ANTIMALARIAL ACTIVITIES OF SOLVENT EXTRACTS AND FRACTIONS FROM *Adansonia digitata* STEM BARK IN *Plasmodium berghei*-INFECTED MICE

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CERTIFICATION

I certify that this work was carried out by Mr. Adeoye, Akinwunmi Oluwaseun in the Department of Biochemistry, University of Ilorin, Nigeria under my supervision.

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DEDICATION

This work is dedicated to the Almighty God, my Creator and to my late father, Mr. Francis Adeboye Adeoye.

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I acknowledge the grace, mercies, protection and blessing of God upon me since I was born. I give all glory, honour and adoration to the Almighty God for helping me thus far. I wish to express my profound gratitude and indebtness to my supervisor Prof. C.O. Bewaji for his immeasurable support, guidance, advice, understanding and suggestions, in making this work a reality. His valuable contributions, effective supervision, unalloyed support and fatherly care are worth a million thanks.

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ABSTRACT

Malaria is caused by a protozoan of the genus *Plasmodium* and it is an infectious disease of humans and other animals. In the process of studying human malaria parasites, rodent parasites are recognized as valuable model parasites for the investigation of parasite-host interactions, biology of malaria parasites, drug testing and vaccine development. Increase in the resistance of malaria parasite to synthetic drugs has led to the continuous search for alternative treatment approach from plant origin. Majority of these plants have biologically active polyphenol components and phytochemicals which have protective and therapeutic properties. These phytochemicals are found as secondary plant metabolites that are explored for their bioactivities with application in medicine. *Adansonia digitata L.* Baobab (Bombacaceae) is rich in these secondary metabolites and is used for the treatment of malaria in Nigeria. The aim of this research was to investigate the possible anti-malarial potential of aqueous and methanolic extracts of *A. digitata* stem bark and its fraction on *Plasmodium berghei* infected mice.

Aqueous and methanolic (AEAD and MEAD) extract was obtained from *Adansonia digitata* stem bark utilizing solvent extraction and the fractions was obtained through solvent partitioning. The free radical scavenging activity of the extract *in vitro* was evaluated. The extract was also evaluated for its antimalarial activity using curative test in mice. Severity of malaria was evaluated by determining the serum and tissue alkaline phosphatase (ALP), aspartate amino transferase (AST) activity, alanine amino tranferase (ALT), the serum C-reactive protein (CRP) and tumor necrosis factor-alpha (TNF-α). Mechanisms of action of the extract were investigated by measuring the degree of tissue peroxidation and tissue antioxidant status, mitochondrial swelling and mitochondria ATPase activity. The efficacy of *Adansonia digitata* stem bark extract in offering protection against experimental malaria was evaluated and its remediation effect when administered after established infection was also examined. The extract fraction was evaluated for its activity against inhibition of β-hematin formation.

The results revealed that *Adansonia digitata* stem bark extracts exhibited DPPH radical scavenging activity, ferric reducing antioxidant power, metal chelating activity and hydroxyl radical scavenging activity comparable ($p<0.05$) with those of standard antioxidants. The HPLC-DAD analysis showed significant levels of flavonoids and phenolic acids in the extracts and fractions of *Adansonia digitata* stem bark. The results revealed that the aqueous and methanolic

extracts were active against *P berghei* NK65 *in vivo* and were not cytotoxic at both 200 and 400 mg/kg body weight. Methanolic extract exhibited higher antimalarial activity causing 70.15%, 90.18% and 100% inhibition in parasite growth at the dose of 400 mg/kg body weight on day 3, 5 and 7 post-inoculation respectively. The extracts showed a significant dose dependent increase in packed cell volume (PCV), at the two doses when administered after established infection compared to control.

Moreover, ethylacetate extract fraction exhibited considerable antiplasmodial activity against inhibition of β-hematin formation (IC_{50} < 50 μg/ml). However, the ability of the extract fraction to inhibit β-hematin formation was significantly lower ($P < 0.05$) than that of chloroquine and artesunate. The extracts significantly reduced ($P < 0.05$) malondialdehyde concentrations in all the tissues investigated compared to the infected untreated mice on day 5 and 7 post-inoculation. Administration of the extract after established infections significantly increased ($P < 0.05$) glutathione concentrations and activities of superoxide dismutase and catalase in a dose-dependent manner compared to the untreated control on day 5 and 7 post-inoculation. The extracts significantly decreased serum creatinine concentrations but significantly increased ($P < 0.05$) serum urea and uric acid concentrations. Administration of the extract reduced serum concentrations of sodium ion, potassium ion, calcium ion and chloride ion compared to control but significantly increased serum concentrations of magnesium ion when compared to control.

A significant increase in the serum concentrations of CRP and TNF-α of the control animals was observed when compared to the baseline (uninfected mice) and the group treated with extract. Significant relationship exists between serum CRP and TNF-α concentration and percentage parasitemia in the control group. Administration of the extracts lowered the CRP and TNF concentrations. Alkaline phosphatase (ALP) activity of the control group increase significantly ($P < 0.05$) in all the tissues investigated compared to the group that received the extract. However, a significant reduction in ALT and AST activity of the control group were observed compared to the group that received the extract. Ethylacetate fraction of *A. digitata* stem bark and the phenolics, quercetin and apigenin induced the opening of mitochondrial membrane permeability transition pore in a concentration dependent manner. The ethylacetate extract fraction also increased ($P < 0.05$) significantly the mitochondrial ATPase activity in a concentration dependent manner.

The results suggest that *Adansonia digitata* protects against *Plasmodium berghei* induced-malaria, and that administration of the extract after established infection reduced malaria progression. A significant relationship was obtained between antioxidant activity and phenolic content indicating that phenolic compounds contribute significantly to antioxidant and antimalarial activity of the plant. Bioactive compounds from *Adansonia digitata* influence mitochondria membrane permeability transition by inducing cell death through mitochondria-mediated pathway of apoptosis. Opening of MMPT pore and induction of apoptotic process could likely be one of the mechanisms of action of antimalarial compound.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the study

Malaria continues to exact a devastating effect on the health of human populations globally and children under five years of age are mostly affected. The prevalence of malaria in the subtropical and tropical regions hasbeen ascribed to consistent high temperatures, rainfall, high humidity and presence of stagnant waters. This provides afavourable environment forthe constant breeding of thisvector (Jamieson *et al.*, 2006). An estimated 1.2 billion people are at high risk of transmission, half of which reside inthe African regions; 80% of such cases areconcerted in 13 countries, andoverhalf in Tanzania, Congo, Kenya, Nigeria and Ethiopia (WHO, 2008).

Nigeria accounts for a quarter ofall malariacases in Africa (WHO, 2008). Transmission in Nigeria is more seasonal in thenorthern part whereas inthesouth, itoccurs throughout the year. Almost all malaria cases inthe country are caused by *P. falciparum,* which is considered tobe the leading cause of deathglobally in 2004, from a singleinfectious agent (WHO, 2008). According tothe WorldHealth Organization, 3.3 billion people are estimated tobe at risk of being infected withmalaria worldwide. An epidemiological data estimates in 2013, reported the occurrence of 198 million cases of malaria in which 584 000 deaths occurred as a result of the disease. The burdento malaria is more pronounce inthe WHO African Region, wherean estimate of 90% of all malaria deathsoccur (WHO, 2014).

In humans, malaria is caused by *Plasmodium falciparum, P. malariae, P. ovale, P. vivax* and *P. knowlesi* (Mueller *et al*., 2007). Among those infected, *P. falciparum* is the most common species identified. *Plasmodium berghei* is a causative agent of rodent malaria and usually

presents similar cerebral infection equivalent to that by *P. falciparum* in human (Albay *et al*., 1999). The major features of *P. berghei* infection are anaemia, splenomegaly, fever and liver damage (Thurston, 1953; Sudhir and Saxena, 1980).

Growth of *P. falciparum* parasite in the red blood cell of human have been reported to require external calcium ion (Ca^{2+}), and this is linked with a markedly increased intracellular calcium ion concentration (Ca^{2+}), of the parasitized red blood cells (Desai *et al*., 1996). The activity of Ca^{2+}-ATPase in the red cell membrane from schizont-infected erythrocytes has been reported to be lowered by 30% compared to normal red cells and the calcium ion content during infection is about 10 to 20 times higher than that of normal red cells (Tanabe *et al*., 1983). The significance of calcium ion signal transduction and its indirect effects on malaria parasite cells has been shown to include regulation of transcription factors, cell cycle arrest and regulation of meiosis (Doerig, 1997).

The host liver is reported to be affected in the early stage of malaria leading to significant changes in host hepatocyte physiology and morphology. Renal involvement in malaria is usually linked with severe disease, shock and electrolyte and hemodynamic disturbances (English *et al*., 1996; Maitland *et al*., 2003). Alterations in electrolyte metabolism are an essential biomarker of malaria-associated disturbance in mineral homeostasis (Ayoola *et al*., 2005). Renal dysfunction is also related with increased number of death in children with cerebral malaria (Enwere *et al*., 1999). The mortality associated with severe renal failure generally occurs in younger children and those with increased creatinine levels and reduce urine output (Sheiban, 1999).

Artemisinin combination therapy (ACT) is adopted asfirst-line treatment for uncomplicated malaria in Africa. The effectiveness of ACTs havebeen reported against asexual parasites and a

number of them havebeen reported tobe considerably effective against the gametocyte stage which reduces transmission to mosquitoes. In Africa, artemether-lumefantrine (AL) ismajorly used. Also, dihydroartemisinin-piperaquine (DP) is effective due toits longer prophylactic period and advantages ofsimpler dosing (Bassat *et al*., 2009).

The potent antimalarial activity of artemisinin and its derivatives is due to its ability to produce free radicals (Efferth *et al*., 2003). This activity depends on the cleavage of an endoperoxide bridge leading to the production of carbon-centered free radicals and oxidative stress (Meshnick *et al*., 1993; Posner *et al*., 1993). Interestingly, free radicals and oxidative stress had been implicated in mitochondrial membrane permeability transition pore opening (Danial and Korsmeyer, 2004). The opening of the pore is the point of no return for apoptosis. Apoptosis is an appropriate defence system for the host to block infection by intracellular pathogens at a very early phase. It is highly specific and does not provoke inflammatory responses (Blaho, 2004).

The mechanism of apoptosis has been reported to involve pathological increase in intracellular free calcium ions [Ca^{2+}]; following inhibition of the cellular Ca^{2+} transport systems, resulting in activation of Ca^{2+}-dependent cytosolic enzymes which include phospholipases, proteases and endonucleases (Bitonti *et al*., 1988). The biochemical markers of apoptosis include DNA fragmentation, nuclear chromatin condensation, loss of mitochondrial membrane potential ($\Delta\Psi m$), and the activation of caspase-like proteases.

However, ACT use is limited due toits high costs, limited production of artemisin in derivatives to Good Manufacturing Practices (GMP) standards and toxicity (Haynes, 2001;

Malomo *et al.,* 2001; Borstnik *et al*., 2002; Adebayo and Malomo, 2002; Afonso *et al.,* 2006; Boareto *et al.,* 2008).

