic forms and the mitochondrial forms and are genetically distinct from each other. They are widely distributed in the human body (Philip, 1994).

Clinical significance:

The activity of AST is important in clinical diagnosis; as such, itis used in monitoring certain disease conditions. AST is found in highest concentrations in the heart when compared to its corresponding values in other tissues like the liver, skeletal muscle and kidney (Mauro *et al*.,

2006). Increased levels of AST is seen in myocardial infarction, hepatic necrosis and active cirrhosis (Wrobleski and La Due, 1955).

1.12.2 Alanine Aminotransferase

Alanine Aminotransferase (ALT, E.C. 2.6.1.2) catalyses the reversible transamination reaction between an amino group from alanine to a α-ketoglutarate, leading to formation of glutamate and pyruvate. ALT is found in the kidney, heart, muscle, pancreases, lungs and is most commonly associated with the liver (Tietz, 1987).

Clinical significance:

ALT is an important maker enzymes for diseases. It is measured as one of the enzymes for liver function tests. Increased serum ALT suggest that there are other medical conditions like congestive heart failure, alcoholic or viral hepatitis, liver damage or biliary duct problems. Increased serum ALT similarly is considered a more sensitive indicator of hepatitis than serum AST because ALT is found in high concentration in the liver than in heart muscle (Reitman and Frankel, 1957).

1.12.3 Alkaline Phosphatase

Alkaline Phosphatase (ALP, E.C. 3.1.3.1) catalyses the hydrolysis of a wide range of phosphomonoesters from a number of organic molecules that include ribonucleotides, deoxy-ribonucleotides, proteins, alkaloids, phosphate esters and anhydrides of phosphoric acid. Alkaline phosphatases are measured primarily to check the possibility of liver disease or bone disease. Since the mucosal cells that sorroundsthe bile system of the liver are the source of alkaline phosphatase, thier free flowthrough the liver and down into the biliary tract and gall bladder are responsible for maintaining the proper level of alkaline phosphatase enzymes in the blood. When the liver, gallbladder or bile ducts system are not functioning properly or are

blocked, this enzyme is not excreted through the bile and alkaline phosphatase is released into the blood stream. Thus, the serum alkaline phosphatase is a measure of the integrity of the hepatobiliary system and flow of bile into the small intestine.

Clinical significance:

ALP has important clinical uses. Its activityis elevated in periphaeral arterial disease, independent of other traditional cardiovascular risk factor (Cheung *et al*., 2009). Increased ALP activity is also observed in some tissues as a result of increase synthesis of plasma membrane proteins (Wright and Plummer, 1974).

1.12.4 Glutamate dehydrogenase

Glutamate dehydrogenase (GDH, E.C. 1.4.1.2) is an hexameric enzyme which catalyses the reversible conversion of glutamate to α-ketoglutarate and ammonia by using $NADP^+$ and converting the later to NADPH. GDH is an important enzyme for distinguishing between acute toxic liver necrosis and acute viral hepatitis or acute hypoxic liver disease, particularly in the case of liver damage with very high aminotransferases. Glutamate dehydrogenase function is also linked to cell signaling processes and redox homeostasis (Duran*et al*., 2012; Jin *et al*., 2015).

Clinical significance:

Elevated serum GDH levels indicate liver damage. Measurement of its activities can play significant role in the differential diagnosis of liver disease, particularly in combination with aminotransferases. Liver diseases in which necrosis of hepatocytes is the predominant event, such as toxic liver damage or hypoxic liver disease, are characterised by high serum GLH levels.

1.12.5 γ-glutamyl Transferase

γ-glutamyl transferase (γ-GT, E.C. 2.3.2.2) is an ectoenzyme with the primary role of metabolizing extracellular glutathione, allowing for precursor amino acids to be assimilated and reutilized for intracellular glutathione synthesis, thereby ensuring the recycling GSH across the plasma membrane (Forman *et al*., 1997). γ-GT is present in the hepatocytes and biliary epithelial cells, intestine, pancreas and renal tubules. However, γ-GT activities are mainly attributed to hepatobiliary system rather than renal tissues where they are more concentrated (Mauro *et al*., 2006).

Clinical significance:

γ-GT transferase isindicated as an index of liver dysfunction and as marker of alcohol intake. Its activity is elevated in chronic and acute alcohol abuse while it isreduced in hypothyroidism, hypothalamic malfunction and low levels of magnesium (Lum and Gambino, 1972).

1.12.6 Lactate dehydrogenase

Lactate dehydrogenase (LDH, EC 1.1.1.27) catalyses the conversion of pyruvate to L-lactate and nicotinamide adenine dinuleotide in the presence of NADH (reduced nicotinamide adenine dinuleotide). It is present in human serum as five isozymes with tetrameric with two types of monomeric subunits (Cahn *et al*., 1962).

Clinical significance:

Elevation of serum LDH activity may reflect internal disease. Itis used for the diagnosis of myocardial infraction. Increase in serum LDH levels are also indicators of disease of the liver, kidneys, blood, skeletal muscle or lungs (Wieme, 1964; Paloheimo and Ikkala., 1965).

1.12.7 Acetylcholine esterase

Acetylcholine esterase (AChE, E.C. 3.1.1.7) catalyses the hydrolysis of acetylcholine, a neurotransmitter, into acetic acid and choline. AChE is primary found in the blood and synapses. Its reaction is necessary for cholinergic neurons to return to their resting state after activation for another set of neurotransmitting reactions.

Clinical significance:

Acetylcholine esterase levels in cells and plasma are required as guide in establishing safety precautions related to exposure to organic phosphate insecticides. Low serum level of AChE is an indicator of exposure to organic phosphate insecticides.

1.12.8 Creatine kinase

Creatine kinase (CK, *E.C.* 2.7.3.2.) catalyses the conversion of creatine to phosphocreatine (PCr) and adenosine diphosphate (ADP) with the consumption of one molecule of adenosine triphosphate (ATP). CK is expressed in various tissues and cell types, especially in heart, skeletal and smooth muscles as well as the brain.

Clinical significance:

The activities of CK in plasma or serum are measured routinely in order to assess injuries to the skeletal muscles or myocardium. Increase in CK levels are pointer of muscular disorders (myopathies). In heart attack, the differentiation of the various isoenzymes of CK are useful indication of myocardial damage (Hekimsoy and Oktem, 2005).

1.12.9 Adenosine Triphosphatases

Adenosine triphosphatases (ATPases) catalyse the hydrolysis of adenosine triphosphate (ATP) to form adenosine diphosphate (ADP) and phosphate (Pi). The hydrolysis release energy for other cellular reactions that include the transport of ion across the plasma membranes,

muscle contraction and removal of toxic ions from cells (Kühlbrandt, 2004). Nobel lauret, Jens Skou, discovered Na^+ K^+-ATPase (Na, K-pump) in the peripheral nerves of shore crab (Skou, 1965). This membrane-bound Na, K-pump mediates the active transport of Na^+ out and K^+ into the cell and has been identified in virtually all animal tissues including the human myocardium. This research work studied Na^+ K^+-ATPase, Mg^{2+}-ATPase as well as Ca^{2+} Mg^{2+}-ATPase in kidney, heart and brain homogenates of experimental animals.

1.12.9.1 Na^+ K^+-Adenosine Triphosphatase

Na^+ K^+-Adenosine triphosphatase (Na^+ K^+-ATPase, EC 3.6.3.9) enzyme pumps $3Na^+$ ions and $2K^+$ ions against concentration gradient with the hydrolysis of one molecule of ATP (Aslihan, 2002). The enzyme is an integral membrane protein located in the basolateral membrane. As such, Na^+ K^+-ATPase maintains this gradient across plasma membrane. Na^+ K^+-ATPase is a heterodimer of α subunit and β subunit (Jørgensen, 1986) both of which are required for its enzymatic activity. The subunit genes are under complex gene regulations (Aslihan, 2002). Several isoforms of the enzyme have been identified. In humans, there exist four isoforms of the α-subunit of Na^+ K^+-ATPase. The α1 is the predominant and ubiquitously expressed; α2 is majorly expressed in heart, skeletal muscle, smooth muscle, brain, lung and adipose tissues (Sweadner, 1989; Lingrel and Kuntzweiler, 1994; Kaplan, 2002). α3 is primarily expressed by neurons and heart cells (Sweadner, 1989; Lingrel and Kuntzweiler, 1994; Kaplan, 2002) while the α4 is expressed only in testes (Shamraj and Lingrel, 1994).

1.12.9.2 Mg^{2+}- Adenosine Triphosphatase

Mg^{2+}- adenosine triphosphatase (Mg^{2+}-ATPase, E.C. 3.6.3.2)is also localized in the cell membrane. Mg^{2+}-ATPase requires magnesium as cofactor for its catalytic action (Szemraj *et al*., 2005). Mg^{2+}-ATPase is important in the control of intracellular Mg^{2+} concentrations

(Cunningham *et al*., 1993), by so doing it also regulates many activities of magnesium (Mg^{2+}) requiring enzymes, which in turn regulates the protein synthesis, and the processes of cell growth (Cunningham *et al*., 1993).

1.12.9.3 Ca^{2+} Mg^{2+}-Adenosine Triphosphatase

Ca^{2+} Mg^{2+}-adenosine triphosphatase (Ca^{2+} Mg^{2+}-ATPase, E.C. 3.6.3.8) regulates the cytoplasmic concentration of Ca^{2+} via the activity of Ca^{2+} pumps which transport Ca^{2+} against a concentration gradient across the cell membrane (Kimura *et al*., 2005). Calcuim signaling is important for a number of events in the life cycle of *Plasmodiumfalciparum* among which are invasion of erythrocytes (Singh *et al*., 2010), synchronization of erythrocytes cycle *in vivo* (Hotta *et al*., 2000), sexual differentiation, motility, invasion in the mosquito gut by ookinetes and sporozoites (Billker *et al*., 2004; Ishino *et al*., 2006; Ono *et al*., 2008). These stress the importance of Ca^{2+} Mg^{2+}-ATPase in regulating calcium homeostasis during malaria infection.

Clinical significance of the ATPases:

Higher concentration of intracellular Na has been observed in cardiomyocytes from heart failure patients and from failing rabbit hearts (Despa *et al*., 2002; Pieske *et al*., 2002) which may be induced by decrease in Na, K-pump activity (Schwinger *et al*., 1999), and also increased Na-influx (Despa *et al*., 2002). Higher concentration of Na may stimulate Na, K-ATPase activity (Crambert *et al*., 2000; Müller-Ehmsen *et al*., 2001), while at resting conditions, only a few percent of the Na, K-pumps have been found to be usually active. On the other hand, only a reduced capacity for Na handling is available on demand. Thus, a Na concentration rise during e.g. ischemia may secondarily limit Ca^{2+} and H^{+} extrusion from myocytes inducing arrhythmias and leading to further progression of heart failure due to cell necrosis.

1.13 Enzymes evaluated in Molecular docking studies

Molecular docking studies and simulations are techniques used in designing and postulating new pharmacophores. They can be used to predict several number of compounds with improved activities based on the ligand-protein interactions obtained from a dock program. They required a form of pre-docking step to validate the production process, where some conditions such as the grid box and torsion centers are set.

1.13.1 Aspartic proteases

Aspartic proteases (Plasmepsins) are a group of proteases so named due to the presence of two highly conserved aspartic acid residues in their active sites for catalytic cleavage of peptide substrates. Aspartic proteases have been reported as potential targets for antimalarials (Russo *et al.,* 2010), therefore new compounds with good ligand-plasmepsin interactions can serve as possible new drugs for malaria therapy. They are homologoues with a sequence identity resembalce of about 60 – 70% between Plasmepsin I and Plasmepsins II, III, and IV. Plasmepsin protein also have sequence homology to cathepsin D, a human related aspartic protease (Ersmark 2006). Their active site includes two aspartic acid residues, which are Asp32 and Asp215. Plasmespsin III is referred to as Histo-aspartic protease (HAP) because the Asp32 residue in the active site is replaced by Histidine (Berry *et al*., 1999). The process of haemoglobin degradation occurs in food vacuole of *Plasmodium* parasite (Francis *et al*., 1997). Plasmepsin I, II, III and IV are involved in the process of haemoglobin degradation in the food vacuole of *P. falciparum* (Coombs *et al.,* 2001; Banerjee *et al*., 2002). KNI-10006 (Figure 11) has been reported as a potent inhibitor of Plasmepsin I (Nezami *et al*., 2003).

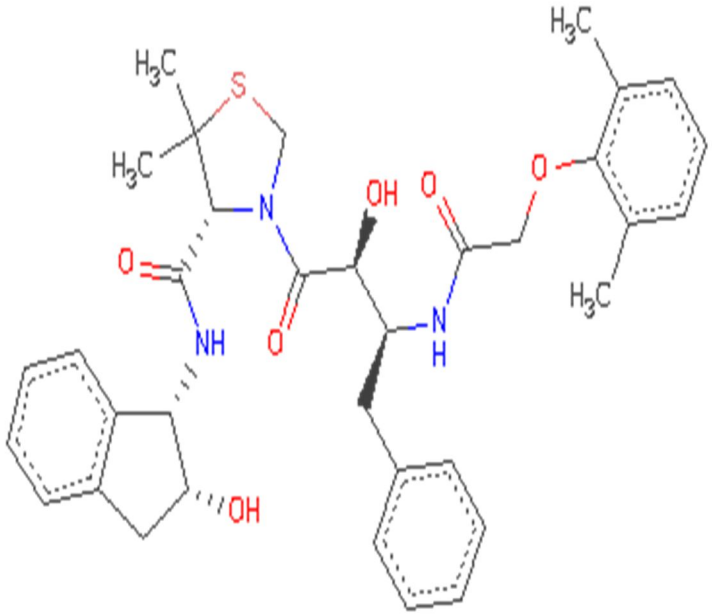


Figure 11: Structure of (4R)-3- [(2S,3S)-3- {[(2,6-dimethylphenoxy)acetyl] amino} -2 –hydroxy -4 -phenylbutanoyl] -N- [(1S,2R) -2- hydroxy-2,3- dihydro -1H-inden-1-yl]-5,5 -dimethyl-1, 3-thiazolidine -4- carboxamide (KNI-10006). KNI-10006 is a known inhibitor of Plasmepsin.

1.13.2 Lactate Dehydrogenase

Lactate dehydrogenase is an important enzyme in the glycolytic pathway. It catalyses the conversion of pyruvic acid to lactatic acid using NADH in the process (Wiwanitkit, 2007). Due to the fact that*Plasmodiumfalciparum* depends solidly on components of the red blood cell and since it lacks mitochondria, Krebs cycle is thus unavailable for the parasite during the erythrocytic stage (Lang-Unnasch and Murphy, 1998). The homology between the species of *Plasmodium*; *P.* vivax, *P.malariae* and *P.ovale* is between 90–92% when compared to *P.falciparum*. NADH (Figure 12) is a known inhibitor of Lactate dehydrogenase.

1.13.3 Falcipain

Falcipain is an important enzyme in the metabolism of *Plasmodiumfalciparum*. It belongs to the cysteine protease group involved in the digestion of haemoglobin in the food vacuole. They have two main domains; the prodomain and mature domain. Four falcipain have been characterize namely; Falcipain 1-4. The homology between falcipain-2 and falcipain-3 exist with up to 67% sequence specificity. Propane-1,2,3-triol (Glycerol) is a unique ligand to Falcipain-2.

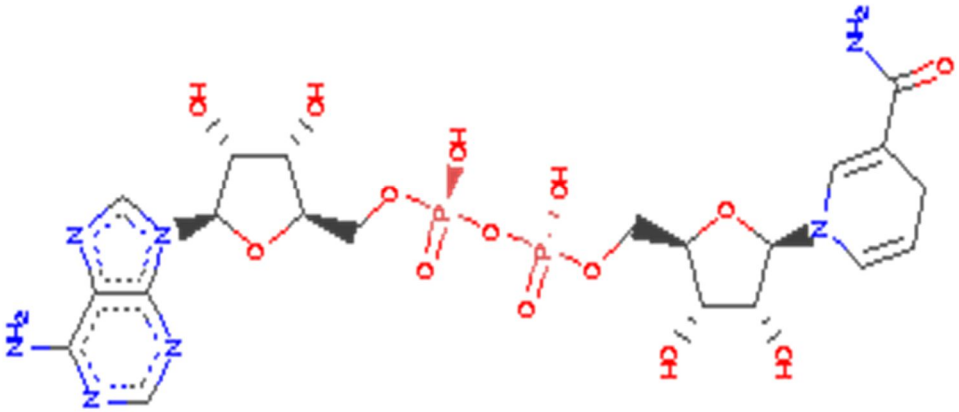


Figure 12: Structure of Nicotinamide Adenine Dinucleotide (NADH).

1.14 Haematological Parameters

The blood isan essential component of the human system. It is a flowing tissue that runs throughout the body. Blood is composed of different components, which are classified based on their structure and functions. The red blood cell carries oxygen and nutrients to all parts of the body and again transport waste products such as carbon dioxide (CO_2) from body tissues for proper disposal. The white blood cells are key components of the immune system; they help to

fight against foreign invaders into the body system. The blood is also important in maintaining body temperature, its body hydraulic fluid and serves as a means for transfer of hormonal messages across the body. The platelets, the white blood cells and the red blood cells are all made from the bone marrow.

Haematological parameters play key role in the diagnosis of several diseases and are also used to assess the effects of chemicals on blood and maybe used to monitor the progress of disease treatments. Red blood cells (RBCs) are the principal sites of infection and all the other clinical manifestations are primarily due to the involvement of red blood cells. Malaria parasite consumes and degrades the intracellular proteins, mainly hemoglobin as a source of nutrient leading to anaemia. In *P. falciparum* malaria, massive destruction of red blood cells accounts for rapid development of anaemia.

The levels of White Blood Cell (WBC), Platelet count (PLT), Neutrophils (NEU), Lymphocytes (LYM), Red Blood Cell (RBC), Packed Cell Volume (PCV), Haemoglobin concentration (Hb), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC) were assayed in Plasmodium-infected and non infected experimental animals.

1.14.1 White Blood Cell

Leucopenia also known as leukocytosis is defined as total white blood cell (WBC) count <4,000/µL. Their main function is fighting infection, defending the body by phagocytosis against invasion by foreign organisms and producing antibodies during immune response to the foreign organisms. The WBC could be nucleated or anucleated. Nucleated cells are call the granulocytes which includes neutrophils, eosinophils, basophils while agranulocytes includes

lymphocytes and monocytes) (Guyton and Hall, 2006). Low levels of white blood cells leads to increased susceptibility to infections and an impaired immune response (Guyton and Hall, 2006).

1.14.2 Haemoglobin concentration

Haemoglobin (Hb) is the red protein molecule found in red blood cell responsible for carrying O_2 from the lungs to other tissues of the body. It is also responsible for returning carbon dioxide, a product of respiration from the tissues to the lungs for excretion. It is composed of four polypeptide chains, a porphyrin ring and iron atoms. Decrease in heamoglobin in circulation is termed aneamia, or dehydration when it is increased (Guyton and Hall, 2006).

1.14.3 Packed Cell Volume

Packed cell volume (PCV) is the total volume of red blood cell in the blood measured in percentages. Red Blood cell and PCV are important parameters used in measuring the oxygen carrying capacity of blood from the lungs to the various body tissues. Low level of PCV is termed anemia, while abnormally high PCV value is termed polycythemia (Guyton and Hall, 2006).

1.14.4 Platelet count

Platelets (PLT) are the smallest form of blood cell in circulation. They are also known as thrombocytes. They provide vital role in coagulation process to prevent excessive bleeding after an injury to a blood vessel. Thrombocytosis suggest dehydration or stimulation of the bone marrow where the cells are produced and thrombocytopenia may indicate an immune system failure, drug reactions, B_{12} or folic acid deficiency (Guyton and Hall, 2006). Thrombocytosis are classified into two; Primary thrombocytosis causes abnormal cells in the bone marrow due to increase in platelet count while the secondary thrombocytosis may be caused by disease

conditions such as anemia, cancer, inflammation in certain infections. Increase in platelet count increases the risk of heart attack or stroke.

1.14.5 Neutrophils

Neutrophils (NEU) also known as neutrocytes. They are the most abundant types of granulocytes and their primary function is to protect the body against infections. High level of neutrophils in circulation indicates the presence of active infection while low level in circulation may indicate a compromised immune system or depressed bone marrow (Dacie and Lewis, 1995).

1.14.6 Lymphocytes

Lymphocytes (LYMP) are integral part of the immune system. They are specific type of white blood cells. Lymphocytes allow the body to respond to foreign invaders. There are three types of lymphocytes namely T cells, B cells and the natural killer cells. T cells develop in the thymus gland and differ from other lymphocytes because they have specific form of T cell receptors. B cells matured in the bone marrow of humans can bedifferentiated also from other lymphocytes by specific proteins on their surface. They all have specific binding sites allowing them to recognize, attach and bind to antigens. Lymphocytes are elevated in active viral infection (Guyton and Hall, 2006).

1.14.7 Red Blood Cell

Red blood cells(RBC)are non-nucleated biconcave disc, which contain haemoglobin. They function include removing carbon dioxide from the body during respiration and transportingit to the lung, which is then released as by-product(Hamasaki and Yamamoto, 2000). RBCs are produced inside the bone marrow and have an average lifespan of 120 days.

1.14.8 Mean Corpuscular Volume

Mean Corpuscular Volume (MCV) is the size of red blood cell expressed by the average amount of space occupied by each red blood cell. Its also known as mean cell volume. The increased value of readings may indicate macrocytic anaemia or vitamin B6 or Folic acid deficiency and decreased values may indicate microcytic anaemia, possibly caused by iron deficiency (Guyton and Hall, 2006).

1.14.9 Mean Corpuscular Haemoglobin

Mean Corpuscular Haemoglobin (MCH) is the average mass of haemoglobin in the red blood cell. Decreased MCH is associated with microcytic anaemia and increased MCH is associated with macrocytic anaemia (Guyton and Hall, 2006).

1.14.10 Mean Corpuscular Haemoglobin Concentration

Mean Corpuscular Haemoglobin Concentration (MCHC) is the average concentration of hemoglobin in a given volume of packed red blood cell. Low MCHC are seen in anemia, indicating that the unit of packed RBC contains less haemoglobin concentration than normal (Guyton and Hall, 2006).

1.15 Justification for the study

Estimates in 2015 showed that there were about 212 million cases of malaria and an estimated 429, 000 deaths (WHO, 2015). 96% took place in low-income countries and 87% in children younger than 5 years of age (Hay *et al*., 2004; WHO, 2008; 2010). Malaria is the twelfth (20th) leading cause of morbidity in the world (WHO, 2008). Most malaria mortality in

Nigeria is caused by *Plasmodiumfalciparum*, the most common species in the highly malaria endemic areas of Africa. Present drugs have become ineffective because *Plasmodiumfalciparum*has developed resistance to most of them, particularly to chloroquine, the cheapest and previously very effective antimalarial drug, and pyrimethamine (Peters, 1998; Wellems and Plowe, 2001). Despite some breakthrough in the synthesis of antimalarial drugs, the dream of a global eradication begins to fade with the growing number of cases of rapid spread of drug resistance in the parasite (Barat and Bloland, 1997; Gu *et al*., 2006) and the serious risk of development of resistance in *P.falciparum*to artemisinin-based combination therapy (ACT).Already, there are reports of clinical resistance to artemisinin derivativesin Cambodia, Vietnam, Thailand and Myanmar (Holt *et al*., 2002; Kiseko *et al*., 2000; Murakami, 2003; Noedi*et al*., 2008; WHO, 2013).These call for development of novel compounds.

The current practice is to deploy antimalarial drugs individually in sequence. When one drug fails, another is introduced. Unfortunately, there are few antimalarial drugs and the evolution of the resistance in *P.falciparum*seems to be outstripping the development of new drugs (White *et al.,* 1999). There are compelling reasons to believe that resistance to the available antimalarial drugs would be slowed down or prevented by the combination of artemisinin or one of its derivativeswith other slow acting drugs, as has been the case with mefloquine. History shows that biodiversity and knowledge of traditional medicine are useful to open new strategies in the field of antimalarial therapy, as it was the case for artemisinin (Quinghaosu Antimalarial Coordinating Research Group, 1979). Thus, some of the phytochemicals of medicinal plants used in treatment of malaria can also be used with artemisinin derivatives in combination therapy.

African pharmacopeia over the years has failed to present an African antimalarial compound with nanomolar antiplasmodial activity (Maranz, 2012). This is a clear indication that the answer may lie in a different direction, perhaps the complexing of active plant principles with a standard antimalarial drug. This present study was thus carried out to synthesize a hybrid molecule from artesunate and a polyphenol (with known antimalarial activity) and evaluate the hybrid compound for its antimalarial activity.

1.16 Objectives of the study

The overall objective of the study was to synthesize a hybrid molecule from procyanidin and artesunate and evaluate its antimalarial, antioxidant and toxicological potentials.

1.16.1 Specific Objectives

The specific objectives of the study were to:

1. Synthesizea hybrid molecule from purified procyanidin (PC) and artesunate (AS);
2. Elucidate the structure of the hybrid molecule synthesized;
3. Determine the characteristics (such as solubility, boiling point, melting point, texture, etc.) of the hybrid compound;
4. Evaluate the *in vitro* antiplasmodial activity and cytotoxicity of the hybrid compound;
5. Evaluate the antimalarial activities of the hybrid compound in *P. berghei* NK65-infected mice;
6. Determine the mechanisms of action of the hybrid compound;
7. Determine the *in vitro* antioxidant activities of the hybrid compound;
9. Determine the antioxidant activities of the hybrid compound in mice; and
10. Evaluate the toxicity potentials of the hybrid compound using selected function indices of the liver, brain, heart and kidney in mice.

CHAPTER TWO

2.0 Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

Artesunate (AT), Procyanidin B_2, and Oligomeric Procyanidins (OPC) containing 5% Procyanidin B_2 were purchased from Zelang Medical Technology, China. Hematin, HEPES, Pyridine, Sodium acetate, Sodium carbonate (Na_2CO_3), Methanol (MeOH-d_4) and Dimethyl Sulfoxide (DMSO-d_6) were obtained from Sigma-Aldrich, Germany. Paracetamol and Ketoconazole were obtained from Glaskosmitkline, Nigeria, and Fabrique, Nigeria, respectively. Other solvents used were of analytical grades.

2.1.2 Parasites

The *in vitro* antiplasmodial tests were performed with blood parasites of *Plasmodiumfalciparum*[W2 clone, chloroquine-resistant parasites] obtained from Laboratorio de Malaria, Instituto de Pesquisas Rene Rachou, FIOCRUZ, Belo Horizonte MG, Brazil. The *in vivo* tests were carried out using a chloroquine-sensitive strain of *Plasmodium berghei* (NK65) obtained from the Institute for Advanced Medical Research and Training (IAMRAT), College of Medicine, University of Ibadan, Oyo state, Nigeria.

2.1.3 Cell lines

Cytotoxicity test were performed using Buffalo Green Monkey Kidney cell line (BGM) obtained from Laboratorio de Malaria, Instituto de Pesquisas Rene Rachou, FIOCRUZ, Belo Horizonte MG, Brazil.

2.1.4 Animals

Adult Swiss albino mice with an average weight of 20.0±2.0 g were obtained from the animal holding unit of the Department of Biochemistry, University of Ilorin, Ilorin, Kwara State. The mice were housed in plastic cages and acclimatized for a minimum of 7 days at temperature range between 25–30°C, relative humidity of between 40–45%, and a 12 h/12 h light-dark cycle with free access to feed and tap water.

2.2 Methods

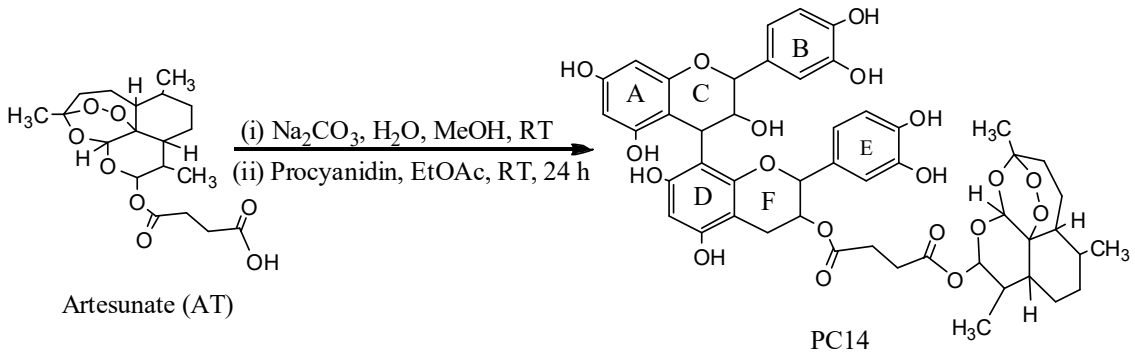
2.2.1 Purification of Procyanidin

Oligomeric Procyanidin (OPC, 5 grams) was dissolved in 300 ml of distilled water and partition thrice with 200 ml of ethyl acetate. Both fractions were concentrated using a lyophilizer for the aqueous fraction and rotary evaporator at 40^{o}C for the ethyl acetate fraction to give the aqueous extract (EA) and Ethyl acetate extract (EAE). EAE was further partition in Diethyl ether and water. One (1) gram of EAE was dissolved in 200 ml of water and extracted thrice with 100 ml of diethyl ether. The diethyl ether fraction was concentrated in vacuo at 40^{o}C to give diethyl ether fraction (DEE) and was stored in a desiccator.

2.2.2 Synthesis of Artesunate-Procyanidin Hybrid

Hybrid molecule (Artesunate-procyanidin, **PC14**, Scheme I) was synthesized using a slightly modified method of de Pilla Varotti *et al*. (2008). Artesunate (600 mg, 1.6 mmol) was dissolved in 20 ml H_2O/MeOH (8:2). Ethyl acetate (20 ml) was added to this solution at room temperature with stirring. Sodium bicarbonate was added in small quantities until the effervescence ceased. The two phases were separated and the organic layer obtained wasdried over anhydrous sodium sulfate and the solvent evaporated. The free base (75 mg) obtained was dissolved in 20 ml ethyl acetate and a solution of Procyanidin (150 mg, 0.26 mmol) in ethyl

acetate was added to it. The mixture was stirred at room temperature for 24h. The solvent was evaporated and purified on a silica column, eluted with Hexane: Ethyl acetate (50:50; 25:75) to give 81 mg of abrownish slurry that was constantly maintained under silica as a drying agent. TLC for prepared samples was performed on Aluminium sheets precoated plate (Merck®, silica gel, type 60, 0.25, Darmstadt, Germany) and revealed using vanillin sulphate stain. HPLC profiles of reactant and products were carried out using a gradient elusion with Methanol and 0.2 % Phosphoric acid on an automated injector, C_{18} equip HPLC with a UV detector (Shimazu, Japan). Artesunate and Procyanidin were best monitored using 254 nm channel while Artesunate-procyanidin (PC14) hybrid was monitored best at the 229 nm channel. A Veego-MPI melting point apparatus was used to determine the uncorrected melting points. Infrared (IR) spectra were recorded on a IRPrestige21, Shimadzu, Japan, while ^{1}H and ^{13}C-NMR spectra were recorded in DMSO-d6 solutions on a Bruker® 400 MHz spectrometer (Bruker, Germany).



Scheme I: Reaction for the Synthesis of Artesunate-procyanidin Hybrid

Ratio Combinations: Different combination ratios of Procyanidin and Artesunate were prepared. Artesunate-Procyanidin combination (Ratio 1:1, **PC01**) was prepared by weighing 0.1 g of Artesunate (0.26 mmol) and 0.15 g of Procyanidin (0.26 mmol) in 5 ml of MeOH:H_2O (9:1) and sonicated until completely soluble. This was occasionally sonicated until it became completely dry. The brown slurry obtained was kept dry at all times in a desiccator. Artesunate-Procyanidin combination (Ratio 2:1, **PC02**) was prepared by weighing 0.2 g of Artesunate (0.52 mmol) and 0.15 g of Procyanidin (0.26 mmol) in 5 ml of MeOH:H_2O (9:1) and sonicated until completely soluble. It was occasionally sonicated until it became completely dry and stored in a desiccator. Similarly, Artesunate-Procyanidin ombination (Ratio 1:2, **PC03**) was prepared by weighing 0.05 g of Artesunate (0.13 mmol) and 0.15 g of Procyanidin (0.26 mmol) in 5 ml of MeOH:H_2O (9:1) and sonicated until completely soluble. It was occasionally sonicated until it became completely dry and the product obtained was kept dry at all times in a desiccator.

2.2.3 Determination of solubility

A Veego-MPI melting point apparatus was used to determine the uncorrected melting points. clog P and clog S were determined using the program ALOGPS 2.1 obtained from www.moldiscovery.com.

Solubility was determined usingturbidimetry method in phosphate buffer saline (PBS) at pH 7.4. The drugs were diluted in 100% DMSO stock before dilution in 0.1M PBS at pH 7.4 to desired concentrations. Drugs were further diluted from the highest concentration (5 mM) to 2.5, 1.25, 0.63, 0.31, 0.16, 0.08 and 0.04mM working concentrations. Another set of dilution was performed in 100% DMSO to determine if the increase in absorbance was due to the compound itself with increase in concentration or due to the formation of a precipitate in respect to solubility properties of the compounds. The PBS tubes were incubated at 37^{o}C for 2 hours

followed by determining the absorbance spectrophotometrically at 595 nm while DMSO tubes were left to stand for 30 minutes before reading the absorbance at 595 nm. Paracetamol (solubility > 14 mg/ml) and Ketoconazole (solubility < 1 mg/ml) were used as reference standards.

2.2.4 *In-vitro* studies

2.2.4.1 *In vitro* Antiplasmodial Studies

The method of Trager and Jensen, (1976) was used in culturing *P. falciparum* parasites in human red blood cells, with minor modifications. Briefly, parasites were cultured in Petri dishes (Corning, Santa Clara, CA, USA) containing RPMI culture medium [supplemented with 1% (v / v) albumax II (Gibco, USA)] with 5% hematocrit. Plates were incubated at 37°C, using the candle jar method. The culture medium was changed daily and parasitaemia monitored in Giemsa-stained smears. The parasites were synchronized by sorbitol solution as described by Lambros and Vandenberg, (1979) to get predominantly ring forms, diluted and incubated in 96 well plates containing the extracts and the standard drug, or culture medium with 0.5% DMSO, used as a positive control of parasite growth.

The SYBR test was used as described by Smilkstein *et al*., (2004) with some modifications. Briefly, serial dilutions of the drugs were incubated at 37°C with the parasite suspensions (0.5% parasitaemia and 2% hematocrit) in "U" bottom 96-wells plates. After 48 hr, the culture supernatant was removed and replaced by 100µL of lysis buffer solution [Tris (20 mM; pH 7.5), EDTA (5 mM), saponin (0.008%; wt/vol), and Triton X-100 (0.08%; vol/vol)] followed by addition of 0.2 µL/mL Sybr Safe (Sigma-Aldrich, Carlsbad, CA, USA). The plate content was then transferred to a flat bottom plate and incubated in the dark for 30 minutes. The

reading was made in a fluorometer (Synergy H4 Hibrid Reader, BioteK) with excitation at 485 nm and emission at 535 mm.

2.2.4.2 Cytotoxicity test

The cytotoxicity test was done using blue green monkey kidney cell line (BGM), and the test performed as described by Aguiar *et al.,* (2012). Cells were cultured in 75 cm^2 plates with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and gentamicin 40 mg/L, at 5% CO_2 atmosphere and 37°C. The cells were trypsinized when the monolayer was confluent, washed with culture medium,distributed in a flat-bottomed 96-well plate (1×10^5 cells/mL) and incubated at 37°C for 18 h to ensure cell adherence. The cells were properly diluted and incubated with 20 µL of the compounds at different concentrations (1-1,000µg/mL) for 24 h in a 5% CO_2 atmosphere at 37°C. The neutral red assay as described by Borenfreund and Puerner, (1985) was used to evaluate cell viability by the accumulation of the dye in the viable cell lysosome. Neutral red solution (4mg/Ml, 200 µL), was added to the plates and incubated for 3 h. The supernatant was carefully removed, followed by the addition of 200 µL of 0.5% v/v formaldehyde and 1%$CaCl_2$ solution. After 5 minutes, the supernatant was removed, then 100 µL of an alcohol- acetic acid (50-1%) solution was added to extract the dye. The absorbance was read at 540nm on an ELISA reader (SpectraMax340PC384, Molecular Devices). Cell viability was expressed as the percentage of control absorbance obtained in untreated cells and the minimum lethal dose for 50% of the cells (MLD_{50}) was determined. Based on the values of cytotoxicity (MLD_{50}) and antimalarial activity (IC_{50}) the selective index (SI) of activity was calculated using the formula:

$$\text{Selective Index (SI)} = \frac{MLD_{50}}{IC_{50}}$$

2.2.4.3 β-Hematin Inhibition Assay

The assay for inhibition of β-Hematin formation was carried out according to the method of Ncokazi and Egan, (2005).

Principle:

β-hematin inhibition assay is based on the ability of hematin to form a low spin complex with aqueous pyridine (5% v/v, pH 7.5), but not with β-hematin at the same conditions.

Procedure:

Serial dilution of the solutions of the drugs in triplicate was prepared in methanol and acidified using 1 M HCl. Drug concentration was also varied from 0 - 10 equivalents relative to hematin in total reaction volume, each containing 33.76 µL in the final mixture. 20 µL of hematin stock solution (1.68mM in 0.1M NaOH) was added to each test well followed by 2.02 µL of test drugs and 11.74 µL of acetate solution (12.9M, pH 5.0, 60ºC). The contents were mixed and incubated at 60ºC for 60 minutes.

The reaction was quenched with 900 µL of pyridine(5% v/v)solution in 200 mM HEPES (pH 7.5) followed by the addition of 1100 µL, pyridine (5% v/v)solution in 20 mM HEPES (pH 7.5). The mixture was shaken to ensure complete dissolution of hematin and allowed to settle at ambient temperature for 15 minutes. The supernatant was transferred to a cuvette without disturbing the precipitate and absorbance was read at 405 nm. The IC_{50} values for β-hematin inhibition were determined by fitting the percentage inhibitions calculated from absorbance data to a sigmoidal dose response curve by non-linear least squares fitting using Origin 7.0 software.

The Fractional Inhibitory Concentrations (FICs) of the various combination ratios were determined using the formula:

$$\text{Step I: FIC50(Combination Ratio A1)} = \frac{\text{IC50 (A1 + B1)}}{\text{IC50 (A)}} \times \text{A1}$$

$$\text{Step II: FIC50(Combination Ratio B1)} = \frac{\text{IC50 (A1 + B1)}}{\text{IC50 (B)}} \times \text{B1}$$

$$\text{Step III: } \Sigma\text{FIC50(Combination Ratio)} = \text{FIC50 (A1)} + \text{FIC50 (B1)}$$

A1 = fraction of A in a given combination

B1 = fraction of B in a given combination

IC_{50} (A1+B1) = IC_{50} of fraction combinations of two drugs A and B

IC_{50} (B) = IC_{50} of fraction containing 100% B

IC_{50} (A) = IC_{50} of fraction containing 100% A

2.2.6 *In vivo* Studies

2.2.6.1 *In vivo* Antimalarial (4-Day Suppressive Test) in Animal Model

A 4- day suppressive test described by Peters (1965) and modified by Carvalho *et al.* (1991) was employed in evaluating the *in vivo* antimalarial activities of hybrid compound and the various combination ratios. Briefly, adult Swiss albino mice averagely weighing 20±2 g were inoculated intraperitoneally with 1×10^5 *Plasmodium berghei*NK65-infected Red Blood Cells (iRBC). The mice were then randomly assign into 23 groups (Group A – W) of five animals per group and treated for three days (D1-D3) consecutively with Artesunate-procyanidin hybrid molecule (PC14 hybrid), Artesunate, Procyanidin and combination ratios (PC01, PC02, PC03) or with only the drug vehicle (5% DMSO), as follows:

Group A:	Infected untreated + 5% DMSO
Group B:	Infected + Artesunate-4 mg/kg body weight
Group C:	Infected + PC14-5 mg/kg body weight
Group D:	Infected + PC14-10 mg/kg body weight
Group E:	Infected + PC14-20 mg/kg body weight
Group F:	Infected + PC14-40 mg/kg body weight
Group G:	Infected + PC14-80 mg/kg body weight
Group H:	Infected + PC01-5 mg/kg body weight
Group I:	Infected + PC01-10 mg/kg body weight
Group J:	Infected + PC01-20 mg/kg body weight
Group K:	Infected + PC01-40 mg/kg body weight
Group L:	Infected + PC01-80 mg/kg body weight
Group M:	Infected + PC02-5 mg/kg body weight
Group N:	Infected + PC02-10 mg/kg body weight
Group O:	Infected + PC02-20 mg/kg body weight
Group P:	Infected + PC02-40 mg/kg body weight
Group Q:	Infected + PC02-80 mg/kg body weight

Group R: Infected + PC03-5 mg/kg body weight

Group S: Infected + PC03-10 mg/kg body weight

Group T: Infected + PC03-20 mg/kg body weight

Group U: Infected + PC03-40 mg/kg body weight

Group V: Infected + PC03-80 mg/kg body weight

Group W: Infected + Procyanidin-10 mg/kg body weight

Blood smears were made from tail blood obtained from the mice on day 4 (D4), day 6 (D6)and day 8 (D8) post-inoculation and fixed with methanol before staining with Giemsa and microscopically examined (x1000magnification) by counting up to 6,000 Red Blood Cells. The mean survival time of the mice in each treatment group was monitor for 30 days.

2.2.6.2 Curative (Rane) Test in Animal Model

Evaluation of the curative potential of the hybrid compound was carried out as described by Ryley and Peters (1970). Briefly, adult Swiss albino mice averagely weighing 21±3 g were inoculated by intraperitoneal route with 1×10^5 *Plasmodium berghei*-(NK65) infected Red Blood Cells (iRBC). The mice were then randomly assigned into 8 groups (A – H) of five animals each. However, unlike the suppressive test, treatment was withheld for 72 h to allow for establishment of infection and commenced when parasitaemia had been established by screening the smears of the tail blood of infected animals for malaria parasites after fixing in methanol and staining with giemsa. The drugs were administered orally once daily for 3 consecutive days (D_3-D_5) as follows:

Group A: Infected untreated + 5% DMSO

Group B: Infected + Artesunate-4 mg/kg body weight

Group C: Infected + PC14-5 mg/kg body weight

Group D: Infected + PC14-10 mg/kg body weight

Group E: Infected + PC14-20 mg/kg body weight

Group F: Infected + PC14-40 mg/kg body weight

Group G: Infected + PC14-80 mg/kg body weight

Group H: Infected + Procyanidin-10 mg/kg body weight

On day 6 (D_6), day 8 (D_8) and day 10 (D_{10}),thin smears were made from tail blood of each mouse, fixed with methanol, stained with giemsa and examined microscopically (Mag: x1000) to determine the parasitaemia. The mean survival time of the mice in each treatment group was also monitored for 30 days.

Calculation:

Parasitaemia was calculated using the following formula:

$$Parasitemia\ (\%)\ = \frac{Infected\ Red\ Blood\ Cells}{Total\ Red\ Blood\ Cells} \times 100\%$$

The inhibition of parasite growth (chemosuppression) in all groups except the negative control was calculated using the formula:

$$\%\ Chemosuppression\ = \frac{P_C - P_{DT}}{P_C} \times 100\%$$

P_C = Parasitaemia in the control (non-treated) group

P_{DT} = Parasitaemia in the drug-treated group

2.2.5 Determination ofAntioxidant Capacity *in vitro*

2.2.5.1 Total Antioxidant Capacity (TAC)

The total antioxidant capacity (TAC)was evaluated using the phosphomolybdenum assay reported by Prieto *et al*. (1999).

Principle:

The method is based on the reduction of Mo(VI) to Mo(V) by the drug sample and different combination ratios and subsequent formation of green phosphate/Mo(V) complex in acidic medium ensured by Tetraoxosulphate (VI) acid.

Procedure:

Briefly, 0.1 mL of drug (2-20 µg/ml) was combined with 1 mL reagent solution containing 0.6 M Tetraoxosulphate (VI) acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. The mixture was incubated at 95 °C for 90 min. Absorbance was measured at 695 nm after cooling to room temperature. Butylated hydroxytoluene (BHT) was used as reference compound.

2.2.5.2 Ferric Reducing Antioxidant Power (FRAP)

The Ferric ion reducing properties was evaluated using the method reported by Girgih *et al*. (2013).

Principle:

The ability of the drug and its combination ratios to reduce ferric ion in solution was measured at a suitable wavelength.

Procedure:

A 250 µL of the drug (2-20 µg/ml) was added to a mixture of 250 µL of 0.2 M phosphate buffer (pH 6.6) and 250 µL of 1% potassium ferricyanide solution. The mixture was incubated at 50 °C for 20 min. After which 250 µL of 10% aqueous trichloroacetic acid (TCA) was added. Then, 250 µL of the drug/TCA mixture was added to a mixture of 50 µL of 1.0% of $FeCl_3$ and 200 µL distilled water. This was allowed to stand at room temperature for 10 min. The mixture was then centrifuged at 1000 g for 20 min. Thereafter, the clear supernatant was transferred into another test tube and absorbance measured at 700 nm. Butylated hydroxytoluene (BHT) was used as reference compound.

2.2.5.3 DPPH Radical Scavenging Assay

The scavenging activity of the drug against 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined according to the method of McCune and Johns (2002).

Principle:

The method is based on the reduction of methanolic DPPH in the presence of a hydrogen-donating antioxidant. DPPH is converted to DPPH-H indicated by a bleached purple colour, the disappearance of which is monitored spectrophotometrically at 517 nm.

Procedure:

A known amount (500 µl) of 0.11 M methanolic DPPH was added to 500 µl of different concentrations (2-20 µg/ml) of the drug and incubated at room temperature in the dark for 10 min. The absorbance of the samples (A_s) and their respective blanks (A_b) were measured at 517 nm. % DPPH scavenging activity was calculated using the formula:

$$\%\ DPPH\ scavenging\ activity = (1 - \frac{A_s}{A_b}) \times 100$$

EC_{50} is the concentration at 50% scavenging effect extrapolated from the standard curve. Butylated hydroxytoluene (BHT) was used as reference compound.

2.2.7 *In vivo*antioxidant studies

The curative test using *Plasmodium berghei* NK65 strain as described by Ryley and Peters (1970) was adopted in inducing oxidative stress. Briefly, 90 mice out of a hundred mice were inoculated intraperitoneally with 1 x 10^5 infected red blood cells on day 0. After 24h, The infected mice were randomly distributed into 9 groups (group B – J) of 10 mice each while the remaining ten (10) uninfected mice (group A) served as control. Drugs were administered orallyonce daily for 3 consecutive daysstarting from day 3 post-inoculation as follows:

Group A:	Control + 5% DMSO
Group B:	Infected untreated + 5% DMSO
Group C:	Infected + Chloroquine-5 mg/kg body weight
Group D:	Infected + Artesunate-4 mg/kg body weight
Group E:	Infected + Procyanidin-10 mg/kg body weight
Group F:	Infected + PC14-5 mg/kg body weight
Group G:	Infected + PC14-10 mg/kg body weight
Group H:	Infected + PC14-20 mg/kg body weight
Group I:	Infected + PC14-40 mg/kg body weight
Group J:	Infected + PC14-80 mg/kg body weight

On day 6 post-inoculation, 5 mice from each group were sacrificed under diethyl ether anaesthesia. Blood was collected into sample bottles (some containing heparin for plasma preparation and the others containing EDTA for the determination of CD4+ count and haematological parameters) and the liver, heart, brain and kidney were excised, cleansed of blood and homogenized in 0.25M sucrose solution (1/5, w/v).The organ homogenates were centrifuged (Eppendorf Centrifuge, Model 5702, Germany) at 1,500 g for 10 mins and the supernatant aspirated into new sample bottles and stored at -80ºC (Ultralow NUAIRE, Germany) until required for use. The blood samples in heparin sample bottleswere centrifuged at 3000 g for 5 minutes; the supernatant and the puffy coat were aspirated out while red blood cells were washed with phosphate buffer (0.1 M, pH 7.4). The red blood cells were then lysed using the repeated freeze-thaw procedure and the lysate was stored at -80ºC until required for use. On day 10 post-inoculation, the remaining 5 mice in each group were sacrificed under diethyl ether anaesthesia and the tissue homogenates and red blood cell lysates prepared as described above.

2.2.8 CD4+ lymphocyte count:

Out of the whole blood samples collected in EDTA tubes, 20 µl of blood was pipettedinto the bottom ofanother sample tube, without allowing blood from pipette tip to touch the tube wall.CD4 mAb PE (20 µl) was pipetted directly into blood sample and mix gently, avoiding sample smears on the tube wall. The mixture was then incubated for 15 minutes at room temperature in the dark. 800 µl of no lyse buffer was added and the sample shaken or swirled gently. The stained blood sample was analysed within two hours after preparation using a Sysmex Partec CyFlow® counter, (Model CY-S-3022, Germany). Haematological parameters such as White Blood Cell (WBC), Haemoglobin concentration (Hb), Packed Cell Volume (PCV), Platelet count (PLT), Neutrophils (NEU) and Lymphocytes (LYMP) were also

determined using the automated haematological analyser, Mindray Machine (Model BC-3200, Germany).2.2.9.1 Determination of Malondialdehyde concentration

Malondialdehyde (MDA) levels in tissue homogenates and erythrocyte lysates were determined using the method described by Varshney and Kale (1990).

Principle:

The method is based on the measurement of thiobarbituric acid reactive substances (TBARS). In acidic medium, malondialdehyde (MDA) generated from membrane fatty acid peroxidation reacts with 2-thiobarbituric acid to yield a MDA-TBA2 adduct, a pink coloured complex that absorbs maximally at 532 nm.

Procedure:

A known volume (0.8 ml) of Tris-KCl (0.15M, pH 7.4)was added to 0.2 ml of the sample and then quenched by addition of 0.25 ml of 30% Trichloroacetic Acid (TCA). 0.25 ml of 0.75% Thiobarbituric Acid (TBA) was added and the reaction mixture incubated for 45minutes at 80^{o}C and then cooled on ice. The resulting pink-coloured reaction mixture was centrifuged at 4000 g for 15 minutes. The absorbance of the clear pink supernatant was then read at 532 nm using distilled water as blank.

Malondialdehyde (MDA) level was calculated using the formula:

$$\text{Malondialdehyde (MDA)} = \frac{\text{Absorbance} \times \text{Volume of mixture}}{E_{532} \times \text{Volume of sample} \times \text{mg protein}}$$

Where E_{532} is molar absorptivity at 532 nm = 1.56×10^{-5}

2.2.9.2 Determination of Nitrite concentration

The nitrite concentration in tissues was estimated using the Griess reaction following the method described by Green *et al*. (1982).

Principle:

The Griess reaction measures nitrite that reflects nitric oxide production rate.

Procedure:

Tissue homogenates and erythrocyte lysates were deproteinised by treating 500 µL of appropriately diluted sample with 100 µL of 5% $ZnSO_4$ and 100 µL of 0.3 M NaOH. The reaction mixture was then centrifuged at 4000 g for 20 minutes. Thereafter, 100 µL of the supernatant was treated with 1000 µL of Griess reagent and the absorbance was read at 540 nm. Griess reagent was used as the blank. A standard calibration curve was prepared using serial dilutions of sodium nitrite (25 – 150 µg/L) working solution, followed by the addition to each test tube, 1000 µL of Griess reagent to and the absorbance read at 540 nm. Each concentration was done in triplicate and the optical densities obtained were plotted against the concentrations. The concentration of nitrite is proportional to the optical density. The nitrite concentration in tissue homogenate was extrapolated from a standard curve prepared using sodium nitrite (Appendix, Figure87).

2.2.9.3 Determination of Superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined according to the method of Misra and Fridovich (1972).

Principle:

Superoxide dismutase (SOD) inhibits the auto-oxidation of epinephrine at pH 10.2. Superoxide anion radical ($O_2^{-\cdot}$) generated by xanthine oxidase reaction causes the oxidation of epinephrine to adenochrome.The yield of adenochrome produced increases per $O_2^{-\cdot}$ introduced with increasing pH (Valerino and McCormack, 1971) and also increases with increasing concentration of epinephrine. These results led to the proposal that auto-oxidation of epinephrine

proceeds by at least two distinct pathways, only one of which is a free radical chain reaction involving super oxide anion radical and hence inhibitable by SOD.

Procedure:

100 µL of the diluted sample was added to 1000 µL of 0.05M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer. The reaction was initiated by addition of 150 µL of freshly prepared 0.3mM epinephrine to the mixture. The reference testtube contained 1000 µL buffer, 150 µL of epinephrine and 100 µL of distilled water. The increase in absorbance at 480nm was monitored every 0.5 min for 2.5 min.

Superoxide dismutase (SOD) activity was calculated using the formula:

$$\% \text{ Inhibition } = \frac{\text{Increase in absorbance for substrate}}{\text{Increase in absorbance of blank}} \times 100$$

Where

$$\text{Increase in absorbance/minute} = \frac{A_5 - A_0}{2.5}$$

A_0 = absorbance after 0.5 min

A_5 = absorbance after 2.5 min

1 unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline to adenochrome during 1 minute.

2.2.9.4 Determination of Glutathione peroxidase activity

The activity of Glutathione peroxidase (GSH-Px) in tissue homogenates and erythrocyte lysates was determined by the method of Rotruck *et al*. (1973).

Principle:

GSH-Px catalyses the splitting of H_2O_2 and other peroxides (-OOH) with concomitant oxidation of reduced glutathione (GSH).

$$H_2O_2 + 2GSH \xrightarrow{GP_x} 2H_2O + GSSH$$

The method is based on the ability of GSH-Px to utilize 2mM of GSH to decompose 10mMof H_2O_2. The reaction is allowed to proceed for a specified period (in minutes) and then terminated by the addition of 10% trichloroacetic acid. The residual GSH in the reaction mixture is then quantified by the addition of Ellman's Reagent (5', 5'-dithiobis- (2-nitrobenzoic acid, DTNB).

Procedure:

The reaction was started by the addition of 200 µL of H_2O_2 (10 mM) to a reaction mixture already containing 800 µL of 0.1 M Tris-HCl buffer (pH 7.4), 400 µL of 2mM GSH, 200 µL of 10 mM sodium azide and 50 µL of appropriately diluted samples made up to 2 ml with distilled water. The assay mixture was incubated at 37^0C for 5 minutes after which the reaction was terminated by the addition of 500 µL of TCA (10%). After centrifuging the assay mixture at 4000 g for 10 minutes, 250 µL of the supernatant was added to a mixture of 1000 µL of DTNB (0.004%) and 250 µL of disodium hydrogen phosphate (0.3 M) solution. The colour developed was read at 420 nm, and a reaction mixture without enzyme source was used as the control.

The glutathione peroxidase activity was expressed in Units per milligram protein (Units/mg protein) as calculated from the formula:

$$\text{Glutathione peroxidase activity} = \frac{\Delta\text{Abs/min} \times \text{GSH Standard} \times \text{Total reaction volume}}{\text{Abs of standard} \times 307.32 \times \text{volume of enzyme source} \times \text{protein conc.}}$$

Where:

Molecular weight of GSH = 307.32 g/mole

Abs = Absorbance at 420 nm

2.2.9.5 Reduced glutathione concentration

The levels of reduced glutathione (GSH) in the tissues and erythrocytes were estimated by the method of Beutler *et al*. (1963).

Principle:

The bulk of cellular non-protein sulphydryl groups are in the reduced form of glutathione, thus, deproteinisation of samples with sulphosalicylic acid is necessary and ensures that no protein cysteine thiol groups can react with the colour reagent. This method relies on the development of a relatively stable yellow colour when 5', 5'-dithiobis- (2-nitrobenzoic acid), DTNB, (Ellman's reagent) is added to sulphydryl compounds. The coloured product resulting from the reaction of Ellman's reagent with the reduced glutathione, 2-nitro-5-benzoic acid absorbs maximally at 412 nm and the amount of GSH in the sample is proportional to the absorbance at this wavelength.

Procedure:

The precipitating reagent 1.5 ml of 4 % sulphosalicylic acid was added to 1 ml of appropraitly diluted sample and then allowed to stand for 10 min at room temperature. The mixture was centrifuged for 4 minutes at 3000 g. 0.25 ml of the supernatant was added to 2 ml of phosphate buffer (0.1 M, pH 7.4). Thereafter, 0.25 ml of 0.04% Ellman's reagent was added and the absorbance was read at 412 nm. The optical density obtained is proportional to GSH concentration in the tissue samples, which was extrapolated from the GSH standard plot (Appendix, Figure88).

2.2.9.6 Glutathione-S-transferase activity

Glutathione-S-transferase (GST) activity in tissues and erythrocytes was estimated according to the method of Habig *et al*. (1974).

Principle:

The principle relies on the ability of Glutathione-S-Transferase to use 1-chloro-2, 4,-dinitrobenzene (CDNB) as second substrate. Conjugation of CDNB with GSH leads to a shift in maximum absorption to a longer wavelength. The absorption increase at the new wavelength of 340 nm provides a direct measurement of GST activity.

Procedure:

The reaction mixture contained 30 µL of GSH, 150 µL of CDNB, 2.79 ml of 0.1 M phosphate buffer (pH 6.5) and 30 µL of sample. The reaction was allowed for 1 min and the absorbances was read against blank at 340 nm. The temperature was maintained at approximately 31^{o}C.

Glutathione-S-transferase (GST) activity is expressed in µmole/min/mg protein as calculated from the formula:

$$\text{Glutathione S} - \text{transferase (GST) activity} = \frac{\text{Absorbance} \times \text{Reaction volume}}{9.6 \times \text{Volume of homogenate} \times \text{Protein conc.}}$$

Where:

Extinction coefficient of CDNB = 9.6 $mmol^{-1}$ cm^{-1}

2.2.9.7 Catalase activity

The method of Sinha (1972) was used to determine catalase (CAT) activities in tissues and erythrocytes samples.

Principle:

This method relies on the reduction of dichromate in acetic acid to chromic acetate when heated in the presence of hydrogen peroxide. The chromic acetate generated is then quantified colorimetrically at 570 – 610 nm. The CAT in the sample of interest is allowed to split hydrogen peroxide over specific minutes after which the reaction is terminated by the

addition of dichromate/acetic acid mixture. The hydrogen peroxide left unsplit is quantified by measuring chromic acetate colorimetrically after heating the reaction mixture.

Procedure:

The assay mixture was made up of 1 ml of H_2O_2 solution (800 µmoles) and 1.25 ml of phosphate buffer (0.1M, pH 7.4), to which 1ml of appropriately diluted enzyme preparation (1:50) was quickly added and mixed by gentle swirling. A 1 ml aliquot of the reaction mixture was withdrawn and added to 2ml dichromate/acetic acid reagent and the absorbance read at 60 sec interval for 3 minutes at 570 nm.

Catalase activity is expressed in Units/mg protein as calculated from the formula:

$$\text{Catalase activity} = \frac{\Delta\ \text{Abs/min} \times \text{Conc. of standard} \times \text{Volume of assay}}{\text{Abs of standard} \times \text{Volume of enzyme} \times \text{Protein conc.}}$$

Where:

Abs = Absorbance at 570 nm

Δ Abs = Change in Absorbance

2.2.10 Molecular docking studies

The various proteins (Plasmepsin I (3QS1), Plasmepsin II (1SME), Plasmepsin III (3FNU), Plasmepsin IV (2ANL), Plasmepsin V (4ZL4), *Plasmodiumfalciparum*Lactate Dehydrogenase PfLDH (1T2C) and Falcipain-2 (1YVB) used in the ducking studies were downloaded from the Protein Databank (http://www.rcsb.org) with these various identification codes. All docking studies were done using AutoDock vina 4.2. (Trott and Olson, 2010). The process were validated using ligands known to bind to the proteins and their affinity energy, best conformation fits, root mean square deviation (RMSD) values and 2D protein-ligand interactions (Discovery Studio Visualizer version 16) were determined and used for comparison in each docking studies. Before validation, the various ligands and water molecules were deleted from

the original protein, no partial charges were added to the structureand the ligands were re-docked into the active site of each protein with the same grid box for each protein. All ligands used which include Artesunate-procyanidin hybrid (PC14), Procyanidin (PC00) and Artesunate (AT00) were flexible structures drawn using Chemdraw Ultra 12.0 and were converted to .pdb format using Chem3D Pro 12.0 (CambridgeSoft Corp., Cambridge, MA) and saved in a .pdbqt file format with appropriate torsion numbers using AutoDockTools v1.5.6.

2.2.11 Erythrocyte lysis assay:

The test was performed using 100 µl of packed RBC (~10^9 cells/ml) obtained from mice, 90 µl PBS (Phosphate Buffered Saline, pH 7) and 10 µl of various concentrations of Artesunate-procyanidin hybrid compound (3.125-50 µg/ml). Also, 10 µl of 1% SDS, Procyanidin, and Artesunate were used as references. The test tubes were incubated at 37ºC for 15 minutes and centrifuged at 13000 rpm for 10 minutes. The supernatant obtained was then diluted with PBS (1/200) in triplicates. The absorbance was read against the PBS blank at 410 nm. % Haemolysis was calculated using the formula:

$$\%\ \text{Haemolysis} = \frac{\text{AbsTest} - \text{AbsSDS}}{\text{AbsPBS} - \text{AbsSDS}} \times 100$$

Where:

AbsTest – Absorbance of test

AbsSDS – Absorbance of SDS

AbsPBS – Absorbance of PBS

2.2.12 Plasma Oxidation Assay:

Plasma samples were obtained from mouse blood by centrifugation at 1200 g for 10 minutes and then diluted up to 40-fold using PBS. The diluted plasma (0.2 mL) was mixed with 0.2 mL of PBS and Artesunate-procyanidin hybrid compound at different concentrations and

incubated at 37°C for 20 minutes with gentle shaking. Cu^{2+} was used to oxidize plasma by adding 0.4 mL of 200 µM $CuSO_4$ and the incubation continued for another 2 h and the absorbance read every 30 minutes at 245 nm to determine the levels of conjugated dienes.2.2.13 Toxicological study

Sixty Swiss albino mice were randomly distributed into 6 groups (A-F) of 10 mice per group. Mice in groups A, B, C, D, E and F were orally administered 0.2 ml of 5% DMSO, 5, 10, 20, 40 and 80 mg/kg body weight of the hybrid molecule respectively for 21 days. Twenty four hours (24h) after the last dose, the animals were sacrificed and the blood, liver, brain, heart and kidney were collected for analysis.

2.2.13.1 Preparation of plasma and tissue homogenate

The mice were sacrificed after they were anesthetized slightly with diethyl ether. Blood was collected into clean, dry test tubes containing appropriate anticoagulant; EDTA for hematological analysis and Heparin for biochemical analysis. The heparinized samples were centrifuged at 1000 g for 15 minutes and the plasma carefully collected using a Pasteur pipette into properly labelled, dry, clean tubes. The samples were stored frozen until needed for analysis. The animals were quickly dissected and the liver, brain, heart and kidney were excised; cleansed of superficial connective tissue, cleaned of blood and weighed. They were then homogenized separately in ice-cold 0.25 M sucrose solution (1:5, w/v). The homogenates were stored frozen overnight before centrifuging. The supernatant obtained after centrifuging was used for the analysis. Organs of two mice from each group were spared and transferred into specimen bottles containing 10 % formalin for histopathological studies.

Organ-body weight ratio

The organ-body weight ratio was calculated using the formula:

$$\% \text{ Organ} - \text{body weight ratio} = (\frac{\text{Weight of the organ}}{\text{Weight of the whole animal}}) \times 100$$

2.2.14 Haematological Analysis

Haematological parameters determined include White Blood Cell (WBC), Platelet count (PLT), Neutrophils (NEU), Lymphocytes (LYM), Red Blood Cell (RBC), Packed Cell Volume (PCV), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH),Mean Corpuscular Haemoglobin Concentration (MCHC) and Haemoglobin concentration (Hb).These were determined using the automated haematological analyser, Mindray Machine(Model BC-3200, Germany).

Principle:

The machine uses whole blood samples. The machine uses cell pack which functions as a detergent and possesses a self-rinsing system to avoid introduction of errors and a stromatolyzer which works on the cells. The stromatolyzer counts the red cells and lyses them thereby releasing the haemoglobin and estimates its concentration using photometric analysis. The machine assumes that all nucleated cells are white and therefore counts all as white cells into their different forms i.e. lymphocytes and neutrophils but will not differentiate between basophils, monocytes and eosinophils and as such, they are recorded as mixed. It lyses the white blood cells based on the size of the nucleus and counts the number of white cells.

2.2.15 Histopathological Studies

Histopathological studies werecarried out according to the method describedby Krause (2001).

Procedure:

This procedure involves fixing of tissues and then it's dehydration through ascending grades of ethanol to 70%, 90%, and 95% absolute alcohol. After which they were cleaned in

xylene, impregnated and embedded in paraffin wax. Sections were cut at 5µm on a rotatory microtome. The cut sections were floated out on clean microscope slides, which had previously been lightly coated with egg albumin preparation (albumerized) to avoid detachment from slides during staining procedures. They were dried for 2 hours at 37ºC (Drury and Wallington, 1973). The slides were then stained in aqueous dyes.The slides together with mounted stained sections were passed through ascending concentrations of 20% -100% alcohol for dehydration and then cleaned with xylene. A permanent mounting medium (basalm) was put on the tissue section. A thin glass cover slip was placed on the covering-mounting medium and underlying tissue sections were allowed to dry and were later observed using Leitz, DIALUX research microscope at x 400 magnification and the photomicrographs were taken in a bright field.

2.2.16 Cardiovascular disease indices

2.2.16.1 Determination of Total Cholesterol Concentration

Total cholesterol concentration was determined according to the method described by Tietz, (1995).

Principle:

Cholesterol concentration is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide (H_2O_2) and 4-aminoantipyrene in the presence of peroxidase and phenol.

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty Acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholestene} - 3 - \text{one} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + 4 - \text{Aminoantipyrene} \xrightarrow{\text{peroxidase}} \text{Quinoneimine} + 4H_2O$$

Procedure:

Three test tubes are labelled standard, sample and blank, to which 10 µl of standard solution, plasma samples, and distilled water were added respectively. 1000 µl of cholesterol reagent [4-aminoantipyrine (0.30 mmol/L), phenol (6 mmol/L), peroxidase (≥0.5 U/ml), cholesterol oxidase (≥0.1 U/ml), cholesterol esterases (≥0.1 U/ml), PIPES buffer (80 mmol/L; pH 6.8)] was added to each of the test tubes. This was thoroughly mixed and incubated for 5 min at 37ºC and the absorbance of the samples and standard was read at 500 nm against the reagent blank.

Cholesterol concentration was calculated using the formula:

$$Cholesterol\ concentration\ = \frac{Absorbance\ of\ sample}{Absorbance\ of\ standard} \times Concentration\ of\ standard$$

2.2.16.2 Determination of Triglyceride Concentration

Triglyceride concentration was determined colorimetrically using the method described by Tietz (1995).

Principle:

The triglyceride concentration is determined after enzymatic hydrolysis with lipases. The indicator is quinoneimine formed from Hydrogen Peroxide (H_2O_2), 4- aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase.

$$\text{Triglycerides} + H_2O \xrightarrow{\text{lipases}} \text{Glycerol} + \text{Fatty Acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol} - 3 - \text{Phosphate} + \text{ADP}$$

$$\text{Glycerol} - 3 - \text{Phosphate} + O_2 \xrightarrow{\text{GPO}} \text{Dihydroxyacetone phosphate} + H_2O_2$$

$$2H_2O_2 + 4 - \text{Aminophenazone} + 4\,\text{Chlorophenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + \text{HCl} + 4H_2O$$

Procedure:

Three test tubes were labelled standard, sample and blank, to which 1000 µl of the working reagent [PIPES-buffer (pH 7.00) 50 mmol/L, p-chlorophenol 5,3 mmol/L, potassium ferrocynate 10 mmol/L, magnesium salt 17 mmol/L, 4-aminoantipyrine 0.9 mmol/L, ATP 3.15 mmol/L, lipoprotein lipase 1800 U/L, glycerol kinase 450 U/L, glycerol - 3- phosphate oxidase 3500 U/L, peroxidase 450 U/ L] was added,after which 10 µl of the sample and standard solution (200 mg/dL) were added appropriately. This was mixed and incubated for 5 minutes at 37ºC. The change in absorbance of standard and sample was read against the reagent blank at 500 nm.

Triglyceride concentration was calculated using the formula:

$$\text{Triglyceride concentration} = \frac{Absorbance\ of\ sample}{Absorbance\ of\ standard} \times Concentration\ of\ standard$$

2.2.16.3 Determination of High Density Lipoprotein-Cholesterol (HDL-cholesterol) Concentration

The concentration of plasma High Density Lipoprotein Cholesterol (HDL-chol) was determined according to the method described by Friedwald *et al*.(1972).

Principle:

Low density lipoprotein and Very low density liproprotein (LDL and VLDL) and chylomicron fractions are precipitated quantitatively by the addition of phosphotungstic acid in the presence of magnesium ions. After centrifugation, the cholesterol concentration in the High Density Lipoprotein (HDL) fraction, which remains in the supernatant, is determined.

Procedure:

To two test tubes labelled standard and sample, 200 µl of standard and sample were added respectively, followed by 500 µl of the reagent [phosphotungstic acid, 0.55 mmol/L; magnesium chloride, 25 mmol/L]. The contents were mixed and allowed to stand for 10 minutes

at room temperature. The mixtures were then centrifuged at 4000 rpm for 10 min and the clear supernatant was aspirated for determination of cholesterol content.

The HDL-C concentration was calculated using the formula:

$$\text{HDL} - \text{C (mmol/L)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \textit{Concentration of Standard}$$

2.2.16.4 Determination of Low Density Lipoprotein-Cholesterol (LDL-cholesterol) Concentration

The LDL-Cholesterol level was calculated using the formula described by Friedwald *et al*. (1972).

$$\text{LDL Cholesterol (mg/dl)} = \text{Total Cholesterol} - \text{VLDL} - \text{HDL} - \text{Cholesterol}$$

2.2.16.5 Determination of Very Low Density Lipoprotein Concentration

Very Low Density Lipoprotein (VLDL) is determined using the formula described by Friedwald *et al.* (1972).

$$\text{VLDL (mg/dl)} = \frac{\textit{Triglyceride}}{5}$$

2.2.16.6 Determination of Atherogenic Index

The atherogenic index (AI) was calculated using the method described by Lamarche*et al*. (1996).

$$\text{Atherogenic index (AI)} = \frac{\text{Total Cholesterol}}{\text{HDL} - \text{cholesterol}}$$

2.2.17 Plasma Electrolytes and Biomolecules

2.2.17.1 Plasma Electrolytes

2.2.17.1.1 Determination of Plasma Calcium Ion Concentration

The method described by Biggs and Moorehead (1974) was used in the determination of plasma calcium ions.

Procedure:

To three test tubes labelled standard, test and blank, 1000 µl of the working reagent (diethylamine 360 mmol/L, O-cresolphalein complex 0.15 mmol/L, 8-Hydroxyquinoline 17.2 mmol/L)was added. 100 µl of standard, test sample and distilled water was added respectively. The Standard contained 10 mg/dL of standard calcium solution.The content of each tube was thoroughly mixed and incubated for 5 min at room temperature. The absorbance of the standard and test was measured against the reagent blank at 578 nm.

The plasma calcium ions concentration was calculated using the formula:

$$\text{Calcium ion concentration (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 10$$

2.2.17.1.2 Determination of Plasma Chloride Ion Concentration

The method described by Thomas (1998) was used in the determination of plasma calcium ions.

Principle:

In an acidic medium, chloride ions and mercury-II- thiocynate form thiocynate ions. These ions react with HNO_3 and Fe^{3+} producing a red colour. The intensity of the colour obtained is directly proportional to the concentration of chloride ions.

Procedure:

To three test tubes labelled standard, test and blank, 1000 µl of the working reagent [mercuric (II) thiocyanate 2 mmol/L, nitric acid 29 mmol/L, ferric nitrate 20 mmol/L] was added and 10 µl of the standard, plasma sample, and distilled water were added to their respective tubes. The content of each tube was mixed and immediately incubated at 37ºC for 1 minute after which the absorbance of the sample and standard was read against the reagent blank at 492 nm. The concentration of chloride ions in the samples was calculated using the formula:

$$\text{Chloride conconcentration (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 5$$

2.2.17.1.3 Determination of Plasma Bicarbonate Ion Concentration

The method described by Forrester (1976) was used in the determination of plasma phosphate ions.

Principle:

Phosphoenol pyruvate carboxylase (PEPC) catalyzes the reaction between phosphoenol pyruvate and bicarbonate to form oxaloacetate and phosphate ion. Oxaloacetate is reduced to malate with simultaneous oxidation of an equimolar amount of reduced nicotinamide adenine dinucleotide (NADH) to NAD^+, catalyzed by malate dehydrogenase (MDH). This results in a decrease in absorbance that is directly proportional to bicarbonate concentration in the sample.

$$\text{Phosphoenol pyruvate} + \text{HCO}_3^- \xrightarrow{\text{PEPC}} \text{Oxalate} + H_2PO_4$$

$$\text{Oxalate} + \text{NADH} + H^+ \xrightarrow{\text{MDH}} \text{Malate} + NAD^+$$

Procedure:

To three test tubes labelled standard, test and blank, 1ml carbon dioxide reagent [PEP 1.8 mM, magnesium sulfate 10 mM, NADH 0.40 mM, MDH (porcine) 1250 U/L, PEPC (microbial)

200 µ/L, sodium oxalate, 2.5 mM] was added and incubated at 37ºC for 3 minutes. Standard solution (30 mmol/L), sample and distilled water, was then added to the tubes respectively. The tubes were gently inverted to mix and incubated at 37º C for 5 minutes. The absorbance (Abs) of standard and tests were then read against reagent blank at 340 nm.

Bicarbonate concentration in the samples was calculated using the formula:

$$Bicarbonate\ ions\ concentration\ (mmol/L) = (\frac{\text{Abs of blank} - \text{Abs of sample}}{\text{Abs of blank} - \text{Abs of standard}}) \times 30$$

2.2.17.1.4 Determination of Plasma Potassium Ion Concentration

The turbidimetric method described by Tietz (1995) was used in the determination of serum phosphate ion concentration.

Principle:

Potassium ion in the sample reacts with sodium-tetraphenylborate to give a turbid potassium-tetraphenylborate complex. The extent of turbidity is proportional to the potassium ion concentration.

$$\text{Na} - \text{tetraphenylborate} + \text{K}^+ \rightarrow \text{K} - \text{tetraphenylborate} + Na^+$$

Procedure:

To two test tubes labelled standard and test, 1000 µl of the potassium reagent (sodium tetraphenylboron (TPS-Na), 0.2 mol/L) was added; 25 µl of the standard solution (5 mmol/L) was added to the standard and 25 µl of sample to the test. The contents of each tube were mixed and incubated for 5 min at 37ºC, after which the absorbance of each sample and standard was read against a distilled water blank at 578 nm.

The concentration of potassium ion (K^+) in the sample was calculated using the formula:

$$\text{Potassium ion conconcentration (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 5$$

2.2.17.1.5 Determination of Plasma Sodium Ion Concentration

The method described by Tietz (1995) was used to determine the sodium ion concentration.

Principle:

Sodium and proteins in the sample are precipitated together by magnesium uranyl acetate as uranyl magnesium sodium acetate salt. Excess of uranyl salt reacts with potassium ferrocyanide to produce a brownish colour, the intensity of which is inversely proportional to the sodium concentration in the sample.

Procedure:

Precipitation was first done by adding 1 ml of precipitating reagent (Uranyl acetate, 19 mmol/L, magnesium acetate, 140 mmol/L) plasma and standard solution (150 mmol/L); this was shaken vigorously and incubated for 5 min at room temperature. The tubes were then centrifuged at 3000 rpm for 2 minutes to obtain a clear supernatant. Then, sodium estimation was done by adding 1ml of colour reagent (ammonium thioglycolate 550 mmol/L, ammonia 550 mmol/L) to all tubes and 20 µl of supernatants were added to their respective tubes and 20 µl of precipitating reagent was added to the blank to make up the volume. The tubes were mixed and allowed to stand at room temperature for 5 minutes. The absorbance (Abs) for test and standard were read against reagent blank at 546 nm.

The concentration of sodium in the samples was calculated using the formula:

$$\text{Sodium ion } concentration\ (mmol/L) = \left(\frac{\text{Abs of blank} - \text{Abs of sample}}{\text{Abs of blank} - \text{Abs of standard}}\right) \times 150$$

2.2.17.1.6 Determination of Phosphate Ion Concentration

Phosphate ion concentration was determined using the method described by Tietz (1995).

Principle:

Ammonium molybdate and sulfuric acid react with phosphate ions in the sample to form a coloured phosphomolybdic complex, the intensity of which is proportional to the concentration of phosphate ions in the sample.

$$\text{Ammonium molybdate} + \text{Sulfuric acid} \xrightarrow{\text{phosphorus}} \text{Phosphomolybdic complex}$$

Procedure:

To three test tubes labelled standard, test and blank, 1000 µl of working reagent (sulfuric acid, 210 mmol/L, ammonium molybdate, 650 mmol/L) was added. A known volume (20 µl) of each of the standard (5 mg/dL) and sample was added to respective test tubes. The content of each tube was mixed and immediately incubated for 1 minute at 37º C and the absorbance of samples and standard was read against the reagent blank at 340 nm.

The concentration of phosphate ion in the samples was calculated using the formula:

$$\text{Phosphorus conconcentration (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 5$$

2.2.17.2 Plasma Biomolecules

2.2.17.2.1 Determination of Total Protein Concentration

The protein concentrations in tissue and plasma were determined using the Biuret method as reported by Gornall*et al.* (1949).

Principle:

Biuret reagent detects the presence of peptide bonds in solution;when compounds containing peptide (-CO-NH-) bonds are treated with alkaline copper sulphate, it results in the formation of a purple complex, the intensity of which is a measure of the protein content in the sample.

Procedure:

To1 ml of sample in a test tube, 4 m1 of Biuret reagent was added and mixed. This was incubated for 30 minutes at room temperature. The blank contained 1 ml distilled water in place of sample. The absorbance of samples was read at 540 nm and protein concentrations of samples were calculated from a standard calibration curve of bovine serum albumin (BSA).The calibration curve used for protein determination was prepared using 0.1-2.0 mg/ml concentrations of bovine serum albumin (BSA) prepared from the stock of 10 mg/ml BSA solution by pipetting volumes of the stock and making up the volume to 1.0 ml with distilled water in test tubes. 4.0 ml of Biuret reagent was added to each of the test tubes. The resulting mixture was mixed thoroughly and allowed to stand for 30 minutes at room temperature for maximum colour development. The blank was made up of 1.0 ml of distilled water and 4.0 ml of Biuret reagent. The absorbance was read at 540 nm against the blank. The absorbance values obtained were used for plotting of the calibration curve for protein determination(Appendix, Figure 89).

2.2.17.2.2 Determination of Plasma Urea Concentration

Urea concentration was determined using the method described by Henry (1963).

Principle:

Urea in sample is broken down to ammonia and carbon dioxide by urease. The ammonia reacts with salicylate in the presence of nitroprusside and hypochlorite to give 2,2-dicarboxyindophenol, a coloured compound; the intensity of which is measured and is proportional to the concentration of urea in the sample.

$$\text{Urea} + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2$$

$$NH_3 + \text{Salicylate} \xrightarrow{\text{Nitroprusside, Hypochlorite}} 2.2 - \text{dicarboxyindophenol}$$

Procedure:

To three clean test tubeslabelled standard, test and blank, 1000 µl of working reagent (Phosphate buffer 60 mmol/L (pH 6.9), urease 20 (U/L) was added. A known volume (10 µl)of each of the standard preparation (13.10 mmol/L), sample and distilled water was added to appropriate test tube. The resulting solutions were mixed and incubated at 37°C for 5 minutes, after which 1000 µl of colour reagent (sodium salicylate, 80 mmol/L, sodium nitroprusside, 4 mmol/L, sodium hypocholorite, 45 mg/dL) was added to all tubes, mixed and incubated again for 5 minutes at 37°C. After the incubation, 1000 µl of distilled water was added to each tube and mixed again. The absorbance of the standard and sample against the reagent blank was read at 600 nm.

Urea concentration was calculated using the formula:

$$\text{Urea concentration (mmol/L)} = \frac{Absorbance\ of\ sample}{Absorbance\ of\ standard} \times Concentration\ of\ standard$$

2.2.17.2.3 Determination of Plasma Uric Acid Concentration

The concentration of uric acid was determined using the method described by Fossati *et al.*(1980).

Principle:

Uricase converts uric acid to allantoin and hydrogen peroxide, which, with the help of peroxidise, oxidizes 3, 5-dichloro-2-hydroxybenzensulfonic acid and 4-aminophenazone to form a red-violet quinoneimine compound, the concentration of which is proportional to the amount of uric acid.

$$\text{Uric Acid} + O_2 + 2H_2O \xrightarrow{\text{Uricase}} \text{Allantoin} + CO_2 + H_2O_2$$

$$2H_2O_2 + 3,5 - \text{Dichloro} - 2 - \text{hydroxybenzensulfonic acid} + 4 - \text{aminophenazone} \xrightarrow{\text{peroxidase}} \text{N} - (4 - \text{antipyryl}) - 3 - \text{chloro} - 5 - \text{sulfonate} - \text{p} - \text{benzo} - \text{quinoneimine}$$

Procedure:

To two test tubes labelled test and standard, 20 µL of each of the sample and standard (0.595 mmol/L uric acid) wereadded respectively and mixed with 1000 µL of working reagent containing (50 mmol/L Hepes buffer, pH 7.0, and 4 mmol/L 3, 5-Dichloro-2-hydroxybenzenesulfonicacid) and followed by reagent containing 0.25 mmol/L 4-aminophenazone, ≥1000 U/I peroxidase and ≥ 200 U/I Uricase. The mixture was incubated at 25ºC for 15 minutes, after which the absorbance of standard and samples was read at 520 nm against reagent blank.

Uric acid concentration was calculated using the formula:

$$\text{Uric concentration (mmol/L)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times Concentration\ of\ standard$$

2.2.17.2.4 Determination of Plasma Creatinine Concentration

The concentration of creatinine was determined according to the method described by Allen (1982).

Principle:

Creatinine reacts with picric acid to produce a coloured compound, creatinine alkaline picrate, of which the intensity is proportional to the creatinine concentration.

Procedure:

Two test tubes are labelled standard and test, to which 1000 µl each of working reagent (sodium hydroxide 300 mmol/L, sodium phosphate 25 mmol/L, picric acid 8.73 mmol/L, surfactant) was added. Then, 100 µl of the standard solution (2 mg/dL) and test were added to appropriate test tubes. The absorbance (T1) of the sample and standard was read after 1 minute against distilled water blank at 505 nm. The second reading (T2) was taken exactly 1 minute after the first reading.

The concentration of creatinine in the samples was calculated using the formula:

$$\text{Creatinine concentration (mg/dl)} = \frac{\text{T2} - \text{T1 of sample}}{\text{T2} - \text{T1 of standard}}$$

2.2.17.2.5 Determination of Plasma Albumin Concentration

The method described by Doumas *et al.* (1971) was used to determine albumin concentration in samples.

Principle:

Albumin reacts with bromocresol-green to produce a change in colour that is proportional to the albumin concentration in a sample.

Procedure:

To three tubes labelled standard, sample and blank, 1000 µl of the working reagent (succinate buffer (pH 4.20) 75 mmol/L, bromocresol green 0.14 g/L) was added. 10 µl of the standard solution (3 g/dL albumin) and sample were added to appropriate test tubes. This was mixed thoroughly and incubation for 1 minute at room temperature. The absorbance of the sample and standard was read against the reagent blank at 630 nm.

The concentration of albumin was calculated using the formula:

$$\text{Albumin Concentration (g/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times Concentration\ of\ standard$$

2.2.17.2.6 Estimation of Plasma Globulin concentration

The estimation of globulin concentration was done using the method described by Tietz (1995).

$$\text{Globulin concentration} = \text{Plasma Total Protein concentration} - \text{Plasma Albumin concentration}$$

2.2.17.2.7 Determination of Plasma Bilirubin concentration

The method described by Walter and Gerard (1970) was used to determine plasma bilirubin concentration.

Principle:

Sulfanilic acid reacts with sodium nitrite to form diazotized sulfanilic acid. Conjugated (direct) bilirubin reacts with diazotized sulfanilic acid to form azo-bilirubin. The intensity of the purple colour formed is proportional to the concentration of bilirubin in the sample. In total bilirubin, diazotized sulfanilic acid reacts with total bilirubin in the presence of total activator bilirubin (TAB; 9mmol/L) to form azobilirubin.

Procedure:

To three test tubes labelled standard, test and blank, 1000 µl of the direct bilirubin reagent (sulfanilic acid 28.9 mmol/L, hydrochloric acid, 165 mmol/L) was added. A known amount (20 µl) of activator direct was dispensed into the standard, 50 µl of plasma and distilled water to the test and blank respectively. The contents of both tubes were mixed and incubated for 5 minutes at room temperature and the absorbance of test was read against reagent blank at 546 nm.

The conjugated bilirubin concentration was calculated using the formula:

$$\text{Conjugated bilirubin concentration} = (\text{Absorbance of test} - \text{Absorbance of reagent blank}) \times 20$$

$$\text{Total bilirubin concentration} = (\text{Absorbance of test} - \text{Absorbance of reagent blank}) \times 29$$

2.2.18 Determination of Enzyme Activities

2.2.18.1 Determination of Aspartate Aminotransferase Activity

Aspartate aminotransferase (AST) activity was determined according to the method described by Reitman and Frankel (1957).

Principle:

Aspartate aminotransferase (AST) activity was measured by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4-dinitrophenylhydrazine.

$$\alpha - \text{ketoglutarate} + \text{L} - \text{aspartate} \xrightarrow{\text{Aspartate aminotransferase}} \text{L-glutamate} + \text{Oxaloacetate}$$

Procedure:

To 100 µl of sample, 500 µl of reagent containing Phosphate buffer (100 mmol/L, pH 7.4), L-aspartate (100 mmol/L), and α-ketoglutarate (2 mmol/L) was added and mixed thoroughly. This was then incubated at 37ºC for 30 minutes and 500 µl of 2,4-dinitrophenylhydrazine (2 mmol/L) was added and incubated at 20ºC for another 20 minutes after which 5000 µl of 0.4 N sodium hydroxide was added and the absorbance read at 546 nm after 5 minutes. The activity of AST in U/mg protein was determined from a calibration curve (Appendix, Figure 90) using the formula:

$$\text{Aspartate aminotransferase activity} = \frac{\text{AST activity from calibration curve x dilution factor}}{\text{Protein concentration}}$$

One unit of AST is the amount of enzyme that generates 1 µmole of glutamate per minute at pH 8.0 at 37°C.

Aspartate aminotransferase (AST) calibration curve was prepared using 0.1 – 1.0 mM concentrations of sodium pyruvate solution. From the sodium pyruvate stock solution of 2 mM, various volumes of the stock was pipette into test tubes and diluted with varying volumes of AST-buffered substrate. 0.2 ml of distilled water and 1.0 ml of 2,4-DNPH (2 mM) were added to the blank and test samples respectively, mixed properly and allowed to stand for 20 minutes. 5.0 ml of NaOH (0.4 N) was added and the mixture was mixed and allowed to stand (for 5 minutes). The absorbance was read against the reagent blank at 546 nm. The values obtained were used in plotting the calibration curve(Appendix, Figure 90).

2.2.18.2 Determination of Alanine Aminotransferase Activity

Alanine aminotransferase (ALT) activity was assayed according to the method described by Reitman and Frankel (1957).

Principle:

Alanine aminotransferase (ALT) activity was measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine.

$$\alpha - \text{ketoglutarate} + \text{L} - \text{alanine} \xrightarrow{\text{Alanine aminotransferase}} \text{L} - \text{glutamate} + \text{Pyruvate}$$

Procedure:

To 100 µl of sample, 500 µl of reagent containing Phosphate buffer (100 mmol/L, pH 7.4), L-alanine (100 mmol/L), and α-ketoglutarate (2 mmol/L) was added and mixed thoroughly. After incubation for 30 minutes at 37 ºC, 500 µl of 2,4-dinitrophenylhydrazine (2 mmol/L) was added and incubated at 37ºC for another 20 minutes after which 5000 µl of 0.4 N sodium hydroxide was added and the absorbance read at 546 nm after 5 minutes. The activity of ALT in U/mg protein was determined from a calibration curve (Appendix, Figure 91) using the formula:

$$\text{Alanine aminotransferase activity} = \frac{\text{ALT activity from calibration curve x dilution factor}}{\text{Protein concentration}}$$

One unit of enzyme activity is defined as the quantity of enzyme that catalyses the reaction of 1 µmole of substrate per minute at 37°C.

Alanine aminotransferase (ALT) calibration curve was prepared using 0.1 – 1.0 mM concentrations of sodium pyruvate solution. From the sodium pyruvate stock solution of 2 mM, various volumes of the stock was pipette into test tubes and diluted with varying volumes of ALT-buffered substrate. 0.2 ml of distilled water and 1.0 ml of 2,4-DNPH (2 mM) were added to the blank and test samples respectively, mixed properly and allowed to stand for 20 minutes. 5.0

ml of NaOH (0.4 N) was added and the mixture was mixed and allowed to stand for 5 minutes. The absorbance was read at 546 nm against the reagent blank. The values obtained were used in plotting the calibration curve (Appendix, Figure 91).

2.2.18.3 Determination of Alkaline Phosphatase Activity

Alkaline phosphatase (ALP) activity in tissue homogenates and plasma was determined using the method described by Wright *et al.* (1972).

Principle:

Enzyme activity was determined by monitoring the rate of formation of p-nitrophenol from the hydrolysis of p-nitrophenylphosphate.

$$\text{p} - \text{nitrophenyl phosphate} + H_2O \xrightarrow{\text{Alkaline phosphatase}} \text{p} - \text{nitrophenol} + \text{Pi}$$

Procedure:

To 10 µl of sample in a test tube, 500 µl of reagent (diethanolamine buffer, 1 mmol/L, pH 9.8, $MgCl_2$, 0.5 mmol/L, and p-nitrophenylphosphate, 10 mmol/L) was added, mixed and the initial absorbance read at 405 nm.The absorbance was read again after 1, 2, and 3 minutes, blanking with air.

Specific activity of alkaline phosphatase (ALP) in Units/mg protein was calculated using the formula:

$$\text{Specific activity of Alkaline phosphatase} = \frac{\Delta\text{Abs/min} \times 2760 \times \text{dilution factor}}{\text{Protein concentration}}$$

One unit of alkaline phosphatase (ALP) activity is the enzyme activity which hydrolyses 1 µmole of 4-nitrophenyl phosphate in 1 minute at 37°C under assay conditions.

2.2.18.4 Determination of Glutamate Dehydrogenase Activity

Glutamate Dehydrogenase (GDH) activity was assayed according to the method of Shimizu *et al.* (1979).

Principle:

The method is based on the reductive amination of α-ketoglutaric acid. In the presence of ammonium ion and reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH), α-ketoglutaric acid is converted to glutamate and NAD^+ or $NADP^+$. The rate of reaction, monitored by decrease in optical density at 340 nm is proportional to the activity of GDH in the samples.

$$\text{Glutamate} + \text{NADP}^+ \xleftrightarrow{\text{Glutamate dehydrogenase}} \alpha - \text{ketoglutarate} + N{H_4^+} + \text{NADPH}$$

Procedure:

The reaction mixture contained 2 ml Tris-HCl Buffer (85mM, pH 7.2), 0.2 ml α-Ketoglutarate (7.6 mM), 0. 2 ml NH_4Cl (0.22 mM), 0.1 ml NADH (0.25 mM) and 0.1 ml EDTA (0.85 mM). The reaction was initiated by the addition of 0.05 ml appropriately diluted sample. The assay was done at room temperature for 5 minutes in a total volume of 2.65 ml. The decrease in optical density at 340 nm was read against blank and the change in optical density per minute calculated.

Glutamate Dehydrogenase (GDH) activity was calculated using the formula:

$$\text{Glutamate dehydrogenase activity} = \frac{\Delta\text{Abs/minute} \times \text{Total volume} \times \text{Dilution factor}}{6.22 \times \text{Volume of sample} \times \text{Protein concentration}}$$

Where:

Millimolar Extinction Coefficient of NADPH (F/micromole) = 6.22

2.2.18.5 Determination of γ-Glutamyl transferase Activity

The activity of **γ-**Glutamyl transferase (**γ-GT**) was determined using the method described by Szasz *et al*.(1969).

Principle:

γ-GT converts L-γ-glutamyl-3-carboxy-4-nitroanilide, in the presence of glycylglycine, to L-γ-glutamylglycylglycine and 5-amino-2-nitrobenzoate. The concentration of 5-amino-2-nitrobenzoate formed can be measured at 405 nm.

$$\mathrm{L-\gamma-glutamyl-3-carboxy-4-nitroanilide + glycylglycine} \xrightarrow{\boldsymbol{\gamma\text{-GT}}} \mathrm{L-\gamma-glutamylglycylglycine + 5-amino-2-nitrobenzoate}$$

Procedure:

Briefly, 0.10 ml of appropriately diluted sample was mixed with 1.00 ml of reagent (100 mmol/L Tris buffer, pH 8.25, 100 mmol/L Glycylglycine and 2.9 mmol/L L-γ-glutamyl-3-carboxy-4-nitroanilide). The initial absorbance was read against air at 405 nm. The absorbance was read again after 1, 2 and 3 minutes.

γ-Glutamyl transferase (γ-GT) activity was calculated using the formula:

$$\gamma - \text{Glutamyl transferase activity} = \frac{\Delta\text{Abs} \times 1158 \times \text{Dilution factor}}{\text{Protein concentration}}$$

2.2.18.6 Determination of Lactate Dehydrogenase Activity

The activity of Lactate Dehydrogenase (LDH) in heart and plasma was determined according to the method described by Wrobleski and La Due (1955).

Principle:

Lactate Dehydrogenase (LDH) catalyses the conversion of pyruvate to L-lactate and nicotinamide adenine dinucleotide in the presence of reduced nicotinamide adenine dinucleotide.

$$\text{Pyruvate} + \text{NADH} \xrightarrow{LDH} \text{L} - \text{lactate} + NAD^+$$

Procedure:

From appropriately diluted sample, 0.02 ml was mixed with 3.00 ml of reagent (50 mmol/L Phosphate buffer, pH 8.25, 0.6 mmol/L Pyruvate, 1.8 mmol/L NADH). The initial absorbance was read against air at 340 nm and again after 1, 2 and 3 minutes.

Lactate Dehydrogenase (LDH) activity was calculated using the formula:

$$\text{Lactate dehydrogenase activity (Units/mg protein)} = \frac{\text{change in absorbance/min} \times 4127}{\text{Protein concentration}}$$

2.2.18.7 Determination of Acetylcholine esterase activity

The method described by Magnottl *et al*. (1987) was used to determine the activity of acetylcholine esterase.

Principle:

The reaction is monitored based on the production of thiocholine by the action of acetylcholine esterase on 5,5'-dithiobis(2-nitrobenzoic acid) to form a yellow colour. The intensity of the product colour which is measured at 412 nm, is proportionate to enzyme activity in the sample.

Procedure:

A test tube was prepared containing 200 µL of calibrator for test sample, and another with 200 µL of distilled water as blank. A known volume (10 µL) of appropriately diluted brain homogenate was also prepared and 190 µL of freshly prepared Working reagent was added to each test tube and mixed gently. The absorbance was read at 412 nm at 2 minutes and at 10 minutes. The specific activity of acetylcholine esterase was calculated using the formula:

$$AChE\ activity = \frac{Abs10 - Abs2}{AbsCal - AbsH20} \times n \times 200\ (U/L)$$

Abs10 = Absorbance at @10 min

Abs2 = Absorbance @2 min

AbsCal = Absorbance of Calibrator @10 min

Abs_{H_2O} = Absorbance of water @10 min

n = Dilution factor

Equivalent activity of calibrator = 200

One unit of AChE catalyses the production of 1 µmole of thiocholine per minute under the assay conditions of pH 7.5 at room temperature.

2.2.18.8 Determination of Creatine Kinase activity

The method described by DiWitt and Trendelenburg (1982) was used to determine the activity of creatine kinase.

Principle:

Kinetic determination of creatine kinase is based on the following reactions:

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow{\text{Creatine kinase}} \text{Creatine} + \text{ATP}$$

$$\text{ATP} + \text{D} - \text{glucose} \xrightarrow{Hexokinase} \text{G} - 6 - \text{P} + \text{ADP}$$

$$\text{G} - 6 - \text{P} + \text{NADP}^+ \xrightarrow{\text{G}-6-\text{PDH}} \text{D} - \text{gluconate} - 6\,\text{phosphate} + \text{NADPH} + H^+$$

Procedure:

In a test tube,200 µl of reagent 1 [imidazole (125 mmol/L, pH 6.7), D-glucose (25 mmol/L), N-acetyl-L-cysteine (25 mmol/L), magnesium acetate (12.5 mmol/L), NADP (2.4

mmol/L), EDTA (2.0 mmol/L), hexokinase (6800 U/L)] and 50 µl of reagent 2 (creatine phosphate, 250 mmol/L, ADP, 15.2 mmol/L, AMP, 25 mmol/L, diadenosine pentaphosphate, 103 µmol/L, glucose-6-phosphate dehydrogenase, 8800 U/L) were mixed and after 25 seconds, 10 µl of appropriately diluted samples was added, mixed and incubated for 2 minutes at 37ºC and the absorbance of sample was read at 340 nm against distilled water. The absorbance was read again at intervals of 1, 2 and 3 minutes. The specific activity of creatine kinase was calculated using the formula:

$$\text{Specific activity (Units/mg protein)} = \frac{\text{Change in absorbance/min x 4127 x dilution factor}}{\text{Protein concentration}}$$

2.2.18.9 Determination of Adenosine triphosphatases (ATPases) Activities

Principle:

The amount of inorganic phosphate cleaved from adenosine triphosphate (ATP) in ionic solution is quantified spectrophotometrically using ammonium molybdate and ascorbic acid system. A yellow colour which is produced when ammonium molybdate is oxidized by concentrated tetraoxosulphuric (VI) acid to molybdic acid which turns blue upon reduction of molybdic acid by ascorbate. The intensity of the final colour developed is proportional to the concentration of phosphate ion cleaved from ATP.

Phosphate Calibration Curve: Varying volumes of 1 mM $NaH_2PO_4.2H_2O$ ranging from 20-120µl were prepared in a final volume of 0.5 ml with distilled water. A known volume (1.5 ml) of reagent C (H_2SO_4-Ammonium molybdate/Ascorbate solution) was added to the varied concentration of $NaH_2PO_4.2H_2O$ solution. The absorbance was read at 820 nm after the mixtures were left at room temperature for 30 minutes. The absorbance was used to plot a calibration curve of inorganic phosphate for the determination of ATPase activity (Appendix, Figure 92).

2.2.18.9.1 Na^+, K^+ ATPase Activity

Na^+, K^+-ATPase activity was determined according to the method of Ronner *et al*. (1977) as modified by Bewaji *et al*. (1985).

Procedure:

In a test tube,400 µl of the working buffer [200 mM NaCl/40 mM KCl/60 mM Tris(pH 7.4), 20 µl of $MgCl_2.6H_2O$ (80 mM), 20 µl of EGTA (20 mM)], 240 µl of distilled water and 20 µl of tissue homogenate were mixed and incubated for 5 minutes at 37ºC. ATP (8 mM, 100 µl) was added, mixed and incubated for 30 minutes at 37ºC. Then, 200 µl of 5% SDS and 2,000 µl of reagent C were added and the mixture was left at room temperature for half an hour for colour development. In the blank, 20 µl of distilled water was used to replace 20 µl of tissue homogenate. The absorbance of the test was read after blanking at 820 nm. Concentration of inorganic phosphate in tissue samples was estimated by extrapolation from the calibration curve. ATPase activity in µmole P_i/mg Prot./hr was calculated using the formula:

$$Na^+\ K^+ATPase\ activity = \frac{[Pi] \times\ 2\ \times\ Dilution\ factor}{1000\ \times\ Protein\ Concentration}$$

Where:

Concentration of inorganic phosphate in µmoles (obtain from calibration Curve) = $[P_i]$

Factor introduced to obtain the amount of P_i released per hour = 2

Factor introduced to convert the P_i release to µmoles = 1000

The actual specific activity of Na^+,K^+-ATPase was obtained by the subtraction of the specific activity of Mg^{2+}-ATPase from that of Na^+,K^+-ATPase.

2.2.18.9.2 Mg^{2+} ATPase Activity

Mg^{2+}-ATPase activity was determined using the method of Ronner *et al*. (1977) as described by Fleschner and Kraus-Friedmann (1986).