Many bioactive agents/naturally occurring substances have the potential to induce or inhibit mitochondrial membrane permeability transition pore opening and its application is valuable in drug development useful in situations of dysregulated apoptosis such as cancer and tissue wastage.

The roles of medicinal plants havebecome indispensable inthe present age, even though one cannot completely overcome the dependence on the synthetic drugs. The medicinal plants and their derivatives have properties which make them alternatives and safer to commercial drugs in many countries. They are not only available at an affordable cost, but are widely distributed and can be propagated by the local population. Majority of these plant derivatives from the roots, leaf, or stem have cellular components with high potential antioxidants, whichare commercially used as herbal medicines. One of such plants is *Adansonia digitata,* aperenial plant, commonly called baobab, dead-rat tree, monkey-bread tree, lemonade tree in English and 'Ose' in Yoruba language. It is found inthe hot, drysavannahs of sub-Saharan Africa and isthe most widespread ofthe *Adansonia* species onthe African continent. *Adansonia digitata* is recognized asan effective treatment for many diseases. It is indigenous inmany African countries (Wickens, 1982; Sidibe and Williams 2002).

Many parts of the plant, especially fruit pulp, seeds and bark fibers and leaves havebeen used traditionally for nutritional and medicinal purposes (Sidibe and Williams 2002; Chadare *et al*., 2009). Previous studiesby Ajaiyeoba, 2005; Musila *et al*., 2013 reported that *Adansonia digitata* hassignificant antimalarialproperties. It is used asan anti-inflammatory, analgesic and anti-

pyretic agent. It alsohas medicinal applications inthe treatment of intestinal and skin disorders (Ramadan *et al*., 1994; Karumi *et al*., 2008). The present study therefore aims at investigating the antimalarialpotentials ofextract and some fractions of *Adansonia digitata* stembark in *Plasmodium berghei* infected mice.

1.2 Statement of Research Problem

Malaria is the major cause of death and illness in children and adults in tropical countries. Mortality, estimated at over a million people per year, has increased in recent years, possibly due to increasing resistance to antimalarial drugs. Currently, about 15% of children with malaria are treated with effective medicines, mainly due to the spread of parasite resistance and high costs of Artemisinin Combination Therapies (ACTs). Due to affordability and or limited accessibility of pharmaceutical medicines in many tropical countries, the majority of the populations depend on conventional medical remedies. This has led researchers to look for alternatives, one of which is investigation of medicinal plants frequently used in the management of the disease. Therefore, this work attempts to investigate the antimalarial activities of aqueous and methanolic extracts of *Adansonia digitata.*

1.3 Research Null Hypothesis

Adansonia digitata stem bark extracts will confer protection against *Plasmodium berghei* infection in mice.

1.4 Alternative Hypothesis

Adansonia digitata stem bark extracts will not confer protection against *Plasmodium berghei* infection in mice.

1.5 Objectives of the Study

The overall objective of this research is to investigate the possible anti-malarial potential of aqueous and methanolic extracts of *A. digitata* stem bark and its fraction on *Plasmodium berghei* infected mice.

The specific objectives are to:

1. determine the secondary metabolites in the extracts and assess the *in vivo* and *in vitro* antioxidant potential of the extracts;
2. investigate the antimalarial effect of the extracts on *Plasmodium berghei* infected mice;
3. investigate the effect of solvent-partitioned fractions on *in vitro* antiplasmodial activity against inhibition of β-hematin formation;
4. investigate the *in vitro* effect of the extracts partitioned fraction on mitochondrial membrane permeability transition and mitochondrial ATPase;
5. identify and characterize the active phytochemical using High performance liquid chromatography and GC-MS.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 MALARIA

Malaria is caused by a protozoan of the genus *Plasmodium* and it is an infectious disease of humans and other animals. The diseaseis transmitted by infective bites froma female Anopheles mosquito or, rarely, through transfusion of infected blood products or inutero from the mother tothe newborn through the placenta orduring delivery (Malhotra *et al*., 2006). These malaria causing parasites are host-specific. The *Plasmodium* parasites infecting humans include *P. falciparum, P.vivax, P.ovale, P.malariae* and *P.knowlesi.* Of these parasites, *P. falciparum* is the most severe in terms of infection rates and the pathology it causes because it produces more merozoites in blood than any other *plasmodium* species (Carrico *et al.,* 2004; Robert *et al.,* 2005). It accounts for over 96% of all malarial infections in the sub-Saharan Africa (Marsh *et al*., 1995; Gal *et al*., 2001). Other *Plasmodium* parasites include *Plasmodium berghei, Plasmodium yoelii, P.vinckei* and *P.chabaudi* are malaria causing parasites in rats (Sinden, 1978).

Malaria isa complex disease thatvaries widely in epidemiology and clinical manifestation invarious parts ofthe globe. This is due to a number of factors such as (1) the species ofmalaria parasites that occur in a given area, (2) the distribution and efficiencyof mosquito vectors, (3) their susceptibility to commonly usedor available antimalarial drugs, (4) the behaviour and (5) the levelof acquired immunity ofthe exposed human populations.

Clinically, the symptoms of malaria includes acute febrile illness withfever, headache, and vomiting tolethal complications like severeanaemia, respiratory distress as a result of metabolic acidosis, orcerebral malaria whichcan ultimately result todeath. The treatment

approach is directed tocure the clinical symptoms and eliminate theacute blood infection (Tripathi, 2006).

Malaria hasa complex geographic distribution within large regions withmalaria-free areas and malaria-afflicted often found close to each other (Greenwood and Mutabingwa, 2002). Globally 3.3 billion people areat risk of malaria infection. Eighty percent ofthe 219 million malaria cases in 2010 and 90% of 660,000 malaria related deaths were from Africa (WHO, 2012).

Malaria transmission occurs infive ofthe six WHO regions, with Europe remainingfree (WMR, 2015). An estimate of 3.2 billion people were reported to be at risk of being infected with malaria. Out of the 214 million cases of malaria reported globally in 2015, 2% from Eastern Mediterranean region, 10% from SE Asia region and 88% were from the African region. It was estimated that 438000 deaths occurred ofwhich 2% is from Eastern Mediterranean region, 7% from SE Asia region and 90% is from Africa. From these estimates, 306000 deaths have occurred in children under 5 years ofage (WMR, 2015). The prevalence of malaria continues to pose serious threat for humanity in every part of the world. Co-existence of malaria and poverty hasbeen reported in most areas, haveabout one fifth average GDP and average growth per capital GDP of those in non-malarious countries. Malaria accounts for about 1.3% reduction in economic growth in areas where it is endemic (Sachs and Malaney, 2002). It is often regarded to as disease of the poor, because it ismore pronounce in world's poorest countries. Sub-Saharan Africa is the region where malaria ismore prevalent with most countries in the region being highly endemic for malaria transmission (UNICEF, 2007).

Malaria mostly affects low income earners wholive in poorly constructed houses (Worral *et al.,* 2005; UNICEF, 2007). Most of these people have low levels ofeducation and live in

unhygienic environment. Malaria burden is felt in resource constrained settings especially in rural areas compared to urban areas dueto socio economic factors such as differences in education and income (Cox, 2002; WHO, 2012).

Despite the currently observed decline in malaria prevalences inseveral malaria endemic regions, malaria still continues tobe a huge social, economical and health problem (WHO, 2011; Yavo *et al*., 2011). One of the major barriers for effective utilization of basic health careservices is poor income as family members of poverty stricken communities cannot afford thecost of insecticide treated nets/long lasting insecticide treated nets (ITNs/LLINs), reliable diagnostic services and expensive orefficacious antimalarial drugs that are either notavailable in public health facilities due topoor infrastructure or inability topay for such services inprivate outlets (Schellenberg *et al*., 2003).

Current treatment practice in malaria cases is based onthe concept of combination therapy. Most especially, the artemisinin-based combination therapy (ACT) hasbeen recommended because of reduction in therisk of treatment failure and developing resistance, enhanced usage convenience and reduced side effects (WHO, 2005). Presently, the production, prescription and application of ACT in the treatment of uncomplicated malarial caseshave significantly increased.

The increase and spread ofmalaria tonew areas and re-emergence of malaria inareas where the disease hadbeen eradicated occurred asa result ofresistance to antimalarial drugs. Population movement has also contributed to increase in parasites resistant to areas previously free of drug resistance (Collins and Jeffery, 1996). This hasencouraged research towards the discovery and development ofaffordable, safe and newanti-malarial chemotherapies.

2.2 RODENT MALARIA

Plasmodium berghei is a protozoan parasite that causes malaria in certain rodents. It is one of four *Plasmodium* species that havebeen described in African murine rodents. These parasites are shown tobe related to the malaria parasites of man and other primates in most essential aspects of structure, physiology and life cycle (Carter and Diggs 1977). *P. berghei* is used as a model organism for studying human malaria because of its similarity to the *Plasmodium falciparum* which cause human malaria. *P. berghei* has a very similar life-cycle to *P. falciparum*, and it causes disease in mice which hassigns similar to those found in human malaria. Importantly, *P. berghei* can be genetically manipulated more easily than *P. falciparum* making it a useful model for research into *Plasmodium* genetics (Franke-Fayard *et al*., 2010).

In the process of studying human malaria parasites, rodent parasites areknown as valuable model parasites forthe investigation of the parasite-host interactions, biology of malaria parasites, drug testing and vaccine development (Menard *et al.,* 1997). *P. berghei* is used in research programs fordevelopment and screening of antimalarialdrugs and for the development of aneffective vaccine againstmalaria (Franke-Fayard *et al*., 2010).

Generally all mammalian *Plasmodium* parasites induce malaria in a similar manner and studies. *P. berghei* is also transmitted by *Anopheles* mosquitoes and the symptoms are to some extentcomparable to symptoms ofcerebral malaria in patients infectedwith the *P. falciparum* parasite (Lin *et al.,* 2000; Franke-Fayard *et al*., 2010). Recent studies demonstrate a high level of conservation of genome organization between rodent and human parasites. The genome of both *P. falciparum* and the four rodent parasites areorganized into 14 linear chromosomes, ranging in size from 0.5-3.8 Mb (Rich & Ayala 2003). Differences in thelife cycle of *P.berghei* and *P.falciparum* or generally differences between various mammalian malaria parasites life cycles

are mainly restricted tothe duration of development and size ofthe different dividing stages (Akawa & Seed, 1980).

2.3 LIFE CYCLE OF THE MALARIA PARASITE

The life cycle of malaria parasite is complex and itinvolves two hosts namely an arthropod (female Anopheles mosquito) and vertebrate (human) hosts andcan multiply in multiple cell types while avoiding clearance bythe host immune system through expression of different surface proteins (Greenwood *et al*., 2008). The development and survival ofthe parasite within the vertebrate and invertebrate hosts, extracellular and intracellular milieu, isdue to a number of genes and some specialized proteins that aid the growth of parasite within the cell and invasion of host immune responses (Laurence *et al*., 2002; Brian et al., 2008). It is believed that the complexity of malarial life cycle reflects aseries of evolutionary adaptations thatoptimized its ability toexploit its hosts (Aravind *et al*., 2003).