Procedure:

In a test tube, 400 µl of 240 mM KCl/60 mM Tris (pH 7.4), 20 µl of $MgCl_2.6H_2O$ (80 mM), 20 µl of EGTA (20 mM), 220 µl of distilled water, 20 µl of tissue homogenate and 20 µl of ouabain (1mM) were mixed and the mixture was incubated for 5 minutes at 37^0C,after which 100 µl of 8 mM ATP was added, mixed and incubated for 30 minutes at 37^0C. Then, 200 µl of 5% SDS and 2,000 µl of reagent C were added and the mixture was left for 30 minutes for colour development at stand at room temperature. The blank was prepared by replacing 20 µl of tissue homogenate with 20µl of distilled water. The absorbance of the test was read against the blank at 820 nm. Concentration of inorganic phosphate in tissue homogenate was estimated by extrapolation from the Pi calibration curve.

ATPase activity in µmole P_i/mg Prot./hr was calculated using the formula:

$$Mg^{2+}\text{ATPase activity} = \frac{[\text{Pi}] \times 2 \times \text{Dilution factor}}{1000 \times \text{Protein Concentration}}$$

Where:

Concentration of inorganic phosphate in µmoles (obtain from calibration curve) = $[P_i]$

Factor introduced to obtain the amount of P_i released per hour = 2

Factor introduced to convert the P_i released to µmoles = 1000

2.2.18.9.3 Ca^{2+}, Mg^{2+} ATPase Activity

Ca^{2+}, Mg^{2+}-ATPase activity was determined according to the method of Ronner *et al*. (1977) as modified Bewaji *et al*. (1985).

Procedure:

To a test tube, 400 µl of 240 mM KCl/60mM Tris (pH 7.4), 40 µl of $CaCl_2$ (4 mM), 20 µl of $MgCl_2.6H_2O$ (80 mM), 220µl of distilled water and 20µl of sample were added in that order, mixed and incubated for 5 minutes at 37ºC. ATP (8 mM, 100 µl) was added, mixed and

incubated for 30 minutes at 37°C. Then, 200 µl of 5% SDS and 2,000 µl of reagent C were added and mixture was left at room temperature for half an hour for colour development. The blank was prepared by replacing 20 µl of tissue homogenate with 20µl of distilled water. The absorbance was read after blanking at 820 nm. Concentration of inorganic phosphate in tissue samples was estimated by extrapolation from the calibration curve.

ATPase activity in µmole P_i/mg Prot./hr was calculated using the formula:

$$Ca^{2+}\ Mg^{2+}\text{ATPase activity} = \frac{[\text{Pi}] \times 2 \times \text{Dilution factor}}{1000 \times \text{Protein Concentration}}$$

Where:

Concentration of inorganic phosphate in µmoles (obtain from calibration Curve) = [P_i]

Factor introduced to obtain the amount of P_i released per hour = 2

Factor introduced to convert the P_i release to µmoles = 1000

2.2.18.10 Histopathological Studies

Histopathological studies were carried out according to the method describedby Krause (2001).

Procedure:

The tissues were fixed in 10% formol saline and then it was dehydrated through ascending grades of ethanol (70%, 90%, 95% and absolute alcohol). They were then cleaned in xylene, impregnated and embedded in paraffin wax. Sections were cut at 5 µm on a rotatory microtome. The cut sections were floated out on clean microscope slides, which had previously been lightly coated with egg albumin preparation (albumerized) to avoid detachment from slides during staining procedures. They were dried for 2 hours at 37°C (Drury and Wallington, 1973). The slides were then stained in aqueous dyes; the slides together with mounted stained sections were passed though ascending concentrations of 20% -100% alcohol for dehydration and then

cleaned with xylene. A permanent mounting medium (basalm) was put on the tissue section. A thin glass cover slip was placed on the covering-mounting medium and underlying tissue sections were allowed to dry and were later examined using Leitz, DIALUX research microscope at x 400 magnification and the photomicrographs were taken in a bright field.

2.2.19 Statistics analysis

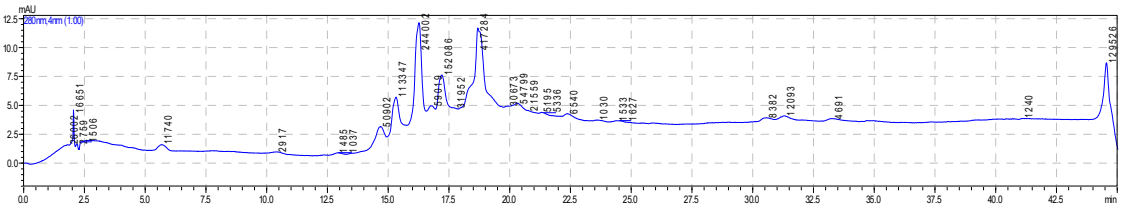
Data are presented as Mean ± SEM of replicates except otherwise stated. The data were analyzed using Analysis of Variance (ANOVA). Significant difference between the treatment was considered at $p<0.05$ using Duncan's Multiple Range Test (SPSS Version 20, SPSS Corporation, Chicago, Illinois, USA).

CHAPTER THREE

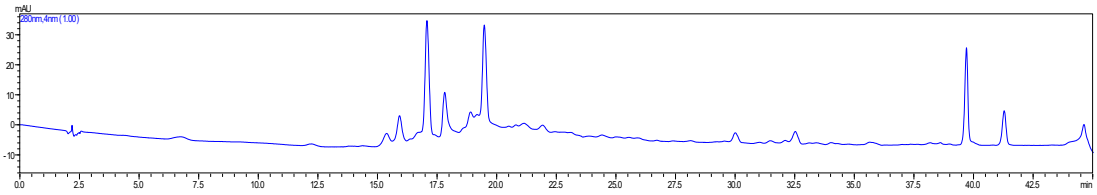
3.0 Results

3.1 Purification of Procyanidin and synthesis

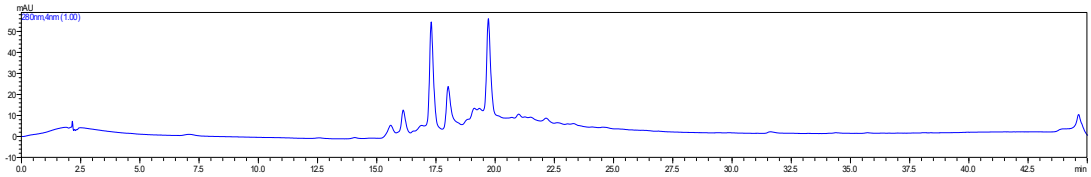
Procyanidin B_2 was obtained from oligomeric procyanidin in three steps partition/extraction procedure (Figure 13). The aqueous-ethyl acetate partition separated the polymeric units from the oligomeric formswhile the diethyl ether partition separated the monomeric units from the dimeric unit. The DEE fraction was washed with distilled water to remove impurities of monomeric units in the fraction.



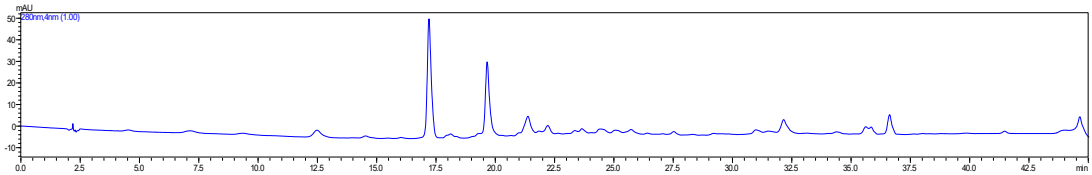
Oligomeric Procyanidins OPC (5%)



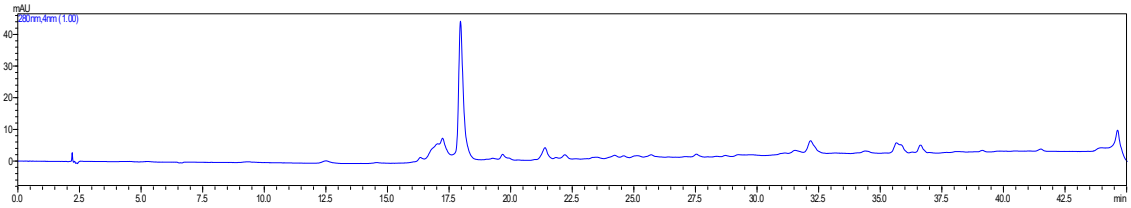
Ethyl Acetate Extract (5%)



B. Aqueous DEE Fraction



A. Diethyl Ether -Fraction



Co-elution (DEE 5% + Procyanidin B_2)

Figure 13: HPLC profile of Oligomeric procyanidin (OPC) separation steps

The reaction schemes were monitored using a TLC plate. The various fractions and reaction products where revealed using vanillin stain. The reactions were terminated once one of the starting materials was exhausted.

The HPLC profile of the reaction at 24^{th} hour showed two peaks (Figure 14). These peaks were separated using a flash chromatography on silica gel, with gradient solvents of hexane ethyl acetate to yield PC14-F2 and PC14-F4 with retention time of 21.94 and 25.35 respectively. The NMR and InfraRed spectra of PC14-F2 were obtained (Table 2, Figure 15).

The percentage free base yield of the hybrid was lower than that of the final hybrid yield (Table 3). Also the hybrid compound had a lower melting point when compared to both procyanidin and artesunate (Table 4).

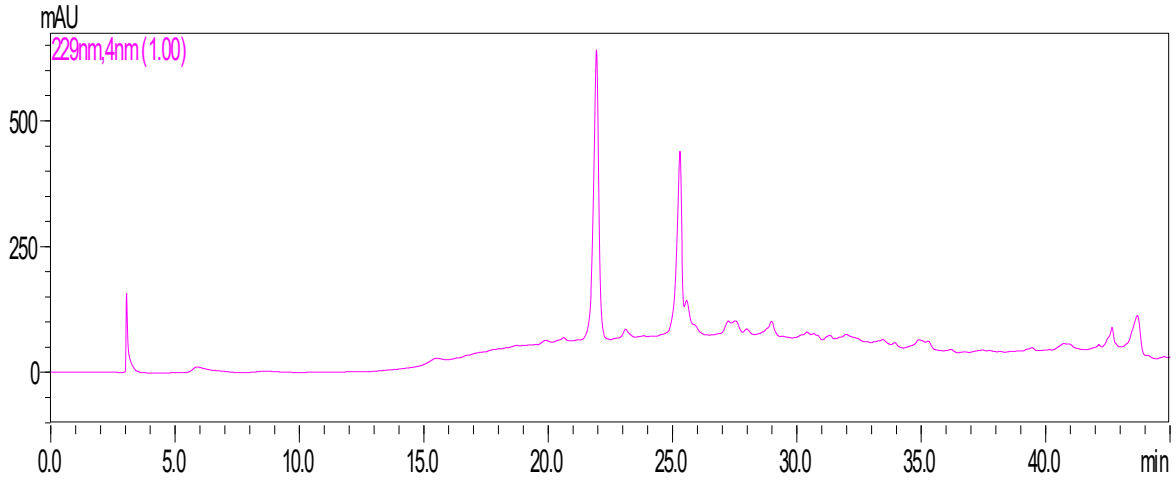
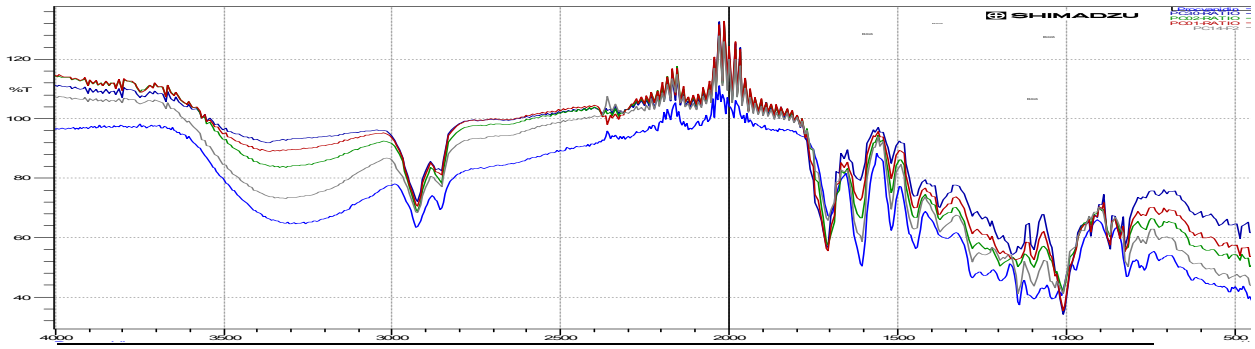


Figure 14: HPLC spectra of PC14 before Purification to give PC14-F2 and PC14-F4

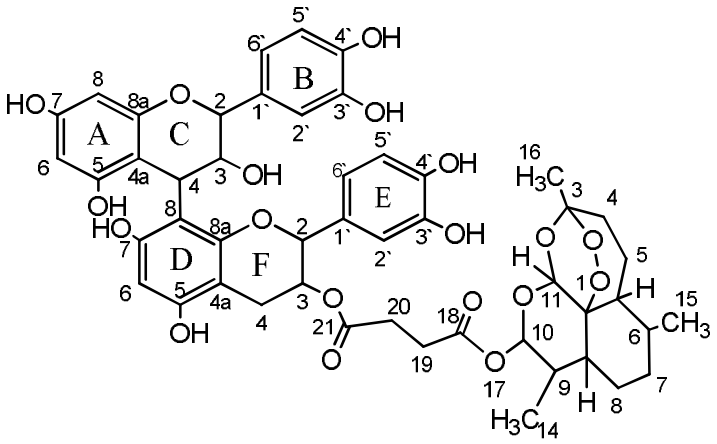
Table 2: NMR data (400MHz) of Artesunate-procyanidin (PC14) in DMSO d_6

Position	^{13}C	^{1}H	Position	^{13}C	^{1}H	Position	^{13}C	^{1}H
1`B	131.0	-	1`E	131.0	-	3	104.0	-
2`B	114.9	6.92 (s,1H)	2`E	114.9	6.88 (s,1H)	4	32.2	1.29 (d,2H)
3`B	156.2	-	3`E	156.2	-	5	21.6	0.89 (m,2H)
4`B	155.8	-	4`E	155.8	-	5a	51.8	3.47 (s,1H)
5`B	115.5	6.72 (m,1H)	5`E	115.5	6.72 (m,1H)	6	36.5	1.03 (s,1H)
6`B	118.9	6.72 (m,1H)	6`E	118.9	6.72 (m,1H)	7	29.3	1.01 (m,2H)
2C	99.0	5.69 (d,1H)	2F	99.0	5.69 (d,1H)	8	28.4	0.99 (m,2H)
3C	66.9	4.91 (d,1H)	3F	60.1	4.73 (s,1H)	8a	45.1	1.24 (d,1H)
4C	34.3	4.91 (d,1H)	4F	25.5	4.49 (d,2H)	9	29.0	2.18 (t,1H)
4aC	94.3	-	4aF	95.6	-	11	91.0	5.55 (s,1H)
5[a]	156.9	-	5D	156.6	-	11a	74.0	-
6[a]	80.4	5.88 (d,1H)	6D	81.5	5.72 (d,1H)	10	92.2	6.58 (dd,1H)
7[a]	157.0	-	7D	156.7	-	14	14.5	0.75 (d,3H)
8[a]	78.5	6.58 (d,1H)	8D	99.5	-	15	19.2	0.93 (d,3H)
8aA	145.3	-	8aD	144.9	-	16	25.7	1.07 (s,3H)
						18	171.5	-
						19	25.0	3.47 (s,2H)
						20	22.7	3.47 (s,2H)
						21	173.8	-



Drug	-OH	-CH	C=O	C=C	C-C
PC14	3655-3025 cm^{-1}	2927 cm^{-1}	1707 cm^{-1}	1605 cm^{-1}	1010 cm^{-1}
PC03	103.24-95.81 %	72.16 %	56.64 %	80.91 %	34.19 %
PC01	104.04-94.92 %	70.53 %	55.53 %	73.55 %	35.38 %
PC02	103.93-92.35 %	68.70 %	57.47 %	66.65 %	41.48 %
PC14	97.75-86.79 %	68.32 %	65.88 %	58.77 %	41.15 %
Proc.	92.93-76.93 %	63.55 %	68.20 %	50.61 %	41.67 %

Figure 15: InfraRed spectra of Artesunate-procyanidin hybrid molecule, Procyanidin and different ratio combinations. Proc.- Procyanidin, PC14- Artesunate-Procyanidin hybrid molecule; Artesunate/Procyanidin Combination: PC01 = Ratio 1:1, PC02 = Ratio 1:2, PC03 = Ratio 2:1



$C_{49}H_{52}O_{19}$

2,2'-bis(3,4-dihydroxyphenyl)-3,5,5',7,7'-pentahydroxy-[4,8'-bichroman]-3'-yl(3,6,9-trimethyldecahydro-3H-3,12-epoxy[1,2]dioxepino[4,3-i]isochromen-10-yl) succinate (PC14);

Figure 16: Elucidated Structure of synthesized Procyanidin-Artesunate Hybrid (PC14)

Table 3: Solvent choice, percentage yield and some characteristics of the artesunate-procyanidin hybrid molecule

Hybrids	Solvent	Starting Material	Free Base Yield (%)	Colour	Duration	Rf (Hex:EtOAc, 2:1)	Rt (mins)	Melting Point (°C)	Hybrid Yield (%)
PC14	Ethylacetate (EtOAc)	AT	12.58	Brown crystal	24 h	0.19	21.94	119-122	33.06

PC14 = Hybrid molecule, AT= Artesunate

3.2 Physical properties of hybrid

The melting point of the Artesunate-procyanidin hybrid molecule (PC14) was lower when compared to the parent materials (ie artesunate and procaynidin alone) (Table 4). The hybrid melted between 199ºC to 122ºC, while procyanidin and artesunate melted between 148 – 152ºC and 165 – 168ºC respectively. The Mp for PC14 hybrid was found to be higher than that of chloroquine (87 – 92ºC). Clog P values for chloroquine was higher than that of artesunate-procyanidin hybrid molecule. Artesunate and Procyanidin had closely related cLog P values. Clog S was however lower for Chloroquine when compared to Artesunate-procyanidin hybrid molecule, Procyanidin and Artesunate.

Solubility of the artesunate-procyanidin hybrid molecule (PC14 hybrid) was similar to that of Artesunate, Procyanidin and the 1:1 combination ratio (Table 4, Figure 17). It was found to be less soluble than paracetamol and the 1:1 combination ratio of artesunate and procyanidin (PC01). Artesunate-procyanidin hybrid molecule was however, more soluble than 1:2 (PC02) and 2:1 (PC03) combination ratios of artesunate and procyanidin.

Table 4: Molecular weight and clog values of Artesunate, Procyanidin and Artesunate-procyanidin hybrid molecule

Compound	Molecular weight (g/mole)	Melting Point (°C)	clog P	clog S
Artesunate	384.42	148 - 152	2.35	-2.75
Procyanidin	578.52	165 - 168	2.29	-3.62
PC14 hybrid	944.31	119 - 122	4.72	-4.12
Chloroquine	319.87	87 - 92	5.28	-4.26

Clog P and Clog S were predicted from ALOGPS 2.1.

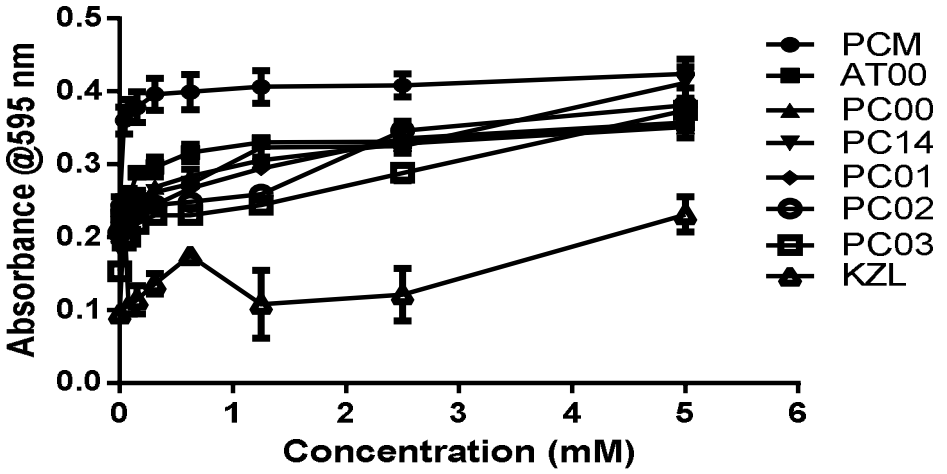


Figure 17: Solubility curves of Artesunate-procyanidin hybrid molecule and combination ratios. Values are Means ± SEM of three determinations. PCM- Paracetamol, AT00- Artesunate, PC00- Procyanidin, PC14- Artesunate-Procyanidin hybrid molecule; Artesunate/Procyanidin Combination: PC01 = Ratio 1:1, PC02 = Ratio 1:2, PC03 = Ratio 2:1, KZL- Ketoconazole

3.3 *In vitro* antiplasmodial activity and cytotoxicity studies

Artesunate-procyanidin hybrid compound was active *in vitro* with an IC_{50}higher than that of artesunate but lower than that of Procyanidin which was found to be greater than 30 µg/mL (Table 5). The various combination ratios were also active with two of them having IC_{50}values lower than that of the hybrid molecule. Artesunate-procyanidin hybrid molecule was less toxic to Buffalo Green monkey kidney cell line (BGM) than the parent compounds and all combination ratios.

3.4 Inhibition of β-Hematin formation Assay result

The various combination ratios and the parent compounds expressed a better β – hematin inhibitoryactivity compared to the Artesunate-procyanidin hybrid molecule(Table 6). However, the hybrid compound was more potent in inhibition ofβ – Hematin formation than chloroquine. Procyanidin and Artesunate were also more potent in inhibition of β–Hematin formation than the hybrid compound. Artesunate-Procyanidin in a 1:1 ratio has a Fractional Inhibitory Concentration (**FIC_{50}**) of 0.96, while it's 2:1 and 1:2 ratios had 1.34 and 1.70 respectively (Table 7). Ratio 1:1 showed a synergistic effect, while the 2:1 and 1:2 ratios showed antagonistic effect.

3.5 *In vivo* antimalarial studies

3.5.1 4-Day suppressive test:

Artesunate, Artesunate-procyaindin hybrid compound, the different combination ratios and procyanidin at the various doses administered exhibitedantimalarial activities which resulted in above 30% chemosuppression on days 4, 6 and 8 (Table 8). Percentage chemosuppression of between 30 and 40% are classified as partially active according to de Souza *et al.*(2014); those having below 30% are classified as inactive while those causing over 40% chemosuppression are

considered active (de Souza *et al*., 2014). Artesunate-procyanidin hybrid molecule exhibitedhigheractivity against *P. berghei* NK65 compared to the combination ratios and procyanidin on days 4 and 6 post-inoculation, comparing favourably with artesunate and procyanidin. The hybrid molecule and the various combination ratios were able to increase the mean survival time in the suppressive test compared to the untreated control (Table 9). The ED_{50} of artesunate-procyaindin hybrid compound was higher than those of the combination ratios on days 4 and 6 post-inoculation bur was lower than those of the combination ratios on day 8 post inoculation (12.95 mg/kg body weight) (Table 10). The ED_{50} values of PC02 on day 4 and day 6 post-inoculation were 14.30 and 13.34 mg/kg body weight which were lower than those of PC01 and PC03 (Table 10).

3.5.2 Rane curative test:

The % chemosuppression of artesunate-procyaindin hybrid molecule compared favourably well with those of to artesunate and procyanidindays 6, 8 and 10 post-inoculation (Table 11). The mean survival time of the mice was increasedat all doses of the hybrid molecule administered (Table 12). Artesunate-procyaindin hybrid molecule hadED_{50} values of 56.98, 48.20 and 56.21 mg/kg body weight on days 6, 8 and 10 respectively (Table 13).

Table 5: *In vitro* antiplasmodial activity and cytotoxicity of Artesunate-procyanidin hybrid compound and different combination ratios

Compounds (µg/mL)		MDL_{50} (µg/mL)* (x ± SD)	IC_{50} (µg/mL)** (x ± SD)	Selectivity Index***	Activity
Artesunate (AT00)		33 ± 9	0.0045 ± 0.0021	7,333	Yes
Procyanidin B2 (PC00)		133 ± 8	≥30	NA	No
Artesunate-Procyanidin (PC14)	Hybrid	155 ± 4	0.0335 ± 0.0021	4,627	Yes
Artesunate-Procyanidin (PC01)		94 ± 20	0.0053 ± 0.0009	17,736	Yes
Artesunate-Procyanidin (PC02)		76 ± 16	0.0122 ± 0.0022	6,230	Yes
Artesunate-Procyanidin (PC03)		41 ± 4	0.0072 ± 0.0019	5,694	Yes

Values are representative of at least two independent experiments.

*Cytotoxicity evaluated by the incorporation of neutral red uptake assay using monkey kidney cell line (BGM).**Antiplasmodial activity evaluated using *Plasmodiumfalciparum*[W2 clone, chloroquine-resistant parasites].***Index obtained from ratio of MDL_{50} and $IC_{50}$where SI ≤ 10 is indicative of toxicity.NA = not applicable, Artesunate/Procyanidin Combination: PC01 = Ratio 1:1, PC02 = Ratio 1:2, PC03 = Ratio 2:1

Table 6: β – Hematin inhibition byartesunate-procyanidin hybrid compound and its combination ratios

Hybrids	IC_{50} µg/ml
Chloroquine	55.53
Procyanidin	31.21
Artesunate	17.79
PC14	42.46
PC01	21.73
PC02	10.41
PC03	18.42

Artesunate-procyanidin hybrid molecule = **PC14,** Ratio 1:1 = **PC01**,Ratio 1:2 = **PC02**,Ratio 2:1 =**PC03**

Table 7: Fractional Inhibitory Concentration (FIC_{50}) for inhibition of β – Hematin formation for various combination ratios

Combinations	Fraction of Artesunate	Fraction of Procyanidin	FIC_{50} Artesunate	FIC_{50} Procyanidin	ΣFIC_{50}
Artesunate	1	0	1	0	1
PC01	0.50	0.50	0.61	0.35	0.96
Procyanidin	0	1	0	1	1
Combinations	**Fraction of Artesunate**	**Fraction of Procyanidin**	**FIC_{50} Artesunate**	**FIC_{50} Procyanidin**	**ΣFIC_{50}**
Artesunate	1	0	1	0	1
PC02	0.66	0.33	1.17	0.17	1.34
Procyanidin	0	1	0	1	1
Combinations	**Fraction of Artesunate**	**Fraction of Procyanidin**	**FIC_{50} Artesunate**	**FIC_{50} Procyanidin**	**ΣFIC_{50}**
Artesunate	1	0	1	0	1
PC03	0.33	0.66	0.52	1.18	1.70
Procyanidin	0	1	0	1	1

Artesunate-procyanidin hybrid molecule = **PC14,** Ratio 1:1 = **PC01**,Ratio 1:2 = **PC02**,Ratio 2:1 **=PC03.** FIC_{50} value less than one (< 1) represent synergistic effect while FIC_{50} value greater than one (> 1) antagonistic effect.

Table 8: Parasitemia and percentage chemosuppression in *P. berghei* NK65-infected mice administered Artesunate-Procyanidin hybrid compound and combination ratios (suppressive test)

Group-Dose (mg/kg bw)	Parasitemia (%) (Chemosuppression, %) DAY 4	DAY 6	DAY 8
Control	0.36	1.24	3.09
Artesunate-4	0.12 (66.67)	0.35 (71.77)	0.57(81.55)
PC14-5	0.07 (80.56)	0.50 (59.68)	1.69(45.31)
PC14-10	0.08 (77.78)	0.45 (63.71)	1.17(62.14)
PC14-20	0.08 (77.78)	0.39 (68.55)	0.88(71.52)
PC14-40	0.11 (69.44)	0.28 (77.42)	0.90(70.87)
PC14-80	0.08 (77.78)	0.53 (57.26)	1.51(51.13)
PC01-5	0.22 (38.89)	0.30 (75.81)	0.46(85.11)
PC01-10	0.16 (55.56)	0.70 (43.55)	1.64(46.93)
PC01-20	0.20 (42.10)	0.62 (50.00)	0.74(76.05)
PC01-40	0.16 (55.55)	0.67 (45.97)	1.63(47.25)
PC01-80	0.23 (36.15)	0.72 (41.94)	1.55(49.84)
PC02-5	0.17 (56.74)	0.68 (45.16)	0.90(70.87)
PC02-10	0.21 (41.67)	0.54 (56.45)	0.91(70.55)
PC02-20	0.23 (36.11)	0.57 (54.03)	0.61(80.26)
PC02-40	0.23 (36.11)	0.72 (42.81)	1.30(57.93)
PC02-80	0.25 (30.56)	0.52 (58.06)	0.67(78.32)
PC03-5	0.06 (83.33)	0.65 (47.58)	1.11(64.08)
PC03-10	0.22 (38.89)	0.83 (33.06)	0.99(67.96)
PC03-20	0.21 (41.67)	0.70 (43.55)	1.28(58.58)
PC03-40	0.25 (30.56)	0.41 (66.94)	0.73(76.38)
PC03-80	0.24 (33.34)	0.52 (58.06)	1.71(44.66)
Procyanidin-10	0.11 (69.44)	0.34 (72.58)	0.94(69.58)

Values are means (n=5); **PC14-** Artesunate-Procyanidin hybrid molecule; Artesunate/Procyanidin Combination: **PC01** = Ratio 1:1, **PC02** = Ratio 1:2, **PC03** = Ratio 2:1

Table 9: Mean Survival Time (MST) of *P. berghei* NK65-infected mice administered Artesunate-Procyanidin hybrid compound and combination ratios (suppressive test)

Groups-Dose (mg/kg bw.)	MST
Infected control	14
Artesunate-4	19
PC14-5	28
PC14-10	22
PC14-20	25
PC14-40	21
PC14-80	27
PC01-5	23
PC01-10	21
PC01-20	27
PC01-40	26
PC01-80	22
PC02-5	26
PC02-10	21
PC02-20	23
PC02-40	19
PC02-80	21
PC03-5	24
PC03-10	24
PC03-20	21
PC03-40	26
PC03-80	25
Procyanidin-10	28

Values are means (n=5);**PC14-** Artesunate-Procyanidin hybrid molecule; Artesunate/Procyanidin Combination: **PC01** = Ratio 1:1, **PC02** = Ratio 1:2, **PC03** = Ratio 2:1

Table 10: ED_{50} of Artesunate-Procyanidin hybrid compound and combination ratios in *P. berghei* NK65-infected mice (suppressive test)

	ED_{50} (mg/kg body weight)		
Compounds	Day 4	Day 6	Day 8
PC14	4.03	3.33	3.25
PC01	10.69	11.61	11.30
PC02	13.85	14.00	7.83
PC03	16.49	11.05	10.03

Values are means (n=5); **PC14-** Artesunate-Procyanidin hybrid molecule; Artesunate/Procyanidin Combination: **PC01** = Ratio 1:1, **PC02** = Ratio 1:2, **PC03** = Ratio 2:1

Table 11: Parasitemia and percentage chemosuppression in *P. berghei* NK65-infected mice administered Artesunate-Procyanidin hybrid compound (curative test)

Group-Dose (mg/kg bw)	Parasitemia (%) (Chemosuppression, %)		
	DAY 6	DAY 8	DAY 10
Control	1.42	3.18	7.09
Artesunate-4	0.17 (88.03)	0.20 (93.71)	0.13 (98.17)
PC14-5	0.14 (90.14)	0.33 (89.62)	0.95 (86.60)
PC14-10	0.21 (85.21)	0.14 (95.60)	0.73 (89.70)
PC14-20	0.27 (80.99)	0.40 (87.42)	1.49 (78.98)
PC14-40	0.45 (68.31)	0.17(94.65)	0.68 (90.41)
PC14-80	0.24 (83.10)	0.11 (96.54)	0.19 (97.32)
Procyanidin-10	0.25 (82.39)	0.11 (96.54)	0.19 (97.32)

Values are means (n = 5); **PC14**- Artesunate-Procyanidin hybrid molecule

Table 12: Mean Survival Time (MST) of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid compound (curative test)

Groups-Dose (mg/kg bw.)	MST
Infected control	12
Artesunate-5	24
PC14-5	18
PC14-10	19
PC14-20	18
PC14-40	26
PC14-80	22
Procyanidin-10	21

Values are means (n = 5); **PC14-** Artesunate-Procyanidin hybrid molecule

Table 13: ED_{50} of Artesunate-Procyanidin hybrid compound in *P. berghei* NK65-infected mice (curative test)

	ED_{50} (mg/kg body weight)		
Compounds	Day 6	Day 8	Day 10
PC14	2.31	2.58	3.45

Values are means (n=5); PC14- Artesunate-Procyanidin hybrid molecule

3.6 Results of Antioxidant Studies

3.7 *In vitro* Antioxidant Activities

3.7.1 Evaluation of Total Antioxidant Capacity (TAC)

Total antioxidant activities of artesunate-procyanidin hybrid molecule, procyanidin and the 1:1 and 2:1 combination ratios were lower compared to those of the 2:1 combination ratio and BHT (Figure 18).

3.7.2 FRAP of compounds

The FRAP of the various combination ratios, Artesunate-procyanidin hybrid molecule and procyanidin compared favourably well withthat of BHT (Figure 19). The hybrid molecule even had higher FRAP than that of BHT.

3.7.3 DPPH Radical Scavenging Activities

Artesunate-procyanidin hybrid molecule had the highest DPPH radical scavenging activities. The DPPH radical scavenging activities of combination ratios 2:1 and 1:1 were similar to that of BHT (Figure 20). They were however lower than those of the combination ratio 1:2, procyanidin and Artesunate-procyanidin hybrid molecule. The various combination ratios had similar IC_{50}values, which were lower than those of Artesunate-procyanidin hybrid molecule and procyanidin (Table 14).

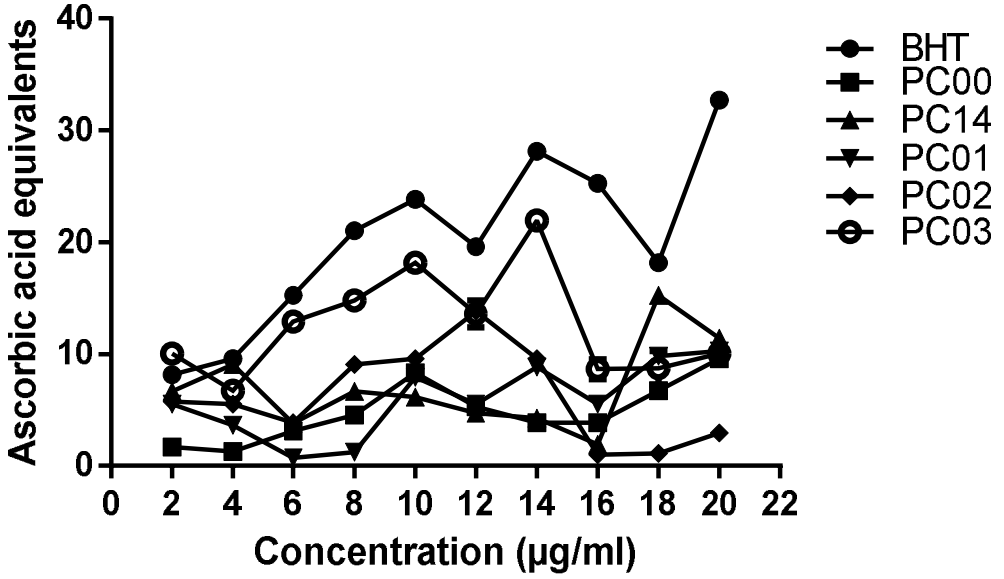


Figure 18: Total Antioxidant Capacities (TAC) of Artesunate-Procyanidin hybrid compound and combination ratios. Values are Means of three determinations. **PC14**-Artesunate-Procyanidin hybrid molecule; **PC00** – Procyanidin; Artesunate/Procyanidin Combination: **PC01** = Ratio 1:1, **PC02** = Ratio 1:2, **PC03** = Ratio 2:1; **BHT**: Butylated hydroxytoluene

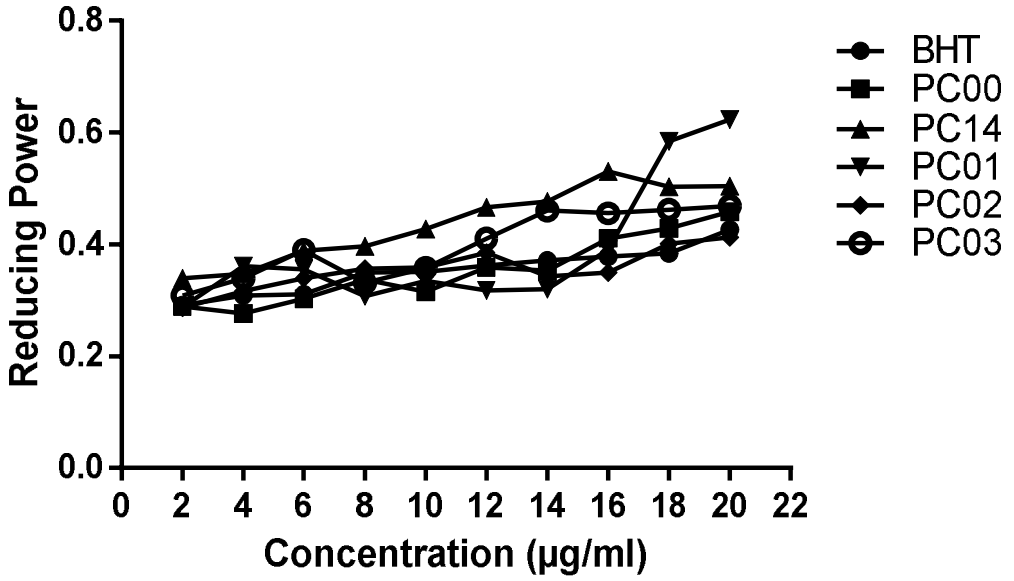


Figure 19: Ferric ion reducing effects of Artesunate-Procyanidin hybrid compound and combination ratios. Values are Means of three determinations. **PC14-** Artesunate-Procyanidin hybrid molecule; **PC00** – Procyanidin; Artesunate/Procyanidin Combination: **PC01** = Ratio 1:1, **PC02** = Ratio 1:2, **PC03** = Ratio 2:1 ; **BHT:** Butylated hydroxytoluene

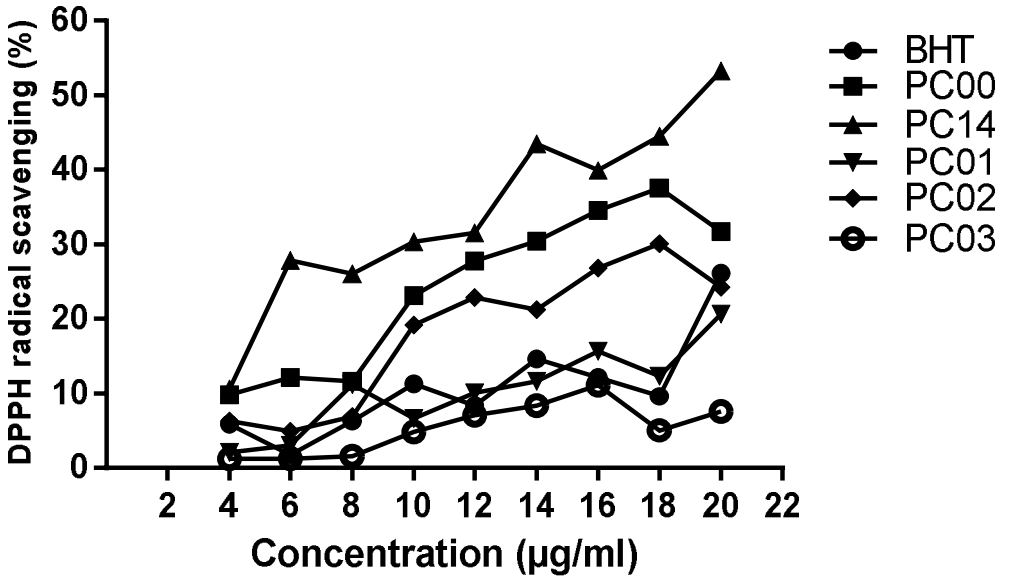


Figure 20: DPPH scavenging activities (%) of Artesunate-Procyanidin hybrid compound and combination ratios. Values are Means of three determinations. **PC14**- Artesunate-Procyanidin hybrid molecule; **PC00** – Procyanidin; Artesunate/Procyanidin Combination: **PC01** = Ratio 1:1, **PC02** = Ratio 1:2, **PC03** = Ratio 2:1 ; **BHT**: Butylated hydroxytoluene

Table 14: IC_{50} values for DPPH scavenging activities of Artesunate-Procyanidin hybrid compoundand combination ratios

	IC_{50}
Compounds	**µg/ml**
BHT	44.21
PC00	19.56
PC14	19.68
PC01	34.42
PC02	34.73
PC03	39.74

PC14- Artesunate-Procyanidin hybrid molecule; **PC00** – Procyanidin; Artesunate/Procyanidin Combination: **PC01** = Ratio 1:1, **PC02** = Ratio 1:2, **PC03** = Ratio 2:1; **BHT**: Butylated hydroxytoluene, **IC_{50}=** Half maximal inhibitory concentration

3.8 Antioxidant Status

3.8.1 Lipid Peroxidation

Erythrocyte Malondialdehyde Levels: On the 6 post-inoculation, the erythrocyte malondialdehyde (MDA) level was not significantly increased ($p>0.05$) in the untreated infected group compared to the uninfected control group and groups treated with chloroquine, artesunate and procyanidin(Figure 21). Groups treated with 5 – 80 mg/kg body weight of Artesunate-procyanidin hybrid molecule had significant decrease ($p<0.05$) in erythrocyte MDA levels when compared to uninfected control group and infected untreated group. On day 10 post-inoculation, there was a significant increase ($p<0.05$)in erythrocyte MDA level of the untreated infected group compared to other groups. Artesunate-procyanidin hybrid, artesunate, procyanidin and chloroquine were able to revert this observed increase in erythrocyte MDA level to the range of the uninfected control (Figure 21).

Hepatic Malondialdehyde Level: On the 6 post inoculation, liver MDA level was significantly increased ($p<0.05$) in the untreated infected groupcompared to the uninfected control. There was no significant difference ($p>0.05$) in hepatic MDA levels of groups treated with chloroquine, artesunate, procyanidin, and artesunate-procyanidin molecule at 5, 20, 40 mg/kg body weight compared to the uninfected and untreated infected groups (Figure 22). However, there was a significant decrease ($p<0.05$) in the hepatic MDA level of mice administered 80 mg/kg body weight of the hybrid molecule compared to untreated infected control. On day 10 post-inoculation, significant increase ($p<0.05$) was observed in hepatic MDA of all treatment groups except those treated withchloroquine and 5 mg/kg body weight of Artesunate-procyanidin molecule compared to the uninfected control group.

Kidney Malondialdehyde Level: On day 6 post-inoculation, there was a significant increase ($p<0.05$) in kidney MDA concentration in the untreated infected group compared to the uninfected control group. Chloroquine, artesunate, procyanidin and Artesunate-procyanidin hybrid molecule at 80 mg/kg body weight were able to significantly ($p<0.05$) revert this increase to the range of the uninfected control (Figure 23). However on day 10 post inoculation, all the groups had their kidney MDA concentration significantlyincreased ($p<0.05$) compared to the uninfected control, with no significant difference ($p >0.05$) between the untreated infected group and the groups treated with drugs (Figure 23).

Heart Malondialdehyde Level: On the 6 post-inoculation, all the groups had their heart MDA concentration significantly increased ($p<0.05$) compared to the uninfected control, with no significant difference ($p >0.05$) between the untreated infected group and the groups treated with drugs (Figure 24). Similarly, on day 10 post inoculation, the heart MDA concentration was significantly increased ($p<0.05$) in the untreated infected group compared to the uninfected control. This increase was significantly reverted ($p<0.05$) to the range of the uninfected control by artesunate, procyanidin and artesunate-procyanidin hybrid molecule at the doses 40 and 80 mg/kg body weight compared to untreated infected control.

Brain Malondialdehyde concentration: On the 6 post-inoculation, all the groups had their heart MDA concentration significantly increased ($p<0.05$) compared to the uninfected control, with no significant difference ($p>0.05$) between the untreated infected group and the groups treated with drugs (Figure 25). On day 10 post-inoculation, a significant increase ($p<0.05$) was observed in brain MDA level in the untreated infected group compared to uninfected control. MDA levels in the groups treated with various drugs were significantly increased ($p>0.05$) compared to uninfected control and untreated infected control, except the group treated with 5

mg/kg body weight of artesunate-procyanidin hybrid molecule, the MDA level of which was not significantly different ($p>0.05$) fromthe untreated infected control.

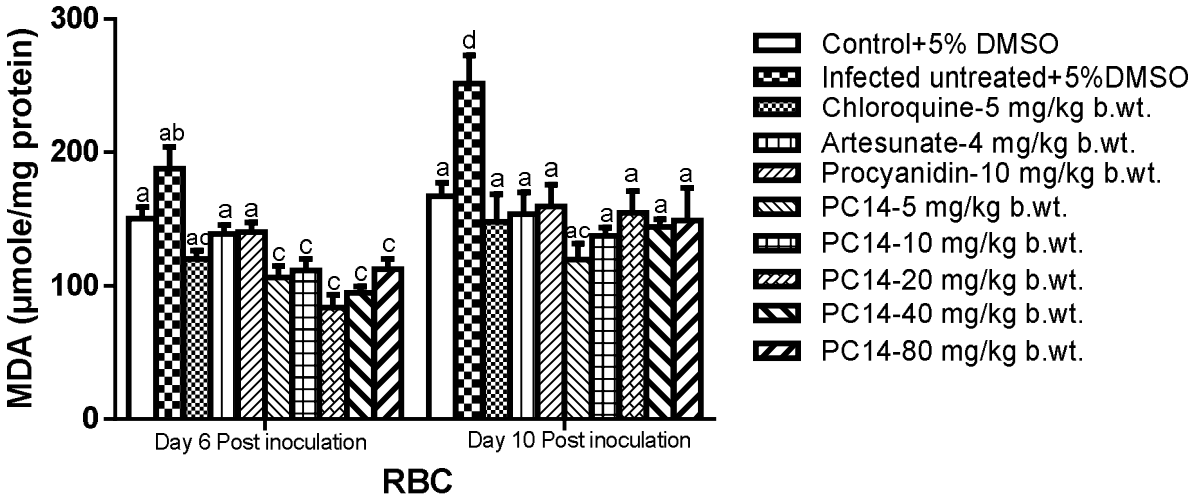


Figure 21: Malondialdehyde concentration in Red blood cell of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

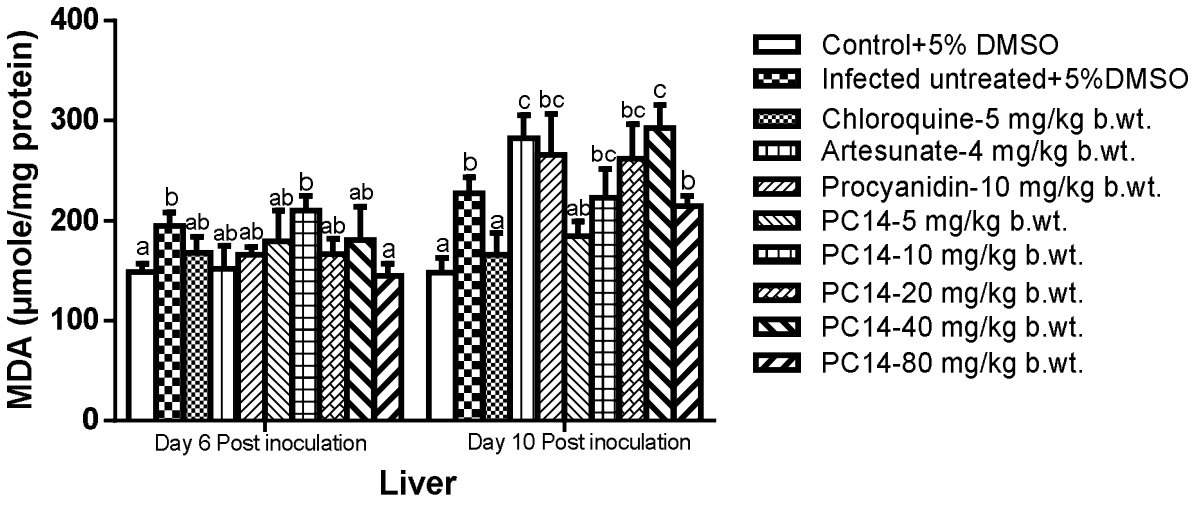


Figure 22: Malondialdehyde concentration in liver of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

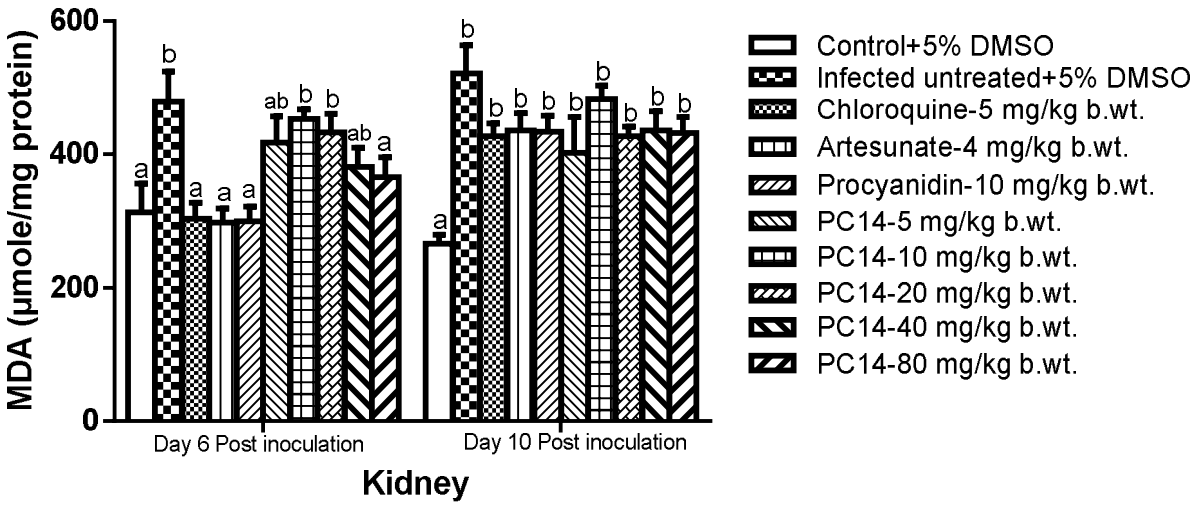


Figure 23: Malondialdehyde concentration in kidney of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

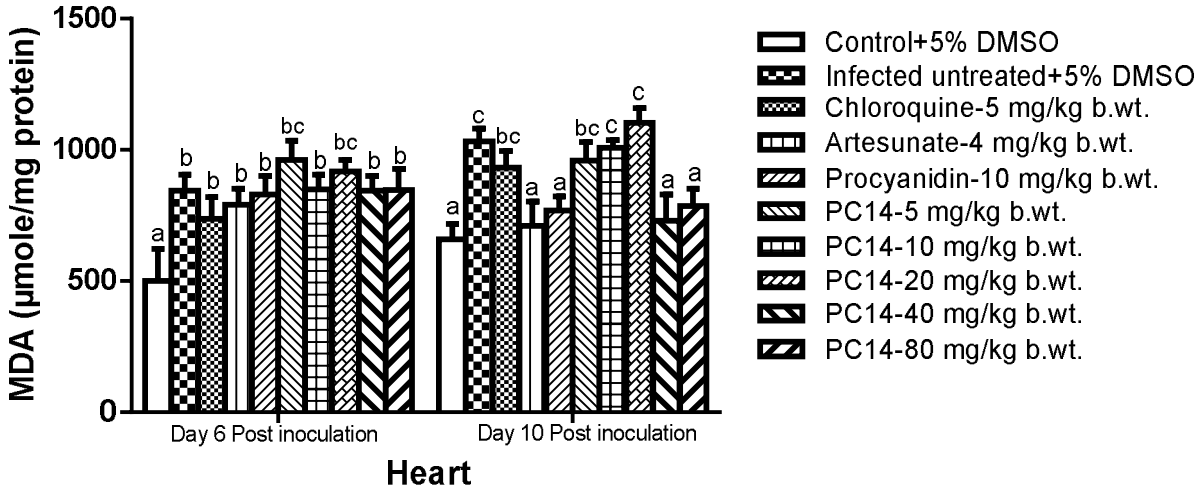


Figure 24: Malondialdehyde concentration in heart of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

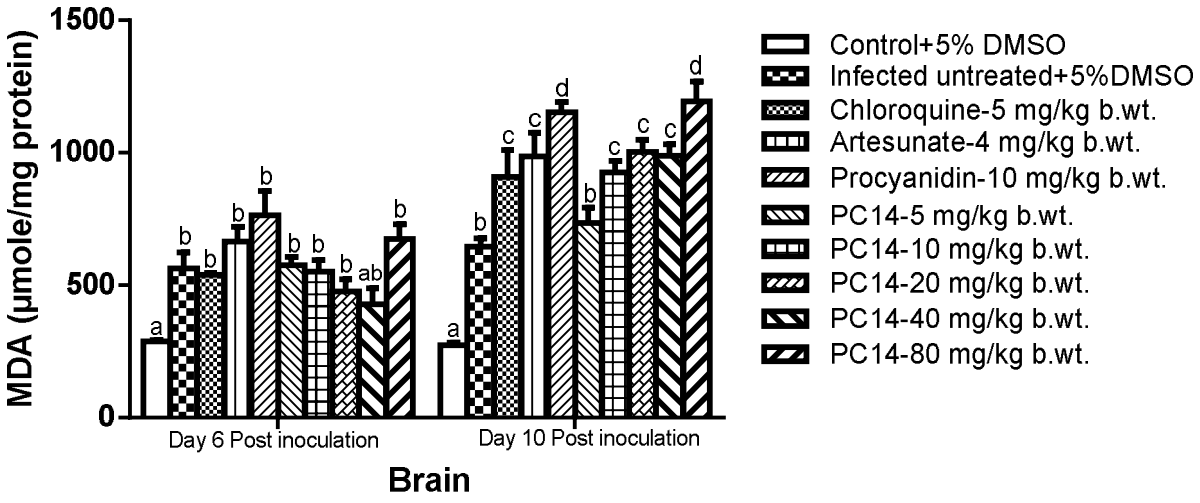


Figure 25: Malondialdehyde concentration in brain of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

3.8.2 Nitrite Levels

Erythrocyte Nitrite Concentration: On day 6 post-inoculation, no significant difference ($p>0.05$)was observed in erythrocyte nitrite concentration in all groups and all doses of Artesunate-procyanidin hybrid molecule compared to uninfected control (Figure 26). Similarly, on day 10 post-inoculation, no significant difference ($p>0.05$) was observed in erythrocyte nitrite concentration of all groups compared to uninfected control.