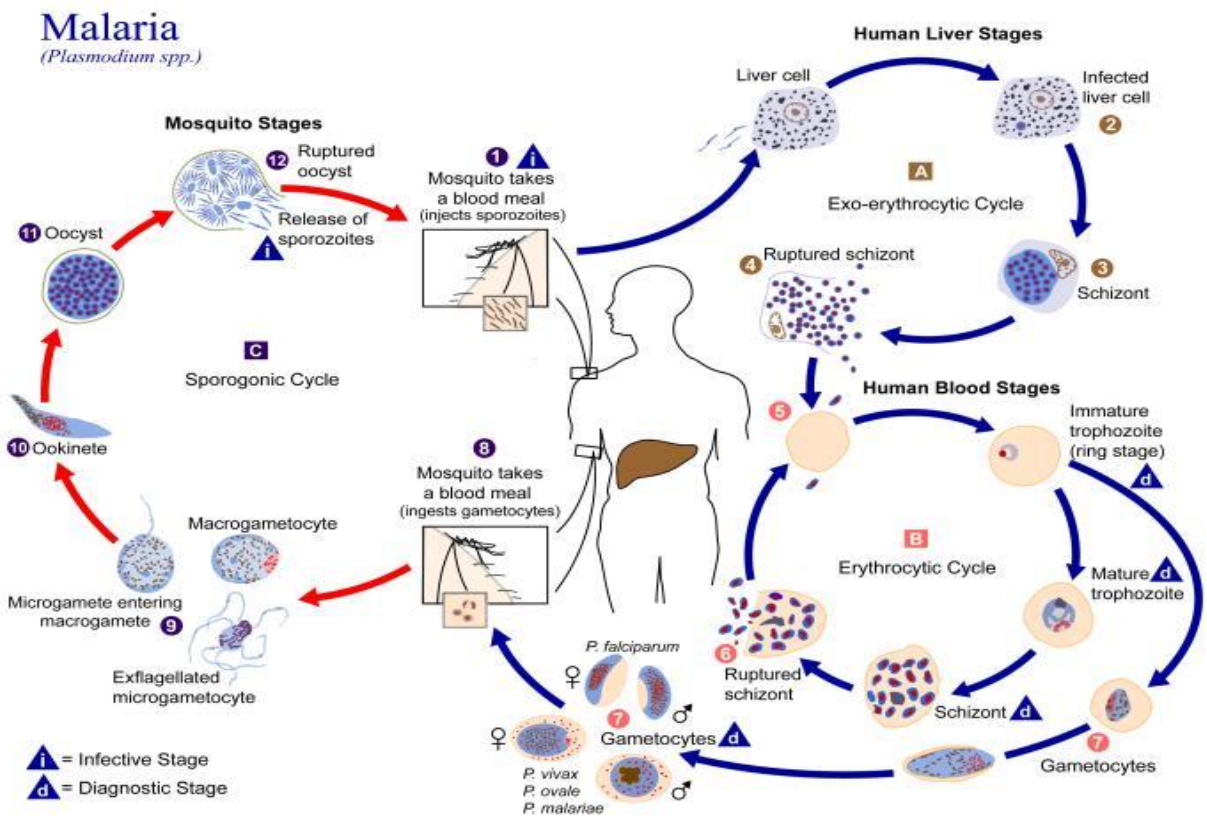


Figure 1: The malaria parasite life cycle (CDC, 1993).

The malaria parasite life cycle involvestwo hosts. A malaria-infected female anopheles *mosquito inoculates sporozoites into the human hostduring a blood meal,* ***(1) a****nd infect the liver cells with sporozoites* ***(2)*** *which mature into schizonts* ***(3)****, and later burst, releasing merozoites* ***(4)****. This is referred to asthe (exo-erythrocytic schizogony* ***A****), (initial replication in the liver). In the (erythrocytic schizogony* ***B****), the parasites go through asexual multiplication inthe erythrocytes. The redblood cell is infected with merozoites* ***(5)*** *and the ring stage trophozoites develop into schizonts, which laterburst and release merozoites. Some parasites are differentiated intosexual erythrocytic stages (gametocytes)* ***(7)****. Manifestations of the disease clinically, depend onthe blood stage ofthe parasites. The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an* anopheles *mosquito during ablood meal* ***(8)****. The sporogonic cyle takes place inthe mosquito where the parasites multiplication occur* ***(C)****.In the mosquito's stomach, the microgametes penetrate the macrogametes resulting into zygotes* ***(9)****. The zygotes become motile and elongated (ookinetes)* ***(10)*** *which invade themidgut wall ofthe mosquito where they develop intooocysts* ***(11)****. The oocysts grow, rupture, and releasesporozoites* ***(12)****, which find theirway into themosquito's salivary glands. Inoculation of thesporozoites* ***(1)*** *into anew human host perpetuates the malaria life cycle.*

2.3.1 Sporogonic stage in mosquito vector

The sexual phase of the life cycle of malaria parasites takes place in the mosquitoes. It is called sporogony and it results in the formation of many forms of parasite that causes disease in human asa result of the mosquito bite. After a blood meal is taking from ahost infected with malaria, the gametocytes (male and female) of theparasite enter the gut ofthe mosquito. (Carolina and Sanjeev, 2005).

The parasite adjust quickly from the warm-blooded human host to the insect host in order to initiate the sporogonic cycle due tothe cellular and molecular changes in the gametocytes. The fusion of male and female gametes in the mosquito gut lead to the formation of zygotes, which subsequently results inthe development of ookinetes that enter into the mosquito midgut wall and formed oocysts. The haploid form called sporozoites isproduced due to growth and division of each oocyst after the sporogonic phase of 8–15 days. Thisleads to the discharge of sporozoites into themosquito body cavity and later move into the mosquito salivaryglands (Heather and Andrew, 2004; Carolina and Sanjeev, 2005; Hill, 2006).

The sporozoites are known to go through severalmetamorphosis during its passage through the midgut and salivary glands of the infected mosquito and the vector canbecome infectious to another mammalian host in about twoweeks after its blood meal infection (Greenwood *et al*., 2008). When the mosquito is loaded with sporozoites and feeds onthe blood ofanother human, the sporozoites in the salivary glands ofthe parasite are deposited into the bloodstream, causing malariainfection in the new host. It has been found that there is a symbiotic relationship between the infected mosquito and the parasite. Thispromotes transmission ofthe infection (Heather and Andrew, 2004; Carolina and Sanjeev, 2005; Hill, 2006).

2.3.2 Schizogony in Humans

Humans are the intermediate hosts for the malaria parasites since theasexual phase of the life cycle of *Plasmodium* occurs in this host. The sporozoites areintroduced by the mosquito into the human host to commence thisphase of the cycle from theliver. This is followed by the developmental stages inthe redblood cells, consequently producing the variousclinical manifestations of the disease.

2.3.2.1 Preerythrocytic stage

Upon entry intothe bloodstream of the mammalian host, sporozoites travel bylymph and blood toinvade specific tissues. In mammals, these stages occur inhepatocytes, divide through a process termed schizogony, and differentiate and mature into thousands ofmerozoites (Aravind *et al*., 2003). For *P. falciparum*, the development of the parasites into schizonts within the liver takes an average of 6.5 days. The schizonts thenrupture releasing thousands ofmerozoites into the hepatic venous circulation and will invade any erythrocyte if it isnot pick upby phagocytic cells (Moorthy *et al*., 2004).

The growth and development of parasite in theliver cells is aided by a favorable environment produced by the circumsporozoite protein ofthe parasite (Miguel *et al*., 2006; Agam *et al*., 2007). The entire pre-eryhrocytic phase occurs between 5 and 16 days depending on the parasite species. The pre-erythrocytic phase isa ‘silent’ phase because fewhepatocytes are affected with little pathology and symptoms (Ashley *et al*., 2008). This phase is asingle cycle which is different from theerythrocytic stage that occurs repeatedly.

2.3.2.2 Erythrocytic Schizogony

The malaria parasite asexual development takesplace in the red bloodcells. Parasitic development occurs with exact periodicity in repeated cycles resulting to the release of hundreds offresh daughter parasites atthe end of eachcycle that invades the red cells. The asexual erythrocytic stage ofthe cycle starts when a single merozoite invades a redblood cell and is enclosed within aparasitophorous vacuole (Susan *et al*., 1997).

The merozoites discharged from theliver recognize, attach, and move intothe redblood cells via multiple receptor–ligand interactions within fewseconds. The rapid movement into the redcells reduces the antigensexposure to thesurface of the parasite and also protects theparasite from the host immune response (Alan and Brendan, 2006; Brian *et al*., 2008; Olivier *et al*., 2008). The entrance ofthe merozoites into the redcells is enhanced by interactions between thehost receptors onthe erythrocyte membrane and the ligands onthe merozoite (David *et al*., 2002; Ghislaine *et al*., 2009).

The specialized apical secretoryorganelles ofthe merozoite, known as micronemes, rhoptries, and dense granules assist theattachment, invasion, and establishment ofthe merozoite into the redcell. As theparasite grow and multiplies inside the redcell, the cytosolic composition and membrane permeability of thehost cell is modified (Kiaran, 2001; Virgilio et al., 2003). The permeation pathways induced by the parasite in the host cell membrane allows theuptake of solutes fromthe extracellular medium, maintenance of electrochemical iongradients and disposal of metabolicwastes. The infected redcells are prevented frompremature haemolysis by the excessive ingestion, digestion, and detoxification ofthe haemoglobin inthe erythrocyte of the host and its subsequent release from theinfected redcells through new permeationpathways. This preserves theosmotic stability ofthe infected redcells (Kiaran, 2001; Virgilio et al., 2003).

The ring stage is immediately followedby the trophozoite stage, a veryactive period during which most of the red bloodcell cytoplasm is consumed. The last form is the schizont stage. The trophozoites undergo several rounds ofbinary divisions leading tothe formation of schizont which actually contains numerous merozoites. The schizonts burst from thehost cell releasing themerozoites which invade new erythrocytes and so initiate anotherround ofthe infection (Susan *et al*., 1997). The duration of erythrocytic cycle varies among plasmodium species: rangingfrom 24hr in simian *P. knowlesi*, 48 hr in *P. falciparum*, *P. vivax* and *P. ovale* to 72 hrs in *P. malariae* (Aravind *et al*., 2003).

Digestion of haemoglobin bythe parasites in red bloodcells leads torelease of toxic metabolite, hemozoin whichis isolated in the parasite's foodvacuole. The intracellular parasites change theerythrocyte inseveral ways. They generate energy fromanaerobic glycolysis of glucose to lactic acid, whichmay contribute to clinical manifestations of hypoglycemia and lactic acidosis (Daily *et al*., 2007).

2.4. Clinical manifestation and pathological features of malaria

The clinical manifestations of malaria result from schizont rupture. Fever, vomitting and/ or diarrhoea are the major signs observed in uncomplicated malaria. In adults, severe infection due to *P. falciparum* in human is distinguished by multiorgan damage, such as renal failure. Severe malarial infection in children could come with prostration, respiratory distress, severe anaemia, and /or cerebral anaemia. Hypoglycemia and acidosis are some additional abnormalities linked with malaria (Greenwood *et al*., 2008). Complications and even deaths have also been reported in non-falciparum malaria but severe malaria is seen in cases of *P. falciparum* infection (Fakhreldin *et al*., 2003; Brian *et al*., 2008).

Genetic factors, intensity of transmission and age of patient could influence susceptibility to cerebral and severe malaria (Greenwood *et al*., 2008). Malaria interact with other infectious diseases (like HIV) to modify the vulnerability and / or severity of either disease. Co-infection with Helminths as well as non-typhoid *Salmonella* spp could affect malarial patients differently (Greenwood *et al*., 2008). The lysis of infected erythrocytes at the end of the schizogony process within the red cells release newly developed merozoites and several waste substances, such as hemozoin pigment, red cell membrane products and other toxic factors like glycosylphosphatidylinositol (GPI) into the blood. They activate macrophages and endothelial cells which secretes cytokines and inflammatory mediators such as tumor necrosis factor, interleukin-1, IL-6, IL-8, interferon-γ, lymphotoxin and macrophage colony-stimulating factor, superoxide, as well as nitric oxide (NO).

Malaria manifestations such as headache, nausea and vomiting, diarrhea and fever have been ascribed to the various cytokines formed in response to parasite (Ian *et al*., 2006). The plasmodial DNA which can also induce cytokinemia and fever is highly proinflammatory. It is presented by hemozoin in order to interact with the Toll-like receptor-9 intracellularly. This result in the release of proinflammatory cytokines and in turn induce COX-2-upregulating prostaglandins leading to induction of fever (Ian *et al*., 2006; Ralf, 2007; Peggy *et al*., 2007). Hemozoin has also been associated to apoptosis induction in developing erythroid cells in the bone marrow, leading to anemia (Gordon *et al*., 2007; Lamikanra *et al*., 2009).

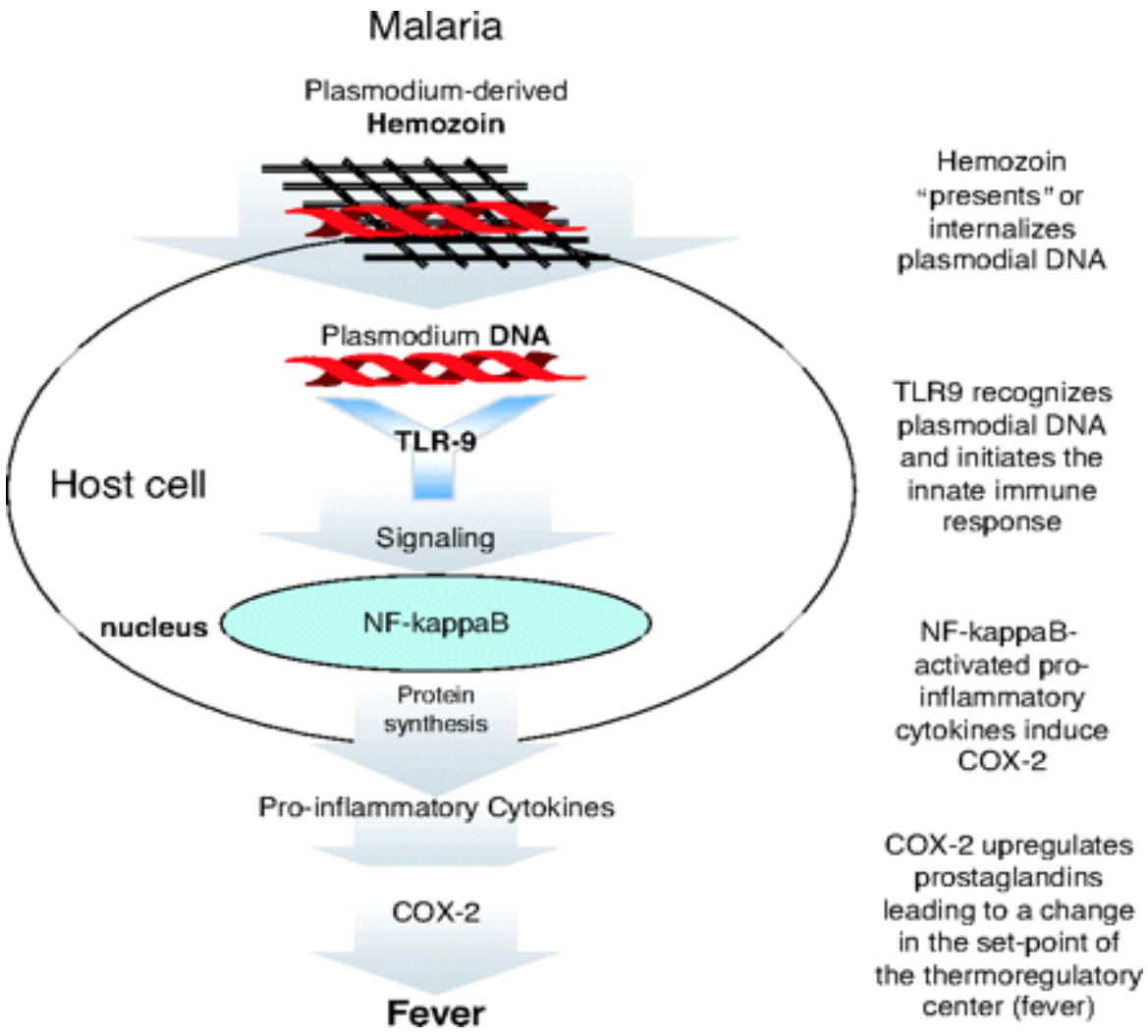


Figure 2: Induction of fever by malaria parasites (Ralf, 2007).

2.4.1. Asymptomatic Malaria

Clinical manifestation of *Plasmodium* infection varies fromasymptomatic to severe and fatalcondition (Marsh and Kinyanjui, 2006; Langhorne *et al*., 2008). Continuous exposure to malaria parasites inhigh transmission areas often results topartial immunity that leads to asymptomatic carriers that canact as potential reservoir of parasites, or gametocyte carriages resulting topersistent transmission (Staalsoe and Hviid, 1998; Bousema *et al*., 2004). Asymptomatic malaria cases refers to situations whereby individuals areoften infected by malarial parasites but donot develop clinicaldisease (Hamad *et al*., 2000).

2.4.2. Uncomplicated Malaria

Any person withsymptomatic malaria with fever and any of the following symptoms; headache, body and joint pains, feeling cold and shivering, loss of appetite and sometimes abdominal pains, diarrhoea, nausea and vomiting and splenomegaly aswell as otherunspecific symptoms isconsidered tohave uncomplicated malaria. A clinical picture ofsuch infection can mimic/resemble that of manyother childhood illnesses.

2.4.3. Severe Malaria

P. falciparum is almost exclusively responsible for almost all forms of severemalaria whereas other non-*falciparum* species arerarely implicated to the most severeforms of the disease (Svenson, 1995). Severe/complicated malaria (SM) is defined as a form ofsymptomatic malaria with signs ofvital organ disturbance complicated byserious organ failures orabnormalities in the patient's blood or metabolism. The clinical manifestation of SM may includecerebral malaria that is associated with abnormal behaviour, impairment of consciousness, seizures, coma orother

neurologicalabnormalities; severe anaemia dueto massive destruction oferythrocytes; haemoglobinuria due to hemolysis; acute respiratory distress syndrome (due to deep breathing as a result of metabolic acidosis); low blood pressure dueto cardiovascular collapse; acutekidney failure; hyperparasitaemia (where more than 5% of the RBCs are infected by malarial parasites); metabolic acidosis (excessive acidity inthe blood and tissue fluids and hypoglycaemia to mention only a few (Svenson, 1995).

2.5. Specific Complications of Severe Malaria Infection

2.5.1. Anaemia

Anaemia is a common cause of mortality and morbidity in malaria infection particularly in children and pregnant women (Nussenblatt and Semba, 2002). Malarial anaemiacould be acute or chronic. Chronic malarial anaemia ismore common. In holo-endemic areas. Acute malarial anaemia occur after enormous erythrocytes lysis dueto increase inparasitemia or todrug-induced or immune haemolysis (Ghosh, 2007). The mechanisms of malarial anaemia may either be due to decrease of RBC production or increaseddestruction of parasitized and un-parasitized erythrocytes. Co-infection with bacteremia, HIV-1 and hookworm, malnutrition and repeated malarial infections in endemic areas may alsocontribute todecrease haemoglobin levels (Nussenblatt and Semba, 2002; Haldar and Mohandas, 2009; Perkins *et al*., 2011).

The immune response plays avital role in the pathogenesis of malarial anaemia; parasitized redcells, hemozoin and malarial antigens activate monocyte and lymphocyte response. Pro-inflammatory and anti-inflammatory mediators, chemokines and growthfactor produced during immune response contributesto anaemia. (Ghosh, 2007; Perkins *et al*., 2011).

2.5.2. Liver involvement

According to the World Health Organization, liverdysfunction is anuncommon occurrence in malaria. Liver is involved inmalaria during the pre-erythrocytic phase and the erythrocytic phase. In the pre-erythrocytic phase, binding ofthe merozoite circumsporozoite protein CSP-A and thrombospondin-related anonymous protein (TRAP) tothe hepatocytes through the heparan sulphate glycosylaminoglycans (GAG) promotes minimal liverdamage (Anand and Puri, 2005). In theerythrocytic phase, jaundice commonlyoccur and it is directly caused by infection such as malarial hepatitis, intravascularhemolysis of parasitized RBC, or by indirect causessuch as G6PD-related hemolysis, antimalarial druginduced-hemolysis. It could also be dueto completely unrelated causesincluding coexisting acuteviral hepatitis and underlying chronichepatitis (Anand and Puri, 2005).

An increase of unconjugated bilirubinemia with mild tomoderate jaundice occuras a result of intravascularhemolysis of parasitized and non parasitized RBC; (Kochar *et al*., 2003) while conjugated hyperbilirubinemia indicates hepatocytedysfunction. The pathogenesis of hepatic dysfunction is not completely known; reduction in portal venous flowas a result of microocclusion of portal venousbranches by parasitized erythrocytes, suppression of bilirubin excretion dueto effect of parasitemia or endotoxemia or metabolic acidosis, intrahepatic cholestasis dueto reticuloendothelial blockage andhepatic microvilli dysfunction, apoptosis and oxidative stress areall mechanisms involved in hepaticdamage (Anand and Puri, 2005; Bhalla *et al*., 2006).

Histopathological findings associated tohepatocyte damage reported inprevious studies includes: deposition of brownmalarial pigment, congestion ofhepatocytes, swollenhepatocytes, Kupffer cell hyperplasia, centrizonal necrosis, portalinfiltration with lymphocytes, steatosis,

parasitized RBC, cholestasis, spotty and submassivenecrosis (Kochar *et al*., 2003; Anand and Puri, 2005).