Hepatic Nitrite Concentration: On day 6 post-inoculation, no significant difference ($p>0.05$) was observed in hepatic nitrite concentration in the various groups except in the procyanidin group where it was significantly increased comparedto the uninfected control(Figure 27). Similarly, on day 10 post-inoculation, no significant difference ($p>0.05$) was observed in hepatic nitrite concentration in various groups, except in groups treated with 40 and 80 mg/kg body weight of artesunate-procyanidin hybrid molecule where it was significantly increased ($p<0.05$) compared to uninfected control (Figure 27).

Kidney Nitrite Concentration: Significant increase ($p<0.05$) in kidney nitrite concentration was observed at 20 and 40 mg/kg body weight of Artesunate-procyanidin molecule while there was no significant alteration ($p>0.05$) in those of other groups compared to uninfected control on day 6 post inoculation (Figure 28). However on day 10 post inoculation, there was no significant difference ($p>0.05$) in kidney nitrite concentrations of the various groups compared to the uninfected control.

Heart Nitrite Concentration: On days 6 and 10 post-inoculation, no significant alteration ($p>0.05$) was observed in heart nitrite concentrations of all groups compared to uninfected control (Figure 29).

Brain Nitrite Concentration: On day 6 post-inoculation, a significant increase ($p<0.05$) was observed in brain nitrite concentration of untreated infected animals with a significant reduction in that of chloroquine treated mice compared to the uninfected control (Figure 30). All the drug-treated groups had significantly higher ($p<0.05$) brain nitrite concentrations than the untreated infected group and the uninfected control, except those treated with 80 mg/kg body weight artesunate-procyanidinmolecule which had similar ($p>0.05$)brain nitrite concentration with that of untreated infected control (Figure 30). On day 10 post-inoculation, a significant increase ($p<0.05$) was observed in brain nitrite concentrations of the various groups except in the chloroquine and artesunate groups where it was significantly reduced ($p<0.05$) compared to the uninfected control (Figure 30).

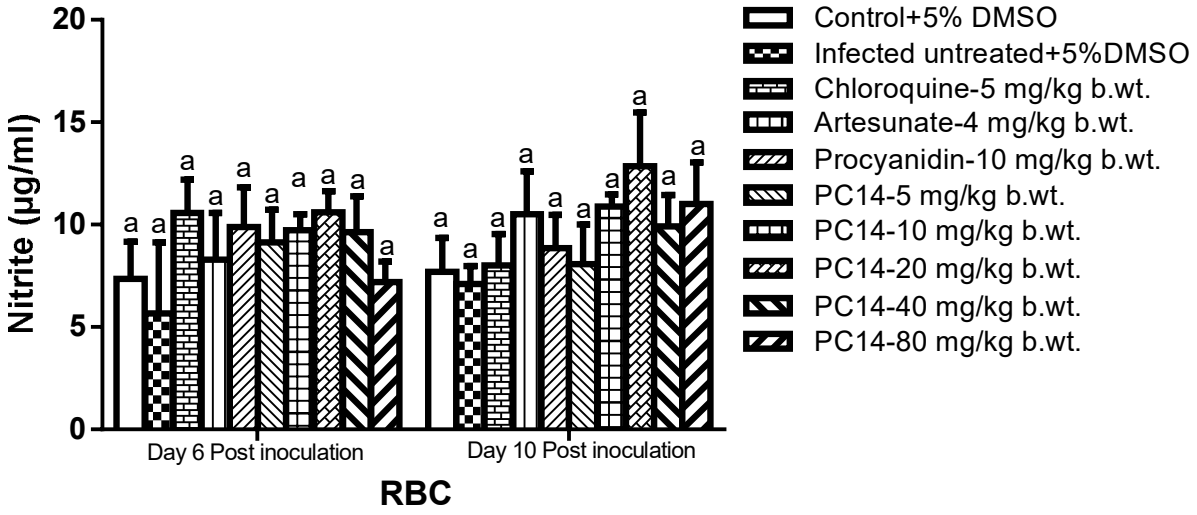


Figure 26: Nitrite concentration in Red Blood Cell of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

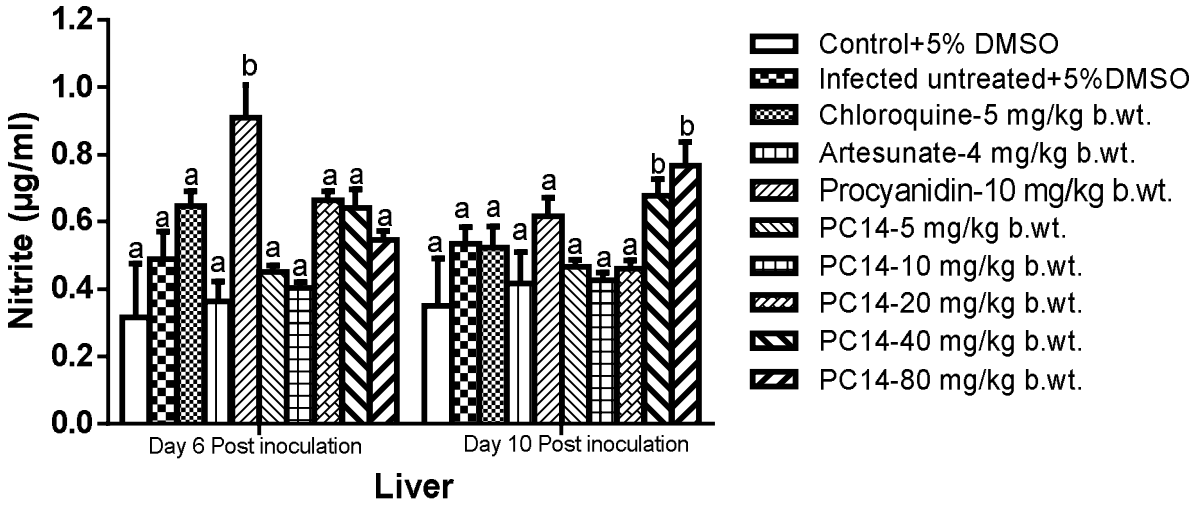


Figure 27: Nitrite concentration in liver of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

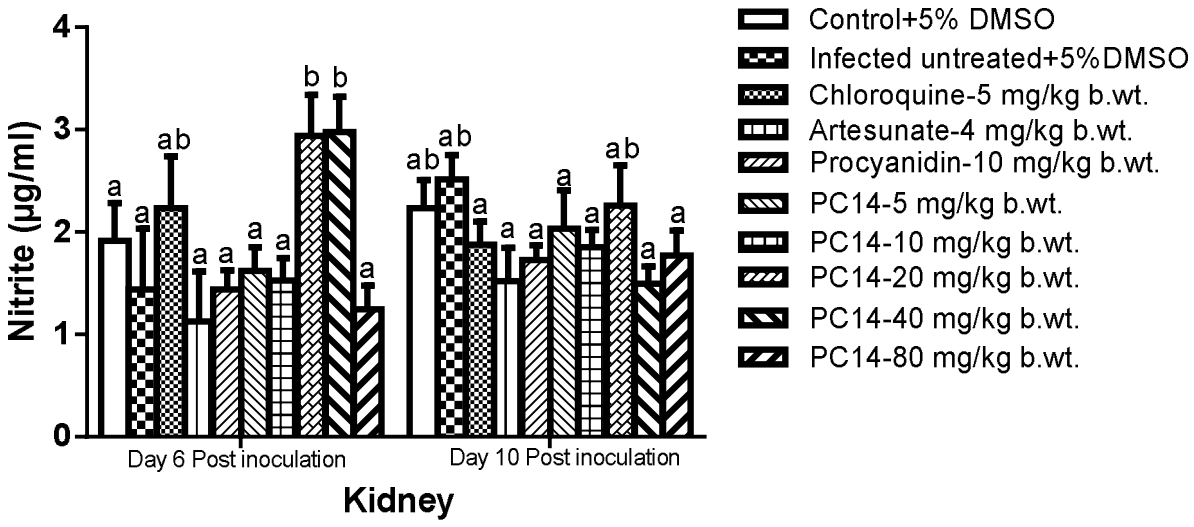


Figure 28: Nitrite concentration in kidney of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

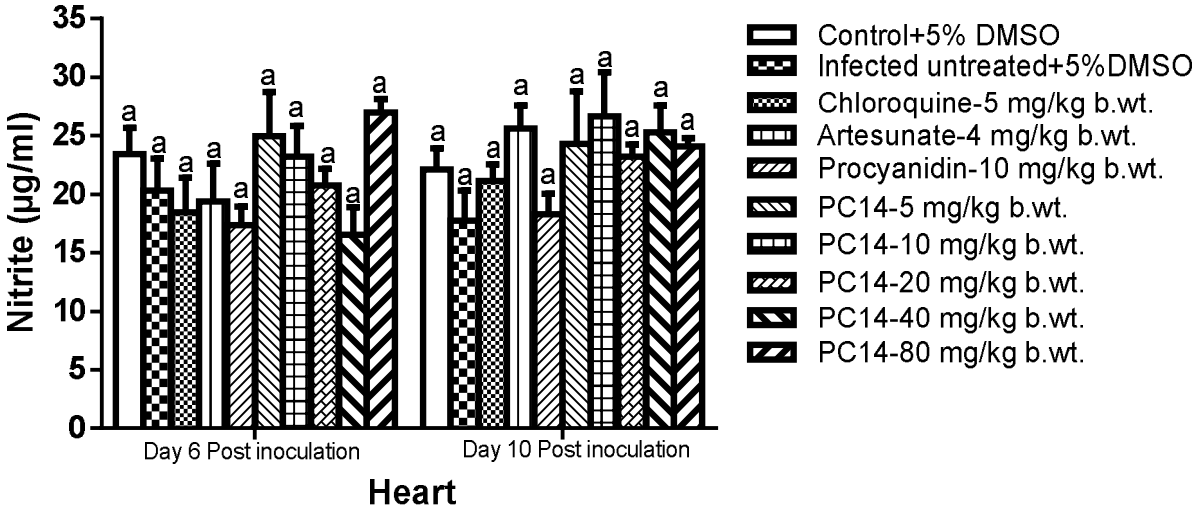


Figure 29: Nitrite concentration in heart of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with same superscripts are not significantly different at $p>0.05$. PC14: Artesunate-procyanidin hybrid molecule.

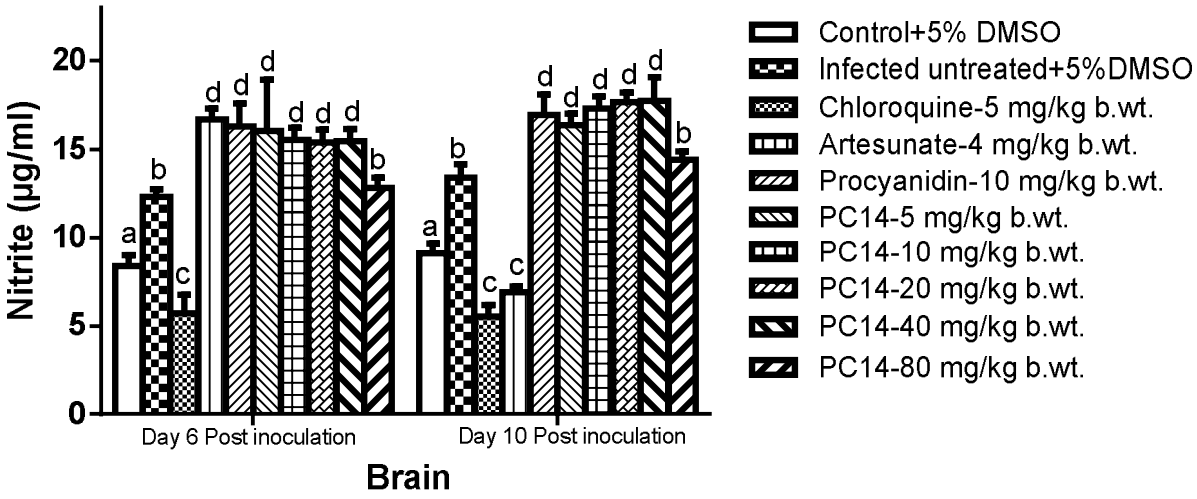


Figure 30: Nitrite concentration in brain of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

3.8.3 Superoxide dismutase activity

Erythrocyte Superoxide dismutase activity: On day 6 post-inoculation, no significant difference ($p>0.05$) was observed in the erythrocyte SOD activities of the groups treated with chloroquine, artesunate, procyanidin and the various doses of artesunate-procyanidin hybrid molecule compared to the uninfected control.However, 40 mg/kg body weight of artesunate-procyanidin hybrid moleculeexhibited a significantly lower ($p<0.05$) erythrocyte SOD activity compared to untreated infected control (Figure 31). On day 10 post-inoculation, erythrocyte SOD activity was significantly increased ($p<0.05$) in the untreated infected group compared to the uninfected control. However, artesunate, procyanidin and artesunate-procyanidin hybrid molecule at doses lower than 80 mg/kg body weight were able to revert the observed increase significantly ($p<0.05$) to the range of the uninfected control (Figure 31).

Hepatic Superoxide dismutase activity: On day 6 post-inoculation, a significant decrease ($p<0.05$) was observed in hepatic SOD activity of untreated infected control compared to the uninfected control.All the drugs at the various doses administered were able to significantly revert ($p<0.05$) the observed decrease to the range of the uninfected control (Figure 32). Similarly, significant decrease ($p<0.05$) was observed in the untreated infected group compared to the uninfected control. All the drugs at the various doses administered were able to significantly revert ($p<0.05$) the observed decrease, though not to the range of the uninfected control except at the dose of 80 mg/kg body weight of the hybrid molecule (Figure 32).

Kidney Superoxide dismutase activity: On day 6 and day 10 post-inoculation, there was significant reduction ($p<0.05$) in kidney SOD activity in the untreated uninfected group compared to uninfected control (Figure 33). All the drugs at the various doses administered were able to revert the observed decrease to the range of the uninfected control (Figure 33).

Heart Superoxide dismutase activity: On days 6 and 10 post-inoculation, , there was significant reduction ($p<0.05$) in heart SOD activity in the untreated uninfected group compared to uninfected control (Figure 34). All the drugs at the various doses administered were able to significantly revert ($p<0.05$) the observed decrease to the range of the uninfected control (Figure 34).

Brain Superoxide dismutase activity: On day 6 post-inoculation, there was significant increase ($p<0.05$) in brain SOD activity of the untreated infected group compared to uninfected control. All the drugs at various doses, except artesunate, were able to significantly revert ($p<0.05$) it to the range of the uninfected control (Figure 35). On day 10 post-inoculation, there was significant increase ($p<0.05$) in brain SOD activities of the untreated infected group and the groups treated with the drugs, except chloroquine, compared to uninfected control (Figure 35).

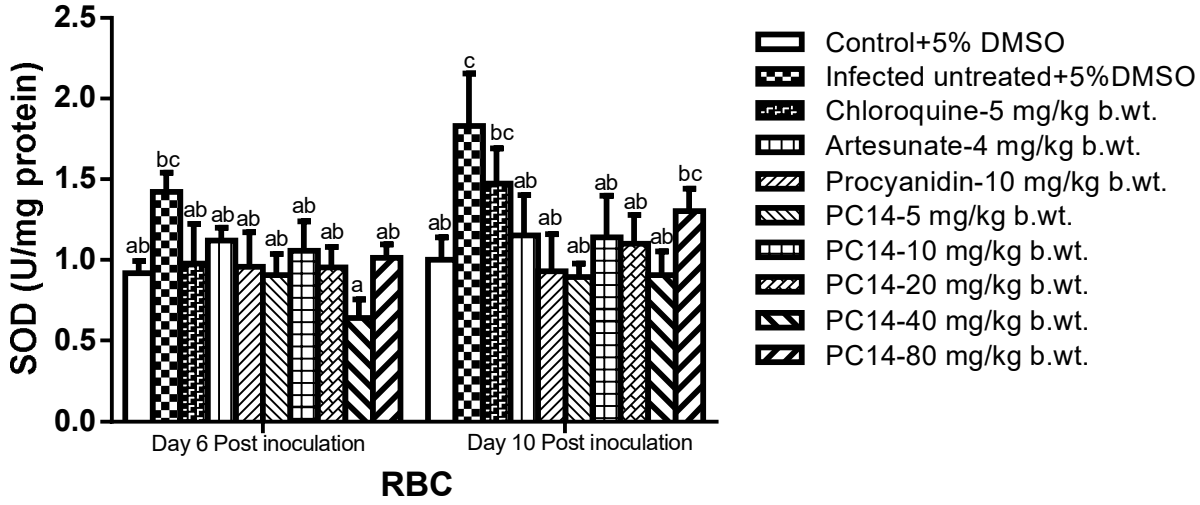


Figure 31: Superoxide dismutase activity in Red blood cell of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

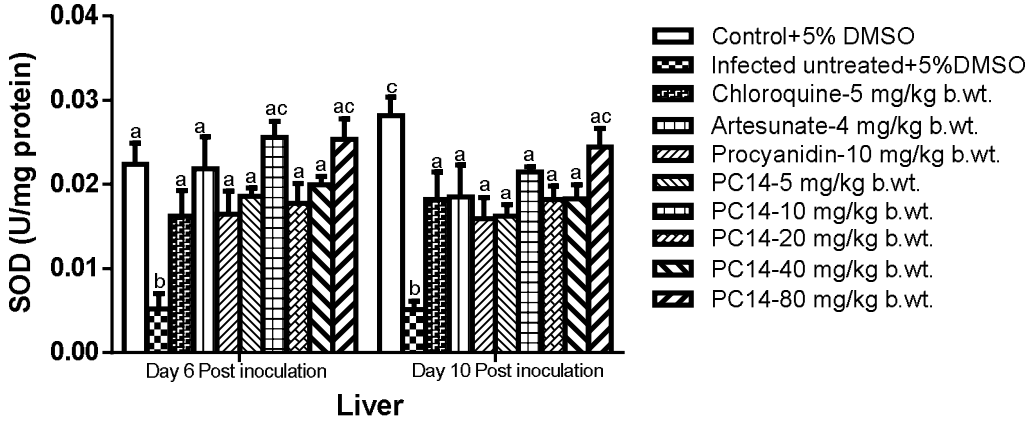


Figure 32: Superoxide dismutase activity in liver of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

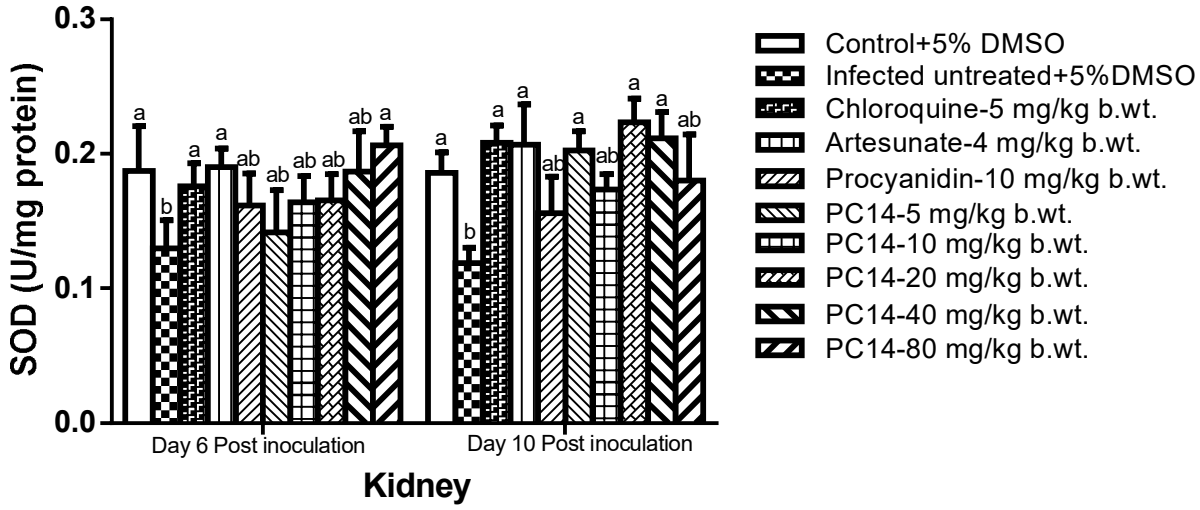


Figure 33: Superoxide dismutase activity in kidney of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Means ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

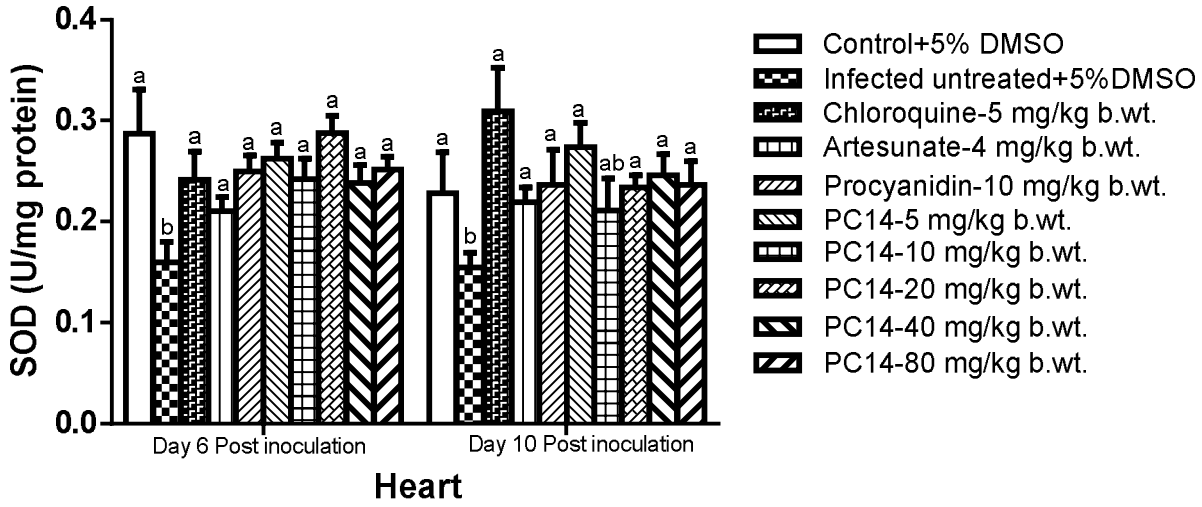


Figure 34: Superoxide dismutase activity in heart of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Means ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

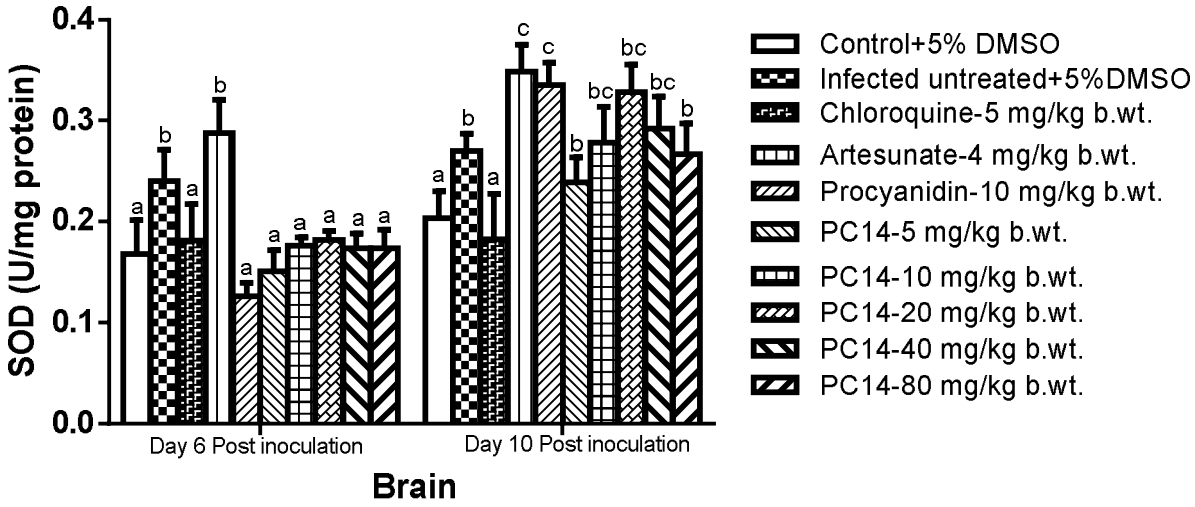


Figure 35: Superoxide dismutase activity in brain of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

3.8.4 Glutathione peroxidase activity

Erythrocyte Glutathione peroxidase (GPx) activity: On days 6 and 10 post-inoculation, there was a significant reduction ($p<0.05$) in erythrocyte GPx activity in the untreated infected group compared to the uninfected control (Figure 36). All the drugs at various doses causedsignificant increase ($p<0.05$) in erythrocyte GPx activity compared to the untreated infected group (Figure 36).

Hepatic Glutathione peroxidase activity: On day 6 post-inoculation, there was no significant change ($p>0.05$) in the hepatic Glutathione peroxidase activities of untreated infected group and the groups treated with chloroquine and 20 and 40 mg/kg body weight of hybrid molecule compared to the uninfected control (Figure 37). Artesunate, procyanidin and other doses of the hybrid molecule caused a significant increase in hepatic GPx activity compared to uninfected control and the untreated infected group (Figure 37). On day 10 post-inoculation, there was significant reduction ($p<0.05$) in the hepatic GPx activity of the untreated infected group and chloroquine treated group compared to uninfected control. All other drugs at various doses were able to revert the reduced hepatic GPx activity to the range of the uninfected control (Figure 37).

Kidney Glutathione peroxidase activity: On day 6 post-inoculation, there was significant decrease ($p<0.05$) in kidney Glutathione peroxidase activities in untreated infected group and the groups treated with drugs except those administered with 20 and 40 mg/kg body weight of artesunate-procyanidin hybrid molecule compared to uninfected control (Figure 38). Similarly on day 10 post-inoculation, there was significant decrease ($p<0.05$) in kidney Glutathione peroxidase activities in untreated infected group and the groups treated with drugs except those administered procyanidin, 20 and 40 mg/kg body weight of artesunate-procyanidin hybrid molecule compared to uninfected control (Figure 38).

Heart Glutathione peroxidase activity: On day 6 and day 10 post inoculation, there was no significant change ($p>0.05$) in heart GPx activity of the untreated infected group compared to uninfected control. There was significant increase ($p<0.05$) in heart Glutathione peroxidase activities of all the groups treated with drugs except chloroquine compared to both uninfected control and untreated infected control (Figure 39).

Brain Glutathione peroxidase activity: On day 6 inoculation, no significant alteration ($p>0.05$) was observed in brain Glutathione peroxidase activities of all the groups compared to the uninfected control (Figure 40). However, on day 10 post inoculation significant decrease ($p<0.05$) were observed at 5, 10 and 20 mg/kg body weight compared to both uninfected control and untreated infected control.

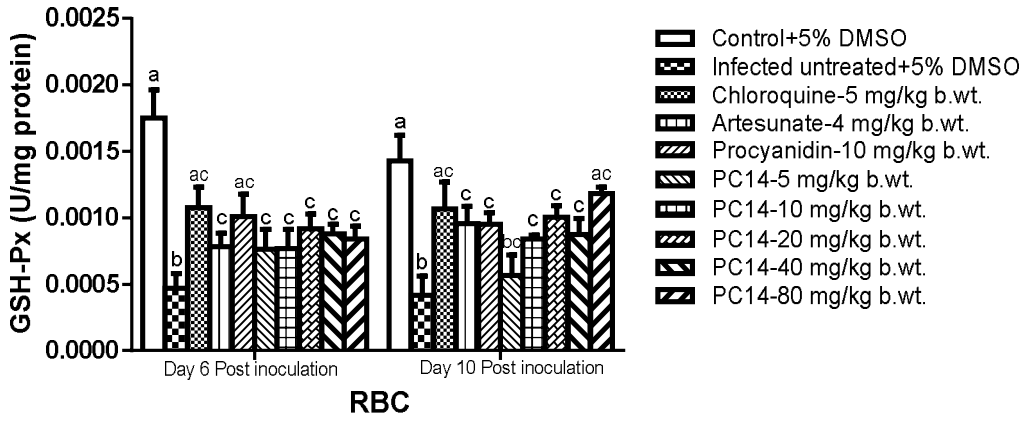


Figure 36: Glutathione peroxidase activity in Red blood cell of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

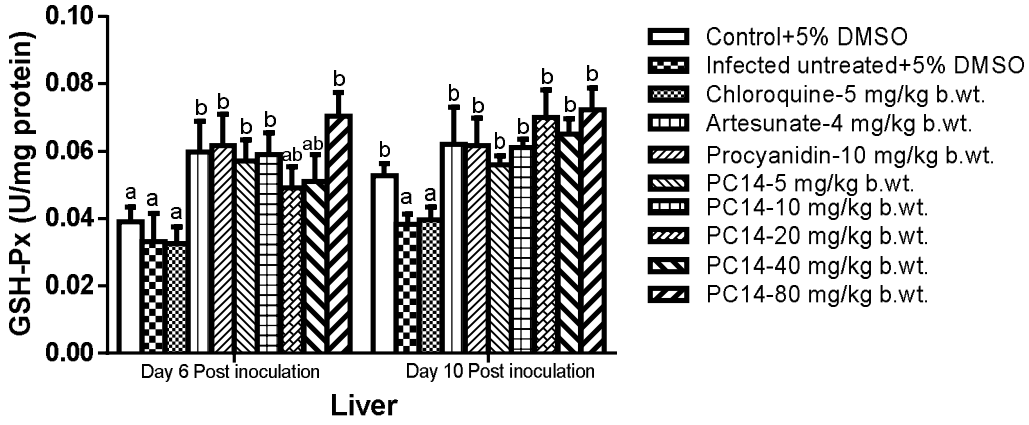


Figure 37: Glutathione peroxidase activity in liver of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

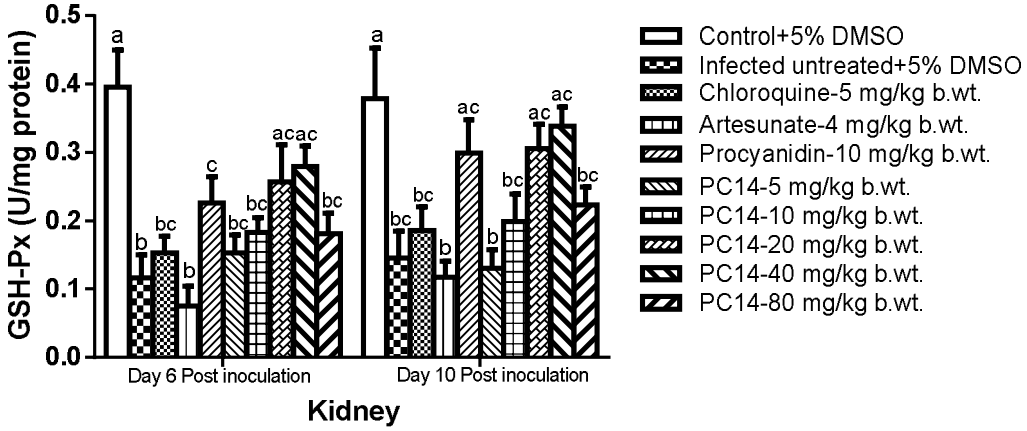


Figure 38: Glutathione peroxidase activity in kidney of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

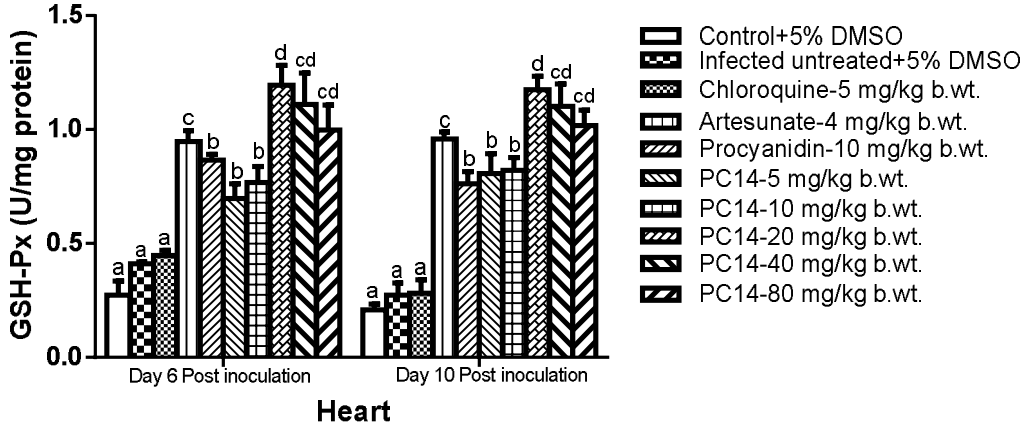


Figure 39: Glutathione peroxidase activity in heart of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

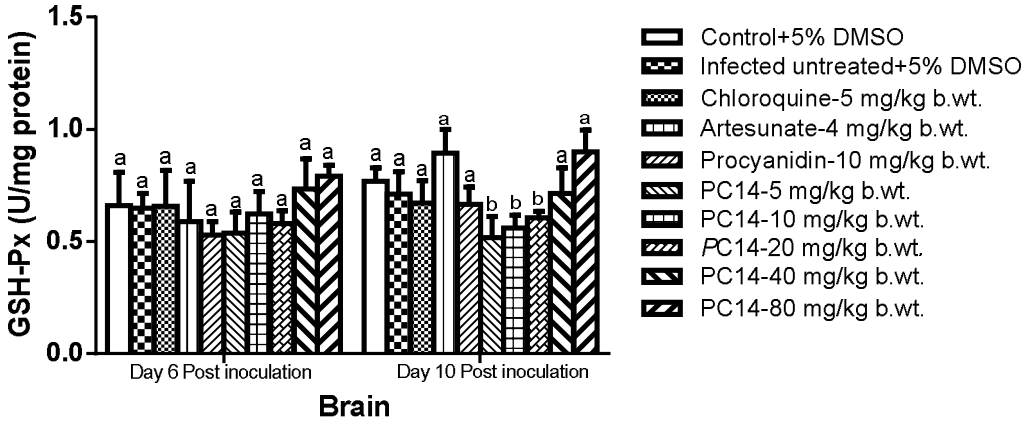


Figure 40: Glutathione peroxidase activity in brain of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

3.8.5 Reduced glutathione concentration

Erythrocyte Reduced glutathione concentration: On day 6 post-inoculation, there was significant reduction ($p<0.05$) in erythrocyte reduced glutathione concentration in untreated infected control compared to uninfected control (Figure 41). Only artesunate, procyanidin and the hybrid molecule at the doses of 5, 40 and 80 mg/kg body weight were able to significantly revert ($p<0.05$) the reduced glutathione concentration to the range of the uninfected control (Figure 41). On day 10 post-inoculation, there was significant reduction ($p<0.05$) in erythrocyte reduced glutathione concentration in untreated infected control compared to uninfected control (Figure 41). The erythrocyte reduced glutathione concentrations of all the drug-treated groups were not significantly different from that of the untreated infected control (Figure 41).

Hepatic Reduced glutathione concentration: On day 6 post-inoculation, there was significant reduction ($p<0.05$) in hepatic reduced glutathione concentration in untreated infected control compared to uninfected control (Figure 42). Only procyanidin and the hybrid molecule at all doses were able to significantly increase ($p<0.05$) the reduced glutathione concentration (though not to the range of the uninfected control) compared to the untreated infected group (Figure 42). On day 10 post-inoculation, there was significant reduction ($p<0.05$) in hepatic reduced glutathione concentration in untreated infected control compared to uninfected control (Figure 42). Only artesunate and the hybrid molecule at all doses were able to significantly increase ($p<0.05$) the reduced glutathione concentration (though not to the range of the uninfected control) compared to the untreated infected group (Figure 42).

Kidney Reduced glutathione concentration: On day 6 post-inoculation, there was significant reduction ($p<0.05$) in kidney reduced glutathione concentration in untreated infected control compared to uninfected control (Figure 43). All the drugs were able to significantly revert

($p<0.05$) the reduced glutathione concentration, with procyanidin and the hybrid molecule at doses lower than 40 mg/kg body weight increasing it beyond the range of the uninfected control. On day 10 post-inoculation, there was significant reduction ($p<0.05$) in kidney reduced glutathione concentration in untreated infected control compared to uninfected control (Figure 43). All the drugs were able to significantly revert ($p<0.05$) the reduced glutathione concentration, with artesunate and the hybrid molecule at all doses increasing it beyond the range of the uninfected control (Figure 43).

Heart Reduced glutathione concentration: On days 6 and 10 post-inoculation, there was significant reduction ($p<0.05$) in heart reduced glutathione concentration in untreated infected control compared to uninfected control (Figure 44). All the drugs at various doses were able to significantly revert ($p<0.05$) the reduced glutathione concentration to the range of the uninfected control (Figure 44).

Brain Reduced glutathione concentration: On day 6 post-inoculation, there was significant reduction ($p<0.05$) in heart reduced glutathione concentration in untreated infected control compared to uninfected control (Figure 45). All the drugs were able to significantly revert ($p<0.05$) the reduced glutathione concentration, with the hybrid molecule at doses higher than 5 mg/kg body weight increasing it beyond the range of the uninfected control. On day 10 post-inoculation, there was significant reduction ($p<0.05$) in heart reduced glutathione concentration in untreated infected control compared to uninfected control (Figure 45). All the drugs were able to significantly revert ($p<0.05$) the reduced glutathione concentration to the range of the uninfected control.

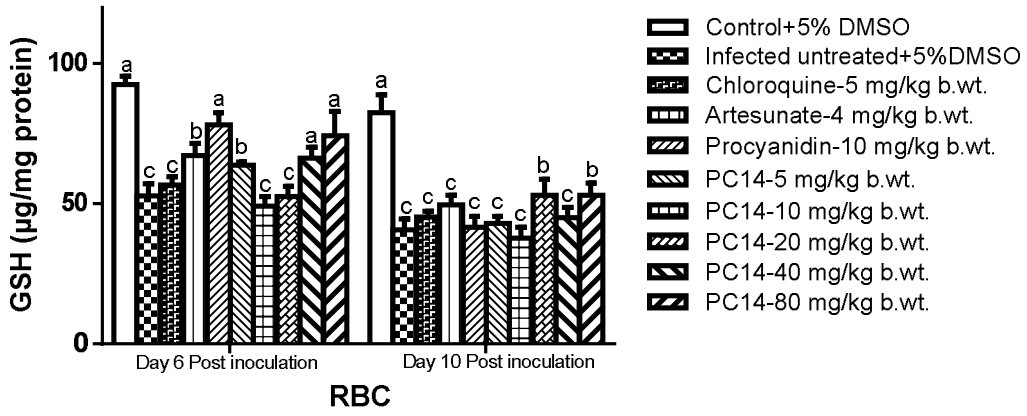


Figure 41: Reduced glutathione concentration in Red blood cell of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

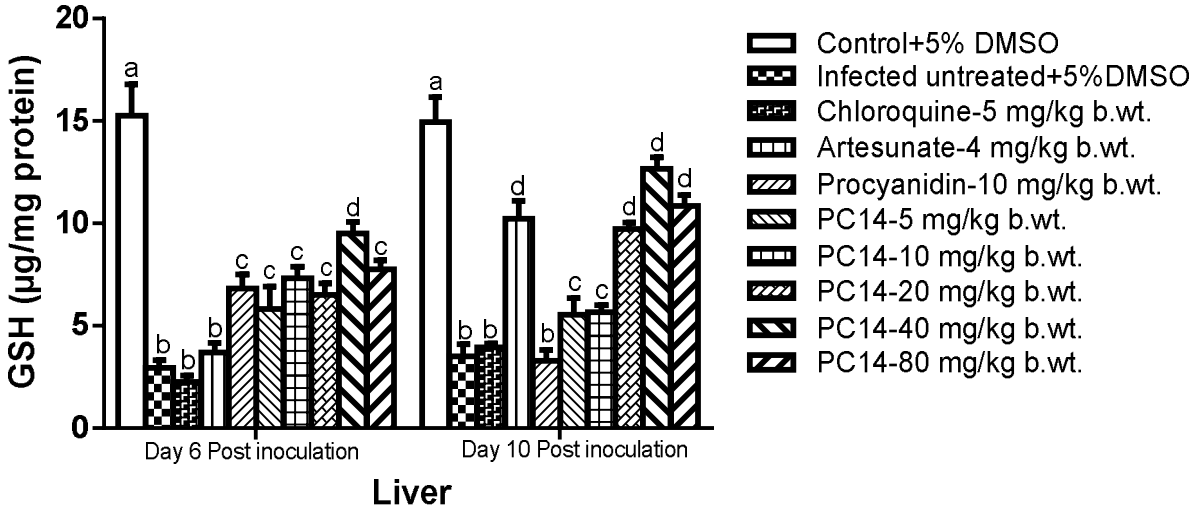


Figure 42: Reduced glutathione concentration in liver of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

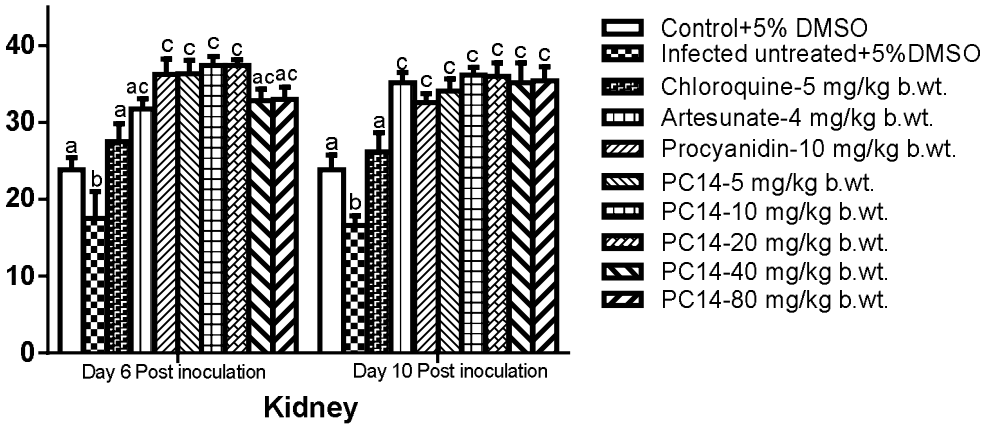


Figure 43: Reduced glutathione concentration in kidney of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

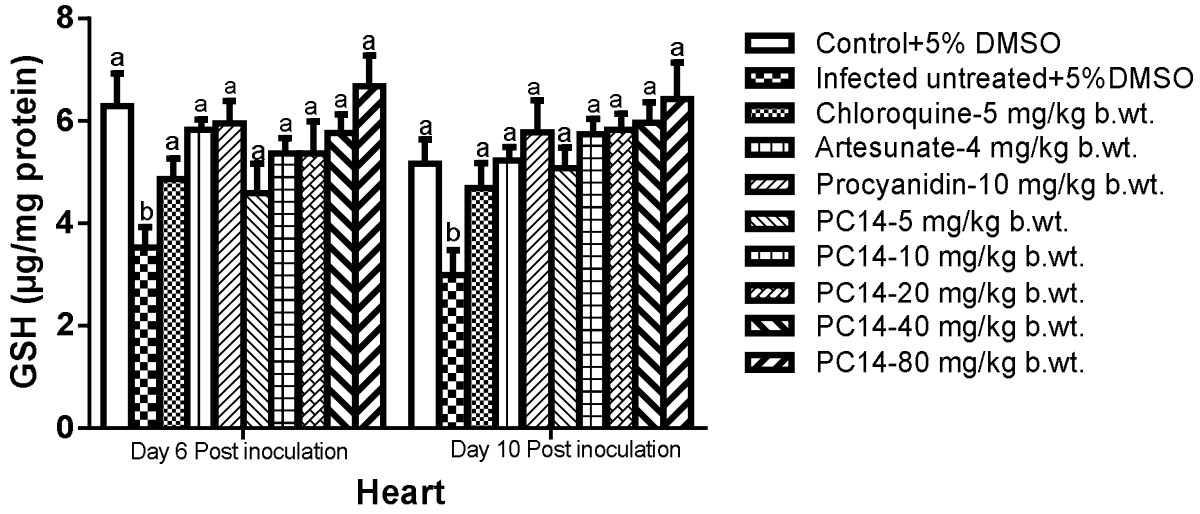


Figure 44: Reduced glutathione concentration in heart of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

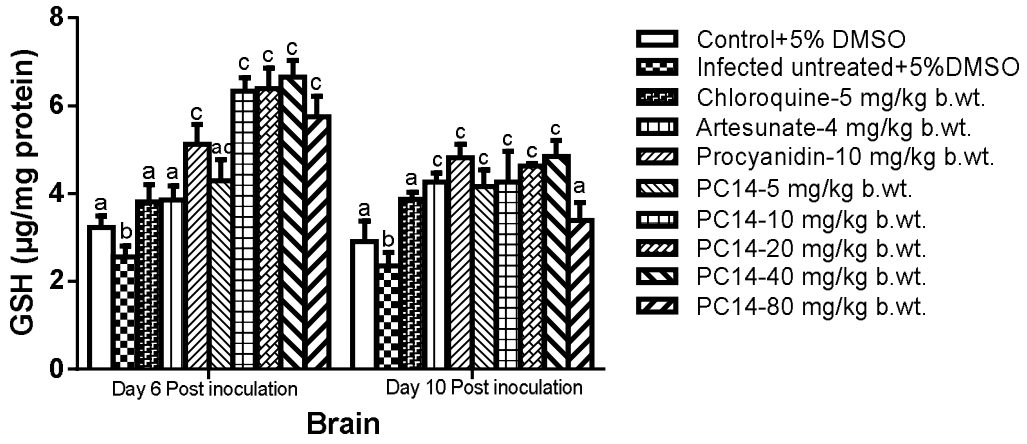


Figure 45: Reduced glutathione concentration in brain of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

3.8.6 Glutathione-S-transferase activity

Erythrocyte Glutathione-S-transferase activity: On day 6 post-inoculation, significant decrease ($p<0.05$) was observed in erythrocyte Glutathione-S-transferase activity of untreated infected animals compared touninfected control (Figure 46). However, chloroquine, artesunate and procyanidin were able to revert the reduced erythrocyte GST activity to the range of the uninfected control. On the other hand, the hybrid molecule at all doses did not cause any significant change ($p>0.05$) in erythrocyte GST activity compared to the untreated infected group. On day 10 post-inoculation, significant decrease ($p<0.05$) was observed in erythrocyte Glutathione-S-transferase activities of untreated infected animals compared to uninfected control (Figure 46). All the drugs at various doses were able to revert the reduced erythrocyte GST activity to the range of uninfected control, with chloroquine significantly increasing ($p<0.05$) it beyond the range.