2.5.3. Kidney involvement

Kidney is involved in malaria aseither acute or chronicdiseases. Acute renal failure (ARF) is one ofthe most challenging diseases intropical countries and malaria plays significant epidemiological role (Naicker *et al*., 2008). It hasbeen reported that high ARF is thecause of mortality in malaria (Mishra *et al*., 2008). Another studyshowed that malaria was the first cause of death in patient with ARF (Rohani *et al*., 2011). In *P. falciparum,* acute renal failure is more common in adults and its pathogenesis is complex which may include mechanicaland immunologic factors, volume depletion, hypoxia, hyperparasitemia andother factors (Ehrich and Eke, 2007; Das, 2008).

Acute renal failure hasbeen associated rarely to rhabdomyolysis in *P. falciparum* and *P. vivax* infections, probably owing to sequestration of parasitized redcells in the skeletal capillaries and asa result of vessels occlusion (Mishra *et al*., 2010). Theuse of non-steroidal anti-inflammatorydrugs, hypovolemia, hypotension, fever and acidosis mayexacerbate the renal function (Reynaud *et al*., 2005; Siqueira *et al*., 2010).

2.6. Haemoglobin digestion in the malaria parasite – an oxidative burden

The oxidative burden in most eukaryotic cells occurs viathe mitochondrial electron transport chain. Redox centres inthis chain leaks electrons to O_2, leading to the production of reactive oxygen species (ROS) (Turrens, 2003). The intra-erythrocytic lifestyle of malaria parasite contributes anadditional oxidative burden,

resulting fromthe digestion of the host haemoglobin (Hb) asthe parasite grows within the infected cell. Hb degradation majorly occurs in the parasite's digestive vacuole (DV) (Klonis *et al*., 2007).

Digestion of Hb iscrucial for the parasite because itprovides amino acids for the parasite to synthesize protein needed forits growth and also aiding the osmotic stability of the host cell (Lew *et al*., 2003; Goldberg, 2005). Digestion of Hb liberatestoxic heme moieties, which hasan iron atom chelated inthe centre of a porphyrin ring. In the acidicenvironment of the digestive vacuole (DV), the heme in oxyHb is oxidised fromthe Fe^{2+} to the Fe^{3+} state. This is followed by the production of O_2, which in the acidic DV isbelieved to dismutate spontaneously to H_2O_2 and O_2 (Becker *et al*., 2004). The parasite mustdestroy or neutralise the heme and H_2O_2, as both species giverise to a varietyof toxic effects through theiroxidation of biomolecules, and in thecase of heme, through additional non-oxidative mechanisms (Fitch, 2004).

The free heme istoxic owing to its ability todestabilize and lyse membranes and also inhibiting the activity of several enzymes. The mechanisms by which heme isdetoxified includes: sequestration of the free heme intohemozoin, orthe malarial pigment; a degradation facilitated by hydrogen peroxide within the foodvacuole; a glutathione-dependentdegradation which occurs in the parasite's cytoplasm; and possibly a heme oxygenase which has been found in *P. berghei* (rodent parasite) and *P. knowlesi* (simian parasite), but not *P. falciparum* (Atamna and Ginsburg, 1995; Egan 2008).

Both the hemozoinformation pathway and the degradative pathways havebeen reported to probably function simultaneously with 25-50% of the free heme beingconverted into

hemozoin and the remainder being degraded (Ginsburg 1999). Some studiesshowed that about 95% of the free iron released during hemoglobin digestion is found in hemozoin (Egan 2008). X-ray crystallography and spectroscopic analysisindicates that hemozoin hasthe same structure as β-hematin (Pagola 2000). β-hematin is a heme dimer formed via reciprocal covalent bonds between carboxylic acid groups on the protoporphyrin-IX ring and the iron atoms of two heme molecules. These dimers interact via hydrogen bonds to form crystals of hemozoin. Therefore, pigment formation isbest described as a biocrystallization, or biomineralization, process (Hempelmann 2007; Egan 2008).

Antimalarial drugs such as chloroquine and other 4-aminoquinolines prevent the detoxification of heme byinhibiting pigment formation aswell as the hemedegradative processes (Ginsburg 1999). The free heme destabilizes thefood vacuolar membrane and other membranes resulting in thedeath of the parasite. Many other antimalarial drugs target the food vacuole whichshows theimportance of this organelle and its various functions to the survival of the parasite (Ginsburg 1999).

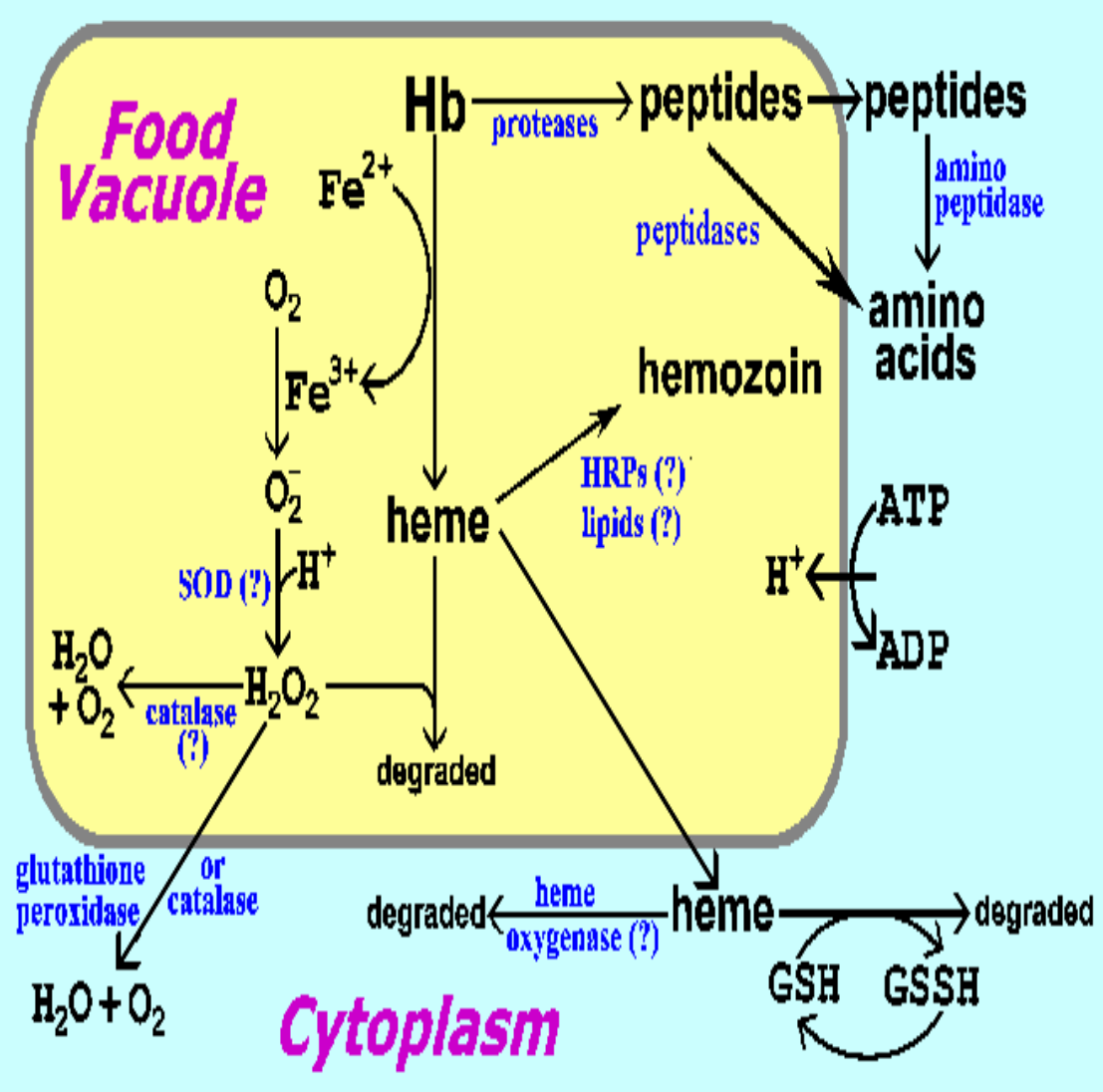


Figure 3: Summary of the activities and functions of the food vacuole (Goldberg *et al*., 1991).

2.7. Redox and antioxidant systems of the malaria parasite

An infection with *Plasmodium* leads to increase in the level of reactive oxygen species in infected RBC (Hunt *et al*., 1990; Schwarzer *et al*., 2003; Ginsburg and Atamna, 2004). Increased level of reactive oxygen speciesresults to premature oxidative damage of the infected RBC. This affects composition of membrane and protein of the infected host cell and aswell increases the production of lipid peroxides. These lipid peroxides affect RBC membrane integrity negatively and alter the host's immune response in infected persons (Skorokhod *et al*., 2010; Aguilar *et al*., 2014). Malaria parasites havestage-dependent high proliferation rates resultingin an increased demand forreducing equivalents. The lifestyle of parasites in prooxidant environments, high metabolic fluxes of proliferating parasites and digestion of haemoglobin lead to anincreased endogenous production of reactive oxygen and nitrogen species. Antioxidant defense and redox regulation playsan important rolein *Plasmodium*, and it represents major targets for chemotherapeutic interventions (Jortzik and Becker, 2012).

It hasbeen reported that during developmental process, malaria parasites are exposed to metabolic and environmental stresses. An important approach to drug development was to raise these stresses by interfering with theantioxidant and redox systems of the parasites, which may be auseful approachto disease intervention (Müller, 2015). *Plasmodium* hasbeen demonstrated to possess a complex redox system comprising glutathione and thioredoxin-based components with overlapping but distinct functions.

Glutathione isan abundant redox active thiol in the parasites thatexist mainly in its reduced form and it allows an effective maintenance of the intracellular reducing milieu of the parasitecytoplasm and its organelles (Müller, 2015). Both glutathione and thioredoxin redox systemsrely on a supply of NADPH requiredas reducing equivalents fortheir disulphide

oxidoreductases thioredoxin and glutathione reductase (GR), respectively (Müller *et al*., 2003; Müller, 2004; Jortzik and Becker, 2012). Pentose phosphate shunt (PPP) usuallyprovide the NADPH which is also thecase in *Plasmodium* (Bozdech and Ginsburg, 2004; Preuss *et al*., 2012).

2.7.1 Generation of reactive oxygen species

The generation of reactive oxygen species (ROS) such ashydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and superoxide anions (O_2^-) are a challenge faced by allorganisms living in an oxygenatedenvironment, as ROS directly or indirectly damage macromolecules such as DNA, proteins and lipids inside cells in various ways (Storz and Imlay, 1999; Imlay, 2003). *Plasmodium* parasite erythrocytic stages live in an environment ofincreased oxidative stress, rich in iron andoxygen, the key components forthe generation of ROS via the Fenton reaction. In the parasite's DV, where largequantities of haemoglobin are digested at acidic pH, the conversion of oxidized oxy-hemoglobin containing ferroprotoporphyrin IX (Fe^{2+}) to met-hemoglobin containing ferriprotoporphyrin IX ($Fe^{3+)}$ and O_2^- is increasedcompared to uninfected RBC, where only 3% of total hemoglobin is converted in this way (Atamna and Ginsburg, 1993; Becker *et al*., 2004).