Hepatic Glutathione-S-transferase activity: For hepatic Glutathione-S-transferase activities of the various groups on day 6 post-inoculation, no significant difference($p>0.05$) was observed compared to uninfected control, except the groups administered40 and 80 mg/kg body weight of artesunate-procyanidin hybrid molecule inwhich hepatic GST activity was significantly increased($p<0.05$) (Figure 47). On day 10 post-inoculation, significant decrease ($p<0.05$) was observed in hepatic Glutathione-S-transferase activities of untreated infected animals compared to uninfected control (Figure 47). All the drugs at various doses were able to revert the reduced hepatic GST activity to the range of uninfected control, with 40 mg/kg body weight of the hybrid molecule significantly increasing ($p<0.05$) it beyond the range.

Kidney Glutathione-S-transferase activity: On day 6 post-inoculation, significant decrease ($p<0.05$) was observed in kidney Glutathione-S-transferase activities of untreated infected

animals compared to uninfected control (Figure 48). All the drugs at various doses were able to revert the reduced kidney GST activity to the range of uninfected control.On day 10 post-inoculation, significant decrease ($p<0.05$) was observed in kidney Glutathione-S-transferase activities of untreated infected animals compared to uninfected control (Figure 48). All the drugs at various doses were able to significantly increase ($p<0.05$) the reduced kidney GST activity above the range of uninfected control,

Heart Glutathione-S-transferase activity: On day 6 and day 10 post inoculation, there was significant reduction ($p<0.05$) in heart Glutathione-S-transferase activityof the untreated infected group compared to the uninfected group(Figure 49). All the drugs at various doses were able to revert the reduced heart Glutathione-S-transferase activity to the range of the uninfected control (Figure 49).

Brain Glutathione-S-transferase activity: On day 6 post-inoculation, significant decrease ($p<0.05$) was observed in brain Glutathione-S-transferase activities of untreated infected animals compared to uninfected control (Figure 50). All the drugs at various doses were able to revert the reduced brain GST activity to the range of uninfected control. On day 10 post-inoculation, significant decrease ($p<0.05$) was observed in brain Glutathione-S-transferase activities of untreated infected animals compared to uninfected control (Figure 50).All the drugs at various doses were able to revert the reduced brain GST activity to the range of uninfected control, with artesunate and 80 mg/kg body weight of the hybrid molecule significantly increasing ($p<0.05$) it beyond the range.

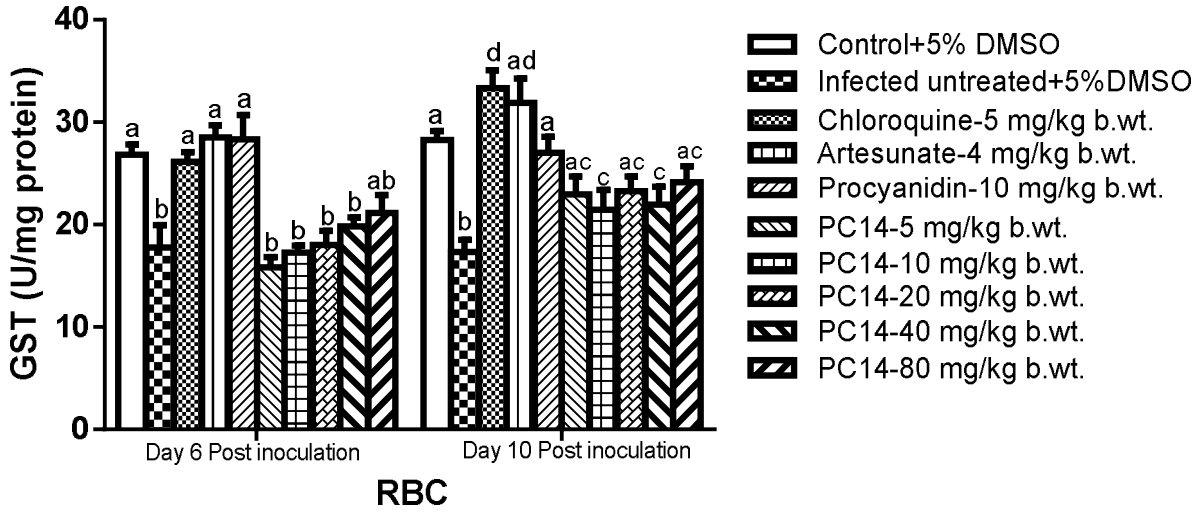


Figure 46: Glutathione-S-transferase activity in Red blood cell of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

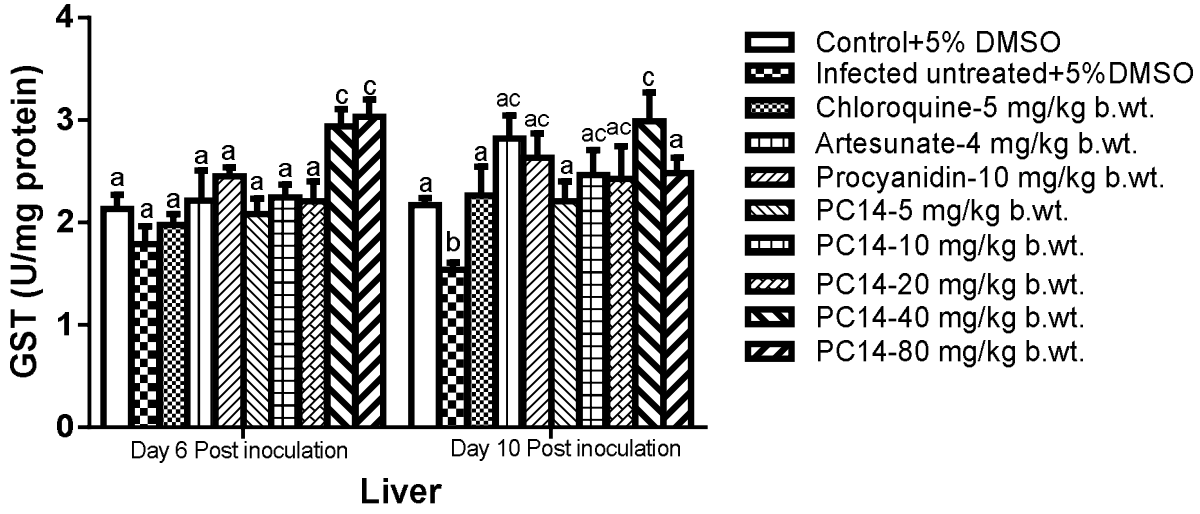


Figure 47: Glutathione-S-transferase activity in liver of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

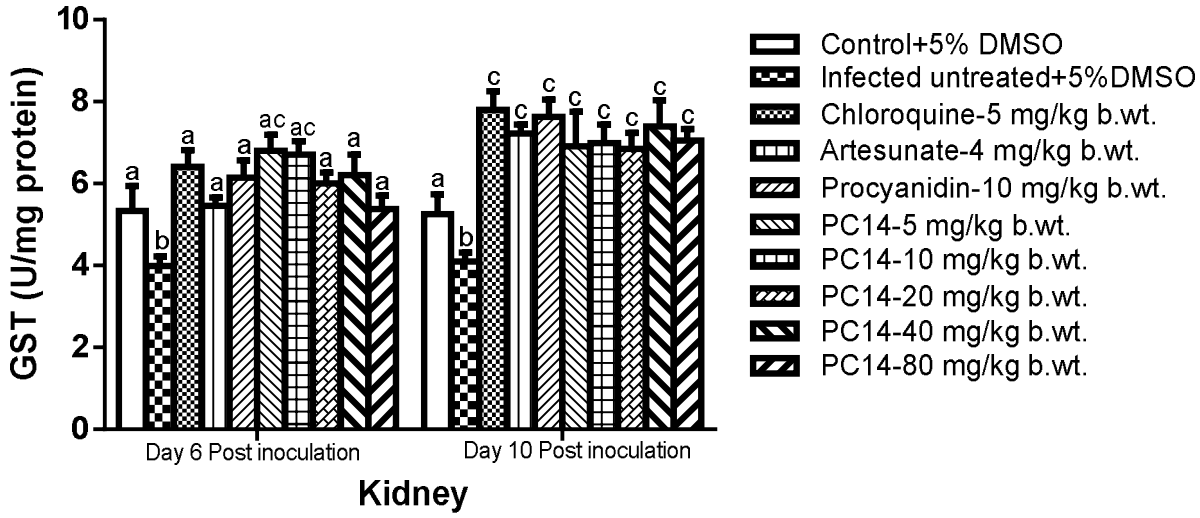


Figure 48: Glutathione-S-transferase activity in kidney of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

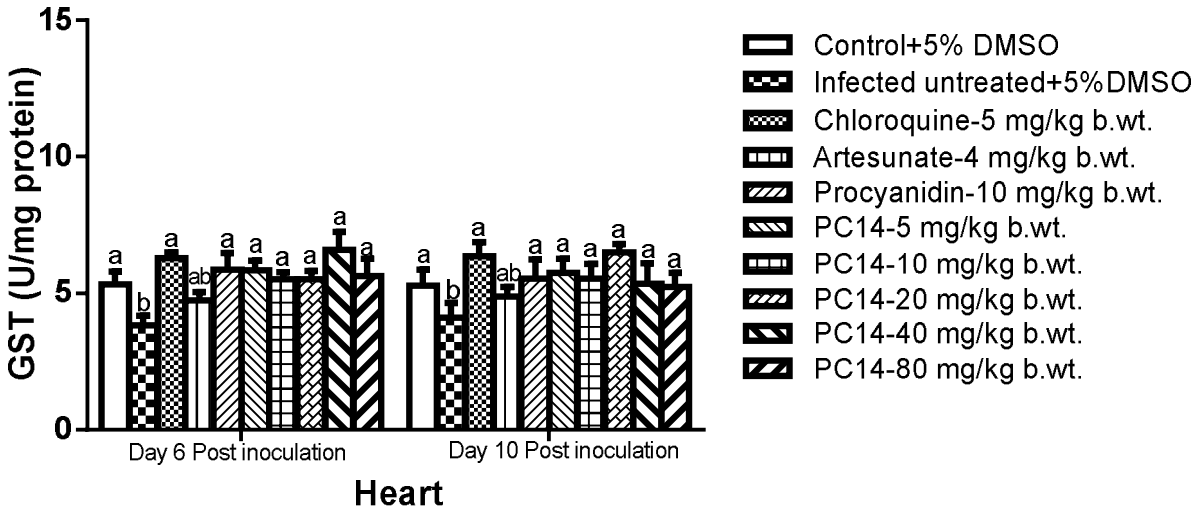


Figure 49: Glutathione-S-transferase activity in heart of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

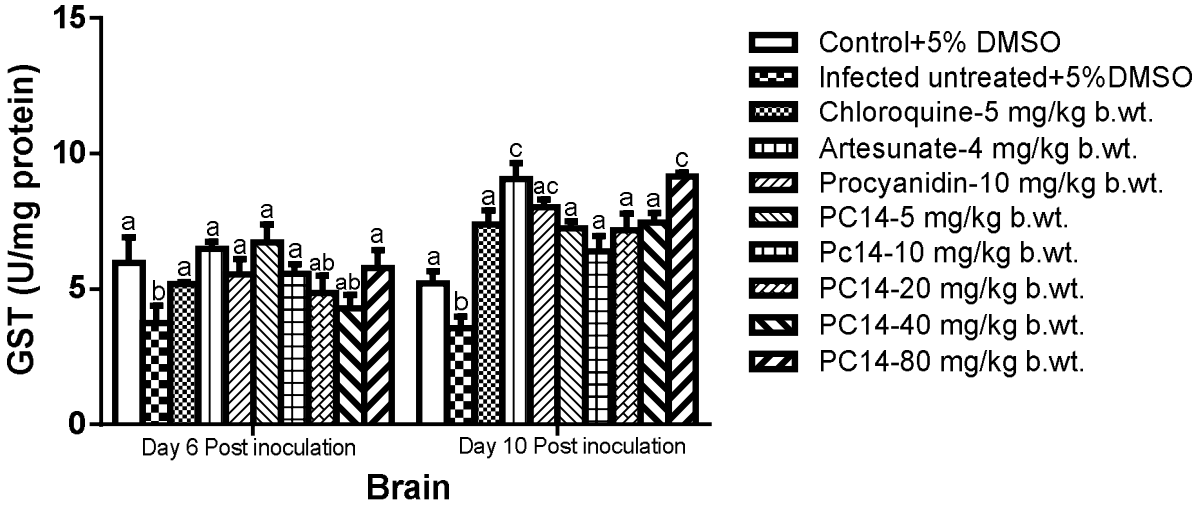


Figure 50: Glutathione-S-transferase activity in brain of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Mean ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

3.8.7 Catalase activity

Erythrocyte Catalase activity: On days 6 and 10 post-inoculation, there was significant reduction ($p<0.05$) observed in erythrocyte catalase activityof the infected untreated group compared to the uninfected control (Figure 51).Only artesunate on day 6 post-inoculation and procyanidin on day 10 post-inoculation were able to revert the reduced erythrocyte catalase activity to the range of the uninfected control. All other drugs did not cause any significant change ($p>0.05$) in erythrocyte catalase activity compared to the untreated infected group (Figure 51).

Hepatic Catalase activity: On day 6 post-inoculation, significant decrease ($p<0.05$) was observed in hepatic catalase activity of untreated infected animals compared to uninfected control (Figure 52). However, the drugs at various doses were able to revert the reduced hepaticcatalase activity to the range of the uninfected control, with 10, 20, 40 mg/kg body weight of the hybrid molecule significantly increasing ($p<0.05$) it beyond the range. On day 10 post-inoculation, significant decrease ($p<0.05$) was observed in hepatic catalase activity of untreated infected animals compared to uninfected control (Figure 52). All the drugs at various doses were able to revert the reduced hepatic catalase activity to the range of uninfected control, with 20 mg/kg body weight of the hybrid molecule significantly increasing ($p<0.05$) it beyond the range (Figure 52).

Kidney Catalase activity: On day 6 post-inoculation, significant decrease ($p<0.05$) was observed in kidney catalase activity of untreated infected animals compared to uninfected control (Figure 53). However, the drugs at various doses were able to revert the reduced kidney catalase activity to the range of the uninfected control, with chloroquine, 5, 40 and 80 mg/kg body weight of the hybrid molecule significantly increasing ($p<0.05$) it beyond the range. On

day 10 post- inoculation, no significant difference ($p>0.05$) was observed in kidney catalase activity of untreated infected animals compared to uninfected control (Figure 53). The kidney catalase activities of the groups treated with chloroquine, artesunate, procyanidin and Artesunate-procyanidin hybrid molecule at the doses of 5 and 40 mg/kg body weight were not significantly different ($p>0.05$) from those of untreated infected group and uninfected control. However, the kidney catalase activities of the groups administered 10, 20 and 80 mg/kg body weight of the hybrid molecule were significantly higher ($p<0.05$) than those of untreated infected group and uninfected control.

Heart Catalase activity: On day 6 post-inoculation, no significant difference ($p>0.05$) was observed in heart catalase activities of all the groups compared to the uninfected control (Figure 54). On day 10 post-inoculation, heart catalase activity of the untreated infected group was significantly increased ($p<0.05$) compare to the uninfected control. However, all the drugs at various doses were able to revert the increased heart catalase activity back to the range of the uninfected control (Figure 54).

Brain Catalase activity: On day 6 post-inoculation, brain catalase activity of the untreated infected group was significantly increased ($p<0.05$) compared to the uninfected control (Figure 55). However, chloroquine, artesunate and the hybrid molecule at the doses of 20 and 40 mg/kg body weight were able to revert the increased brain catalase activity back to the range of the uninfected control (Figure 55). On day 10 post-inoculation, brain catalase activity of the untreated infected group was significantly increased ($p<0.05$) compared to the uninfected control. However, procyanidin and the hybrid molecule at the doses of 40 and 80 mg/kg body weight were able to revert the increased brain catalase activity back to the range of the uninfected control (Figure 55).

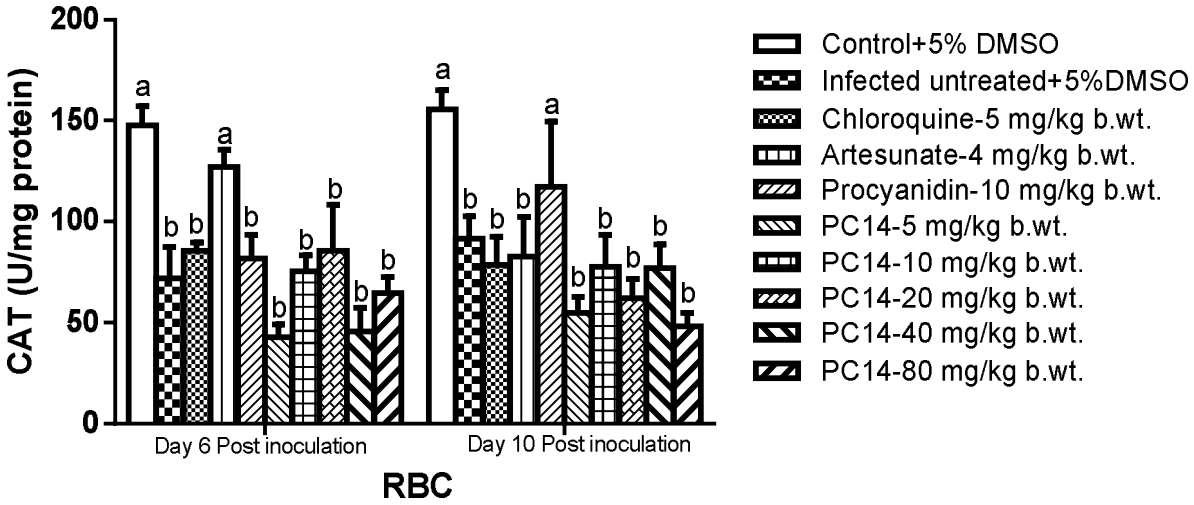


Figure 51: Catalase activity in Red blood cell of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Means ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

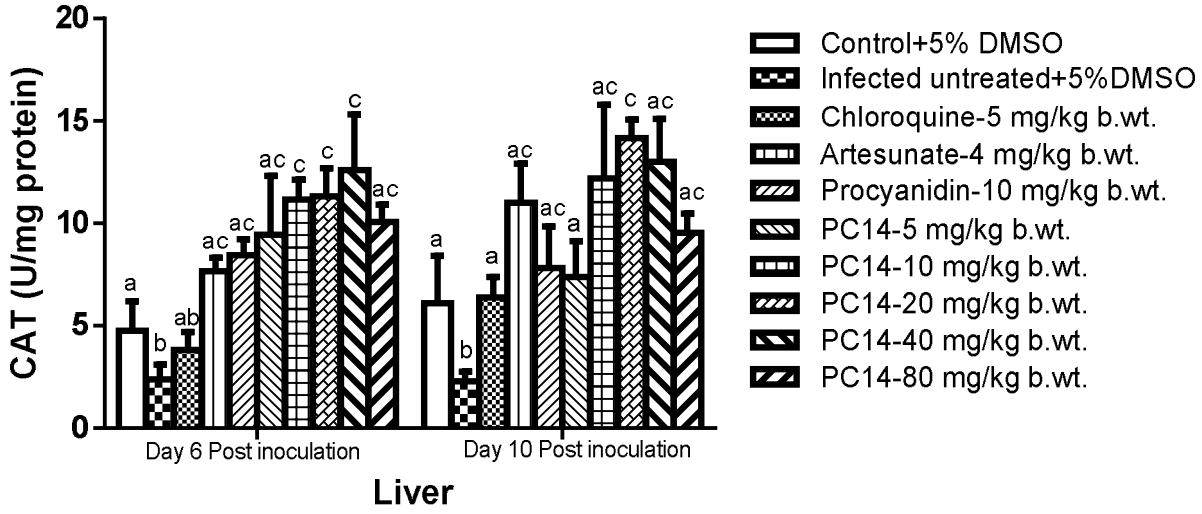


Figure 52: Catalase activity in liver of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Means ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

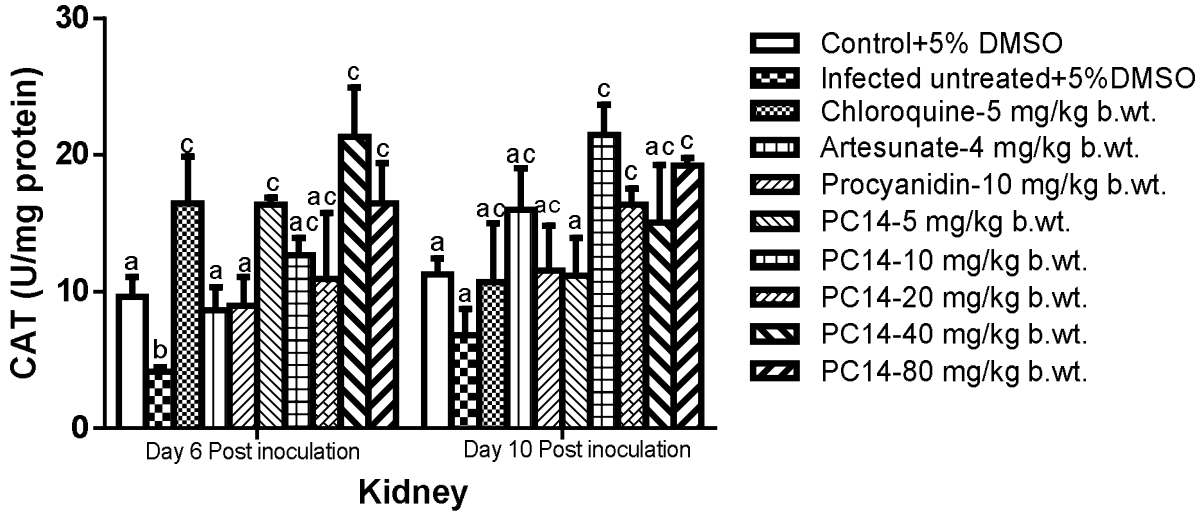


Figure 53: Catalase activity in kidney of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Means ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

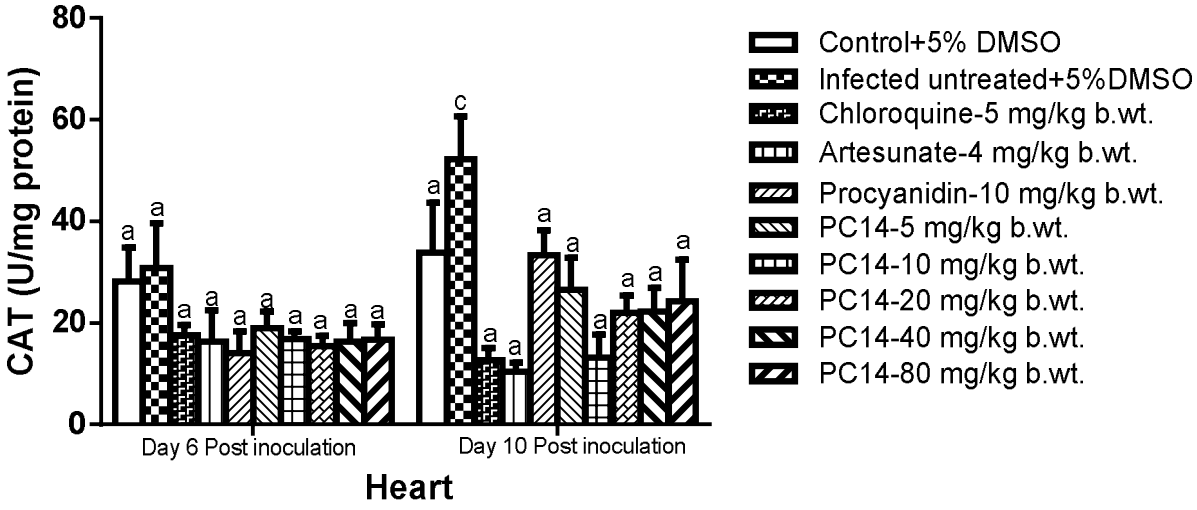


Figure 54: Catalase activity in heart of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Means ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

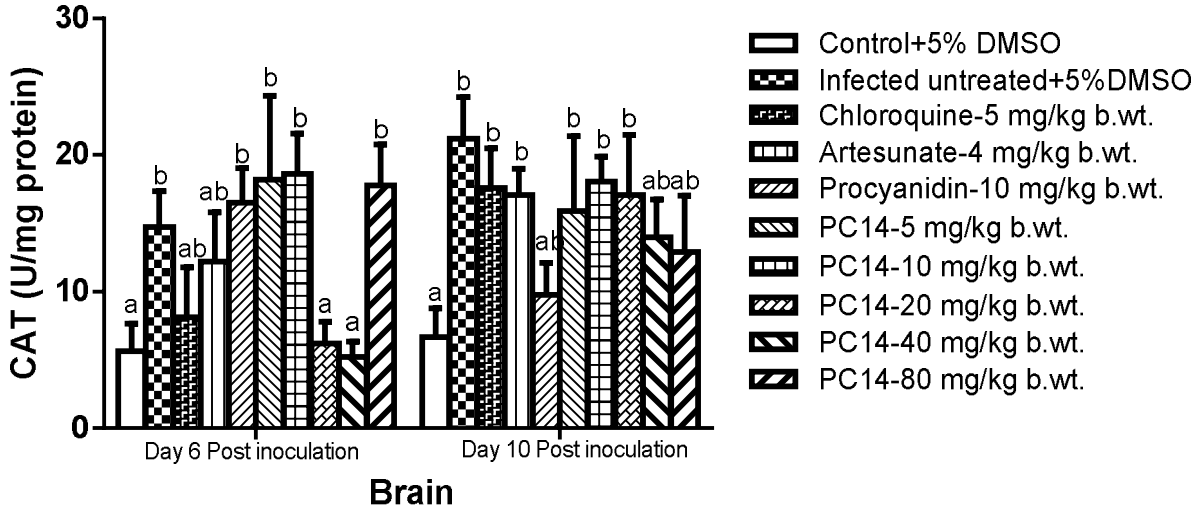


Figure 55: Catalase activity in brain of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 and day 10 post-inoculation. Values are Means ± SEM, n=5, Values for each day with different superscripts are significantly different at $p<0.05$. PC14: Artesunate-procyanidin hybrid molecule.

3.9 Haematological Parameters

On day 6 post-inoculation, significant increase ($p<0.05$) was observed in WBC of *P. berghei* NK65-infected mice administered the drugs at various doses compared to untreated infected control (Table 15). Similarly, a significant increase ($p<0.05$) in PCV was observed at various doses of the drugs except at 40 mg/kg body weight hybrid molecule compared to the untreated infected group. No significant difference($p>0.05$) was observed in the Haemoglobin concentrations of all the groups compared to the uninfected control. However, there was significant increase ($p<0.05$) in the levels of Neutrophilin the various groups compared to uninfected control. On day 10 post-inoculation, significant increase ($p<0.05$) was observed in WBC, PCV, Hb, and % Neutrophils in groups treated with chloroquine, artesunate, procyanidin and all doses of artesunate-procyanidin hybrid compound compared to untreated infected control (Table 16). A significant decrease ($p<0.05$) was also observed in lymphocyte levels of the groups treated with Chloroquine, Artesunate and Procyanidin and all doses of artesunate-procyanidin hybrid compound compared to that of the untreated infected group.

The CD4+ lymphocyte count of the untreated infected control was significantly reduced($p<0.05$) on day 6 post-inoculation compared to the uninfected control (Figure 56). All the drugs at various doses (except 20 mg/kg body weight of the hybrid molecule) significantly increased CD4+ lymphocyte count, though not to the range of the uninfected control.On day 10 post-inoculation (Figure 57), the CD4+ lymphocyte count of untreated infected control and all drugs at various doses was significantly reduced ($p<0.05$) compared to uninfected control.

Table 15: Haematological indices of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 6 post-inoculation

Groups-Doses (mg/kg body weight)	WBC (/µL)	Hb (g/dL)	PCV (%)	PLT ($\times 10^3$/µL)	NEU (%)	LYMP (%)
Control	2583.0±56.0^{a}	16.0±0.8^{a}	41.2±0.5^{a}	836.0±8.0^{a}	12.0±1.0^{a}	88.0±0.9^{a}
Infected Untreated	1576.0±31.0^{b}	13.0±0.7^{ab}	25.0±0.9^{b}	468.0±10.0^{b}	8.0±1.0^{b}	92.0±5.1^{d}
Chloroquine – 5	34230.0±867.0^{f}	14.0±1.2^{a}	33.0±1.3^{c}	165.0±9.0^{c}	31.0±3.0^{c}	69.0±1.9^{e}
Artesunate- 4	33100.0±756.0^{f}	14.0±0.9^{a}	34.2±0.8^{c}	623.0±10.0^{a}	25.0±2.0^{c}	75.0±1.4^{d}
Procyanidin-10	47412.0±99.0^{f}	13.0±0.9^{a}	33.6±0.9^{c}	578.0±8.0^{a}	40.0±2.0^{d}	60.0±4.5^{e}
PC14-5	8241.0±14.0^{e}	14.0±1.0^{a}	33.2±1.1^{c}	639.0±11.0^{a}	55.0±1.0^{e}	45.0±1.5^{c}
PC14-10	8250.0±11.0^{e}	13.0±0.4^{a}	33.4±0.6^{c}	468.0±7.0^{b}	35.0±1.0^{cd}	65.0±1.9^{e}
PC14-20	6239.0±143.0^{d}	14.0±0.9^{a}	34.8±0.5^{c}	272.0±12.0^{c}	28.0±2.0^{c}	72.0±1.2^{f}
PC14-40	2962.0±15.0^{c}	16.0±1.1^{a}	26.8±1.0^{b}	274.0±10.0^{c}	61.0±5.0^{f}	39.0±1.9^{b}
PC14-80	3423.0±14.0^{c}	13.0±0.4^{a}	33.0±1.1^{c}	968.0±8.0^{a}	24.0±1.0^{c}	76.0±0.4^{d}

Values are Mean ± SEM, n=5, Values with different superscript are significantly different at $p<0.05$; **PC14:** Artesunate-procyanidin hybrid molecule.

Table 16: Haematological indices of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid molecule on day 10 post-inoculation

Grouping/Doses (mg/kg body weight)	WBC (/µL)	Hb (g/dL)	PCV (%)	PLT ($\times 10^3$/µL)	NEU (%)	LYMP (%)
Control	2883.0±45.0^{a}	15.0±1.7^{a}	39.2±0.8^{a}	844.0±10.0^{a}	12.0±1.0^{a}	88.0±2.0^{a}
Infected Untreated	1360.0±31.0^{b}	10.0±0.8^{b}	20.0±1.3^{c}	522.0±15.0^{b}	9.0±2.0^{a}	91.0±4.2^{c}
Chloroquine - 5	2833.0±34.0^{a}	13.0±1.0^{a}	32.0±1.6^{a}	341.0±11.0^{c}	32.0±4.0^{b}	68.0±3.0^{d}
Artesunate - 4	3100.0±82.0^{a}	12.0±0.7^{a}	30.0±0.8^{a}	522.0±09.0^{b}	29.0±5.0^{b}	71.0±1.9^{d}
Procyanidin - 10	4522.0±71.0^{c}	13.0±1.0^{a}	32.8±0.9^{a}	500.0±12.0^{b}	42.0±5.0^{c}	58.0±7.3^{d}
PC14-5	4820.0±44.0^{e}	13.0±1.0^{a}	30.2±1.3^{a}	592.0±17.0^{e}	34.0±3.0^{b}	66.0±4.0^{d}
PC14-10	5755.0±37.0^{e}	13.0±0.5^{a}	31.0±0.5^{a}	546.0±07.0^{b}	33.0±2.0^{b}	67.0±3.2^{d}
PC14-20	5210.0±49.0^{e}	13.0±0.7^{a}	31.2±1.1^{a}	427.0±15.0^{d}	30.0±4.0^{b}	70.0±3.8^{d}
PC14-40	3271.0±40.0^{a}	14.0±0.5^{a}	27.0±1.2^{ab}	342.0±15.0^{c}	55.0±6.0^{c}	45.0±3.2^{b}
PC14-80	4052.0±29.0^{d}	12.0±0.8^{a}	29.0±1.0^{ab}	781.0±13.0^{a}	25.0±4.0^{b}	75.0±4.9^{d}

Values are Mean ± SEM, n=5, Values with different superscript are significantly different at $p<0.05$. **PC14**: Artesunate-procyanidin hybrid molecule.

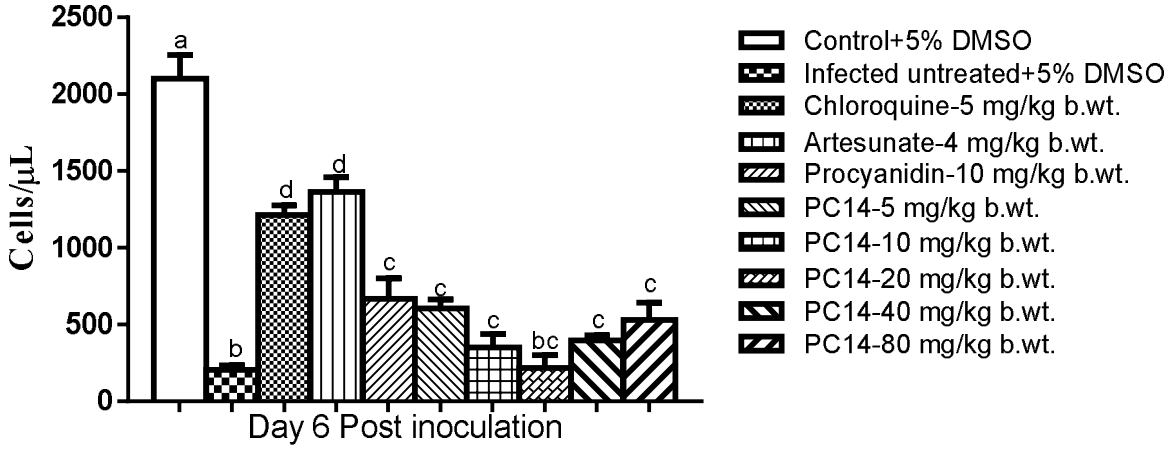


Figure 56: CD4+ lymphocyte count in blood of *P. berghei* NK65-infected mice administered Artesunate-procyanidin hybrid compound on day 6 post-inoculation. Values are Mean ± SEM, n=5, Values with different superscript are significantly different at $p<0.05$. **PC14**: Artesunate-procyanidin hybrid molecule.

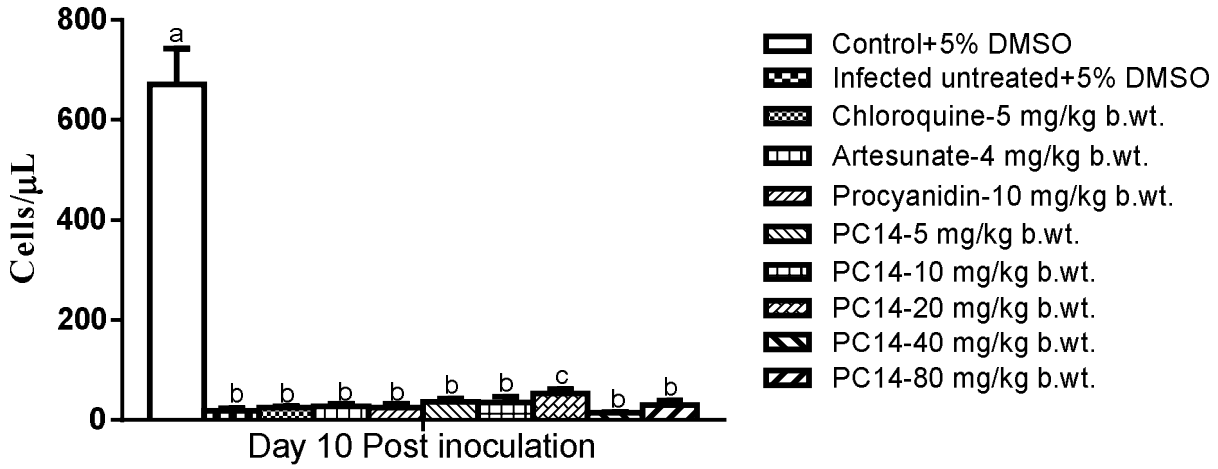


Figure 57: CD4+ lymphocyte count in blood of *P. berghei* NK65-infected mice administered Artesunate**-procyanidin hybrid compound on day 10 post-inoculation.** Values are Mean ± SEM, n=5, Values with different superscript are significantly different at $p<0.05$. **PC14:** Artesunate-procyanidin hybrid molecule.

3.10 Molecular docking studies

All molecules re-docked into their native protein showed good RMSD values as well as good binding affinities. NADH in the PfLDH binding pocket seems to be a superimposed conformation on the native inbound ligand (Figures 58-59). The various conformations had RMSD values as low as 1.84 and binding affinity of -10.4 kcal/mol (Table 18). The hybrid compound had a -9.6 kcal/mol binding affinity, which was lower than that of the native ligand and an RMSD value ranging between 2.46 - 21.41, while Procyanidin and Artesunate had binding affinity of -8.0 and -6.2 kcal/mol with RMSD values ranging between 1.11 - 28.48 and 2.05 - 21.76 respectively. The molecules interacted with different amino acids in the binding pocket of PfLDH (Table 17). For Artesunate-procyanidin hybrid molecule, no amino acid was similar to the native ligand interaction in the most active conformations. NADH in the binding pocket acted as both H-bond donor and acceptor while PC14 hybrid and Procyanidin acted mainly as H-bond donor and Artesunate as H-bond acceptor.

Best dock poses of Artesunate-procyanidin hybrid compound in Plasmepsin I and III had lower affinity energy compared to the native ligands used in the validation process but was however higher than the affinity energy of Procyanidin and Artesunate (Figures 60-61, Table 18). The native ligand (KNI-0006) and Procyanidin were docked into the S4 subunit, while Artesunate-procyanidin hybrid and Artesunate where docked into S3 and S1 respectively. The native ligand was found to interact with Arg307, Pro304 and Ile221 as seen for procyanidin(PC00), while hybrid molecule (PC14) interacted with Phe242, Ala111, and Met283 as seen for the native ligand. Artesunate-procyanidin hybrid compound in Plasmepsin II binding pocket interacted with Ile290 and Ala219 in similar manner to the native ligand (STA). The affinity energy for Artesunate-procyanidin and procyanidin were 9.5 and -8.2 kcal/mol

respectively. Plasmepsin III (HAP) showed good affinity energy of -9.6, -9.0 and -7.7 kcal/mol for Artesunate-procyanidin hybrid molecule, Procyanidin, Artesunate respectively but was, however, higher than the affinity energy of the native ligand KNI-10006 which was -10.2 kcal/mol. The docked pose of Artesunate's interaction with the amino acids wasmuch more similar to that of the native ligand. Artesunate-procyanidin hybrid molecule in the binding site of Plasmepsin IV interacted with Gly78, Ile294, Ile310, Leu131 and Val292 in a similar manner to JE2, which was the molecule used in the validation process (Figures 62-63). The most active pose of Procyanidin and Artesunate only interacted with Ser79 and Gly78 with affinity energy of -7.8 kcal/mol and -7.1 kcal/mol respectively. Plasmespsin V, in docking process, was validated using the native ligand WeHi. Artesunate-procyanidin hybrid molecule interacted with Arg167, Asn204, Pro196, Pro200 and Ser194. The binding affinity energy was lower (-9.5 kcal/mol) compared to that of Procayindin (-9.2 kcal/mol). The binding affinity energy for Artesunate-procyanidin hybrid molecule, Procyanidin and Artesunate in Falcipain-2 binding pocket was -7.3 kcal/mol, -7.4 kcal/mol, -6.3 kcal/mol respectively (Figures 64 and 65).

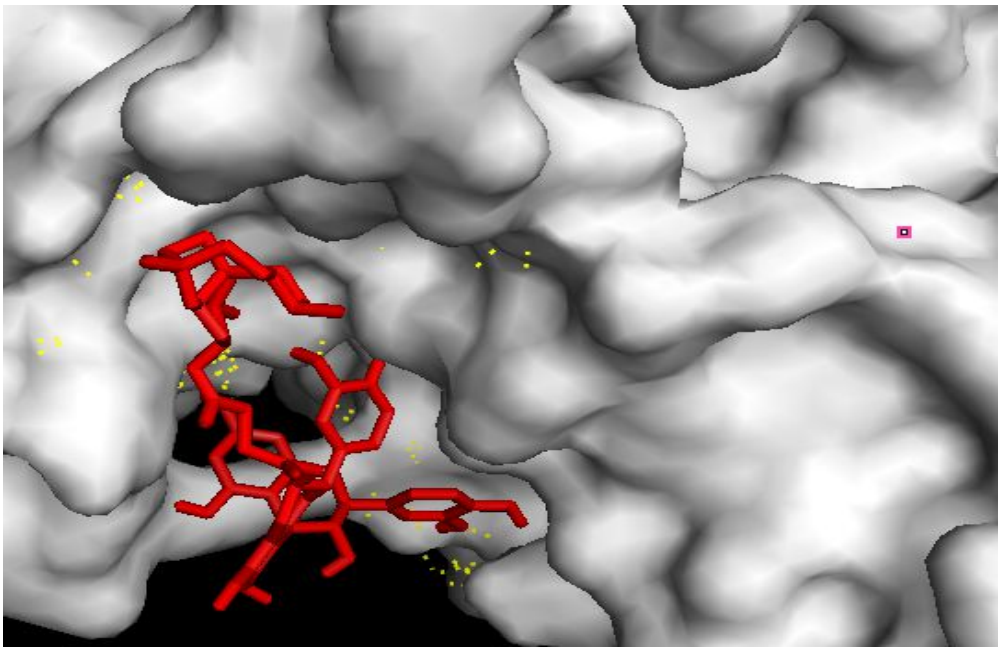


Figure 58: Artesunate-procyanidin hybrid molecule most active pose in pfLDH binding pocket

(Artesunate-procyanidin hybrid molecule in red)

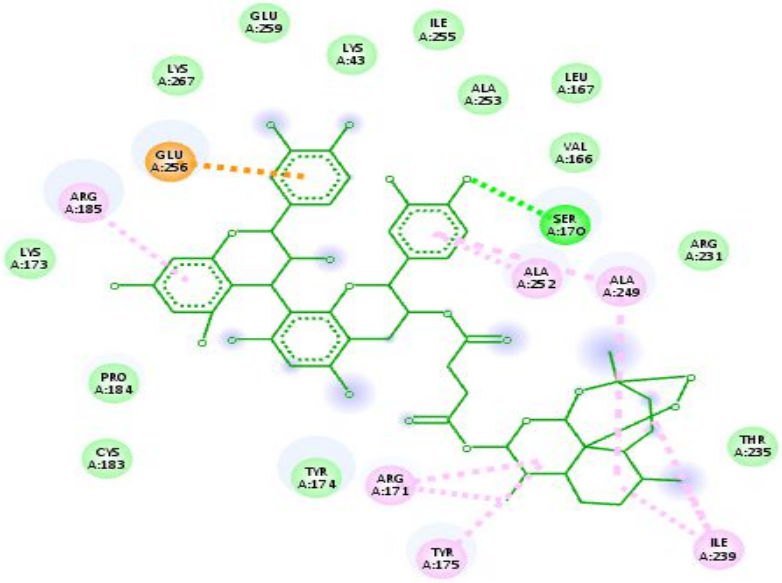


Figure 59: Artesunate-procyanidin hybrid molecule-protein interactions in pfLDH binding pocket

(Showing interactions with (A) Ala249, Ala252, Arg17, Arg185, Glu256, Ile239, Ser170, Tyr175)

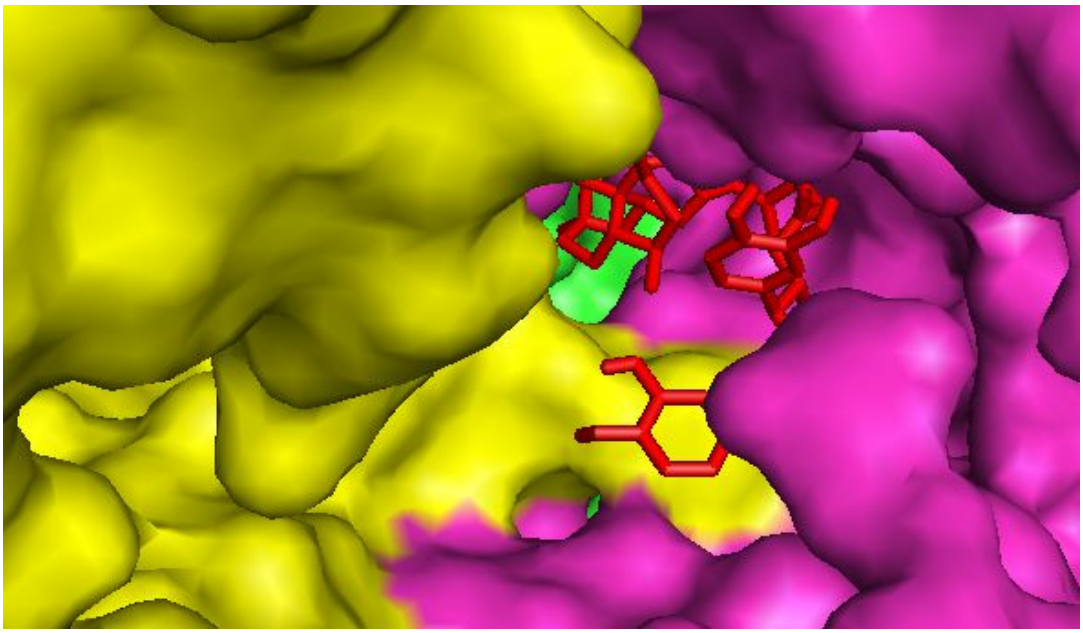


Figure 60: Artesunate-procyanidin hybrid molecule most active pose in Plasmepsin I binding pocket
(Artesunate-procyanidin hybrid molecule in red)

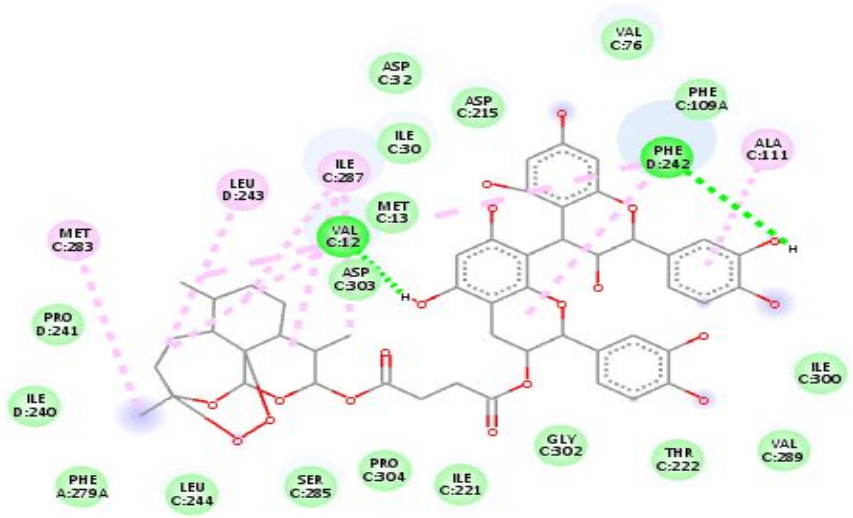


Figure 61: Artesunate-procyanidin hybrid molecule - protein interaction in Plasmepsin I binding pocket
(Showing interactions with Ala111C, Ile287C, Leu243D, Met283C, Phe242D, Val12C)

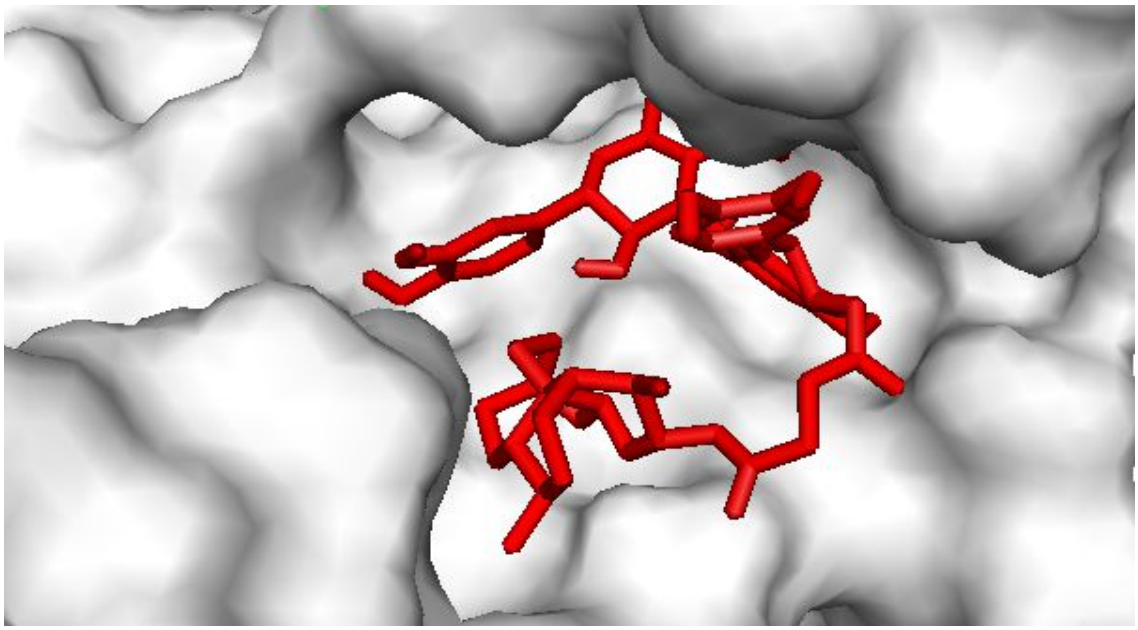


Figure 62: Artesunate-procyanidin hybrid molecule most active pose in Plasmepsin II binding pocket

(Artesunate-procyanidin hybrid molecule in red)