In infected RBC, not only the parasite but also thehost cell itself issubject to increased oxidative stress and oxidative damage. Several bloodcell disorders such as α- and β-thalassaemia and sickle cell anaemia aswell as glucose 6- phosphate dehydrogenase deficiency cause a naturally higherlevel of oxidative stress in the RBC andconfer a certain protection against infection with *Plasmodium* (Kwiatkowski, 2005; Williams, 2006; Aslan and Freeman, 2007; Ho *et al*., 2007; Fibach and Rachmilewitz, 2008;). This may either beas a result ofimpaired infection

and growth of the parasites in these host cells due toincreased oxidative stress or byearlier damage induced alterations to the red blood cell membranes during the development of the parasite and earlierrecognition of the infected cells by the immune system (Cappadoro *et al*., 1998; Giribaldi *et al*., 2001; Williams *et al*., 2002).

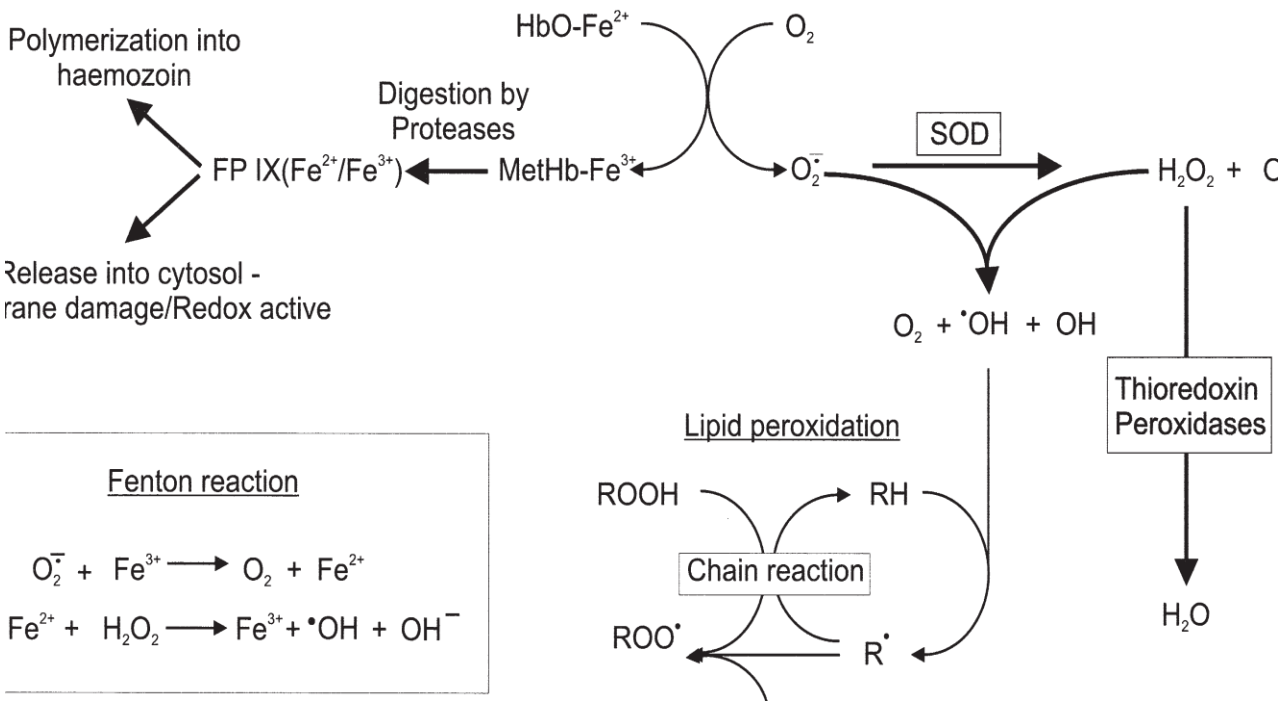


Figure 4: Sources of reactive oxygen species in Plasmodium (Imlay, 2003)

2.7.2. Superoxide mutases

Superoxide dismutases (SOD) are metalloproteins responsible for the detoxification of superoxide anions. Eukaryotes havemitochondrial Mn-dependent SODs and cytosolic Cu/Zn dependent SOD, while protozoan parasites usually contain Fe-dependent SODs (Fridovich, 1995). These differences probably make theparasite SODs attractive therapeutic targets for a wide range of infectious diseases (Turrens, 2004). *P. falciparum* is made up of two distinct Fe-dependent SODs, localized in thecytosol and mitochondrion (Gratepanche *et al*., 2002; Sienkiewicz *et al*., 2004). Both enzymes convert superoxide anions produced by the electron transport chain and other metabolic processes to molecular oxygen and hydrogen peroxide. It hasbeen suggested previously that large amounts of the host cell Cu/Zn-SOD taken up into the DV can contribute to the detoxification of superoxide anions generated in this organelle before the enzyme is digested (Fairfield *et al*., 1983). However, the dismutation of superoxide anions can occur spontaneously in this organelle and might be efficiently accelerated by the low pH and not in need of enzymatic catalysis (Ginsburg and Atamna, 1994). No gene for a catalase hasbeen identified in *P. falciparum.*

2.7.3 Glutathione

Glutathione is the main thiol redoxbuffer in almost all eukaryotes and it determines the intracellular redox status. Glutathione tripeptide (γ-glutamyl-cysteinyl-glycine) is a co-factor for antioxidant and detoxification enzymes such as glutathione S-transferases (GST) and glutathione peroxidases (GPx). Its abundance and redox state helps the cell todefend itself against external and internal stresses. Generally, glutathione is highly abundant as an enzyme co-factor and an

antioxidant. It is active inits reduced form as GSH, which is maintained by the NADPH-dependent glutathione reductase (Krauth-Siegel *et al*., 1996). The ratio of GSH to GSSG is usually high (>30:1) to ensure maintenance of theintracellular reducing environment. Therefore, GSH levels and GSH/GSSG ratio give an appropriate indicator of cellular redox homeostasis and oxidative stress (Mullineaux and Rausch, 2005; Deponte, 2013).

It hasbeen reported that *Plasmodium* species also possess γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GS), the enzymes responsible for the *de novo* biosynthesis of GSH. Availability of the amino acid precursors (glutamate, cysteine, and glycine) ascertains a constantsource of the tripeptide in theparasites cytoplasm in the GSH biosynthetic pathway (Lüersen *et al*., 2000; Meierjohann et al., 2002; Patzewitz et al., 2013). GSH biosynthesis is important in *Plasmodium*-infected RBC. Rapid loss or turnover of glutathione is constantly replenished via their biosynthetic pathway (Lüersen *et al.*, 2000; Barrand *et al*., 2012; Patzewitz *et al*., 2012). The rapid glutathione turnover was suggested to be as a result of GSSG generated in the parasite which may be excreted into the host RBC, and apparently has a decreased or loss of ability to synthesize GSH *de novo.* This provides theco-factor to its host cell, containing glutathione reductase whichreduces GSSG back to GSH (Atamna and Ginsburg, 1997).

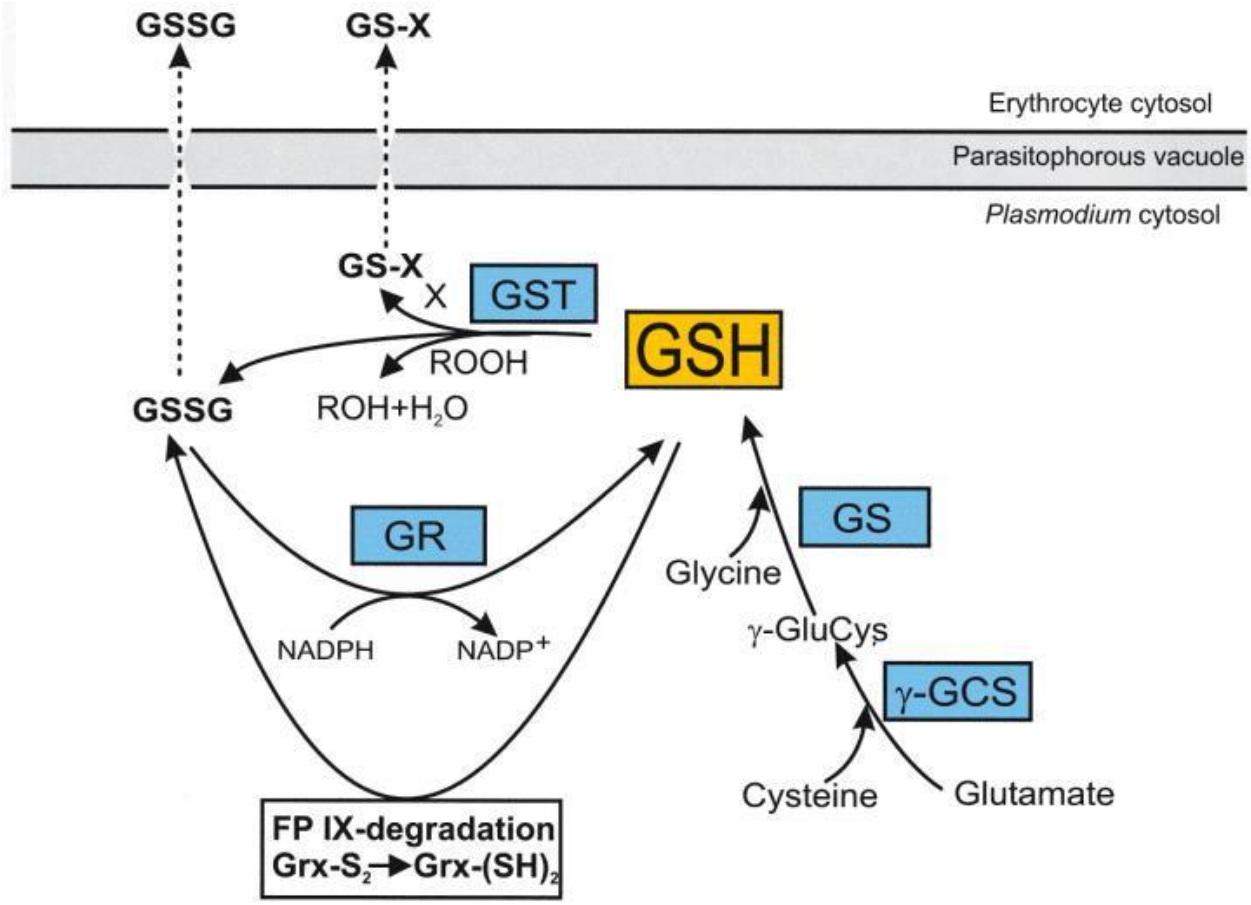


Figure 5: GSH metabolism of *P. falciparum* (Müller 2004).

GSH is synthesized in two consecutive steps by theenzymes γ-glutamylcysteine synthetase (γGCS) and glutathione synthetase (GS). GSH is used by the single glutathione-S-transferase found in P. falciparum to label xenobiotics (X) for export (GS-X). GS-X adducts are excreted from cells throughpumps such as multidrug resistanceproteins. GST can also use GSH to detoxifyperoxides whichresults in the formation of oxidized glutathione disulphate (GSSG). GSSG is alsoproduced by the reduction of glutaredoxins (Grx-S2 to Grx(SH)2) and thedegradation of ferriprotoporphyrin IX (FPXI). GSSG is either excreted from the parasite or reduced to GSH by glutathione reductase (GR).

2.8. Immunity against malaria

Acquisition of immunity to malaria is a gradualprocess and a state of sterile immunity is never attained. In malaria endemic regions where individuals are constantly exposed to infections; clinical malaria immunity is usually acquiredleaving children under the age of five as the mostlyaffected group (Hviid, 2005). Host immune responseplays an important role in conferringantiparasite and disease immunity and this hasbeen reported to be age and transmission intensity dependent (Marsh and Kinyanjui, 2006; Langhorne *et al*., 2008). The mechanisms by whichimmunity against malaria is acquired is notwell understood but there is ampleevidence suggesting that antibody repertoires raised against malaria parasites' variant surface antigens (VSAs) over time areresponsible for the gradual development of immunity (Day and Marsh; 1991; Staalsoe and Hviid, 1998; Bousema *et al*., 2004). Host immuneresponse can be directed againstmany stages of the parasites' life cycle (Langhorne *et al*., 2008).

Protective antibodies directed tospecific malaria antigensprevent erythrocytes invasion by merozoites (Blackman *et al*., 1990). Cytophilic antibodies such as IgG1 and IgG3 (Bouharoun-Tayoun *et al*., 1995) also mediate theantibody dependent parasitekilling by facilitating opsonisation followed byphagocytosis through the interaction between antibodies and parasite proteins (Bull *et al*., 1998). Development of an effective vaccine againstmalaria hasbeen attributed to the complexinterplay of parasite proteins with the immunesystem of the host. Immunity againstmalaria can beclassified into innate or natural immunity and adaptive or acquired immunity.

2.8.1. Natural or innate immunity

The innate immunity is found in the host and doesnot depend on any previousinfection. Alterations or modification inthe structure of haemoglobin or in certain enzymes havebeen shown toconfer protection against either the infection or its severe manifestation which areoften found in areas of high malaria transmission (Richard and Kamini, 2002; Denise *et al*., 2009). Innate immunity to malaria is naturally present in the host that prevents theestablishment of the infection or inhibitory responseagainst the introduction of the parasite. The immune response to the parasite is multifaceted and it is basically both species and stage specific (Stevenson and Riley, 2004). The activation of components of theinnate immune system is crucial tocontrol parasitereplication, contributing tothe subsequent elimination and resolution of the infection (Urban *et al*., 2005). Neutrophils, monocytes/macrophages, natural killer (NK) cells, NKT cells, dendritic cells, and gamma T cells are all the cells of theinnate immune system in charge of controlling the earlyprogression of the disease throughphagocytosis and/or production of inflammatory mediators. The consequences of the inflammatory responsecreated by the cells of the innate immune system which arestimulated by parasites or their products at the rupture of the late stage infected erythrocytes producedvarious symptoms of malaria attacks such as fever, nausea, headaches, and others (Taramelli *et al*., 2000; Stevenson and Riley, 2004).

An imbalance between the production of pro- or anti-inflammatory cytokines, such as TNF-α, IL6, IL1β, or IL-10 or mediators, like nitric oxide, maycontribute to the pathogenesis of the severe form of the disease. Elevated levels of TNF-α are found in the serum of severe patients and havebeen associated with cerebral malaria (CM) (Kwiatkowski *et al*., 1990). The cells of native immunity have a receptor, known as pathogen recognition receptors (PRRs). This receptor triggers the response upon the recognition of specific parasite molecules called pathogen

associated molecular patterns (PAMPs) (Janeway, 2002). Malaria PAMPs include the hemozoin (malaria pigment) or protein anchor, glycosyl-phosphatidyl inositol (GPI). PRRs related to malaria are the membrane bound Toll-like receptors (TLRs), the cytosolic and soluble receptors (Coban *et al*., 2005; Krishnegowda *et al*., 2005; Shio *et al*., 2009).

2.8.2. Acquired or adaptive immunity

Acquisition of specific immunitydue to repeated exposure to theparasite occurs inareas where malaria is pronounced (Riley *et al*., 2006; Langhorne *et al*., 2008). Acquired immunity againstmalaria is seen after infection and its protective efficacy varies depending on the number of infections suffered, place of stay, characteristicsof the host etc. Acquired immunity are classified as anti-parasite immunity, anti-disease immunity, and sterilizing immunity (protects against new infections by maintaining a low-grade, asymptomatic parasitemia; also called premunition) (Richard and Kamini, 2002; Denise *et al*., 2009).

The innate system provides the mechanisms for malaria immunity which is subsequently followed by the development of acquiredimmunity. People living in malaria endemic areas gradually acquire mechanisms due to repeated infections. Parasites have been shown to maintain a balancedrelationship with their human hosts. They can escape partially from the host effector mechanisms, while hosts are capable of developing partial immunity against the parasite. This type of immunity takes years to develop and usually lasts shortly but requiresrepeated infections, (Riley *et al*., 2006; Langhorne *et al*., 2008).

In stable endemic region, a greatburden of morbidity and mortality falls onyoung children. Children born to immune mothersappear to be relatively immune tomalaria for a period (Fried *et al*., 1998; Urban *et al*., 2005). This is as a result of the prenatal or postnatal transfer of

protective antibodies from mother to child. The acquired immunity is mediatedby specific antibodies toseveral conserved and polymorphic proteins, and to the highly variableprotein; *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) expressed by the parasite at the trophozoites and schizonts stages and exported on the surface of infected erythrocytes (Miller, *et al*., 2002).

The acquired anti malaria immunity doesnot last long. The acquired immunity becomes ineffective and theindividual is again vulnerable to the full impact of a malarial infection when they leavemalarious environment. The immunity during pregnancy also becomeless effective, dueto the physiological immunosupression aswell asthe cytoadherence of erythrocytes tothe newly available Chondroitin Sulfate A receptors on theplacenta. The loss of acquired immunity predisposes pregnantwoman to malaria and its complications (Richard and Kamini, 2002; Denise *et al*., 2009). Immunosupression in HIV/AIDS also increasesthe risksof clinical malaria, its complications and death (Laith *et al*., 2006).

2.9. Malaria diagnosis

Accurate and reliable diagnosis is one of the major interventions of the malaria control strategy and thekey to effective disease management. It is of greatconcern that poor diagnosis continues to hinder effective malaria control. This may bedue to various factors including, high prevalence of asymptomatic infection in some areas, lack of resources and inadequate access to trained health care providers and healthfacilities (WHO, 1993).

The laboratory diagnosis of malaria dependexclusively on microscopy, a valuable technique when carried outcorrectly but unreliable and wasteful when poorly performed. The development of rapid diagnostic tests (RDTs) for malariausing immunochromatographic test

strips, offer a valid alternative to complement microscopy (WHO, 1996). Various RDTs have been tested in clinical and field situations.

2.9.1. Clinical/presumptive diagnosis and treatment

Clinical diagnosis has been the only feasible option particularly in rural areas where laboratory support to clinical diagnosis does not exist. It requires nospecial equipment or supplies and inexpensive tocarry out. Clinical diagnosis is based on the signs and symptoms of patients aswell as physical findings at examination. The symptoms of malaria vary and are nonspecific. It includes fever, headache, diarrhea, nausea, vomiting, anorexia, weakness, myalgia, chills, dizziness and abdominal pain, (Looareesuwan *et al*., 1999).

Clinically, the diagnosis of malaria is still consider as a challenge owingto the imprecise nature ofthe signs and symptoms, which tosome extent overlap with other common potentially life-threatening diseases (Mwangi *et al*., 2005; McMorrow *et al*., 2008). A diagnosis of malaria on clinical basis alone is unreliable, and this couldbe confirmed by laboratory tests when possible. Due to lack ofspecificity, in some settings, disease management based onclinical diagnosis alone is justifiable.

2.9.2. Laboratory diagnosis of malaria

Microscopy of thin and thickstained blood smears is believedto be the gold standard for malaria diagnosis (Moody and Chiodini, 2000). Thick smears are used to detect infections whereas thin smears are important for species identification, quantification of parasitaemia as well as assessment for thepresence of schizonts, gametocytes and malaria pigments in neutrophils and monocytes. The diagnostic accuracy of microscopy dependsmajorly on the

quality of blood smears and experience of the laboratory personnel preparing and reading the blood slides (Wongsrichanalai *et al*., 2007).

The diagnostic advantages of microscopy is that it is comparatively inexpensive; requires little laboratory infrastructure; allows definitive identification of infecting species and mixed infections; can be used to determine the magnitude of parasitemia and can be used for serial examinations to monitor the efficacy of therapy. With the advancement in diagnostic technologies, diagnostic tools with better sensitivity and specificity for malaria havebeen developed. Amongst others, these include, fluorescence microscopy of parasite nuclei stained with acridine orange, rapid diagnostic test (RDT), polymerase chain reaction (PCR), serology and real-time quantitative nucleic acid sequence based amplification (real time QT-NASBA) (Mens *et al*., 2006; Mens *et al*., 2007; de Oliveira *et al*., 2009).

The performances of these new tools havebeen shown to be similar or even better than thatof microscopy in diagnosing very low parasite densities. However, many of the highly sensitive diagnostic tools such as PCR and real time QTNASBA are usually utilised in epidemiological studies rather than in facilitating treatment (Mens *et al*., 2006; Mens *et al*., 2007), leaving RDTs as the only feasible diagnostic complement for malaria diagnosis in resource constrainedsettings. Rapid diagnostic tests aresimple to perform, easy to interpret, require minimal laboratory training and capitalinvestment or electricity and above all, if managed well RDTs can perform equallywell or even betterthan microscopy (Dhorda *et al*., 2012; Endeshaw *et al*., 2012).

2.10. Treatment of malaria

2.10.1. General treatment

Antimalarial drugs work by disrupting processes or metabolic pathways in different subcellular organelles and most of them target the erythrocytic stages. For effective treatment, antimalarial drugs must be fast acting, highly potent against blood stageparasites, with minimal toxicity and should be readilyavailable and affordable to inhabitants of endemicregions (Greenwood *et al*., 2008).

Early malaria case detection and prompt treatmentwith safe and effective antimalarial drugs still remains themainstay of malaria casemanagement (Winstanley, 2000). If not properly managed, eitherdue to missed or delayed diagnoses, malaria may progress frommild through complicated to severe disease. Case management usually depends on the severity of infection, age, costs and availability of drugs and therapeuticefficacy of the antimalarial drug (White, 1996). Gestational age is also animportant issue to consider prior to prescription of anydrugs due to potential risks of harming theembryo/foetus (Nosten *et al*., 2006).