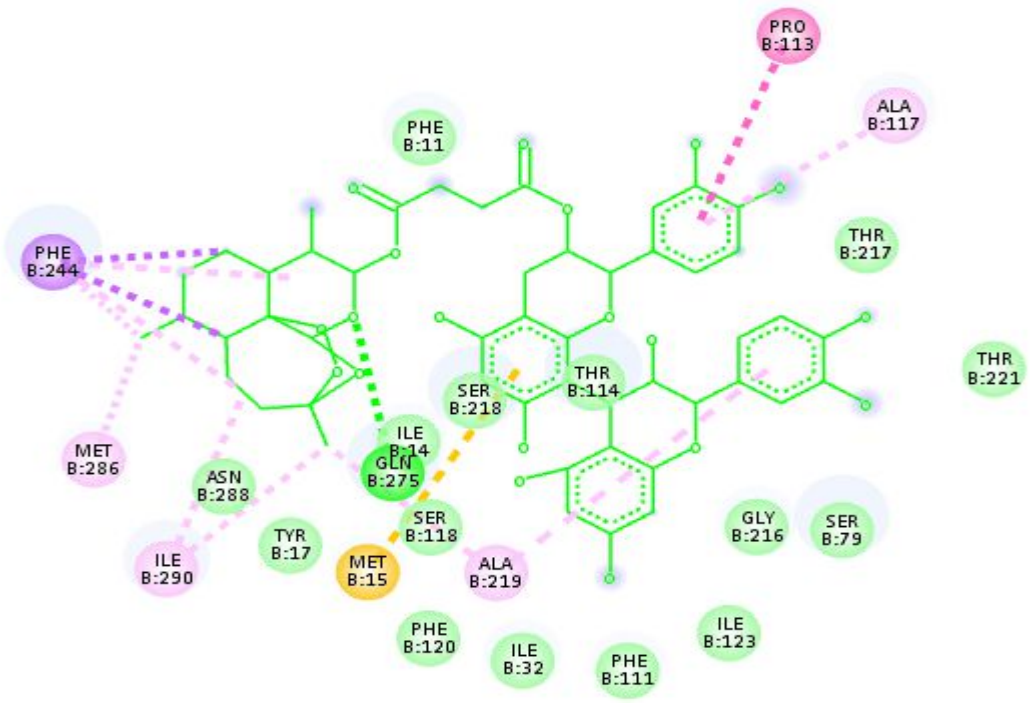


Figure 63: Artesunate-procyanidin hybrid molecule -protein interaction in Plasmepsin II binding pocket

(Showing interaction with (B) Ala117, Ala219, Asn288, Gln275, Ile290, Met15, Met286, Phe244, Pro113)

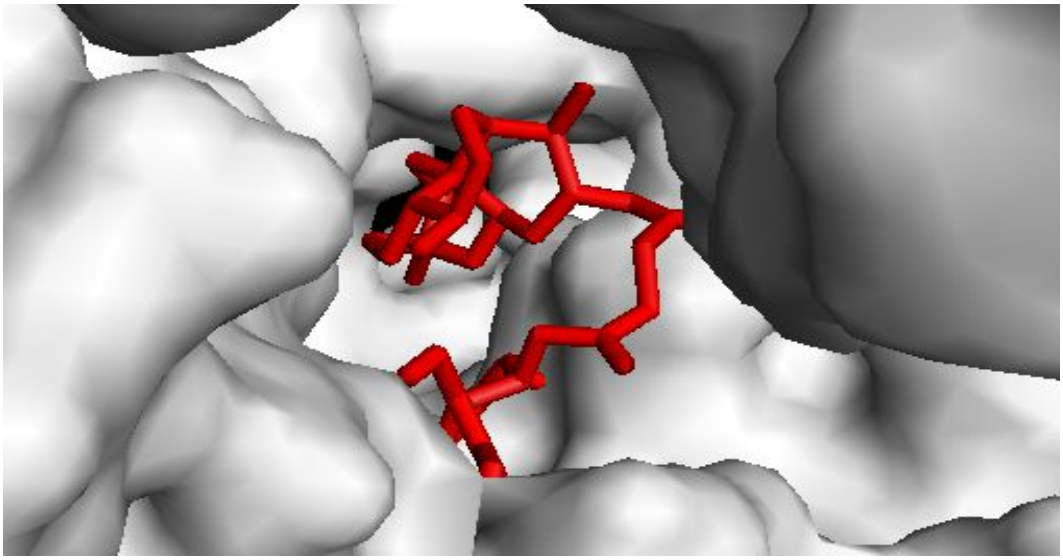


Figure 64: Artesunate-procyanidin hybrid molecule active pose in Plasmepsin III binding pocket

(Artesunate-procyanidin hybrid molecule in red)

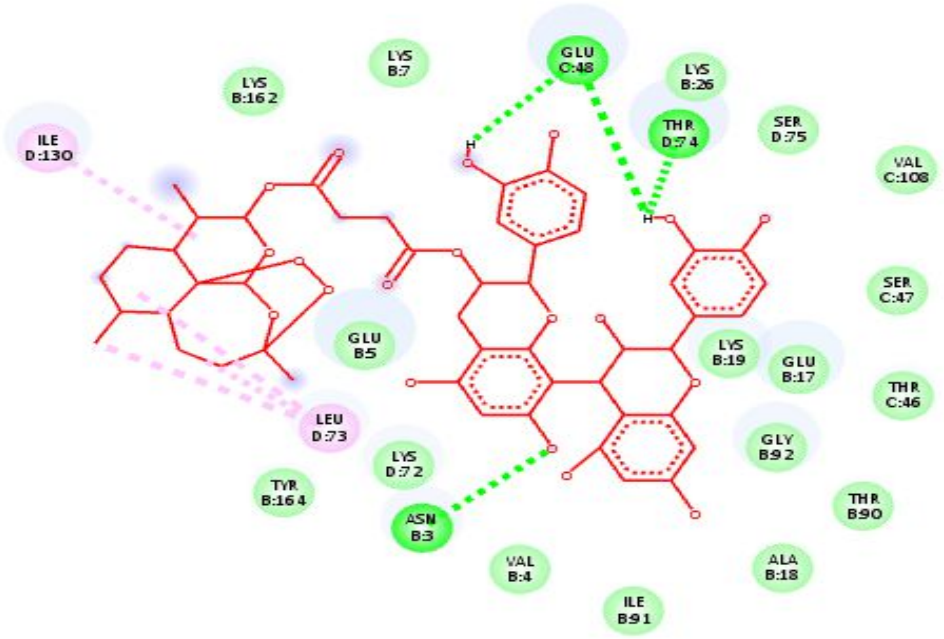


Figure 65: Artesunate-procyanidin hybrid molecule -protein interactions in Plasmepsin III binding pocket

(Showing interaction with Asn3B, Gln48C, Ile130D, Leu73D, Thr74D)

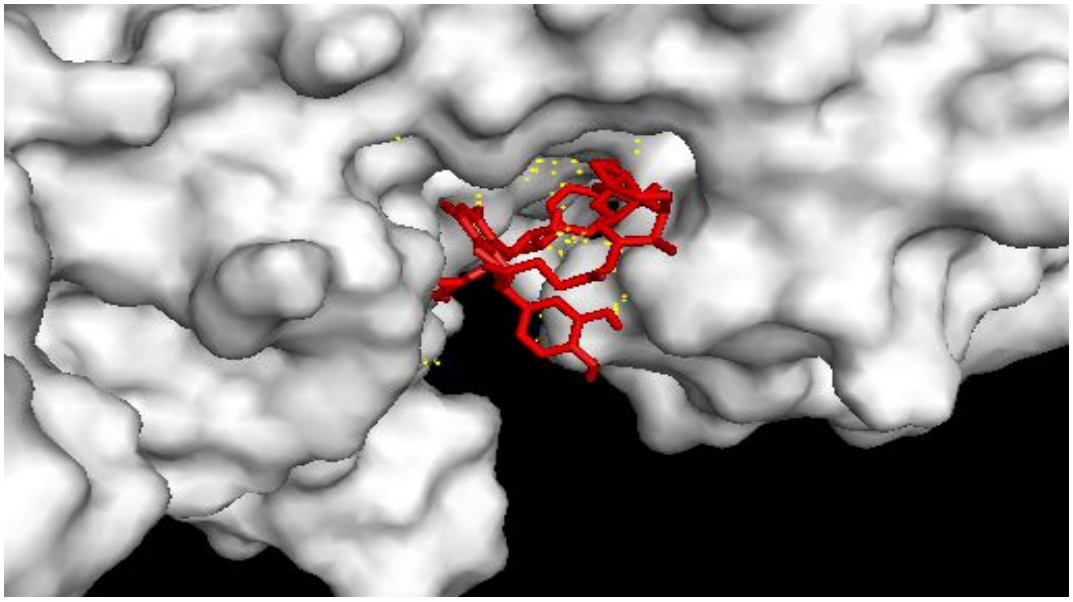


Figure 66: Artesunate-procyanidin hybrid molecule most active pose in Plasmepsin IV binding pocket

(Artesunate-procyanidin hybrid molecule in red)

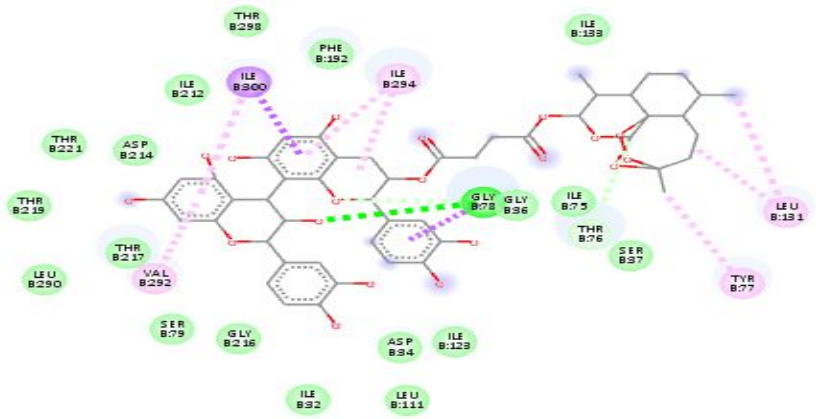


Figure 67: Artesunate-procyanidin hybrid molecule –protein interactions in Plasmepsin IV binding pocket

(Showing interactions with (B) Gly78, Ile294, Ile300, Leu131, Thr76, Tyr77, Val292)

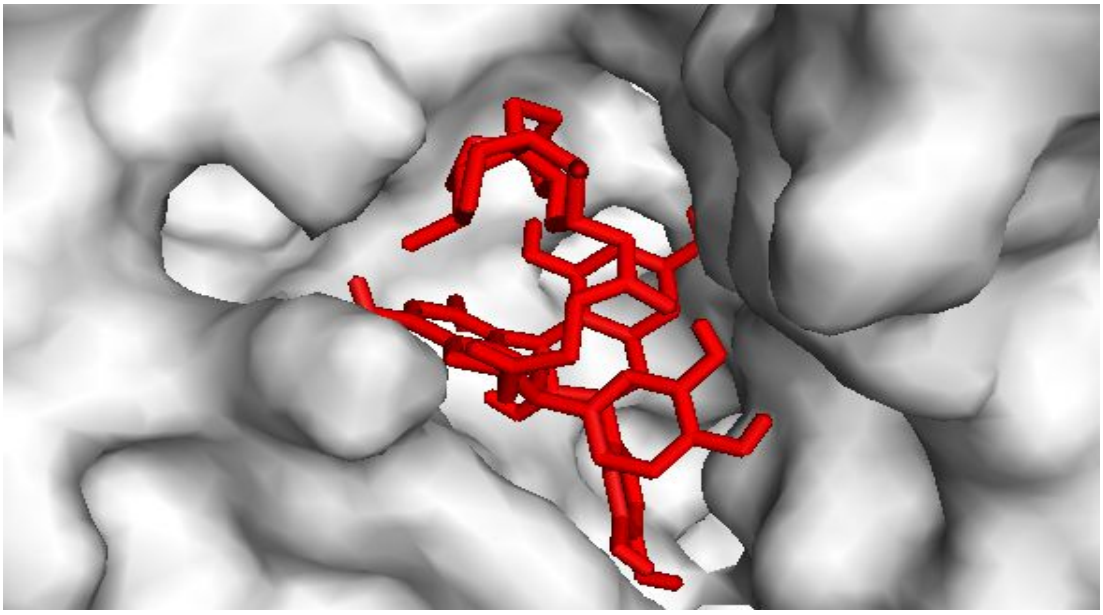


Figure 68: Artesunate-procyanidin hybrid molecule most active poses in Plasmepsin V binding pocket

(Artesunate-procyanidin hybrid molecules in red)

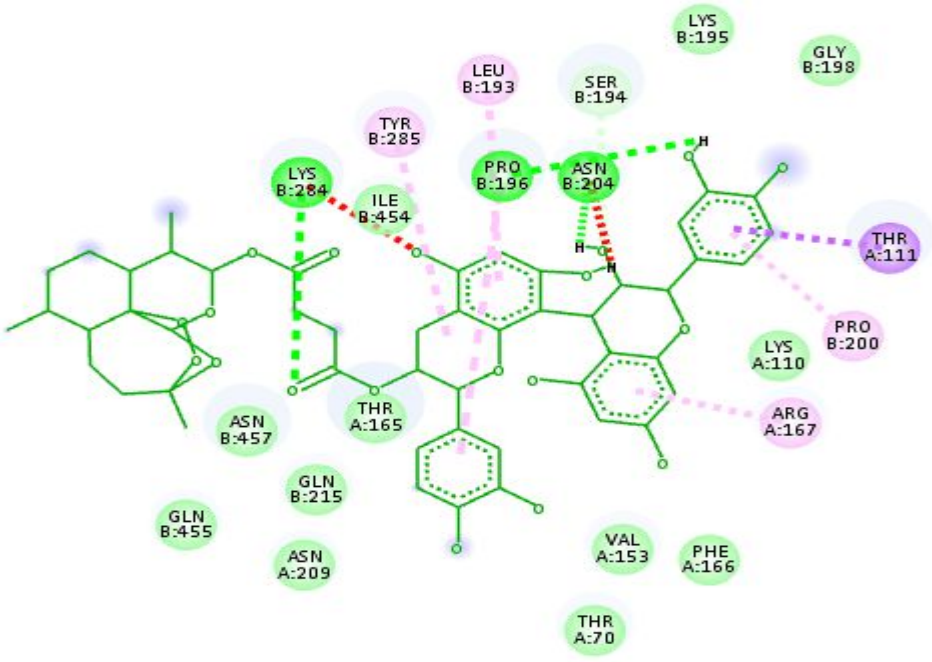


Figure 69: Artesunate-procyanidin hybrid molecule –protein interactions in Plasmepsin V binding pocket

(Showing interactions with Arg167A, Asn204B, Leu193B, Lys284B, Pro196B, Pro200B, Ser194B, Thr111A, Tyr285B)

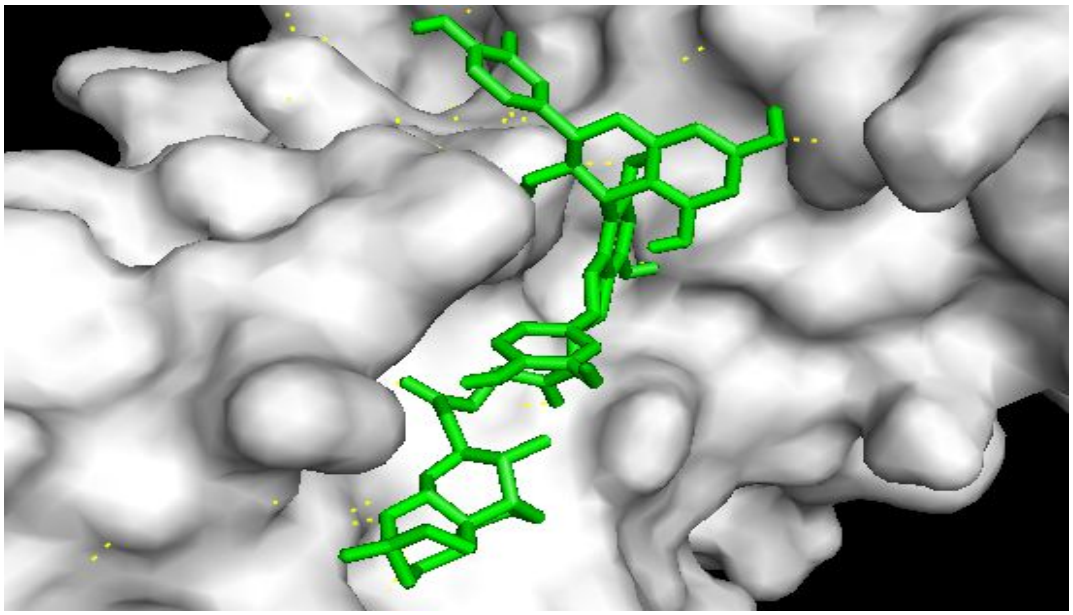


Figure 70: Artesunate-procyanidin hybrid molecule most active pose in Falcipain-2 binding pocket
(Artesunate-procyanidin hybrid molecule in green)

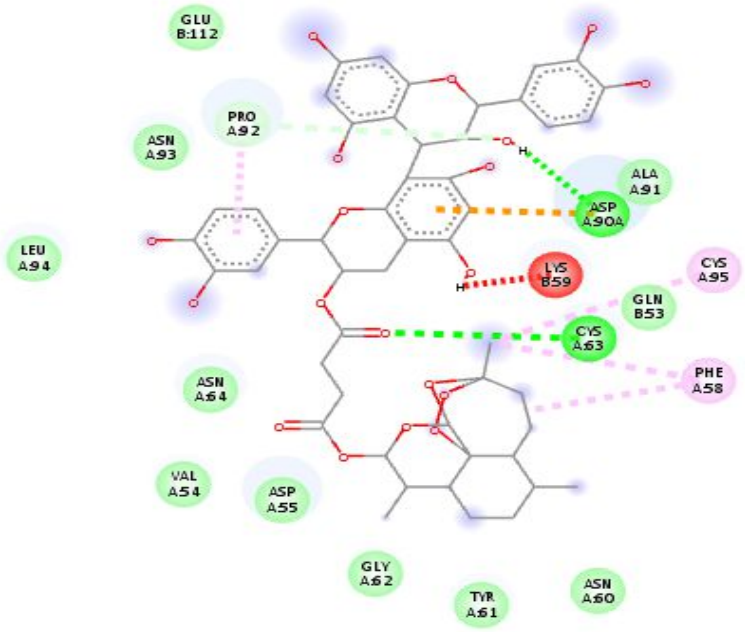


Figure 71: Artesunate-procyanidin hybrid molecule-protein interactions in Falcipain-2 binding pocket

(Showing interactions with Asp90A, Cys63A, Cys95A, Gln53B Lys59B, Phe58A, Pro92A)

Table 17: Summary of Ligand-Amino acid interactions in various Docking studies

S/No	Protein	PC14 interaction	PC00 interaction	AT00 interaction
1.	Plasmepsin I	Ala111C, Ile287C, Leu243D, Met283C, Phe242D, Val12C	Arg307D, Asp215D, Ile221D, Phe242C, Pro304D	Gln178A, Gly177A, Leu324A, Tyr267A, Tyr309A, Val266A
2.	Plasmepsin II	Ala117B, Ala219B, Asn288B, Gln275B, Ile290B, Met15B, Met286B, Phe244B, Pro113B	Arg307B, Asn13B, His276B, Lys163B, Tyr272B, Val160B	Ile14B, Ile32B, Met15B, Phe11B
3.	Plasmepsin III	Asn3B, Gln48C, Ile130D, Leu73D, Thr74D	Asn3B, Glu17B, Leu73D, Lys72D, Lys93B, Phe1B, Thr46C, Thr74D	Ile80C, Leu73C, Met104C, Phe109C, Phe111C, Trp39C, Tyr112C, Val120C
4.	Plasmepsin IV	Gly78B, Ile294B, Ile300B, Leu131B, Thr76B, Tyr77B, Val292B	Ala117B, Ala118B, Glu119B, Leu114B, Met15B, Phe120B, Ser79B	Gly78B, Leu111B, Leu114B, Phe120B, Thr217B, Tyr77B
5.	Plasmepsin V	Arg167A, Asn204B, Leu193B, Lys284B, Pro196B, Pro200B, Ser194B, Thr111A, Tyr285B	Leu115B, Leu169B, Pro123B, Pro200B, Ser112A, Ser113B	Leu64A, Lys51A, Val157A
6.	*Plasmodiumfalciparum* Lactate Dehydrogenase (pfLDH)	Ala249A, Ala252A, Arg17A, Arg185A, Glu256A, Ile239A, Ser170A, Tyr175A	Gly99A, Ile31A, Met30A, Phe100A, Pro246A, Thr101A, Thr97A	Leu201A, Lys198A, Lys314A, Phe229A, Val200A, Val233A
7.	Falcipain-2	Asp90A, Cys63A, Cys95A, Lys59B, Phe58A, Pro92A	Arg23B, Asn106B, Asp139A, Gln107B, Pro103B	Gly62A, Leu94A, Pro92A, Ser57A, Val54A,

PC14: Artesunate-procyanidin hybrid molecule, **PC00:** Procyanidin B_2, **AT00:** Artesunate

Table 18: Summary of Binding affinities (Kcal/mol) in various Docking studies

S/No	Protein	PC14	PC00	AT00
1.	Plasmepsin I	-10.3	-8.5	-6.6
2.	Plasmepsin II	-9.5	-8.2	-6.4
3.	Plasmepsin III	-9.6	-9.0	-7.7
4.	Plasmepsin IV	-10.2	-7.8	-7.1
5.	Plasmepsin V	-9.5	-9.2	-6.6
6.	pfLDH	-9.6	-8.0	-6.2
7.	Falcipain-2	-7.3	-7.4	-6.3

PC14: Artesunate-procyanidin hybrid molecule, **PC00:** Procyanidin B_2, **AT00:** Artesunate

3.11 Results of Erythrocyte lysis and Plasma Oxidation Assays:

Artesunate-procyanidin hybrid compound did not cause any erythrocyte lysis at the various concentrations tested (Table 19). The Artesunate-procyanidin hybrid compound at 50 µg/mlwas able to protect against the formation of conjugate dienes in the plasma. This protection was at similar magnitude compared to Procyanidin and the various combination ratios. However, this was at a lower magnitude compared to Butylated hydroxytoluene and Ascorbic acid. Lower concentrations of Artesunate-procyanidin hybrid compound had a better protective effect against the formation of plasma dienes compared to its higher concentrations (Figures 71-72).

Table 19: Effects of Artesunate-Procyanidin hybrid compound and combination ratios on erythrocyte lysis

TEST	% Haemolysis
Control (1% SDS)	100.00
Artesunate	0.00
Procyanidin	0.00
PC14	0.00
PC01	0.00
PC02	0.00
PC03	0.00

PC14- Artesunate-Procyanidin hybrid molecule; Artesunate/Procyanidin Combination: **PC01** = Ratio 1:1, **PC02** = Ratio 1:2, **PC03** = Ratio 2:1

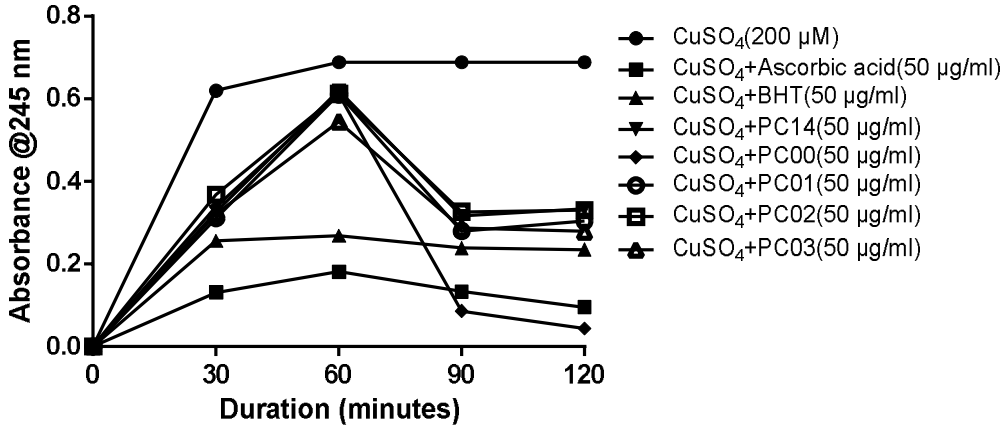


Figure 72: Protective effects of Artesunate-Procyanidin hybrid compound and combination ratios against $CuSO_4$-induced accumulation of conjugated dienes in plasma. Values are means of three determinations. **PC14**- Artesunate-Procyanidin hybrid molecule; **PC00** – Procyanidin; Artesunate/Procyanidin Combination: **PC01** = Ratio 1:1, **PC02** = Ratio 1:2, **PC03** = Ratio 2:1; **BHT**: Butylated hydroxytoluene

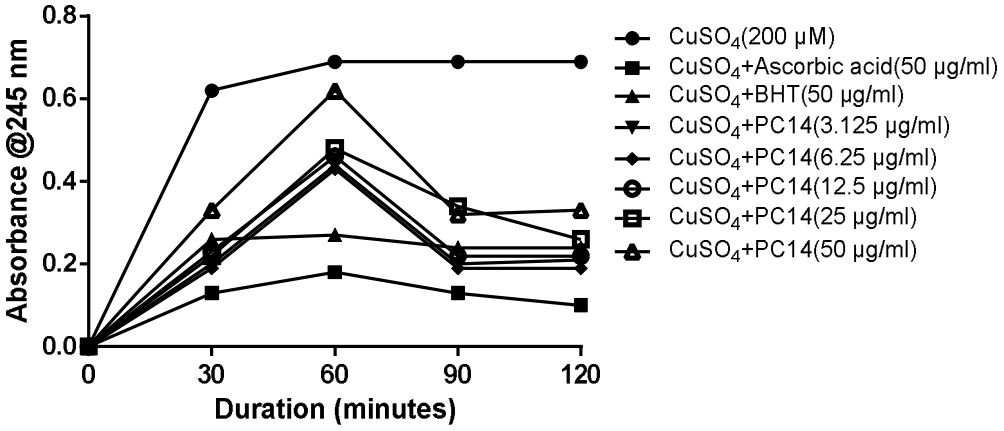


Figure 73: Protective effects of Artesunate-Procyanidin hybrid compound against $CuSO_4$-induced accumulation of conjugated dienes in plasma. Values are means of three determinations. **PC14**- Artesunate-Procyanidin hybrid molecule; **BHT:** Butylated hydroxytoluene

3.12 Results of Toxicological studies

3.13 Organ-body weight ratio

Artesunate-procyanidin hybrid compound at all doses administered did not cause any significant increase ($p>0.05$) in the % organ-body weight of the liver, kidney, heart and brain of experimental animals compared to controls (Table 20).

3.14 Haematological Analysis

The hybrid molecule did not cause any significant change ($p>0.05$) in white Blood Cell (WBC) count, platelet count (PLT), Neutrophils (NEU) and lymphocyte (LYM) after 21 days of administration at various doses compared to controls (Table 21). The hybrid molecule caused no significant change ($p>0.05$) in Red Blood Cell count (RBC), Packed Cell Volume (PCV), Haemoglobin concentration (Hb), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC) after 21 days of administration at all doses range compared to control (Table 22).

Table 20: Percentage Organ-Body weight ratio of mice after 21 days administrations with Artesunate-procyanidin hybrid compound

Treatment (mg/kg bwt.)	Liver (%)	Kidney (%)	Heart (%)	Brain (%)
Control	4.16±0.56[a]	1.35±0.09[a]	0.60±0.04[a]	1.57±0.21[a]
PC14-5	4.75±0.22[a]	1.40±0.11[a]	0.57±0.06[a]	1.51±0.07[a]
PC14-10	4.53±0.08[a]	1.50±0.45[a]	0.52±0.01[a]	1.78±0.10[ab]
PC14-20	4.39±0.22[a]	1.23±0.04[a]	0.52±0.02[a]	1.74±0.11[ab]
PC14-40	3.47±0.36[a]	1.09±0.38[ab]	0.52±0.12[a]	1.56±0.07[a]
PC14-80	4.82±1.19[a]	1.61±0.17[ac]	0.66±0.14[a]	1.59±0.02[a]

Values are Mean ± SEM, n=5, Values with different superscript are significantly different at $p<0.05$. **PC14**: Artesunate-procyanidin hybrid molecule.

Table 21: White blood cell indices and platelet count of experimental animals after 21 days treatment with Artesunate-Procyanidin hybrid compound

Treatment (mg/kg b.wt.)	WBC (/μL)	PLT ($\times 10^3$/μL)	NEU (%)	LYMP (%)
Control	4624.0±121.0^a	572.0±78.0^a	27.0±1.0^a	73.0±3.0^a
PC14-5	3450.0±189.0^a	491.0±28.0^a	28.0±2.0^{ab}	71.0±4.0^a
PC14-10	4069.0±167.0^a	408.0±49.0^a	35.0±2.0^b	65.0±5.0^a
PC14-20	3408.0±462.0^a	528.0±33.0^a	30.0±1.0^b	70.0±6.0^a
PC14-40	4134.0±412.0^a	477.0±56.0^a	34.0±2.0^b	66.0±7.0^a
PC14-80	3370.0±220.0^a	516.0±27.0^a	33.0±1.0^b	67.0±7.0^a

Values are Means ± SEM, n=5. Values with different superscripts are significantly different at $p<0.05$, PC14 – Artesunate-procyanidin hybrid molecule.

Table 22: Red blood cell indices of experimental animals after 21 days treatment with Artesunate-Procyanidin hybrid compound

Treatment (mg/kg b.wt.)	RBC ($\times 10^{12}$/L)	PCV (%)	Hb (g/dL)	MCV (fL)	MCH (pg)	MCHC (g/dL)
Control	8.3±0.3[a]	43.2±2.2[a]	14.5±0.4[a]	54.9±1.3[a]	16.4±0.6[a]	32.7±0.9[a]
PC14-5	7.6±0.4[a]	42.8±1.2[a]	14.9±0.5[a]	55.0±0.4[a]	16.2±0.6[a]	33.2±0.7[a]
PC14-10	8.3±0.3[a]	42.8±1.2[a]	14.5±0.4[a]	55.7±0.4[a]	16.0±0.9[a]	31.8±0.4[a]
PC14-20	7.5±0.3[a]	41.6±1.0[a]	14.7±0.5[a]	55.7±0.4[a]	16.8±0.5[a]	31.8±0.4[a]
PC14-40	7.9±0.1[a]	41.4±0.9[a]	14.5±0.4[a]	55.3±0.4[a]	15.7±0.9[a]	32.0±0.5[a]
PC14-80	7.4±0.4[a]	41.8±1.1[a]	16.5±1.9[a]	55.6±0.6[a]	15.7±0.7[a]	31.8±0.4[a]

Values are Means ± SEM, n=5. Values with the same superscripts are not significantly different at $p>0.05$. PC14 – Artesunate-procyanidin hybrid molecule.

3.15 Liver function indices

The hybrid molecules at all doses did not significantly ($p>0.05$) alter the levels of total protein, globulin, total and conjugated bilirubin after 21 days of administration compared to control (Table 23). However, a significant increase ($p<0.05$) was observed in albumin concentration at 5 mg/kg body weight of the hybrid molecule, with no significant alteration ($p>0.05$) at other dosescompared tocontrol.

3.16 Lipid profiles

Table 24 presents the plasma lipid profile of mice administated different dose of artesunate-procyanindin hybrid molecule. A significant ($p<0.05$) increase was observed in triglyceride level at 5 mg/kg body weight of the hybrid molecule, with no significant alteration ($p>0.05$) at other doses compared to control.The levels of total cholesterol, HDL-cholesterol, LDL-cholesterol and VLDL in the plasma as well as the atherogenic index were not significantly altered ($p>0.05$) at all doses of the hybrid molecule administered compared to controls.

3.17 Kidney function indices

The effects of the administration of artesunate-procyanidin hybrid molecule for 21 days on some electrolytes and biomolecules in the plasmaare shown in Table 25.Administration of the hybrid molecule at doses lower than 80 mg/kg body weight caused a significant increase ($p<0.05$) in plasma chloride ion concentration, with no significant alteration ($p>0.05$) at 80 mg/kg body weight compared to control. Similarly, a significant ($p<0.05$) increase was observed in plasma potassium and uric acid concentrations at 5 mg/kg body weight of the hybrid molecule, with no significant alteration ($p>0.05$) at other doses compared to controls (Tables 25 and 26). The concentrations of calcium, bicarbonate, sodium and phosphate ions were not significantly altered ($p>0.05$) at all doses of the hybrid molecule administered compared to

control. Similarly, there was no significant change ($p>0.05$) in urea and creatinine concentrations at all doses of the hybrid molecule compared to controls.

Table 23: Selected liver function indices of mice after 21 days of administrationofArtesunate-procyanidin hybrid compound

Treatment (mg/kg bw)	Total Protein (g/L)	Albumin (g/L)	Globulin (g/L)	Total bilirubin (µmol/L)	Conjugated bilirubin (µmol/L)
Control	26.87±5.74[a]	11.50±0.50[a]	10.0±2.50[a]	6.05±0.75[a]	4.10±0.35[a]
PC14-5	20.08±1.55[a]	18.00±3.00[b]	6.55±2.05[a]	6.85±0.25[a]	3.80±0.92[a]
PC14-10	23.78±2.38[a]	11.50±1.50[a]	6.45±1.95[a]	5.65±0.55[a]	3.05±0.55[a]
PC14-20	19.05±1.89[a]	11.50±2.50[a]	7.90±2.60[a]	5.25±0.55[a]	2.90±0.33[a]
PC14-40	23.58±2.58[a]	10.00±1.00[a]	8.95±2.55[a]	5.30±0.90[a]	2.85±0.75[a]
PC14-80	19.46±1.56[a]	10.00±0.50[a]	7.90±3.60[a]	5.00±0.10[a]	3.25±0.15[a]

Values are Means ± SEM, n=5, Values with different superscript are significantly different at $p<0.05$. **PC14**: Artesunate-procyanidin hybrid molecule.

Table 24: Effects of Artesunate-procyanidin hybrid molecule on plasma lipid profile of mice after 21 days of administration

Treatment (mg/kg bw.)	Total Cholesterol (mmol/L)	Triglycerides (mmol/L)	High Density Lipoproteins (mmol/L)	Low Density Lipoproteins (mmol/L)	Very Low Density Lipoproteins (mmol/L)	Atherogenic index
Control	2.05±0.05^{ab}	1.00±0.12^{a}	0.65±0.05^{a}	0.95±0.15^{a}	0.20±0.00^{a}	3.18±0.32^{a}
PC14-5	2.25±0.15^{a}	1.50±0.11^{b}	0.70±0.15^{a}	0.90±0.10^{a}	0.30±0.00^{a}	3.21±0.21^{a}
PC14-10	2.05±0.05^{ab}	1.05±0.15^{ab}	0.65±0.05^{a}	0.95±0.05^{a}	0.20±0.00^{a}	3.18±0.32^{a}
PC14-20	1.95±0.15^{ab}	1.00±0.20^{a}	0.60±0.12^{a}	0.95±0.25^{a}	0.20±0.00^{a}	3.39±0.81^{a}
PC14-40	1.90±0.10^{b}	1.15±0.05^{ab}	0.60±0.15^{a}	0.80±0.10^{a}	0.20±0.00^{a}	3.17±0.11^{a}
PC14-80	1.90±0.15^{b}	0.90±0.10^{a}	0.65±0.05^{a}	0.80±0.10^{a}	0.20±0.00^{a}	2.94±0.22^{a}

Values are Means ± SEM, n=5, Values with different superscript are significantly different at $p<0.05$. **PC14**: Artesunate-procyanidin hybrid molecule.

Table 25: Selected kidney function indices of mice administered Artesunate-procyanidin hybrid compound for 21 days

Treatment (mg/kg bwt.)	Ca^{2+} (mg/dL)	Cl^{-} (mmol/L)	HCO_3^{-} (mmol/L)	K^{+} (mmol/L)	Na^{+} (mmol/L)	PO_4^{3-} (mg/dL)	Urea (mmol/L)	Creatinine (µmol/L)
Control	8.50±0.57^{a}	14.50±1.50^{a}	9.00±3.00^{a}	35.75±7.25^{a}	71.50±12.50^{a}	15.50±0.45^{a}	162.09±37.37^{a}	14.10±2.30^{a}
PC14-5	9.25±.074^{a}	34.50±3.50^{c}	10.50±0.50^{a}	80.00±1.00^{b}	51.00±4.00^{a}	15.50±0.98^{a}	174.91±18.34^{a}	21.15±6.85^{a}
PC14-10	8.00±1.20^{a}	21.50±4.50^{b}	9.50±2.50^{a}	39.25±7.25^{ab}	85.00±5.00^{a}	16.00±0.49^{a}	165.10±17.74^{a}	13.55±3.85^{a}
PC14-20	9.50±1.00^{a}	26.50±1.50^{b}	7.50±1.50^{a}	50.75±3.00^{ab}	53.00±8.00^{a}	16.00±0.90^{a}	172.64±17.21^{a}	24.15±0.95^{a}
PC14-40	8.57±0.97^{a}	25.00±1.00^{b}	13.50±1.50^{a}	62.00±2.00^{ab}	61.00±3.00^{a}	17.00±0.48^{a}	166.61±08.21^{a}	19.50±2.70^{a}
PC14-80	9.70±1.90^{a}	16.00±2.00^{a}	10.50±0.50^{a}	26.00±2.00^{a}	83.00±3.00^{a}	16.50±0.66^{a}	140.98±10.43^{a}	10.05±5.35^{a}

Values are Means ± SEM, n=5, Values with different superscript are significantly different at $p<0.05$. **PC14**: Artesunate-procyanidin hybrid molecule.

Table 26: Effects of artesunate-procyanidin hybrid compound on plasma uric acid levels in mice after 21 days administration

Treatment (mg/kg bwt.)	Uric Acid (µmol/L)
CONTROL	296.50±31.50[a]
PC14-5	492.00±73.00[b]
PC14-10	303.50±53.50[a]
PC14-20	281.00±88.00[a]
PC14-40	282.50±29.50[a]
PC14-80	221.50±44.50[a]

Values are Means ± SEM, n=5, Values with different superscript are significantly different at $p<0.05$. **PC14**: Artesunate-procyanidin hybrid molecule.

3.18 Enzyme Studied

3.18.1 Aspartate Aminotransferase

Administration of Artesunate-procyanidin hybrid molecule at various doses for 21 days did not cause any significant change ($p>0.05$) in the specific activities of aspartate aminotransferase in the liver, heart and plasma compared to controls (Figure 73).

3.18.2 Alanine Aminotransferase

There was no significant change ($p>0.05$) in the activity of alanine aminotransferase (ALT) in the plasma after 21 days of administration of various doses of artesunate-procyanidin hybrid molecule compared to control (Figure 74). However, there was significant increase ($p<0.05$) in liver ALT activity at various doses of the hybrid molecule compared to control. There was significant decrease ($p<0.05$) in heart ALT activity at 5 and 10 mg/kg body weight of the hybrid molecule with no significant alteration ($p>0.05$) at other doses compared to control (Figure 74).

3.18.3 Alkaline Phosphatase

There was no significant change ($p>0.05$) in alkaline phosphatase activities in the liver, kidney, heart, brain as well as the plasma after 21 days of administration of various doses of artesunate-procyanidin hybrid molecule compared to controls (Figure 75).

3.18.4 Glutamate Dehydrogenase Activity

After 21 days of administration of artesunate-procyanidin hybrid molecule to experimental animals, no significant change ($p>0.05$) was observed in the liver and plasma Glutamate dehydrogenase (GDH) activities of miceat all doses compared to control (Figure 76).

3.18.5 γ-Glutamyl transferase Activity

After 21 days of administration of various doses of Artesunate-procyanidin hybrid molecule to experimental animals, no significant change ($p>0.05$) was observed in γ –Glutamyl transferase activities of the liver and kidney compared to controls (Figure 77). A significant increase ($p<0.05$) in plasma γ –Glutamyl transferase activity was observed at 5 mg/kg body weight of hybrid molecule with no significant change ($p>0.05$) at other doses compared to control.

3.18.6 Lactate Dehydrogenase

Administration of Artesunate-procyanidin hybrid molecule for 21 days to experimental animals did not cause any significant change ($p>0.05$) in heart and plasma lactate dehydrogenase (LDH) activities at all doses compared to control (Figure 78).

3.18.7 Acetylcholine esterase activities

No significant change($p>0.05$) was observed in acetylcholine esterase activity in the brain after 21 days of administration of various doses of artesunate-procyanidin hybrid molecule compared to control (Figure79).

3.18.8 Creatine kinase activities

No significant change($p>0.05$) was observed in creatine kinase activities in the heart and plasma of mice after 21 days of administration of various doses of artesunate-procyanidin hybrid molecule compared to controls (Figure 80). A significant increase ($p<0.05$) was observed in brain creatine kinase at 40 mg/kg body weight of hybrid molecule with no significant alteration ($p>0.05$) at other doses compared to control.

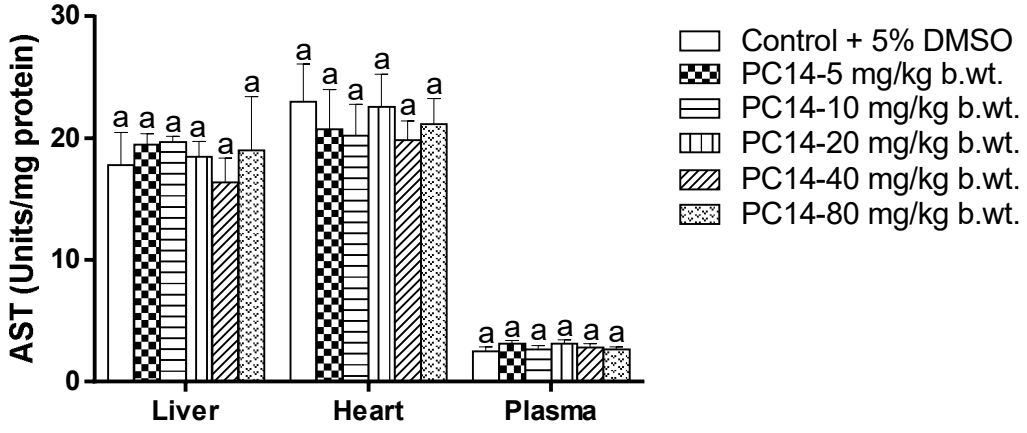


Figure 74: Aspartate aminotransferase activities in plasma and tissues of mice after 21 days administration of Artesunate-procyanidin hybrid compound. Values are Means ± SEM, n=5, Values with different superscript are significantly different at $p<0.05$. **PC14**: Artesunate-procyanidin hybrid molecule.

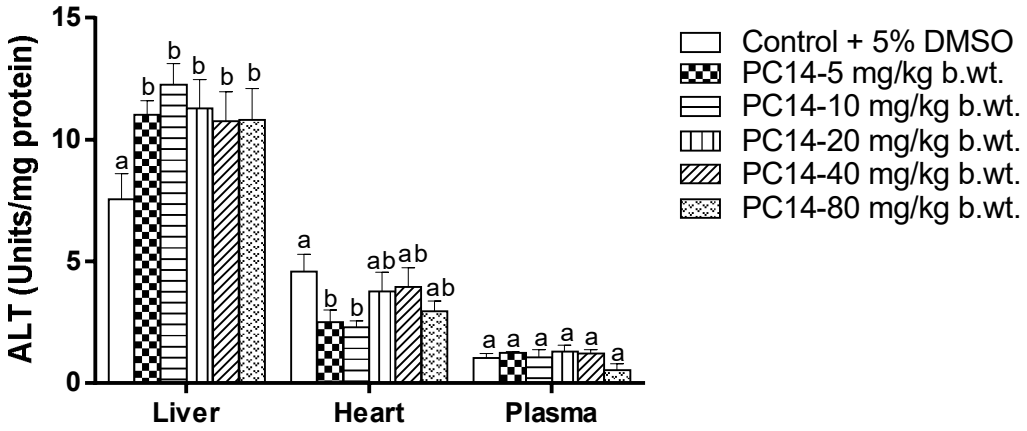


Figure 75: Alanine aminotransferase activities in plasma and tissues of mice after 21 days administration of Artesunate-procyanidin hybrid compound. Values are Means ± SEM, n=5, Values with different superscript are significantly different at $p<0.05$. **PC14**: Artesunate-procyanidin hybrid molecule.

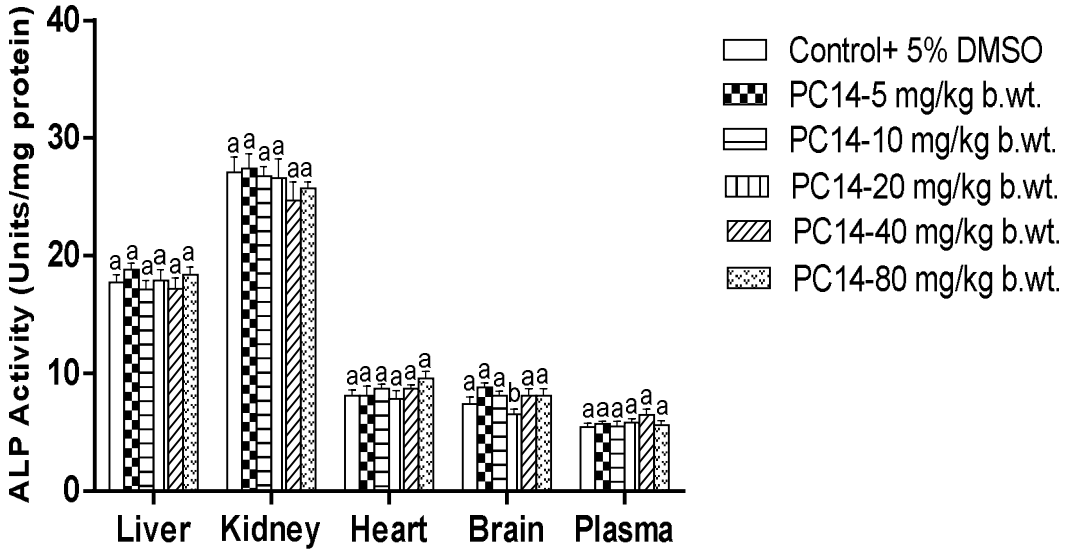


Figure 76: Alkaline phosphatase activities in tissues and plasma of mice after 21 days administration of Artesunate-procyanidin hybrid compound. Values are Means ± SEM, n=5, Values with different superscript are significantly different at $p<0.05$. **PC14**: Artesunate-procyanidin hybrid molecule.

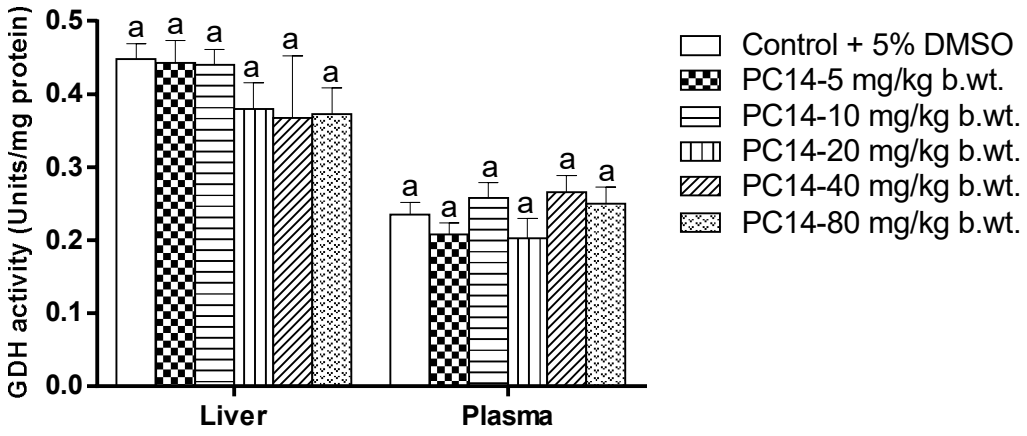


Figure 77: Glutamate dehydrogenase activities in tissues and plasma of mice after 21 days administration of Artesunate-procyanidin hybrid compound. Values are Means ± SEM, n=5, Values with different superscript are significantly different at $p<0.05$. **PC14**: Artesunate-procyanidin hybrid molecule.

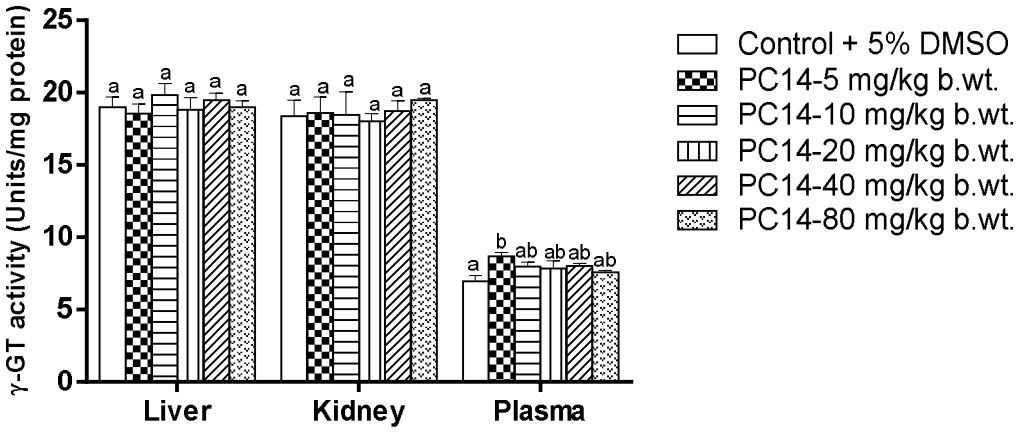


Figure 78: γ –Glutamyl transferase activities in tissues and plasma of mice after 21 days administration of Artesunate-procyanidin hybrid compound. Values are Means ± SEM, n=5, Values with different superscript are significantly different at $p<0.05$. **PC14**: Artesunate-procyanidin hybrid molecule.

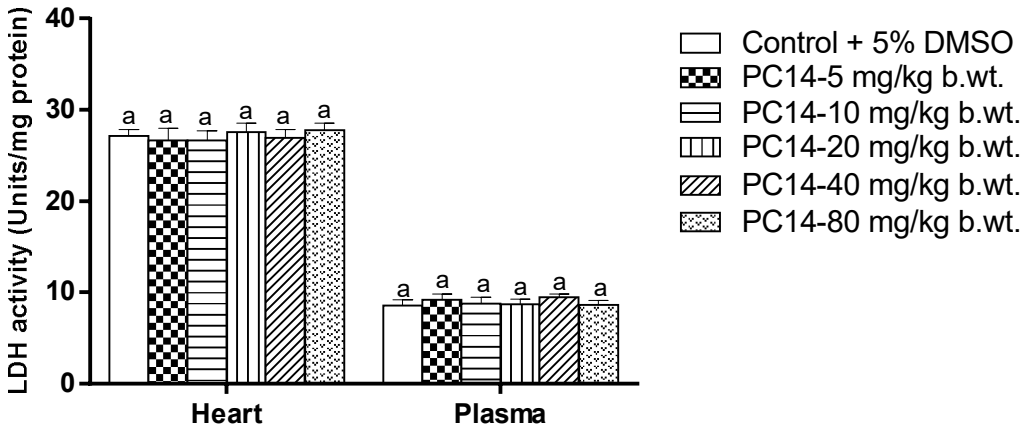


Figure 79: Lactate dehydrogenase activities in tissues and plasma of mice after 21 days administration of Artesunate-procyanidin hybrid compound. Values are Means ± SEM, n=5, Values with different superscript are significantly different at $p<0.05$. **PC14**: Artesunate-procyanidin hybrid molecule.

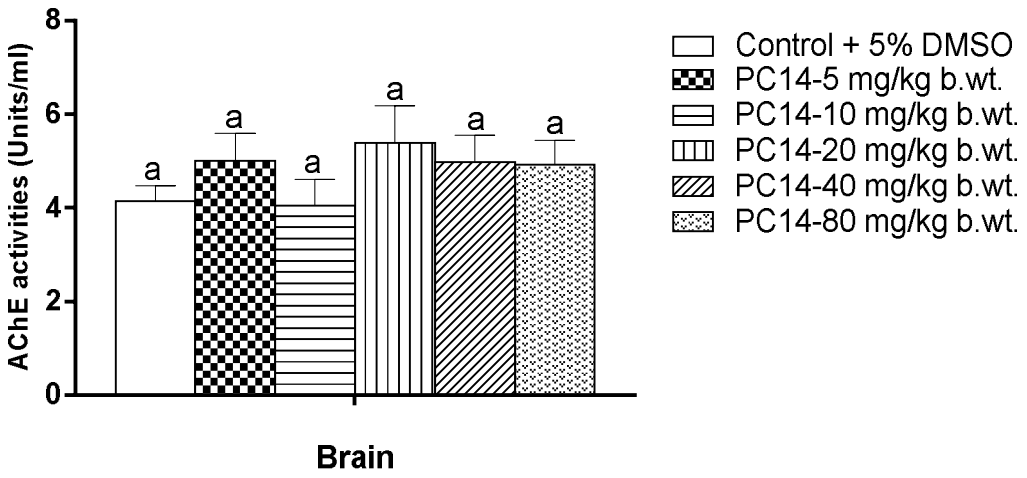


Figure 80: Acetylcholine esterase activities in brain of mice after 21 days administration of Artesunate-procyanidin hybrid compound. Values are Means ± SEM, n=5, Values with different superscript are significantly different at $p<0.05$. **PC14**: Artesunate-procyanidin hybrid molecule.

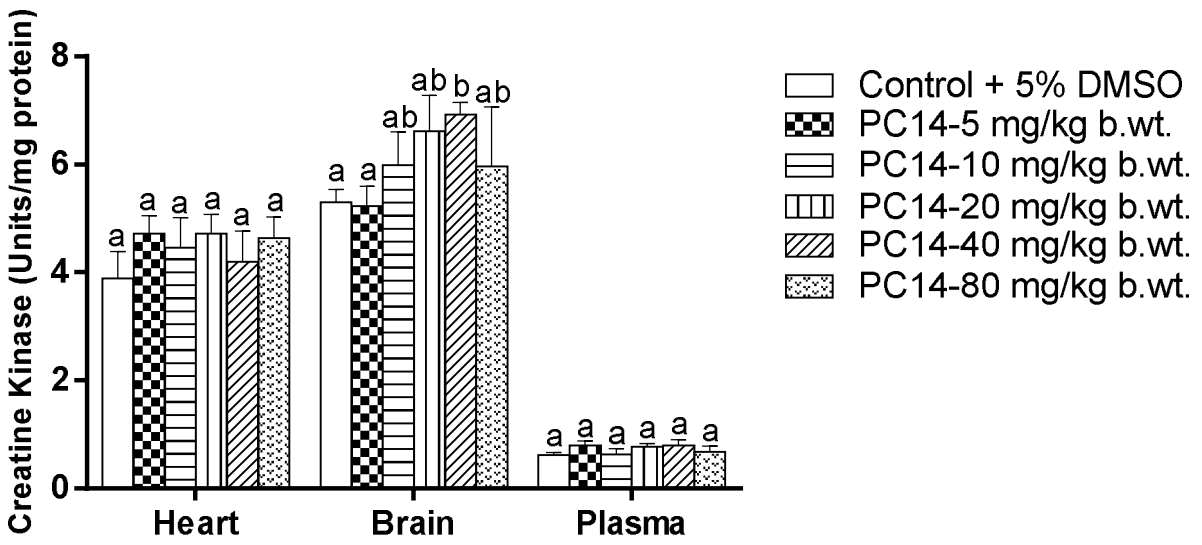


Figure 81: Creatine kinase activities in tissues and plasma of mice after 21 days administration of Artesunate-procyanidin hybrid compound. Values are Means ± SEM, n=5, Values with different superscript are significantly different at $p<0.05$. **PC14**: Artesunate-procyanidin hybrid molecule.

3.18.9 ATPase activities

3.18.9.1 Mg^{2+} - ATPase

The activities of Mg^{2+} -ATPase in kidney and heart after 21 days of administration ofvarious doses of artesunate-procyanidin hybrid molecule were not significantlychanged ($p>0.05$) compared to controls (Figure 81). There was significant decrease ($p<0.05$) in brain Mg^{2+} -ATPaseat all doses of artesunate-procyanidin hybrid molecule compared to control (Figure 81).

3.18.9.2 Ca^{2+}, Mg^{2+} - ATPase

No significant change ($p>0.05$) was observed in kidney and brain Ca^{2+}, Mg^{2+} - ATPase activities after 21 days of administration of various doses of artesunate-procyanidin hybrid molecule compared to controls (Figure 82). However, a significant decrease ($p<0.05$) in heart Ca^{2+}, Mg^{2+} - ATPase activity was observed at 5 and 10 mg/kg body weight of thehybrid molecule with no significant change ($p>0.05$) at higher doses compared to control.

3.18.9.3 Na^{+}, K^{+} - ATPase

Artesunate-procyanidin hybrid molecule did not cause any significant change ($p>0.05$) in Na^{+}, K^{+} - ATPase activity in the kidney at all doses administered compared to control (Figure 83). There was no significant change ($p>0.05$) in heart Na^{+}, K^{+} - ATPase activityat 20, 40 and 80 mg/kg body weight of the hybrid molecule compared to control. A significant decrease ($p<0.05$) in Na^{+}, K^{+} - ATPase activity in the brain was observed at all doses of the hybrid molecule compared to control.

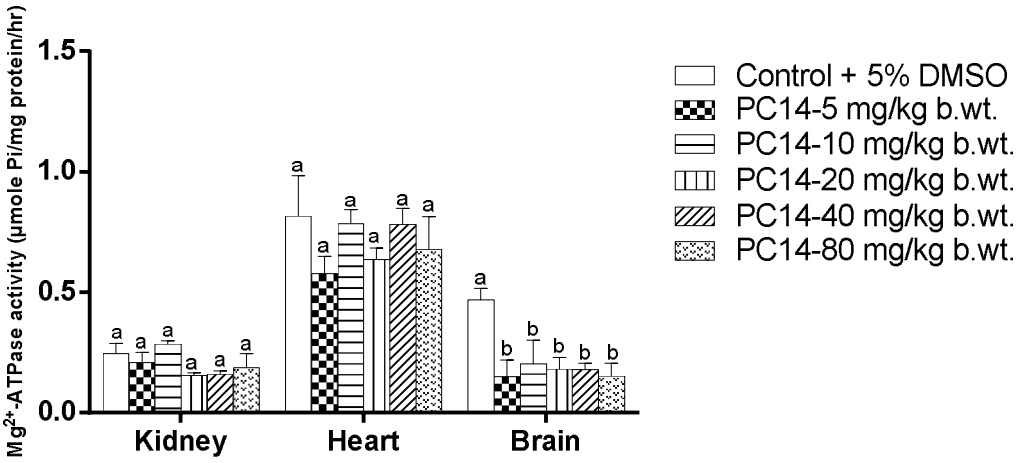


Figure 82: **Mg^{2+}-ATPase activities in selected tissues of mice after 21 days of administration of Artesunate-procyanidin hybrid compound.** Values are Means ± SEM, n=5, Values with different superscript are significantly different at $p<0.05$. **PC14**: Artesunate-procyanidin hybrid molecule.

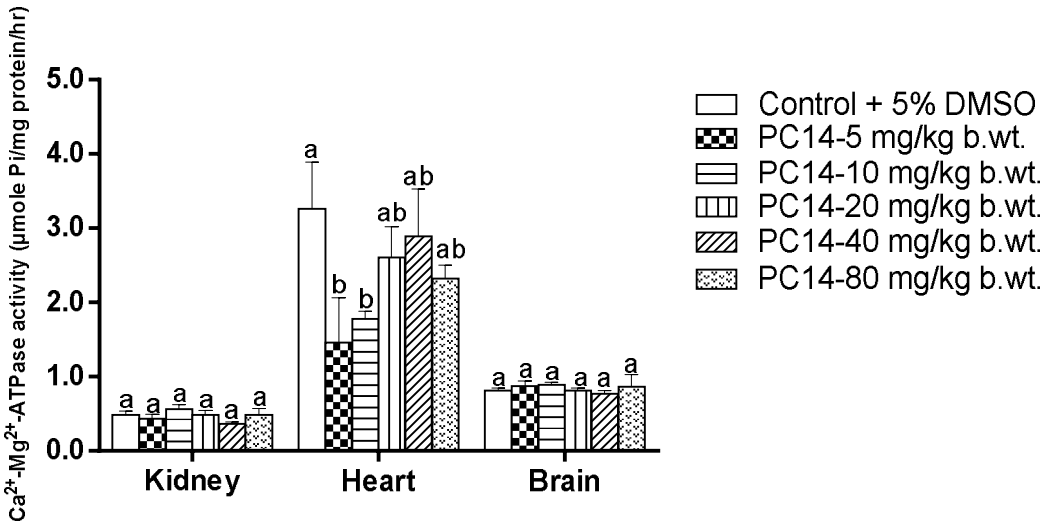


Figure 83: Ca^{2+}-Mg^{2+}-ATPase activities in selected tissues of mice after 21 days of administration of Artesunate-procyanidin hybrid compound. Values are Means ± SEM, n=5, Values with different superscript are significantly different at $p<0.05$. **PC14**: Artesunate-procyanidin hybrid molecule.

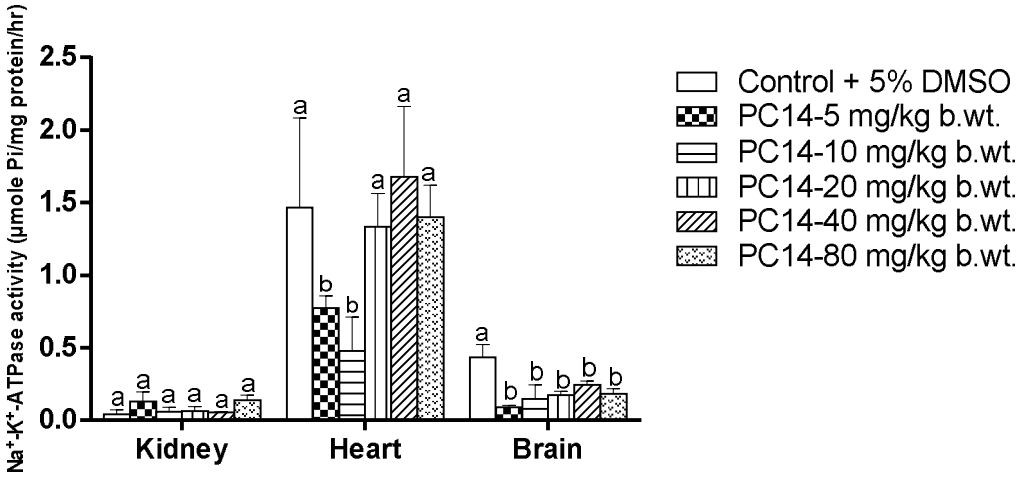


Figure 84: Na^+-K^+-ATPase activities in selected tissues of mice after 21 days of administration of Artesunate-procyanidin hybrid compound. Values are Means ± SEM, n=5, Values with different superscript are significantly different at $p<0.05$. **PC14**: Artesunate-procyanidin hybrid molecule.

3.18.10 Results of Histopathological Studies

There was no structural alteration observed in the cross-sections of the various tissues (including the liver, kidney, heart and brain) of mice administered the various doses of artesunate-procyanidin hybrid molecule compared to those of control (Plates 1-4).

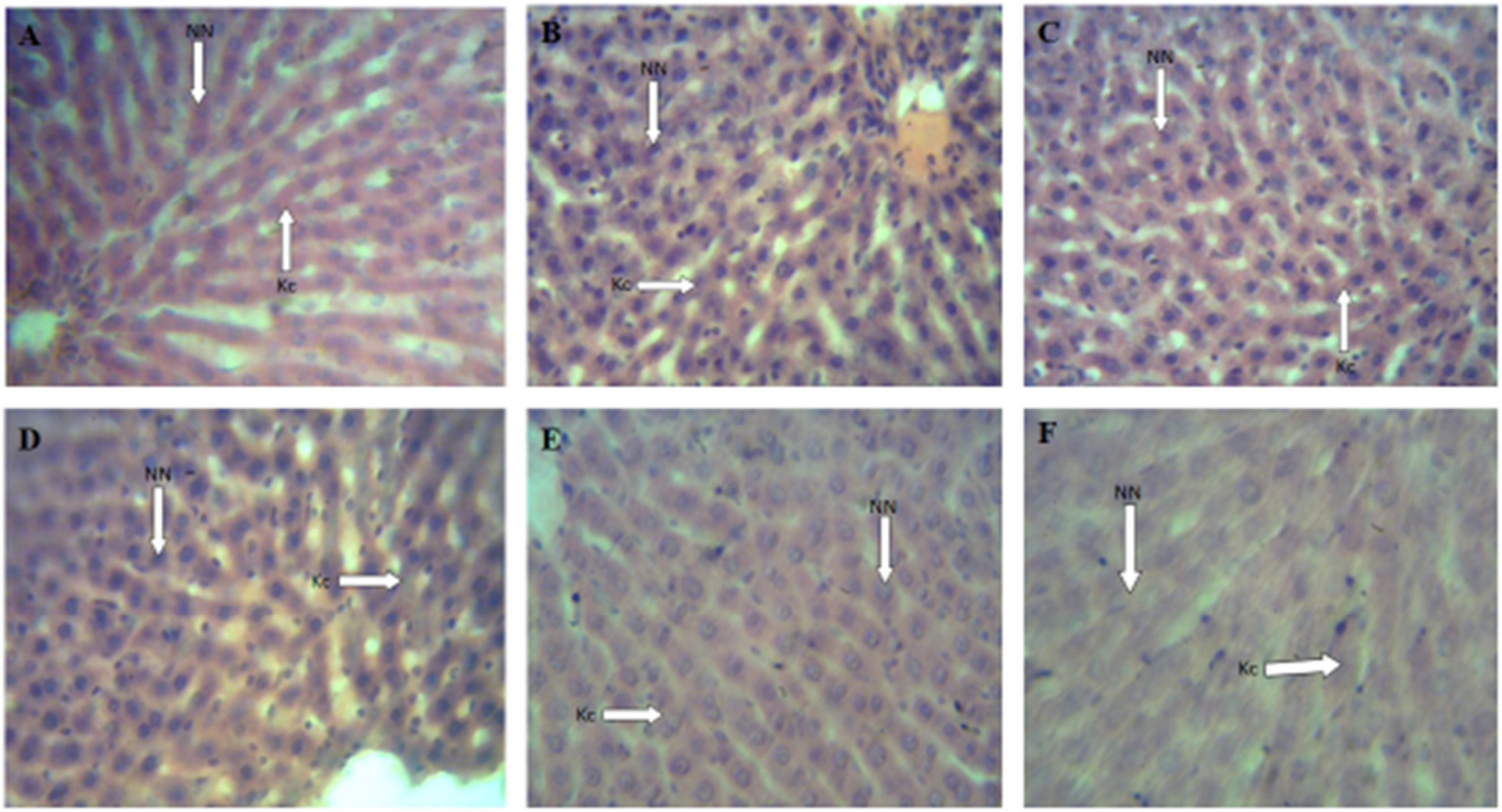


Plate 1: Photomicrograph of the liver of mice administered various doses of Artesunate-procyanidin hybrid molecule for 21 days (H and E; x400). A: 5% DMSO; B: 5 mg/kg body weight; C: 10 mg/kg body weight; D: 20 mg/kg body weight; E: 40 mg/kg body weight; F: 80 mg/kg body weight respectively. NN = normal nuclei, Kc = Kupffer cell. The photomicrograph revealed no visible change in the architecture of the tissues when compared to control animal.

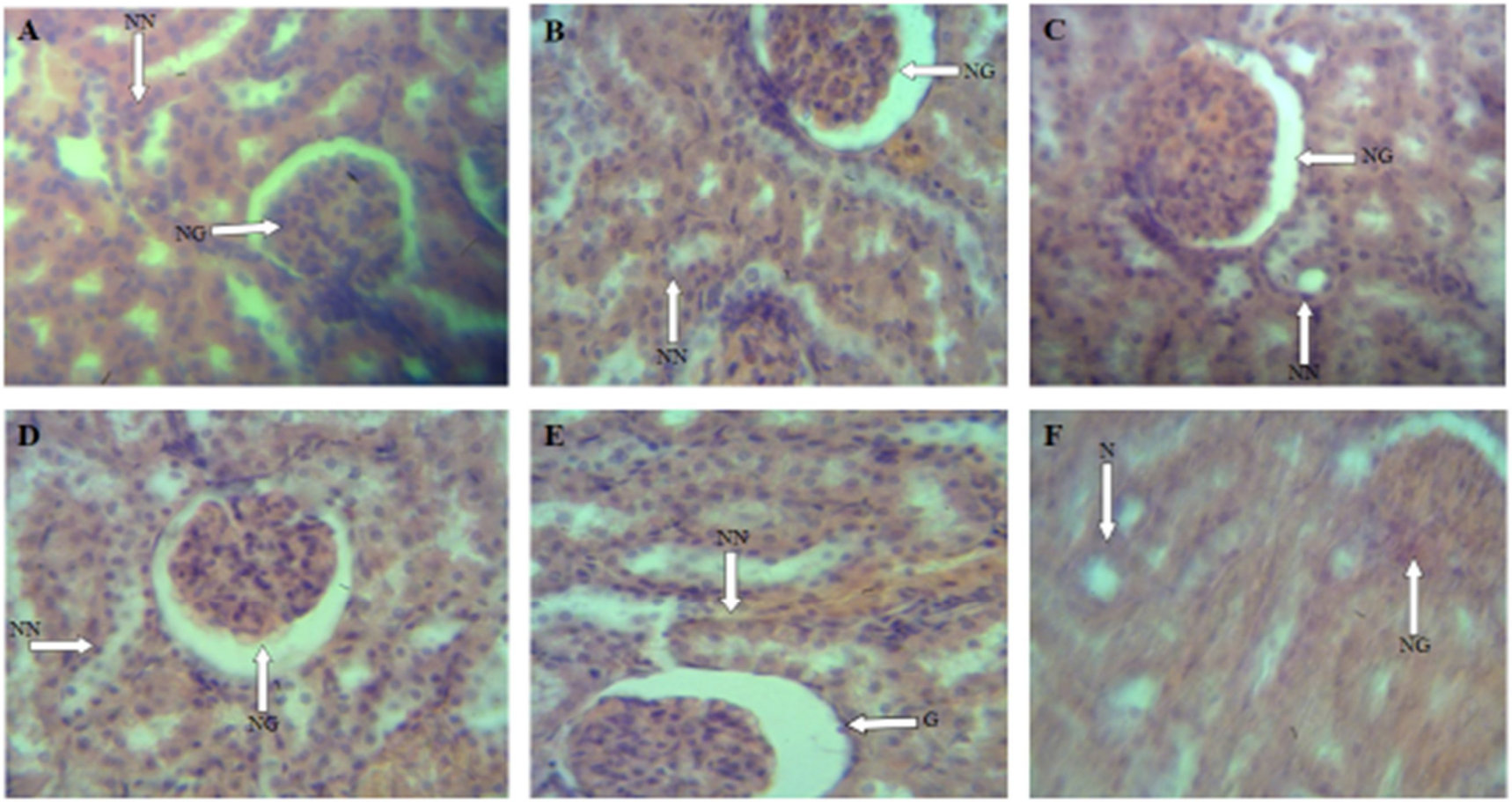


Plate 2: Photomicrograph of the kidney of mice administered various doses of Artesunate-procyanidin hybrid molecule for 21 days (H and E; x400). A: 5% DMSO; B: 5 mg/kg body weight; C: 10 mg/kg body weight; D: 20 mg/kg body weight; E: 40 mg/kg body weight; F: 80 mg/kg body weight respectively. NN = normal nuclei, NG = normal glomeruli, G = glomeruli. The photomicrograph revealed no visible change in the architecture of the tissues when compared to control animal.

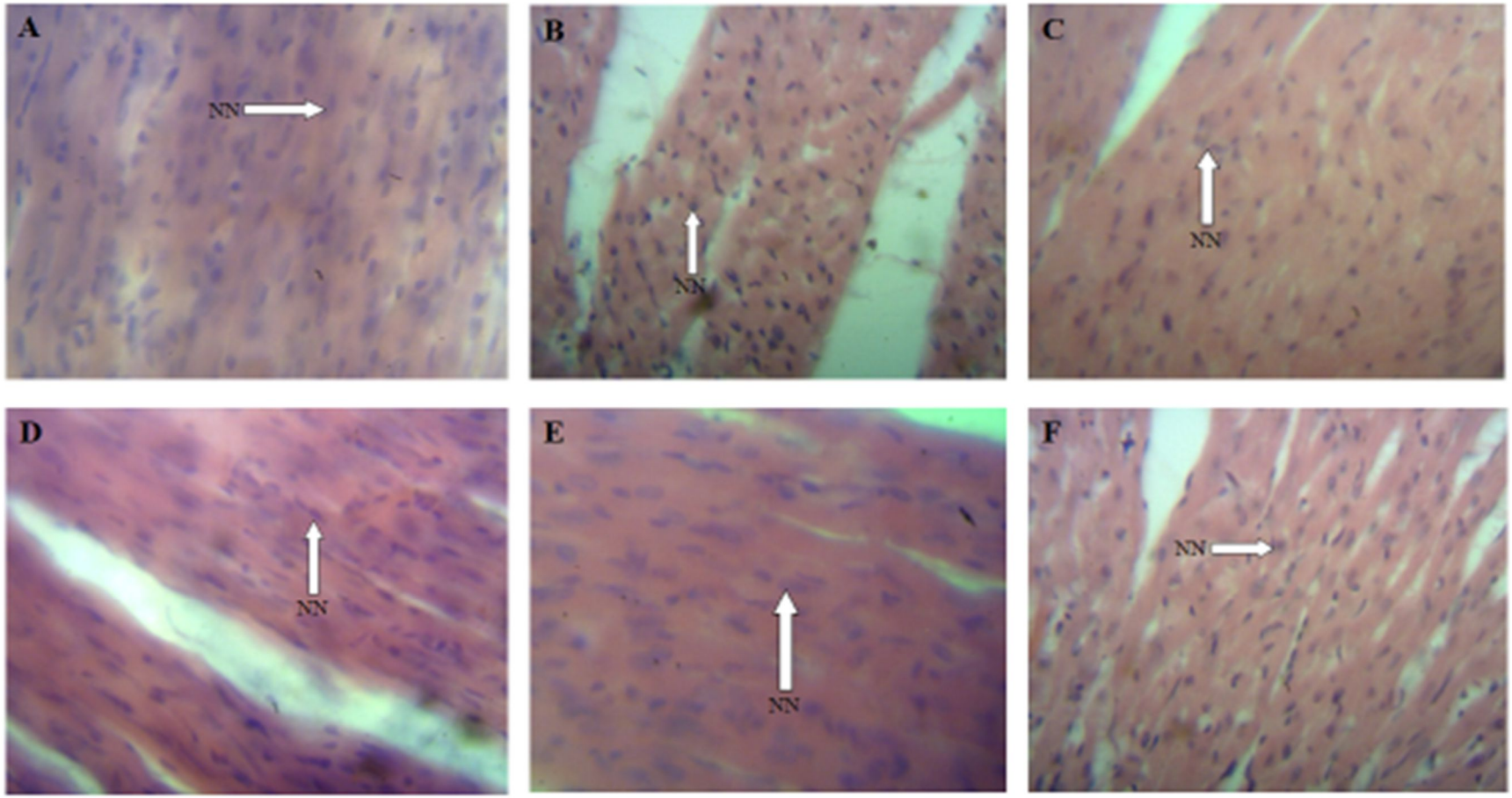


Plate 3: Photomicrograph of the heart of mice administered various doses of Artesunate-procyanidin hybrid molecule for 21 days (H and E; x400). A: 5% DMSO; B: 5 mg/kg body weight; C: 10 mg/kg body weight; D: 20 mg/kg body weight; E: 40 mg/kg body weight; F: 80 mg/kg body weight respectively. NN = normal nuclei. The photomicrograph revealed no visible change in the architecture of the tissues when compared to control animal.

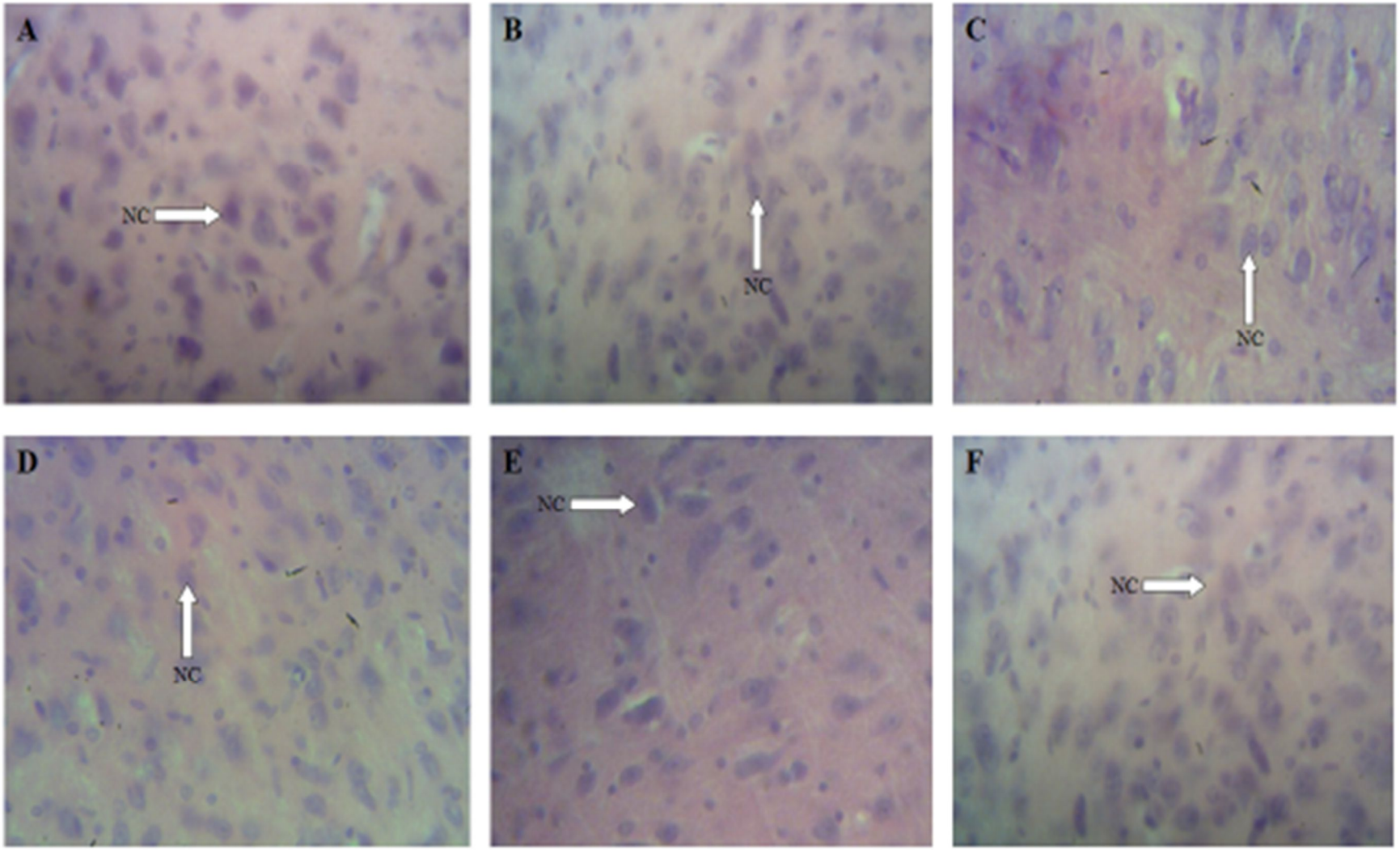


Plate 4: Photomicrograph of the brain of mice administered various doses of Artesunate-procyanidin hybrid molecule for 21 days (H and E; x400). A: 5% DMSO; B: 5 mg/kg body weight; C: 10 mg/kg body weight; D: 20 mg/kg body weight; E: 40 mg/kg body weight; F: 80 mg/kg body weight respectively. NC = normal brain cell. The photomicrograph revealed no visible change in the architecture of the tissues when compared to control animal.

CHAPTER FOUR

4. 0 Discussion

4.1 Synthesized artesunate-procyanidin hybrid molecule.

The NMR spectra showed that the endoperoxide bridge of the hybrid compound remained intact while the IR spectra indicated the presence of the hydroxyl groups and a functional carbonyl group. This suggests that the involvement of the endoperoxide bridge of artesunate in the antimalarial activity of the compound was not adversely affected. One of the mechanisms of action of artesunate proposed by Meshnick (2002) involve heme-catalyzed cleavage of the endoperoxide bridge forming a carbon-centred radical followed by specific and selective alkylation of some parasite proteins (Yang *et al.,* 1993).

The new hybrid compound expressed a slight displacement in the transmittance of hydroxyl group, carbonyl group and the carbon-carbon double bond. However, the carbon-hydrogen, carbon-carbon single bond and most part of the finger print region seem to be unaffected by the process of preparing theartesunate-procyanidin (PC14) hybrid molecule. The results also revealed that they two molecules were linked together by an ester bond in a 1:1 stoichiometry. This is of a great advantage because having another stoichiometry higherthan this may adversely affect the absorption the drug in the intestine.

4.2Physical properties of hybrid molecule

4.2.1 Melting Point and clog

The hybrid compound had a lower melting point compared to Procyanidin and Artesunate (Table 4), but higher than that of chloroquine. The compound had a similar sharp melting point noticed in the parent compound (procyanidin). The melting point of the hybrid molecule suggests that it is a stable drug at room temperature.

An ideal drug candidate according to Lipinski's rule must possess characteristics such as molecular weight less than 500 g/mol, calculated Log P value less than 5, Number of hydrogen bond acceptors (sum of N and O atoms) less than 10, Number of hydrogen bond donors (sum of OH and NH groups) less than 5 and Number of rotatable bonds not more than 10 (Araújo de Brito, 2011). Similarly, Clog P value is among the parameters that affect drug absorption, distribution, metabolism, excretion and toxicity (ADMET); others include Distribution coefficient (Log D), hydrogen bonding, ionizability (pKa), lipophilicity, permeability and solubility. Clog P depends on molecular weight of a compound, its polarity or hydrogen bonding properties. High clop P depicts poor solubility (high lipophilicity) and it is associated with poor permeability which in turn affects absorption of a drug. A moderate log P ranging between 0 – 3 is desirable for good gastrointestinal absorption and good oral bioavailability of a drug (Kerns and Di, 2008). Artesunate and Procyanidin had good clog P values that were below 3.0 (Table 4) but the hybrid molecule however had a higher value similar to that of Chloroquine. This suggests that the parent compounds are less lipophilic than the hybrid compound.

4.2.2 Solubility

A highly soluble drug (paracetamol) with solubility greater than 100 µM and a poorly soluble drug (Ketoconazole) with solubility of lower than 2 µM were used in assessing the solubility of the synthesized hybrid compound. The solubility of Artesunate-procyanidin hybrid molecule was similar to that of Artesunate, Procyanidin and the 1:1 combination ratio (Figure 17). Increase in the ratio of any of the starting material decreased solubility of the compound. The hybrid molecule can be considered as a soluble compound based on the values obtained in the solubility assay. Its water solubility was above the recommended minimum value (>20 µg/ml) for drug-like compounds for oral administration (Lipinski *et al*., 1997). Drug solubility is

required for choice of the route of administration of the drug. Most antimalarial drugs are administered through oral route to maximize compliance and reduce auxiliary care for patients (Ray, 2010). The results obtained for the hybrid molecule suggests that the oral route will be the preferred route of administration of the drug.

4.3 *In vitro* Antiplasmodial Activity

Artesunate is a semisynthetic drug that was obtained from a two-step synthesis from artesmisinin using succinic acid as an alkyl group. Due to its fast action as an antimalarial drug, it has been recommended by WHO for use in combination therapy. Procyanidin is a flavonoid, and contains hydroxyl groups, which contribute to its antioxidant activities (Ayoola *et al.,* 2008). Flavonoids have also been shown to exhibit antiparasitic activity, including antiplasmodial activity (Perez-Victoria *et al.,* 2001; Lehane and Saliba, 2008). However, from the results obtained in this study, procyanidin was found to be inactive against *P. falciparum* W2 (choloroquine-resistant) *in vitro*, because its IC_{50} was greater than the 25 µg/mL reference value (Table 5) (Adebayo and Krettli, 2011).However, artesunate, the hybrid molecule and the three combination ratios were active against *P. falciparum* W2 *in vitro*. This suggests that the hybrid molecule may be active against chloroquine-resistant strains as artesunate. Moreover, the hybrid molecule was less toxic to Buffalo Green Monkey kidney cell line (BGM) than the parent compounds and the combination ratios. Its high selectivity index qualifies it to be considered as a candidate for rational drug design. The combination ratios also had high selectivity indices with the 1:1 ratio having the highest. This suggests that they may also be considered for rational drug design.

4.4 Inhibition of β-Hematin Formation

Malaria parasitedepends mainly on host haemoglobin as its amino acid source. The catabolism of haemoglobin leads to the release of heme (which is toxic to the parasite) which the parasite polymerizes to form hemozoin in its food vacuole.It has been demonstrated that at least 95% of the heme released in the parasite is converted to hemozoin (Egan, 2008). Blocking this detoxification pathway kills the parasite (Egan, 2008). The results of this study revealed that artesunate, procyanidin, the hybrid molecule and the combination ratios were more effective in inhibiting β-hematin formation than chloroquine (Table 6). However, the parent compounds and the combination ratios were more effective that the hybrid molecule. Nevertheless, the results still suggest that the hybrid molecule may have the inhibition of hemozoin formation as one of its mechanism of action.

The evaluation of synergistic/antagonistic or additive interactions of the drug combinations (Berenbaum *et al*., 1980; Ohrt *et al* 2002; He *et al*., 2010), revealed that a synergetic activity was observed in the combination ratio 1:1 with a Fractional Inhibitory Concentrations (FIC) value of less than 1 (Table 7). The other combination ratios of 1:2 and 2:1 expressedFIC values which were higher than 1, suggesting antagonistic interaction.

4.5 *In vivo* antimalarial activity

Two models (a 4-day suppressive test and Rane curative test) were employed in assessing of the *in vivo* antimalarial activities of Artesunate-procyanidin hybrid molecule and the various ratios. The drugs exhibited good chemosuppression at the different doses used (Tables 8-11). Artesunate is an established antimalarial compound known for effective action against the erythrocytic stage of the disease. It is also widely used in combinations with other drugs in form of ACTs. Artesunate inhibits DNA synthesis which reduces the growth of the parasite; it also

reduces the energy process of the parasite by inhibiting respiratory process of the parasite (Yi *et al*., 1987). Major concerns arising from the use of artesunate or other members of the artemisinins include possible development of resistance by the parasite. This has prompted researchers to search for new hybrid compounds with better antimalarial activity as well as ability to delay development of resistance to the artemisinins. In this study,artesunate-procyanidin hybrid compound exhibited a better suppressive antimalarial activity than the combination ratioswhile it favourably compared with artesunate and procyanidin (Table 8). The results suggest that the mechanisms of action of the parent compounds may still be retained by the hybrid molecule, though it may also exhibit new ones.Structures of both parent compounds are key to their antimalarial activities. Artesunate has an endoperoxide bridge which when destroyed renders its antimalarial activity void. In addition, procyanidin is a flavonoid with aromatic rings and about 10 hydroxyl groups. Increase in hydroxyl groups implies increase in antioxidant activity. Thus, thesemoieties of the parent compounds may be responsible for the antimalarial activity of the hybrid molecule.

The hybrid molecule was also active in the curative test comparing favourably well with artesunate and procyanidin (Table 11). The results obtained in this study corroborate earlier reports that procyanidin exhibited antimalarial activity against multidrug pyrimethamine/chloroqiune resistant strains (Attioua *et al*., 2011). Other flavonoids such as diglycosides, monoglycosides and myristicyclins A and Bhave also been reported to retard malaria parasite life cycle, inhibiting various stages ofthe parasite (Murakami, 2003).

4.6 Antioxidant activity

4.6.1 *In vitro* Antioxidant Activity

Progression in malaria conditions is associated with increase in generation of reactive oxygen species (ROS). Antioxidants are helpful in neutralizing the possible negative effects of excess ROS or RNS. Researchers have reported the important roles antioxidants play in the prevention of several diseases including atherosclerosis, cardiovascular diseases and cancer (Hercberg *et al*., 1998; Johnson, 2002; Trumbeckaite *et al*., 2006). In this study, the ability of the hybrid molecule to scavenge free radicals was evaluated. The various combination ratios, artesunate-procyanidin hybrid molecule and parent compounds exhibited good antioxidant activity*in vitro* (Table 14). Their antioxidant properties were comparable to the reference compound, butylated hydroxytoluene (BHT). The hybrid molecule had lower TAC compared to the reference compound. However, the hybrid molecule exhibited higher Ferric reducing powerand DPPH radical scavenging activity compared to BHT. These strong *in vitro* antioxidant properties of the hybrid molecule could derive its source from the functional groups of the individual parent compounds, most especially the hydroxyl groups of procyanidin. Procyanidins havehigh antioxidant activity, being good scavengers of reactive oxygen species (Ariga and Hamano, 1990; Hagerman *et al*., 1998). Procyanidin-rich fractions, found in grape seed or pine bark extract, consumed over a longtimegave rise to high plasma antioxidant capacity (Busserolles *et al*., 2006) with no harmful effects on normal human cells (Ugartondo *et al*., 2007). Factors modulating these antioxidant activities include number of hydroxyl groups, methoxy esters, carbohydrate moieties and phenolic units (Kelly *et al*., 2002; Kumar *et al*., 2013).

4.6.2 *In vivo* antioxidant activity

The imbalance between the productions of oxidants and responses of the antioxidant defenses system is termed oxidative stress.*Plasmodium* species infections are associated with oxidative stress with depletion of the antioxidants (Das *et al*., 1993). Overproduction of reactive oxygen species (ROS)can be toxic to cells leading to oxidation of important macromolecules including lipids, proteins and nucleic acids and cellular and tissue damage (Bowen, 2015). Induction of malaria in this study, led to increasein MDA levels in all the tissues and RBC on days 6 and 10 post inoculation. Earlier reports have indicated this increase during malaria episodeseven with treatment with artesunate and chloroquine (Iribhogbe *et al*., 2012). The hybrid molecule was able to significantly reverse the increase in MDA level in the RBC at all doses administered compared to uninfected control on days 6 and 10. The hybrid molecule was also able to alleviate the increase in MDA level in the kidney and liver at 80 mg/kg body on day 6 post-inoculation and in the heart at 40 and 80 mg/kg body weight on day 10 post-inoculation (Figure 21-25). The results suggest that the pronounced protective effect of the hybrid molecule observed in the RBC compared to other tissues may be due to access of RBC to higher concentration of the hybrid molecule.The ability of the drug to directly kill the parasite in the RBC may prevent generation of free radicals that will lead to lipid peroxidation.Secondly, flavonoids exhibit protective effect against oxidative damage(Kumar*et al*., 2013; Kumar and Pandey, 2012). Thus, the procyanidin moiety of the hybrid molecule may also contribute immensely to this protection against lipid peroxidation. Procyanidins provide high antioxidant capacity, at concentrations, which are not harmful to normal human cells (Ugartondo *et al*., 2007). Procyanidin B2 scavenges hydroxyl radical and superoxide anions better than epicatechin, its building unit (Li and Jiang, 2007). The free 3-hydroxyl group on the aromatic

ring is key to their antioxidant properties as reports have indicated that the removal of the free 3OH group annuls the co-planarity and conjugation properties of flavonoid units, which compromises the scavenging ability (Bors *et al*., 1990).

Nitrite and nitrate are final products of nitric oxide oxidation pathway, and are used as index of systemic nitric oxide production (Giustarini *et al.*, 2008). Nitrite levels in the RBC and heart were not significantly changedthroughout the experimental periodcompared to controls (Figures 26 and 29). Earlier reports revealed no relationship between plasma nitrite and malaria (Agbenyega *et al*., 1997). However, significant increase was observed in nitrite level of the brain of the untreated infected mice compared to control which the hybrid molecule at all doseswas unable to reverse but exacerbate compared to control. This suggests that nitric oxide playeda prominent role in the oxidative stress imposed on the brain, which was made evident by increased brain MDA level.The increased nitrite level at higher doses of the hybrid molecule in the liver on day 10 post-inoculation and in the kidney on day 6 post-inoculation without corresponding increase in those of the untreated uninfected group suggests another mechanism by which nitric oxide is generated apart from the infection at those doses (Ono *et al*., 2003; Ramiro *et al*., 2005; Hämäläinen *et al*., 2007).

Antioxidant status remains one of the frequently assessed parameters in malaria conditions; they are used as makers during the progress or otherwise of the infection. SOD is the first line of enzymic antioxidant defense against oxidant in the body. They catalyse the dismutation of highly reactive superoxide anions ($O_2^{\bullet-}$) into O_2 and hydrogen peroxide (H_2O_2) (López-Jaén *et al*., 2013), which is less reactive, hence averting the deleterious effects of superoxide radical (Zelko *et al*., 2002). In this study, the heart, liver and kidney, superoxide dismutase activities were significantly reduced in the untreated infected animals compared to

uninfected control (Figures 31-35). This suggests that the buffering system required to induce the synthesis of more of the enzyme in order to offset the oxidative stress imposed by the infection has been overwhelmed (Hunt and Stocker, 1990).Artesunate-procyanidin hybrid molecule was able to revert the decrease in SOD activity in the heart, liver and kidney in most cases to the range of the uninfected controls on days 6 and 10 post-inoculation. This may be due to its inherent antioxidant activity and its ability to induce the synthesis of the enzyme. SOD activity was increased in the RBC and brain of untreated infected animals on days 6 and 10 post-inoculation compared to uninfected controls. However, the hybrid molecule was able to revert this increase to the range of the controls. This suggests that the buffering mechanism in synthesizing more SOD in the brain in order to offset the oxidative stress of the untreated infected mice has not been overwhelmed and the antioxidant activity of the hybrid molecule might have contributed to the alleviation of the oxidative stress, thereby reducing the magnitude of the induction of the synthesis of the enzyme in the brain. This kept the SOD activity of the mice treated with the hybrid molecule in the range of the control. However, the hybrid molecule was not able to revert the increase in SOD activity in the brain to the range of the control on day 10 post-inoculation.

Glutathione peroxidaseis also a major enzyme in the radical scavenging antioxidant system, which is responsible for the detoxification of cellular hydrogen peroxide (H_2O_2) (Dreher *et al.,* 1997). Increase in glutathione peroxidiseactivity has been reported in the blood of patients with malaria (Claudio *et al.,* 2008). In this study, GPx activity was reduced in the RBC and kidney of untreated infected mice on days 6 and 10 post-inoculation and in the liver of untreated infected mice on day 10-post-inoculation. This suggests that the buffering system required to induce the synthesis of more of the enzyme in order to offset the oxidative stress imposed by the

infection has been overwhelmed. The increase in RBC, liver and kidney GPx activities of the hybrid molecule-treated groups to the range of the uninfected control and even beyond in some cases may be due to the activation of the enzyme *in situ* or induced synthesis of the enzyme effected by the drug (though the latter is not applicable to RBC since it lacks nucleus in its matured form). The increase in heart GPx activity of the drug treated groups compared to the untreated infected and uninfected groups suggests activation of the enzyme *in situ* or induced synthesis of the enzyme effected by the drugs (Barry and Feely, 1990). In the various groups, it was observed that there was no significant change in brain GPx activities compared to that of the uninfected control. This suggests that the scavenging of the increased concentration of hydrogen peroxide produced by increased SOD activity was mainly left for catalase to handle in the brain of the drug treated groups. This supports earlier reports that catalase acts to handle higher concentrations of hydrogen peroxide while GPx acts on lower concentrations (Messner*et al*., 2012).

Reduced glutathione (GSH) is synthesized from the amino acids L-glutamate, Lcysteine and L-glycine. GSH is a major endogenous antioxidant and the most abundant non-protein thiol in mammalian cells (López-Jaén *et al*., 2013). It protects the cell against the deleterious effects of free radicals by directly reacting with them or through its use by GPx to reduce peroxides (Sies, 1999). There was reduction in the levels of GSH in RBC,liver, kidney, heart and brain of untreated infected mice compared to the uninfected controls. This reduction was reverted in the kidney, heart and brain to the range of the uninfected control and even beyond in some cases by the drugs. This suggests induction of the synthesis of the enzymes responsible for the production of the tripeptide by the drugs to alleviate the oxidative stress imposed by the infection (Figures 36-40). The reduction was not alleviated in the RBC of the drug-treated mice on day 10 post-

inoculation, suggesting the depletion of glutathione in RBC and inability of the drugs to induce the synthesis of the enzymes responsible for its production due to lack of nucleus in the matured RBC. The reduction in liver reduced glutathione concentration was also alleviated by the hybrid compound but could not revert it to the range of the uninfected control.

It has been known that glutathione S-transferases (GSTs) can reduce lipid hydroperoxides through their Se-independent glutathione peroxidase activity and that the enzymes can also detoxify lipid peroxidation end products such as 4-hydroxynonenal (4-HNE) (Sharma *et al*, 2004). GST activities in the RBC, liver, brain, heart and kidney of untreated infected mice were significantly reduced compared to uninfected control. This suggests that the buffering system required to induce the synthesis of more of the enzyme in order to offset the oxidative stress imposed by the infection has been overwhelmed. The drugs at various doses were able to revert the reduced GST activities in the various tissues/cells to the range of the uninfected controls and even beyond it in some cases except the hybrid molecule in the RBC on day 6 post-inoculation. The reversal caused by the drugs may be due to direct killing of the parasite, induction of the synthesis of the enzyme or activation of the enzyme *in situ* (Srivastava*et al*., 1999).

Catalase (CAT) prevents the conversion of H_2O_2 into more active oxidant species such hydroxyl radicals ($^{\bullet}OH$) by converting it to H_2O and O_2. The infection reduced CAT activity in RBC of untreated infected mice compared to uninfected mice. The reduction was not alleviated in the RBC of the hybrid molecule-treated mice on days 6 and 10 post-inoculation, suggesting the depletion of CAT in RBC and inability of the drug to induce the synthesis of the enzyme due to lack of nucleus in the matured RBC. The reduction in liver and kidney CAT activities in the untreated infected group were reverted to the range of the control and even beyond at some

doses by the hybrid molecule (Figures 51-55). Catalase has been demonstrated to be a major determinant of hepatic antioxidant status (Mishra *et al.,* 2003). Thus the ability of the hybrid molecule to restore CAT activity in the liver of infected animals is an important added advantage to its antimalarial activity. The hybrid molecule was able to maintain CAT activity in the heart of infected mice in the range of the uninfected control. Brain CAT activity of the mice treated with various doses of hybrid molecule mainly higher than those of the uninfected control, falling in the range of the untreated infected group. This is necessary because of the oxidative stress in the brain of the infected mice, made evident by increased MDA and nitrite levels in the brain. This led to an increased brain SOD activity in the hybrid molecule treated mice, thus requiring commensurate increased catalase activity.

Procyanidin B2 has the ability to significantly reverse the adverse effect of CCl_4 induce oxidative hepatic injury on CAT, SOD, GPx and MDA (Yang *et al*., 2015). Thus, the ability of the hybrid molecule to induce the synthesis of antioxidant enzymes observed in various tissues may be dependent on the procyanidin moiety.

4.7 Haematological Parameters

Haematological changes in malaria are common complications, which play a major role in the pathogenesis of the disease. These changes affect the major cell types such as erythrocytes, leucocytes and thrombocytes (Maina *et al*., 2010; Bakhubaira, 2013; van Wolfswinkel *et al*., 2013; Warimwe *et al*., 2013). The life cycle of *P.falciparum* occurs both in human host and anopheles mosquito. In the asexual stage in the human host, *P.falciparum* infects red blood cells (RBCs). The RBC then undergoes notable morphological and rheological changes while the parasite is transformed from the ring stage to trophozoite and finally schizont stage, constituting a 48 h asexual reproduction cycle (Maier *et al*., 2009).

Platelets have also been shown to mediate clumping of *P.falciparum* infected erythrocytes (Pain *et al*., 2001). Change in platelet count is notable in patients with high parasitemia; increasein *P.falciparum* loads results in a decreased platelet count (Kotepui *et al*., 2014; Kotepui *et al*., 2015). Immune-mediated destruction of circulating platelets has been postulated as a cause of thrombocytopenia seen in malaria infection (Kotepui *et al*., 2014). This may be responsible for reduced platelet count on days 6 and 10 post-inoculation in the infected *P. berghei* NK65 mice (Tables 15-16). Artesunate-procyanidin hybrid molecule improved platelet count at 80 mg/kg body weight on days 6 and 10 post-inoculation, suggesting that the bone marrow was stimulated at this dose to produceplatelet in the bone marrow (Vadhan-Raj et al., 1997; Chandra and Choudhry, 2009).

Significant decrease in lymphocyte and monocyte count in patients with high parasitemia has been reported (Kotepui *et al*., 2014; Kotepui *et al*., 2015). However in this study, a significant increase was observed in lymphocyte count of *P. berghei* NK65-infected mice, though with reduction in WBC compared to uninfected controls. The ability of artesunate-procyanidin hybrid molecule to increase significantly the WBC in infected micecompared to the untreated infected group suggests that it may stimulate the production of WBC in mice. Thus, it may not cause immunosuppression which has been reported for some antimalarials (Salmeron and Lipsky, 1983). This is also an added advantage in combating the infection.

The digestion of RBC haemoglobin by *Plasmodium*species and lysis of RBC bythe parasite causes reduction in haemoglobin level in the blood. RBC and Hb are significantly reduced in patients with high parasitemia (Kotepui *et al*., 2014; Kotepui *et al*., 2015). No significant difference in haemoglobin level was observed between the various groups and the uninfected control on day 6 post-inoculation, though there was significant decrease in PCV in

the untreated infected group; however, on day 10 post- inoculation, a significant reduction in haemoglobin level and PCV was observed only in untreated infected group compared to uninfected control.The observed anaemia(haemoglobin concentration <11g/dl) will lead to reduced oxygen carrying capacity of blood in the infected mice (Adebayo *et al.,* 2005; Yakubu *et al.,* 2006). The results thus suggest that the hybrid molecule may alleviate the reduced oxygen carrying capacity of blood during *Plasmodium* infection.

Neutrophil count has been reported to be significantly lower in malaria-infected patients (Kotepui *et al*., 2014). The increased lymphocyte count observed on day 6 and day 10 in untreated infected control (Tables 15-16) differs from earlier reports by Kotepui *et al*. (2014, 2015). This suggests that the infection stimulated the adaptive immune systemin the mice. The lymphocyte count were still increased in the artesunate-procyanidin hybrid molecule-treated mice compared to uninfected mice, suggesting that the lymphocytes produced as a result of the infection have not been cleared.

4.8 CD4+ lymphocyte response

Flavonoids such as catechins, epicatechins and procyanidins have been reported to initiate the CD4+ T-cell proliferation in malaria infection and were able to initiate protective immune response in malaria infected mice (Aladesemipe *et al*., 2013). This was replicated in artesunate-procyanidin hybrid molecule which was able to significantly increase the CD4+ lymphocyte count on day 6 post-inoculation except at 20 mg/kg body weight (Figure 56). On day 10 post-inoculation, only artesunate-procyanidin hybrid molecule at 20 mg/kg weight was able to increase the CD4+ lymphocyte count (Figure 57).

4.9 Molecular docking studies

Docking studies can predict the possible interactionsof specific amino acids in a protein-binding site. An important protein in a pathway can also be targeted at during a docking study to identify which ligand can tightly bind to the active site of the protein in order to inhibit the important function of that protein. The aspartate proteases (Plasmepsins I, II and V) and the cysteine proteases (Falcipains 1, 2, and 3) are potential targets for antimalarials (Russo *et al.,* 2010); therefore, compounds with good binding affinity will in turn inhibit the functions of the enzyme.This results in the death of the *Plasmodium* parasite.

4.9.1 Plasmepsins

Plasmepsins act by degrading host haemoglobin in the food vacuole of the parasite. Phenylalanine and Leucine at positions 33 and 34 in the globin chain of hemoglobin are the first target for cleavage by Plasmepsin I during degradation (Moon *et al*., 1997). In docking studies, various native ligands have been identified in theplasmepsinswhich have good binding affinity, expressing their various abilities to tightly bind to the proteins (Table 18). KNI-10006 as used in plasmepsin I docking studies, is a potent inhibitor of the protein (Nezami *et al*., 2003). Compounds such as Pepstatin as used in Plasmepsin II docking studies, has been demonstrated to bind tightly to target protein and killing *P.falciparum* parasite *in vitro* most likely by blocking haemoglobin degradation (Bailly *et al*., 1992; Francis *et al*., 1994; Liu *et al*., 2009).

In this study, it was observed that artesunate-procyanidin hybrid molecule had the highest binding affinity compared to procyanidin and artesunate, indicating that it a more potent inhibitor for plasmepsins I, II, III, IV and V.

4.9.2 *Plasmodium falciparum* Lactate Dehydrogenase

Docking pose obtained forArtesunate-procyanidin hybrid molecule in *Plasmodiumfalciparum* Lactate Dehydrogenase (pfLDH) enzyme revealed that the hybrid moleculesnugly fitted in the binding pocket compared to the human Lactate Dehydrogenase (hLDH) enzyme, indicating that the hybrid molecule is more specific for plasmodial LDH rather than the human enzyme (Data not shown). However, the compound interacted as hydrogen donor and acceptor in the binding site of both proteins. The hybrid molecule also had higher affinity for the enzyme than artesunate and procyanidin.

4.9.3 Falcipain-2

Falcipains 1 and 2 are among the cysteine proteases found in the food vacuole of *P.falciparum*which are responsible for the digestion of host haemoglobin and provision ofamino acids for the parasite's survival and development (Rosenthal *et al*., 2002). Artesunate-procyanidin hybrid molecule in falcipain 2 binding pocket had a good binding affinity (Table 18) interacting with Asp90A and Cys63A through conventional hydrogen bonding (Figure 70). It also interactedwith Cys95A through an alkyl interaction with the methyl group of Artesunate moiety. Nitrites are among the inhibitors of cysteine proteases. They form a reversible thioimidate intermediatefrom the nucleophilic attack of the catalytic cysteine residue (Greenspan *et al*., 2003; Altmann *et al*., 2004; Ehmke *et al*., 2011a, Ehmke *et al*., 2011b; Ehmke *et al*., 2012) and have been reported to display excellent inhibitory activity against falcipain-2 and against cultured *P.falciparum* (Coterón *et al*., 2010). The interaction with cysteine residues of falcipain-2 could be one of the mechanism by which Artesunate-procyanidin hybrid molecule exhibit its activities. Artesunate-procyanidin hybrid molecule contains both aromatic and aliphatic as well as alkyl side groups which could provide excellent interaction with amino acids in the binding

pocket of falcipain-2. All these put together may be responsible for the higher binding affinity of the hybrid molecule for the enzyme compared to artesunate and procyanidin.

4.10 Erythrocyte lysis and Plasma Oxidation Assay:

Erythrocytes are key to survival of malaria parasite, and are susceptible to oxidation due to their high content of polyunsaturated lipids, rich oxygen supply and the presence of iron, copper and other transition metals (Delmas-Beauvieux *et al*. 1995; Mennen *et al*. 2004). The invasion of malaria parasite in host red blood cell during malaria infection is one of the main sources of oxidants; the cells become hemolysed when the oxidation stress imposed on them is too high. This induces antioxidant resistance of host erythrocytes (D'Souza *et al*., 2009). Similarly, accumulation of conjugated dienes in plasma is serious indications of lipid peroxidation. Malaria infection increase lipid peroxidation due to intracellular generation of reactive oxygen species, leading to increased erythrocyte-conjugated diene concentration. Deficiency of Glucose-6-phosphate dehydrogenase (G-6-PD) has been reported to result in RBC lysis (Wiese *et al*., 1995), indicating its preventive role in oxidative stress conditions. The ability of Artesunate-procyanidin hybrid molecule to protect against the generation of conjugate dienes may be attributed to the reach antioxidant properties of Procyanidin and can serve as a mechanism to cushion the adverse effect that could be occasioned by progressive generation of lipid peroxidation products. Its inability to cause hemolysis is also an indication that perturbing the erythrocytes membrane is not one of its mechanisms of action. The monomeric units of Procyanidin (catechin and epicatechin) have been reported to possess good protective effects against APPH induced erythrocyte lysis in a similar manner with procyanidin,though lower compared to ascorbic acid (Qin *et al*., 2005).

4.11 Organ-body weight ratio

Increase in organ-body weight ratio or its decrease are associated with inflammation or cell constriction respectively (Moore and Dally, 1999). The percentage organ-body weight ratios of the various tissues in all treatment doses where not affected by treatment with Artesunate-procyanidin hybrid molecule after 21 days of administration. This suggests that there was no inflammation in the liver, kidney, heart and brain nor were there visible cell constrictions during the toxicological (Table 20).

4.12 Liver function indices

An organism is made of a dynamic system in which the activities are regulated to serve specific purposes. The liver is one of such organs with specific and important metabolic functions, which include protein synthesis, glucose homeostasis and detoxification of toxic metabolites. The liver also ensures the utilization and recycling of important nutrients in the system (Dwivedi1 *et al*., 2011). Results obtainedfrom this study suggest that the synthetic capacity of the liver was not affected by administration of the hybrid, since no significant change was observed in the plasma total protein, albumin and globulin concentration (Table 23). Albumin is the main transport molecule of plasma fatty acids. Thus, the metabolite transportation by albumin may not be affected at the doses administered. Albumin also reduces the oxidant effects through ROS scavenging actions, by modifying redox abnormalities and mediating proinflammatory responses (Peters, 1996; Quinlan *et al*., 2005). Bilirubin is a product of haemoglobin catabolism in the red blood cells. It is conjugated in the liver with glucoronic acid to form excretable compound (Burtis *et al.*, 2008). Total and conjugated bilirubin concentrations where not significantly altered after 21 days of administration of Artesunate-procyainidin hybrid molecule at all doses. Thus, the excretory role of the liver was not

compromised. This result also corroborates earlier results that the hybrid molecule did not cause haemolysis(Yakubu *et al.,*2009).

Alkaline phosphatase is used as an indicator of plasma membrane integrity (Akanji et al, 1993). Alkaline phosphatase activities in the tissues and plasma were not significantly altered by administration of the hybrid molecule at all doses throughout the duration used in the study (Figure 76), suggesting that the plasma membrane integrity in the various tissues was not compromised. AST and ALT are important transaminases normally localized within the cells of tissues. They are considered to be sensitive indicators of hepatocellular damage and within limit can provide a quantitative evaluation of the degree of damage to the liver (Appidi *et al.,* 2008). Earlier studies have indicated significant increase in activities of AST, ALT and ALP in the liver with Artesunate treatment (Nwanjo and Oze, 2006; Ngokere *et al*., 2004; Woodrow *et al*., 2005; Udobre *et al*., 2009; Omotuyi *et al*, 2008) while some reports have also reported that there was nosignificant change in these enzyme activities (Hamman *et al*., 2011; Anyasor and Olorunsogo, 2011). However, compared to other antimalarial drugs, toxic effects of artemisinins are less frequently reported (Taylor and White, 2004). The most common effects such as nausea, vomiting, anorexia and dizziness are attributed to malaria rather than the artemisinin drugs (Price *et al*., 1999). Activities of AST were not significantly altered in the liver and plasma at all the doses administered throughout the study (Figure 74). However, a significant increase in ALT activities was observed in the liver at all doses but not in the plasma compared to controls (Figure 75),suggesting that the hybrid molecule may have induced the synthesis of the enzyme in the liver as a mechanism to offset the stress impose on the liver (Malomo *et al.,* 1995). The increase in ALT activity may also be as a result of the activation of the enzyme by the hybrid molecule *in situ*. The hybrid molecule may not further complicate the increased levels of AST

and ALT during the treatment of malaria infection as observed with treatmentusing Artesunate alone.

GDH catalyses an important reaction involving the oxidative deamination of glutamate to α-ketoglutarate which is further metabolized in the Krebs cycle to generate energy inform of ATP (Andreas *et al*., 2017). The enzyme is a marker for liver damage especially that which involves damage to the mitochondria. In this study, the hybrid molecule caused no significant change in the liver and plasma GDH activities (Figure 77). This corroborates the fact that there was no damage done to liver. Similarly, there was no significant increase the γ-GT activities except at 5 mg/kg body weight dose of the hybrid molecule when compared to the control (Figure 78). γ-GT is present in hepatocytes and biliary epithelial cellsand plays major role in glutathione metabolism and reabsorption of amino acids from the glomerular filtrate and intestinal lumen (Kaplan, 1972). They help in the differentiation between liver diseases and bony disorders when ALP values are elevated (Rosalki and Mcintyre, 1999; Jarikim *et al.,* 2002). The results revealed that liver and plasma γ-GT activities were not significantly changed, still suggesting that there was no liver damage.

4.13 Kidney Function indices

The kidney is tasked with the function of removing waste products from metabolic reactions which are carried in the blood for subsequent excretion in urine. The nephrons are key to this function and thus the renal function test indices are used to assess the normal functioning capacity (Guyton and Hall, 2006). To assess this, the plasma levels of electrolytes, urea, uric acid and creatinine were estimated (Tables 25-26).

Large quantities of inorganic electrolytes could occur in cellular fluids and they can dissociate readily into their constituent ions or radicals and are measured in extracellular and

intracellular compartments (Zilva et al., 1991). Electrolyte imbalanceare likely to occur due to excessive ingestion and diminished excretion of an electrolyte or diminished ingestion and excessive elimination of an electrolyte. The most common cause of electrolyte disturbance is renal failure (Guyton and Hall, 2006). Calcium, bicarbonate, sodium and phosphate ions were not significantly altered by the hybrid compound at the doses administered in this study.

Calcium ion is absorbed with the help of vitamin D in the body. It is involved in several normal day to day body activities such as muscle contraction, transfer of nerve impulse and most importantly cardiac function. Lack of significant alteration in calcium values after treatment with the hybrid molecule for 21 days suggests that the hybrid molecule did not impair intestinal absorption of calcium ions or affect the conversion of vitamin D to 1, 25-dihydroxy vitamin D_3, the active form which serves as primary hormone to mediate calcium absorption in the intestine (Malomo *et al.,* 2006). Bicarbonate and phosphate ions are essential components of acid/base balance in the body.There was nosignificant alteration in the concentrations of bicarbonate and phosphate ions,suggesting that the body's buffering capacity may not be affected and also that their reabsorption in the kidney may not be adversely affected. The level of sodium ion in the plasma in the hybrid molecule treated mice was found within the range of the control (Table 25), indicating that reabsorption of Na^+ in the kidney, most especially the activity of the sodium pump (Na^+, K^+-ATPase) in the kidney was not adverselyaffected as evident from the results obtained in this study (Fig. 85).

Chloride ionis the most abundant anion in extracellular fluid. The significant increase in plasma chloride ion concentration at 5, 10, 20 and 40 mg/kg body weight of artesunate-procyanidin hybrid moleculesuggests increased reabsorption of chloride ions in the kidney (Hall and Guyton, 2016).The high level of plasma potassium ion observed at 5 mg/kg body weight of

the hybrid molecule compared to control suggests that the compound at this dose may predispose subjects to cardiac arrhythmias after prolonged administration at this dose.

Urea excretion is the primary method of nitrogen elimination. Its concentration reflects a balance between its production by the liver and elimination by the kidney. Creatinine is the final product of muscle creatine metabolism (Gao *et al*., 2010), which is excreted also by the kidney. High plasma values of creatinine or blood urea are indication that the kidney is not effectively eliminating the excess. Lack of significant alteration in plasma creatinine and urea concentrationsat all doses of the hybrid molecule suggests that glomerular filtration in the kidney was not compromised.

Elevated uric acid is seen in renal failure, chronic disease and other diseases associated with nucleic acid metabolism. High values are associated with low solubility of uric acid in the extracellular environment that could lead to the formation of crystals and their deposition in certain tissues (de Oliveira and Burini, 2012). Positive correlation exists between the concentration of uric acid and inflammatory response during malaria infection and may serve as useful biomarker for severe malaria (Lopera-Mesa *et al*., 2012). The non-significant alteration in plasma uric acid concentration after drug administration except at the dose of 5 mg/kg body weight (Table 26) suggests that the hybrid molecule may not adversely affect DNA catabolism and the antioxidant role of plasma uric acid (Chernecky and Barbara, 2001).

The hybrid molecule did not cause any significant alteration in the activities of ALP, γ-GT and all ATPases in the kidney. This suggests that the integrity of the kidney membranes and the role of the ATPases in reabsorption of electrolytes in the kidney were not adversely affected (Figures 76, 78, 82-84).

4.14 Cardiovascular indices

Alterations in the concentrations of major lipids like total cholesterol, HDL- cholesterol, LDL- cholesterol and triacylglycerol in the blood can give useful information on possible existence of cardiovascular diseases (Chawla, 1999; Abolaji *et al*., 2007). Elevation of all the lipids except HDL is associated with an increased risk of atherosclerosis (Ng *et al*., 1997). Total cholesterol levels in experimental animals treated with Artesunate-procyanidin hybrid molecule were not significantly different from the control (Table 24), suggesting that the hybrid molecule may not predispose subjects to cardiovascular diseases, possibly due to the protective role of procyanidin(Tebib *et al*., 1994). HDL-C and LDL-C are the major lipoproteins in the plasma where they play major roles in cholesterol transport. Their concentrations are reduced during malaria infection unlike the triglycerides that are moderately increased (Nilsson-Ehle and Nilsson-Ehle, 1990; Mohanty *et al*., 1992). However, Faucher *et al*. (2002) reported otherwise about the lipoproteins. No correlation was established between the severity of malaria and extent of HDL-C decrease (Kittl *et al*., 1992).Elevation of all the lipids except HDL (tagged "good cholesterol") is associated with an increased risk of atherosclerosis (Ng *et al*., 1997). The hybrid molecule did not significantly alter the concentrations of HDL-C and LDL-C at all doses tested (Table 24), still suggesting that it may not predispose the heart to risks of atherosclerosis. The protective effect ofprocyanidin moiety of the hybrid molecule may also contribute to this. Procyanidins are capable of inhibiting the progress of atherosclerosis (Yamakoshi *et al*., 1999) and could prevent the increase in LDL-C as well as their oxidations (Tebib *et al*., 1994).

However, the significant increase observed in triglyceride level at the lower dose of 5 mg/kg body weight of the hybrid molecule suggests increased triglyceride synthesis and their subsequent incorporation into lipoproteins by the hepatocytes (Adebayo *et al.,* 2007).

Alkaline phosphatase is a "marker" enzyme for assessing the integrity of plasma membrane and endoplasmic reticulum (Wright and Plummer, 1974; Akanji *et al.,* 1993). There was no significant change in heart ALP activities (Figure 76), suggesting that the hybrid molecule did not adversely affect the integrity of the heart cell membrane, leading to no leakage of the enzymes into extracellular fluid. Increase in plasma ALT and AST activities are important in the diagnosis of heart and liver damages caused by drug toxicity, infection or heart attack (Nelson and Cox, 2000). The hybrid molecule did not cause any significant alteration in heart AST activities compared to control. However, a significant decrease was observed in heart ALT activities at 5 and 10 mg/kg body weight (Figure 75), suggesting suppression of the synthesis of the enzyme or inhibition of the enzyme by the hybrid molecule.

No significant alteration was observed after the administration of the hybrid molecule at all doses for 21 days in Mg^{2+}-ATPase activities (Figure 82) suggesting that the fluidity and integrity of the membrane was maintained (Reinhart 1988). Calcium ion is required in cell signaling and by neurons to control a number of functions which include cellular differentiation, synaptic maturation, neurotransmsitter release and in cell death (Mata and Sepulveda, 2010). Calcium ion is also required for cardiac muscle contraction. The decrease observed in Ca^{2+}-Mg^{2+}-ATPase activity at 5 and 10 mg/kg body weight of the hybrid molecule (Figures 83) suggests inactivation of the enzymes *in situ* or suppression of the synthesis of the enzyme by hybrid molecule. This may adversely affect transmission of nerve impulse at the neuromuscular junction and muscle contraction.

Lactate dehydrogenase catalyses the conversion of pyruvate to lactate. Increase in lactate dehydrogenase in the plasma in an indicator of myocardial infarction. Lack of significant change in the plasma and heart LDH activities (Figure 79) suggests that the hybrid molecule

may not predispose subjects to myocardial infarction and may not aggravate the complications malaria may impose on the heart (Ehrhardt *et al.,* 2004; Ehrhardt *et al.,* 2005).Creatine kinase is reported to be lower in malaria patients compared to normal patients (Baloch *et al.,* 2010). The hybrid molecule caused no significant alteration in cardiac and plasma CK activities (Figure 81), still suggesting that the hybrid molecule may not predispose subjects to myocardial infarction.

4.15 Brain indices

Acetylcholine esterase (AChE) is primarily found in the blood and nerve synapses. It helps in degrading acetylcholine after nerve impulse transmission at the post-synaptic neuron. Treatment with intravenous artesunate has been recommended for severe and cerebral malaria. However, neurotoxic effects of the artemisinins are of great concern. Their administration at higher doses has been reported to cause severe and irreversible changes in the brain of laboratory animals (Brewer *et al*., 1994). There was no significant change in brain AChE during treatment with hybrid molecule at all doses (Figure 80),suggesting that inactivation of acetylcholine at the postsynaptic cholinergic neurons after transmission of nerve impulse may not be adversely affected.

The activity of creatine kinase (CK) in plasma or serum is a measured routinely in order to assess injuries to the heart and brain, which can be differentiated through their isozymes.Marked abnormalities have been reported in the activity of CK during malaria (Davis *et al.,* 2000). Increased activity of CK was observed at 40 mg/kg body weight of Artesunate-procyainidin hybrid compound (Figure 81). This increase was only seen in the brainwith no corresponding incresase in the plasma. The increase may be because of the induction of the synthesis of the enzyme or activation of the enzyme *in situ* at this dose.

Alkaline phosphatase activity in the brainwas significantly reduced at 20 mg/kg body weight dose of the hybrid molecule (Figure 76). This may be because of the inactivation of the enzyme rather than damage to the cardiac cell membrane. Mg^{2+} ATPase and Na^{+},K^{+}-ATPase activities in the brain of hybrid molecule–treated mice were reduced at all doses compared to control, suggesting that the hybrid molecule may adversely affect nerve impulse transmission in the brain.

4.17 Histology of tissues

Histological studies using the hematoxylin and eosin (H & E) stain revealed no alterations in the architecture of the liver, kidney, heart and brain respectively, indicating that the Artesunate-procyainidin molecule did not adversely affect the structure of these organs. Artesunate and Procyanidin B_2 have been reported to have beneficial effect on hepatic architecture in CCl_4 induced acute liver damage (Wu*et al*., 2006; Yang *et al.*, 2015). Procyanidin B_2 was able to reverse the lost hepatic structure and condensed nuclei that were observe in non Procyanidin B_2 administered animals.

Conclusion

Results from the studies indicate that:

1. Artesunate-procyanidin hybrid molecule, a brownish crystal compound synthesized from Artesunate and Procyanidin possesses two pharmacopores linked by an ester bond;

2. Artesunate-procyanidin hybrid molecule has a lower melting point compared to procyanidin but has a higher melting point compared to Artesunate;

3. The synthesized hybrid molecule was less active against *P.falciparum*W2 *in vitro*than artesunate and the various combination ratios of artesunate and procyanidin that were evaluated;

4. The hybrid molecule was more active against *P. berghei* NK65 *in vivo* than artesunate and the various combination ratios that were evaluated;

5. The mechanism of action does not involve perturbation of the membrane of infected red blood cells and may entail inhibition of hemozoin formation, exhibiting higher inhibitory activity than chloroquine;

6. The hybrid compound possesses *in vitro* antioxidant activity and induces the antioxidant system *in vivo;*

7. The hybrid compound exhibited a superior ligand-protein interaction with pfLDH, Falcipain-2, Plasmepsins I,II, IV and V compared to the parent compounds.

8. The hybrid molecule had no effect on organ-body weight ratio of the liver, kidney, heart and brain of throughout the duration of the study;

9. The hybrid molecule did not adversely affect the normal glomerular filtration and reabsorption of the nephrons of the kidney;

10. It may adversely affect nerve impulse transmission in the brain after prolonged use; and

11. No adverse effect of the hybrid molecule was observed in the cardiovascular and hepatic function indices, except for the reduction in Ca^{2+},Mg^{2+} ATPaseactivity in the heart.

Results suggest that the hybrid molecule may be a better therapeutic option than artesunate, possessing the dual capability of killing the parasites and alleviating the induced oxidation stress which is responsible for the secondary complications of malaria. However, it may adversely affect nerve impulse transmission in the brain after prolonged use. Thus, the abuse of the drug in form of prolonged use should be avoided.

Recommendations

1. Further studies should investigate the immune response to the hybrid molecule during treatment in malaria.

2. The duration of treatment should be increase in a chronic toxicity in order to investigate the effect of the hybrid molecule in case of drug abuse.

3. The functional groups of the hybrid molecule should be modified in order to alleviate its observed toxicity.

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APPENDIX

Preparation of Some Reagents and Solutions

Vanillin stain

6 g Vanillin and 1 ml of concentrated H_2SO_4 were dissolve in 100 ml of absolute ethanol. This was stored and used when required.

Biuret Reagent

1.5 g of Cupric sulphate ($CuSO_4.5H_2O$) and 6.0 g of Sodium potassium tartrate were dissolved in 500 ml of distilled water and transferred to a 1 litre flask. 1 g of potassium iodide (KI) and 300 ml of 10% NaOH solution were added. The resulting solution was made up to the 1 litre mark with more distilled water.

1% Bovine Serum Albumin (BSA) Standard

1 g BSA was dissolved in little quantity of 0.5 N NaOH and made up to 100 ml in a volumetric flask with the same 0.5 N NaOH solution.

2,4-Dinitrophenylhydrazine (1mM)

200mg of 2,4-dinitrophenylhydrazine was dissolved in hot 1N HCl and allowed to cool. The solution was then made up to 1,000ml, using more 1N HCl, in a standard flask.

AST-Buffered substrate solution (0.1M, pH 7.4)

1.5g K_2HPO_4 and 0.2g KH_2PO_4 were dissolved in 10ml distilled water after which 1.32g L-aspartic acid was added and then 0.03g α-ketoglutarate. 60ml of distilled water was then added and the pH adjusted to 7.4 with 0.4N NaOH solution and the resulting solution was made up to 100ml using distilled water.

ALT-Buffered substrate solution (0.1M, pH 7.4)

1.5g K_2HPO_4 and 0.2g KH_2PO_4 were dissolved in 10ml distilled water after which 1.78g DL-alanine was added and then 0.03g α-ketoglutarate. 60ml of distilled water was then added and the pH adjusted to 7.4 with 0.4N NaOH solution and the resulting solution was made up to 100ml using distilled water.

0.4N NaOH

16g of NaOH pellet was dissolved in a little amount of distilled water and then made up to 1,000ml in a standard volumetric flask.

Pyruvate standard solution for AST and ALT (2mM)

0.022g of sodium pyruvate was dissolved in distilled water and made up to 100ml with more distilled water.

Standard Bovine Serum Albumin (BSA, 10mg/ml)

0.5g of bovine serum albumin was dissolved in a little amount of 0.5N NaOH and then made up to 50ml with the same solution.

200mM NaCl/40mM KCl tris buffer (pH 7.4)

2.922g of NaCl, 0.745g of KCl and 1.8165g of Tris were dissolved in 200ml of distilled water in a beaker. The pH was adjusted to 7.4 using HCl and the solution was then made up to 250ml with distilled water In a standard volumetric flask.

240mM KCl/60mM Tris buffer (pH 7.4)

4.473g of KCl and 1.8165g of Tris were dissolved in 200ml of distilled water in a beaker. The pH was adjusted to 7.4 using HCl and the solution was made up to 250ml with distilled water in a standard volumetric flask.

8mM ATP solution

0.22g of ATP was dissolved in distilled water and the solution was diluted to 50ml in a standard volumetric flask.

20mM EGTA solution

0.3804g of EGTA was dissolved in distilled water and made up to 50ml in a standard volumetric flask.

80mM $MgCl_2.6H_2O$ solution

0.8132g of $MgCl_2.6H_2O$ was dissolved in distilled water and made up to 50ml.

5% Sodium Dodecyl Sulphate (SDS) solution

5g of SDS was dissolved in distilled water and made up to 100ml

Ammonium molybdate / Suphuric acid solution (Reagent A)

2g of ammonium molybdate was dissolved in 310ml of distilled water, after which 9ml of conc. H_2SO_4 was added and thoroughly stirred.

9% Ascorbic acid solution (Reagent B)

9g of ascorbic acid was dissolved in distilled water and made up to 100ml.

Reagent C

This was prepared by mixing **reagent A** and **reagent B** in ratio 8:2 (v/v).

1mM sodium dihydrogen phosphate solution.

0.015g of $NaH_2PO_4.2H_2O$ was dissolved in distilled water and made up to 100ml.

Buffered substrate 42nmM (pH 10.00)

5ml of 6% sodium lactate was added to 125ml of 0.1M glycine buffer. 75ml of 0.1N NaOH was then added and Store in the refrigerator.

Standard pyruvate

11mg of sodium pyruvate was dissolved in a little quantity of Sorensen glycine and than made up 100ml with more of the buffered solution using a volumetric flask. The resulting solution containing 1.1 umole pyruvate/ml was used immediately.

4N Sodium hydroxide

8.0g of sodium hydroxide was dissolved in a little distilled water and made up to 500ml in a volumetric flask with distilled water.

0.001M 2,4-dinitrophenyl hydrazine, DNPH

200mg of DNPH was dissolved in a little quantity of hot 1N hydrochloric acid and made up to 1 litre with the acid in volumetric flask.

Preparation of inoculation size

Step i. Preparation of Citrate Glucose Solution

3.8 g of of Sodium citrate and0.5 g of Glucose were weigh and dissolved in 100 mL of distilled water. Keep refrigerated until required.

Step ii. Determine the percentage parasitemia from the donor mouse.

$$\% \text{ Parasitemia} = \frac{Number\ of\ Infected\ RBC}{Total\ Number\ of\ RBC} \times 100$$

Step iii. The donor mouse-tail was sterilize with cotton wool soak with ethanol. 1 mL of Citrate Glucose solution was measure into a sterile bottle, and another 1 mL in a 2 mL syringe. The volume in the syringe was use to collect drops of blood from the donor mouse (about 10 – 15 drops, depending on the number of mice to be inoculated) and transfer to the sterile bottle. The syring and it content were keptin cold ice all through the procedure.

Step iv. Appropriate volume was introduce into the Haemocytometer counting chamber and view under ×10 magnification. Counting cells in 5 different square boxes. Multiply the total value obtain by 5 and 10,000.

Example:

Number of counted RBC = 616

Total number of RBC/mL = 616 × 5 × 10,000 = 3,080,000 RBC/ml

Parasitemia = 10%

Total number of infected RBC/mL $= \frac{10}{100} \times 3{,}080{,}000$ = 308,000 iRBC/ml

Step v. If the required inoculum size is 1×10^5 infected RBC, then the required volume in appropriate 200 µL need to be calculated i.e.

100 µL = 308,000 iRBC

200 µL = 616,000 iRBC

Step vi. The required dilution factor will be 1 mL of stock inoculum in 5.16 mL of Citrate Glucose solution. The mice will be inoculate at 45^{o} using a strile niddle, preferably at the second nipple in female mouse.

Calculations for drug administation

Preparation of 5% DMSO

0.5 ml of DMSO dissolve in 9.5 ml of distilled water to be administer to the control group.

For 5 mg/kg body weight group

Step i: Calculate the average weight of mice in the group plus extra mice in case of spillage.

Number of mice per group = 6

Average weight of mice for a group = 21.11 g

Step ii: Determine the appropriate volume to dissolve the drug.

Volume of extract to be administered per mouse = 0.2ml

Volume of extract to be administer for 5 mg/kg bwt. group = 1.2 ml

Step iii: Calculate weight of drug to be dissolve per group.

5 mg = 1,000 g mice

105.55 mg= 21.11 g mice

Step iv: Multiply the calculated weight by the number of mice by the duration of administration.

105.55 mg × 6 mice × 3 days

1899.9 mg of drug was dissolve in 1.2 ml of 5% DMSO

Step v: Divide the weight of mice by the average weight of the group and multiplied by total volume for the group to obtain the precise volume per mouse.

$$\text{Thus } \frac{\text{weight of mouse}}{\text{Average weight of mice in the group}} \times 0.2 \text{ ml}$$

Therefore, a mouse of 20.00 g in the group was administerd 0.19 ml of prepared drug solution.

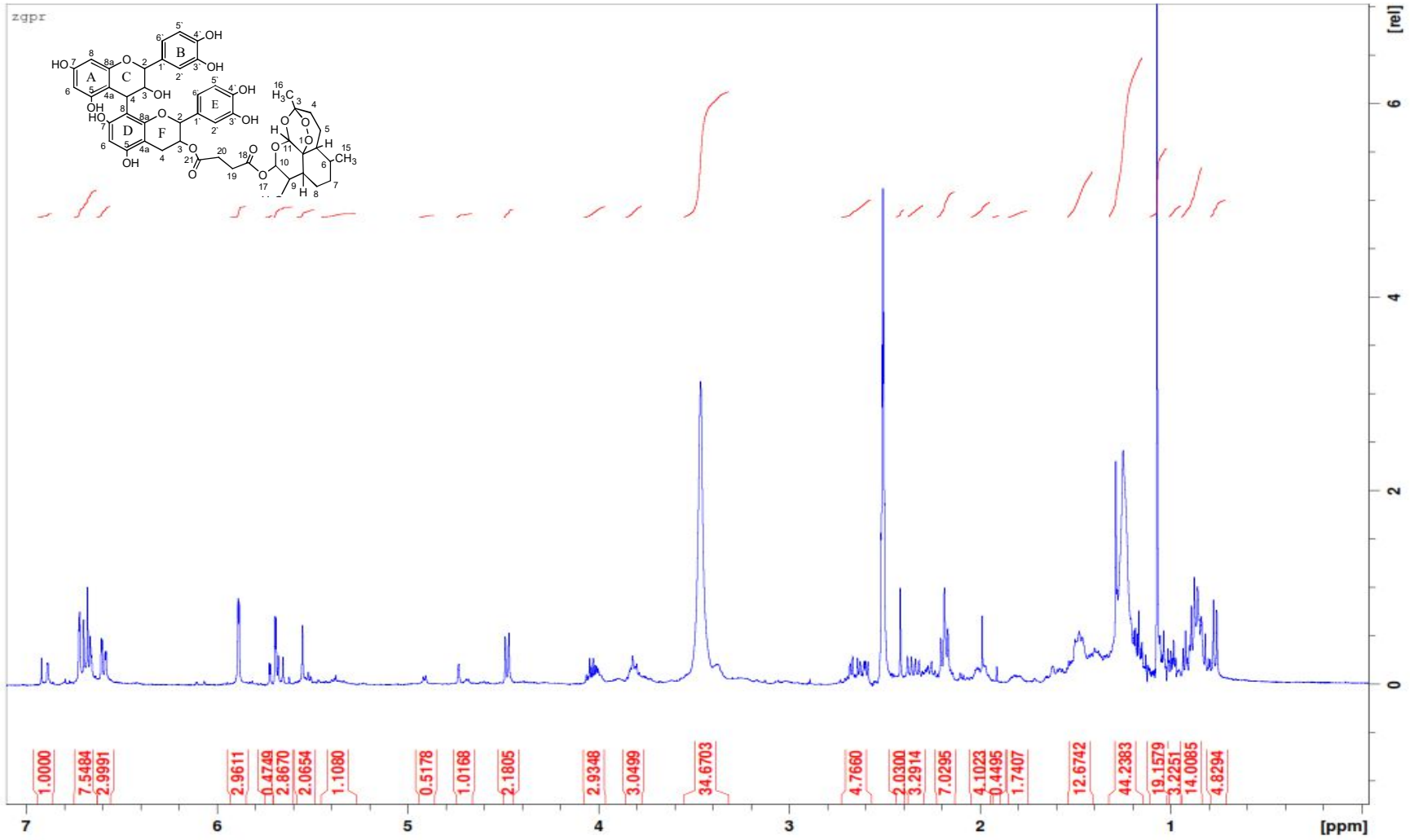

Figure 85: ^{1}H NMR Spectra (400MHz) of Artesunate-procyanidin hybrid molecule (PC14) in DMSO d_6

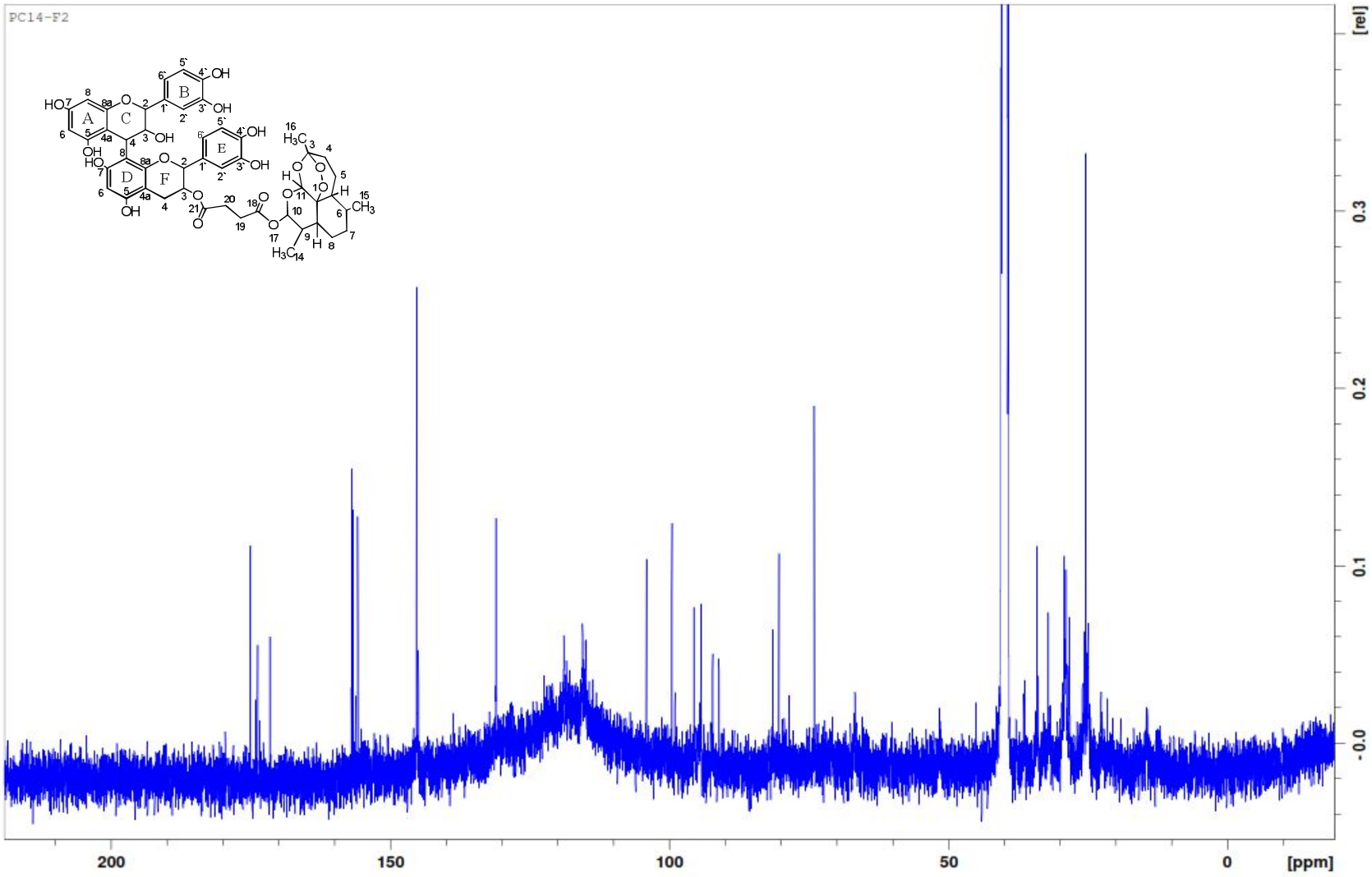

Figure 86: ^{13}C NMR spectra (400MHz) of Artesunate-procyanidin hybrid molecule (PC14) in DMSO d_6

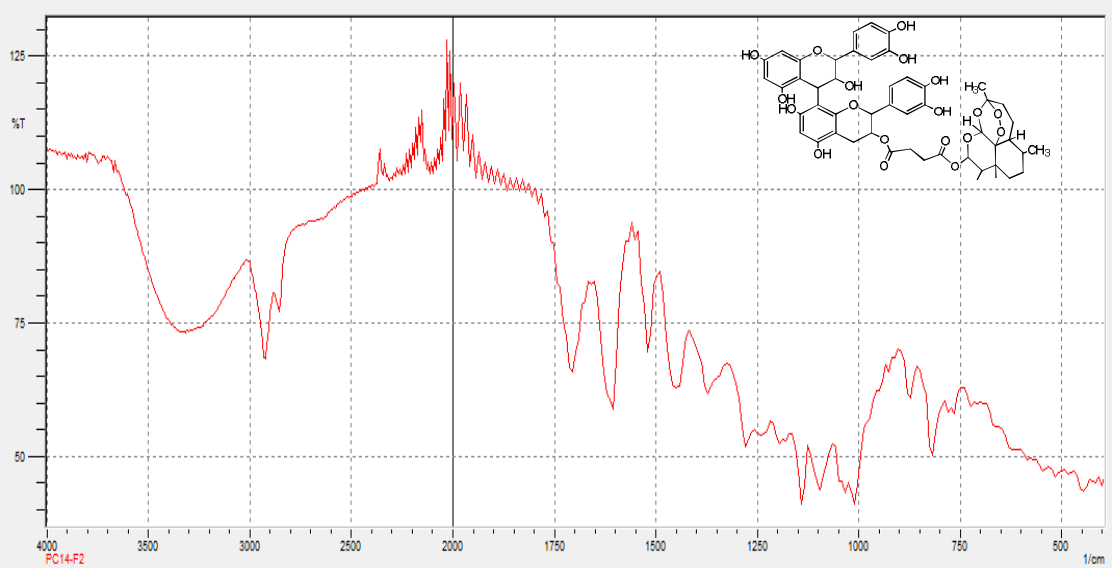

Figure 87: Infrared spectra of Artesunate-procyanidin hybrid molecule (PC14)

Table 27: Amino acid codes as used in docking studies

Code	Amino acid
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic Acid
Cys	Cysteine
Gln	Glutamine
Glu	Glutamic Acid
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

CALIBRATION CURVES

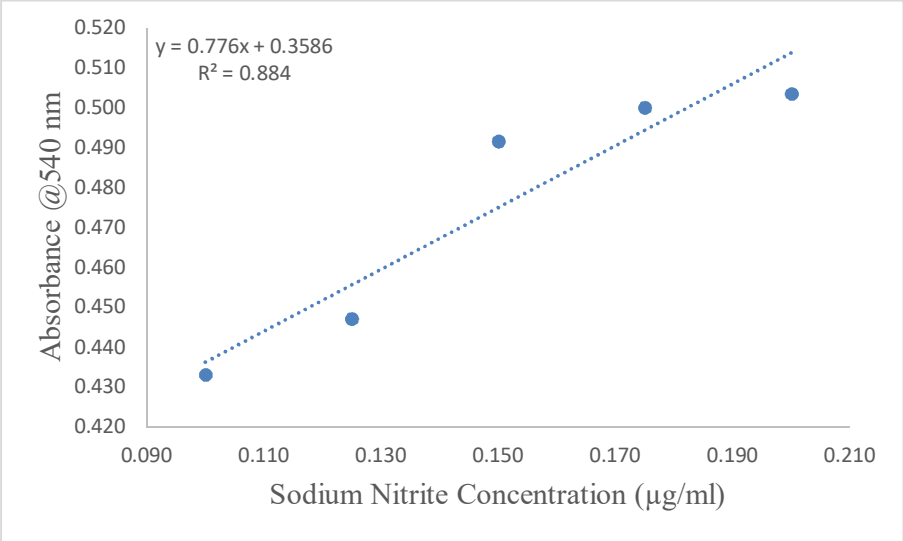

Figure 88: Calibration curve for determination of Nitrite

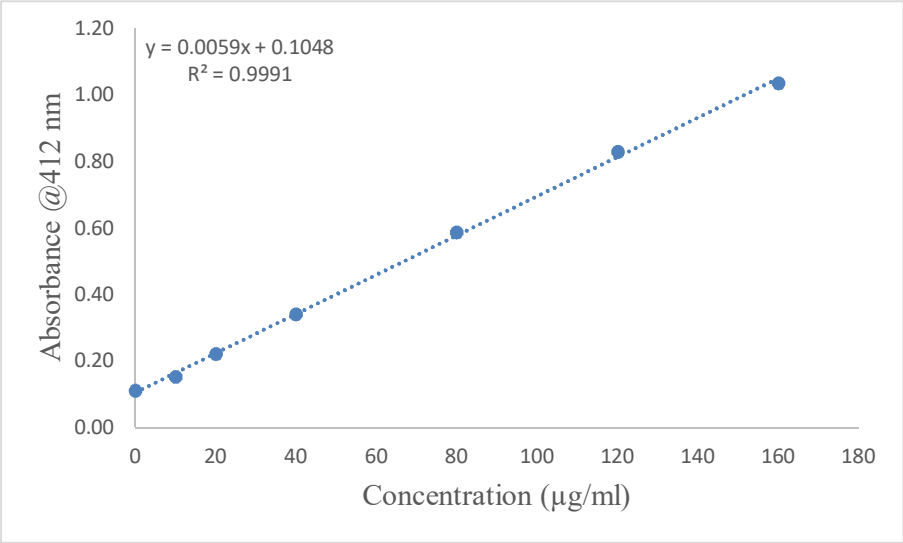

Figure 89: Calibration curve for GSH determination

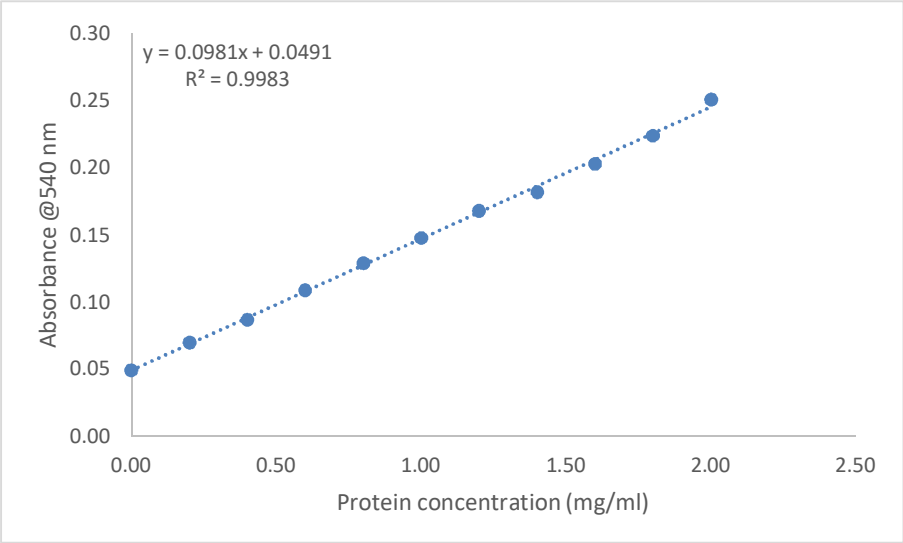

Figure 90: Calibration curve for protein determination

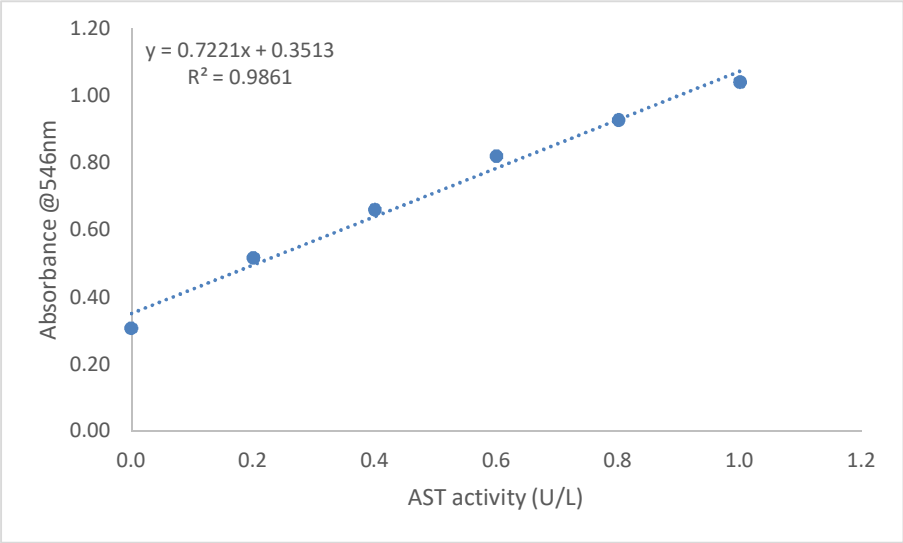

Figure 91: Calibration curve for determination of Aspartate aminotransferase activity

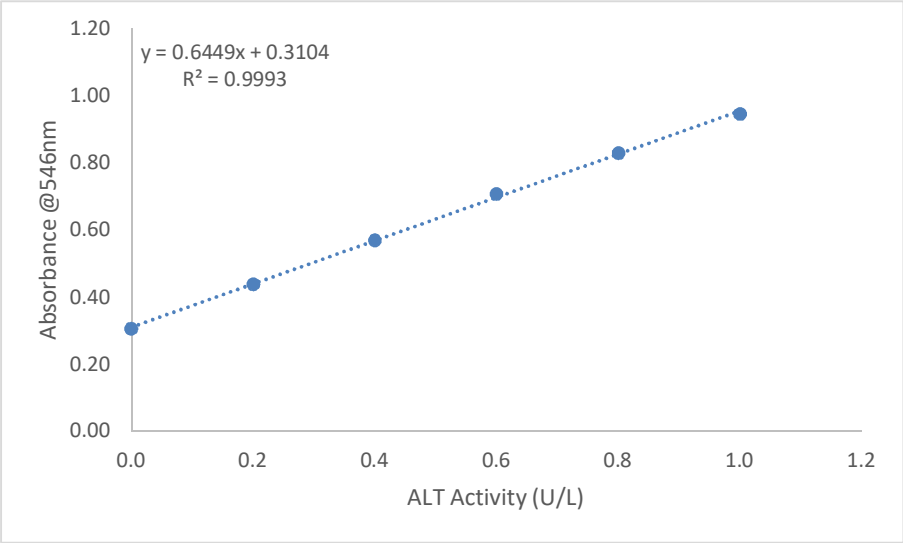

Figure 92: Calibration curve for determination of Alanine aminotransferase activity

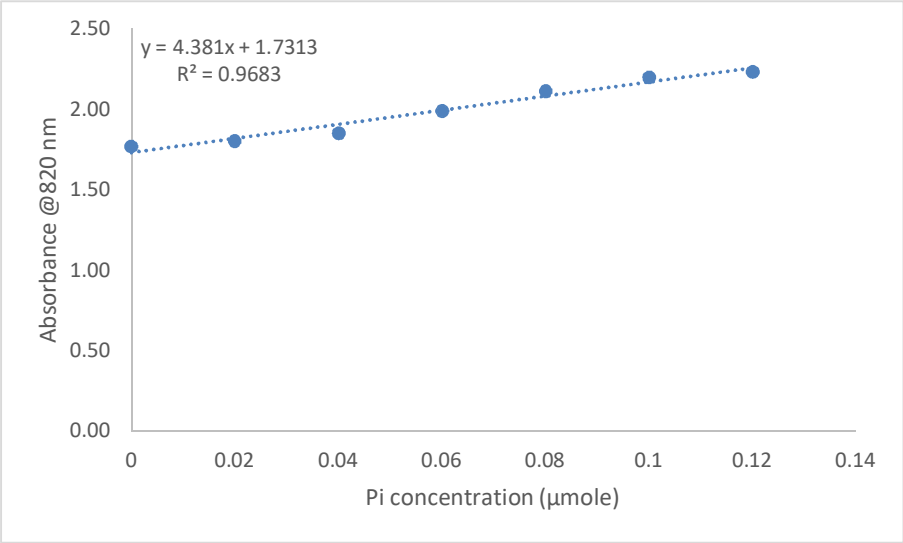

Figure 93: Calibration curve for inorganic phosphate