2.10.2. Antimalarial drugs

Some drugs are used prophylactically to prevent malaria, while others are directed towards treating acute attacks. In general, antimalarial drugs are classified interms of the action against the different stages of the life cycle of the parasite. Blood schizonticidal drugs is the most antimalarial drugs thattarget the asexual erythrocytic stages of the parasite. Tissue schizonticidal drugs targetthe hypnozoites in the liver whereas gametocytocidal drugs terminatethe sexual stages of the parasite in the bloodstream.

Limited number of antimalarial compounds canbe used to treat or prevent malaria. The most commonlyused are quinine and its derivatives (chloroquine, amodiaquine, primaquine and mefloquine), antifolate (proguanil, chlorproguanil, pyrimethamine, and trimethoprim) and sulfa (dapsone, sulfalene, sulfamethoxazole, sulfadoxine, and others) combination drugs, as well as artemisinin and its derivatives (artesunate, arteether, artelinate, artemether, dihydroartemisinin). Antibiotics such as tetracycline and its derivatives (doxycycline) can also be used for treatment and prophylaxis in combination with quinine.

Due towidespread of drug resistance, the mosteffective treatment for malaria are artemisinin-based combination therapies (ACTs) which is nowcentral to the first-line treatment of malaria. Many malaria prevalent regions haveshifted to artemisinin combination therapy (ACT) where artemisinin is partneredwith a drug with long half life such as mefloquine or lumefantrine (Mutabingwa and Adam, 2013). ACTs compensate for thepoor pharmacokinetic properties of the artemisinins, enhance treatmentefficacy and are believe to reducethe emergence of drug-resistant parasites (Petersen *et al*., 2011).

Other effective drug combinations such as atovaquone-proguanil (Malarone) are also available but theiruse is compromised by high costs. Theuse of combination therapy, especially when partnerdrugs have different mechanisms of action hasthe potential of acting synergistically allowing rapid parasite clearance and at the same time inhibitingthe development of resistance to either of the components.

2.11. Antimalarial drug resistance

Resistance to antimalarial drug hasemerged as one of the major obstacles facingthe global effortto control malaria (Yusuf *et al*., 2010). Resistance to nearly all antimalarials currently in

use hasbeen developed by *P. falciparum*, although thedistribution of resistance to any single antimalarial drug variesgreatly in different geographical locations (Mockenhaupt, 1995; Looareesuwan *et al*., 1997). As a result of extensive use of antimalarial compounds, a massive selection pressure hasbeen subjected to the parasites that havein turn developed mechanisms of resistance (White, 2004; WHO, 2010).

The important distinction to make is that not all treatment failures aredue to drug resistance as several other factors canalso contribute to treatment failures such aswrong diagnosis, incorrect choice of drugs, sub-optimal regimen (dose, schedule, duration), non-compliance, sub-optimal absorption (nausea, diarrhea, vomiting, malabsorption), idiosyncratic pharmacokinetics, poor quality drugs, resistance of thepathogen to the drug etc. Antimalarial drug resistance spreads because itconfers a survival advantage in thepresence of the antimalarial drugs and hence with time it results in a greater probability of transmitting resistantthan sensitive parasites. Resistant strains can easilyrecrudesce and also mayhave a greater chance of generating adequate gametocytes that are transmitted and perpetuate its population (White *et al*., 2009).

2.11.1. Mechanisms and spread of antimalarial drug resistance

The spread of antimalarial resistance occurs whenparasites are exposed to the selective window of drug concentrations adequate todestroy sensitive but not resistantparasites (Barnes *et al*., 2008). The advantage of drugs with longer terminal elimination half-lives is that it provides a longer post-treatment prophylactic effect, which appears tobe important fortheir action in intermittent preventive therapy (IPT) in vulnerablegroups such as pregnant women, infants and young children. However, the disadvantage of these long acting antimalarials is the residual

concentrations inhibitingsensitive parasites far longer than resistant parasites, consequently fuelling thespread of resistance (Barnes *et al*., 2008).

Mutations and/or changes inthe copynumber of genes encoding or related to the drug's parasite target or influx/efflux pumps mayaffect intra-parasitic concentration of the drug. The process might require only a single genetic event or an array of multiple unlinked events (White, 2004). Generally, resistance appears to occur viaspontaneous mutations which confer decreased sensitivity to agiven drug or class of drugs (Mahajan and Umar, 2004).

Numerous factors contributing to theinitiation, spread and intensification of drug resistance exist, although theirrelative contribution to resistance is unknown. Factors that havebeen linkedwith antimalarial drug resistance include human behavior, vector and parasite biology, pharmacokinetics and economics. The conditions leadingto malaria treatment failure may also beresponsible for the development of resistance (Austin and Anderson, 1999). The emergence of antimalarial drug resistance in *Plasmodium* depends on multiple factors such as the mutation rate of the parasite, overall parasite load, drug selection, treatment compliance and the spread of those resistant alleles toother individuals (Petersen *et al*., 2011).

2.11.2. Molecular basis of antimalarial resistance

Chloroquine (CQ) resistance to malaria parasites isassociated topoint mutations in the chloroquine resistance transporter (PfCRT [encoded by *pfcrt*, located on chromosome 7]) (Van *et al*., 2011). *Pfcrt*-K76T mutation confers resistance *in vitro* and is themost reliable molecular marker for CQ resistance (Sidhu *et al*., 2002). Polymorphisms and point mutations in *P. falciparum* multidrug resistance (pfmdr-1) play a modulatoryrole in CQ resistance, which appears to be a parasite strain-dependent phenomenon (Valderramos and Fidock, 2006). There is

a suggestion that *pfmdr*-1 mutationassociated with chloroquine resistance may also account for reduced susceptibility to quinine. Available evidence suggeststhe roles of the multidrug resistance gene 1 (pfmdr1) on the resistance of *P. falciparum* to a quitenumber of blood schizonticides including chloroquine, mefloquine, quinine and artemisinin.

Antifolate agents used for the management of malarial infection acton the folate metabolism of the parasite. Malaria parasites resistance to antifolates is mediatedby mutations in thetarget proteins dihydrofolate reductase (DHFR) and dihydropteroate synthetase (DHPS). Pyrimethamine, proguanil and chloproguanil bind andinhibit dihydrofolate reductase (DHFR) and the sulfa drugs (sulfadoxine and dapsone) inhibit dihydropteroate synthetase (DHPS) by actingas analogues of p-aminobenzoic acid (PABA), a folate precursor (Triglia and Cowman, 1994). The *dhfr* mutations modify theshape of the active site cavity wherethe DHFR inhibitors bind the enzyme, leading to differential binding affinities forthe different drugs. Both the *dhfr* and *dhps* gene mutations occur in a progressive, stepwise fashion, with higher levels of *in vitro* resistance occurring in thepresence of multiple mutations (Plowe *et al*., 1997).

Specific gene mutations encoding for resistance to both DHPS and DHFR havebeen identified and the combinations of these mutations arerelated to varying degrees of resistance to antifolate combination drugs (Wang *et al*., 2005). Emergence of decreased sensitivity of the parasite to artemisinin hasput at risk the entire strategy for malaria treatment (Ward and Boulton, 2013). Mechanisms of resistance to artemisinin are poorly understood, although mutations in some parasite genes havebeen partly linked with resistance (Vernet *et al*., 2014).

The major molecular marker of artemisinin resistance proposed is the ATP-consuming calcium-dependent *P. falciparum* SERCA ortholog, PfATP6. Earlier studies in transfected Xenopus laevis oocytes provided evidence that PfATP6 could be specifically inhibitedby

artemisinin, an effect that couldbe antagonized by the mammalian SERCA inhibitor thapsigargin (Eckstein-Ludwig *et al*., 2003). Recent study however reports that theassociation of mutations in the *pfatp*6 (target of artemisinins) and *pfmdr*1 genes might be the main contributor to artemisinins resistance (Cui and Su, 2009; Ding *et al*., 2011).

2.12 Apoptosis

Apoptosis otherwise regarded as programmed cell death is a normalcomponent of the development and health of multicellular organisms. Cells die in response to various stimuli and during apoptosis they do so in a controlled, regulated fashion. This makes apoptosis distinct from anotherform of cell death known as necrosis in which uncontrolled cell death leads tolysis of cells, inflammatory responses and, potentially, to serious health problems. Apoptosis, by contrast, is a process in which cells play an activerole in their own death. Hence, apoptosis is often referred to as cell suicide) (Lockshin, 1964).

Apoptosis is an essential physiological process that plays avital role intissue homeostasis, differentiation, regulating growth, development and immune response, and clearing redundant or abnormal cells in organisms. Alteration in the apoptosis is linkedto a number of pathological conditions. Deficiency in the apoptotic processs could lead to cancer, autoimmune diseases and viral infections, while excessive or enhanced rate of apoptosis results in neurodegenerative disorders, AIDS and ischemic diseases (Launay *et al*., 2005).

Mitochondria plays animportant role in ROS production and this is relevant under pathophysiological conditions such as cancers, ischemia and diabetes. The considerable damage leads toincrease in mitochondrial permeability capable of moving solutes up to 1.5kDa,

membrane depolarization, uncoupling ofoxidative phosphorylation, ATP depletion and ultimately cell death .

The complex role of mitochondria in mammalian cell apoptosis became knownwhen biochemical studies identified several mitochondrial proteins usually resident in the intermembrane spaceof mitochondria that areable to trigger cellular apoptotic programs directly (Liu *et al*., 1996; Susin *et al*., 1999; Du *et al*., 2000; Li *et al*., 2001). These proteins are released into the cytosol and/or the nucleus in response to avariety of apoptotic stimuli. They promote apoptosis eitherby activating caspases and nucleases or by neutralizing cytosolic inhibitors of this process (Li *et al*., 2001). Mitochondria may be the source of three relatively independent lethal signals thattrigger or switch cell death pathways: ATP, ROS, and the apoptogenic/necrogenic pathway.

2.12.1 Initiators and executors of apoptosis

The caspases, regarded as cysteine proteases are homologous to *C. elegans* ced-3. They areimportant in the apoptotic signalling networks and areactivated in most cases of apoptotic cell death (Bratton *et al*., 2000). Caspases are derivative of cysteine-dependent aspartate-specific proteases. About fourteen (14) different members of the caspase-family havebeen described in mammals, with caspase-11 and caspases-12 only identified in mouse and sevenare identified in *Drosophila*, and (Denault, 2002).

Caspases are synthesized in the cell as inactive zymogens, so theyare regarded as procaspases. The proapoptotic caspases are grouped into initiator caspases including procaspases-2, 8, 9 and 10, and executioner caspases including procaspases 3, 6 and 7. The executioner caspases hasonly short prodomains while the initiator caspases possess long

prodomains, having death effector domains (DED) (such as procaspases 8 and 10) or caspase recruitment domains (CARD) (such as procaspase 9 and procaspase 2). The initiator caspases are recruited and activated at death inducing signaling complexes either inresponse to signals that originates fromthe cell (intrinsic apoptosis pathways) or in response tothe binding of cell surface death receptors (extrinsic apoptosis pathways) through their prodomains.

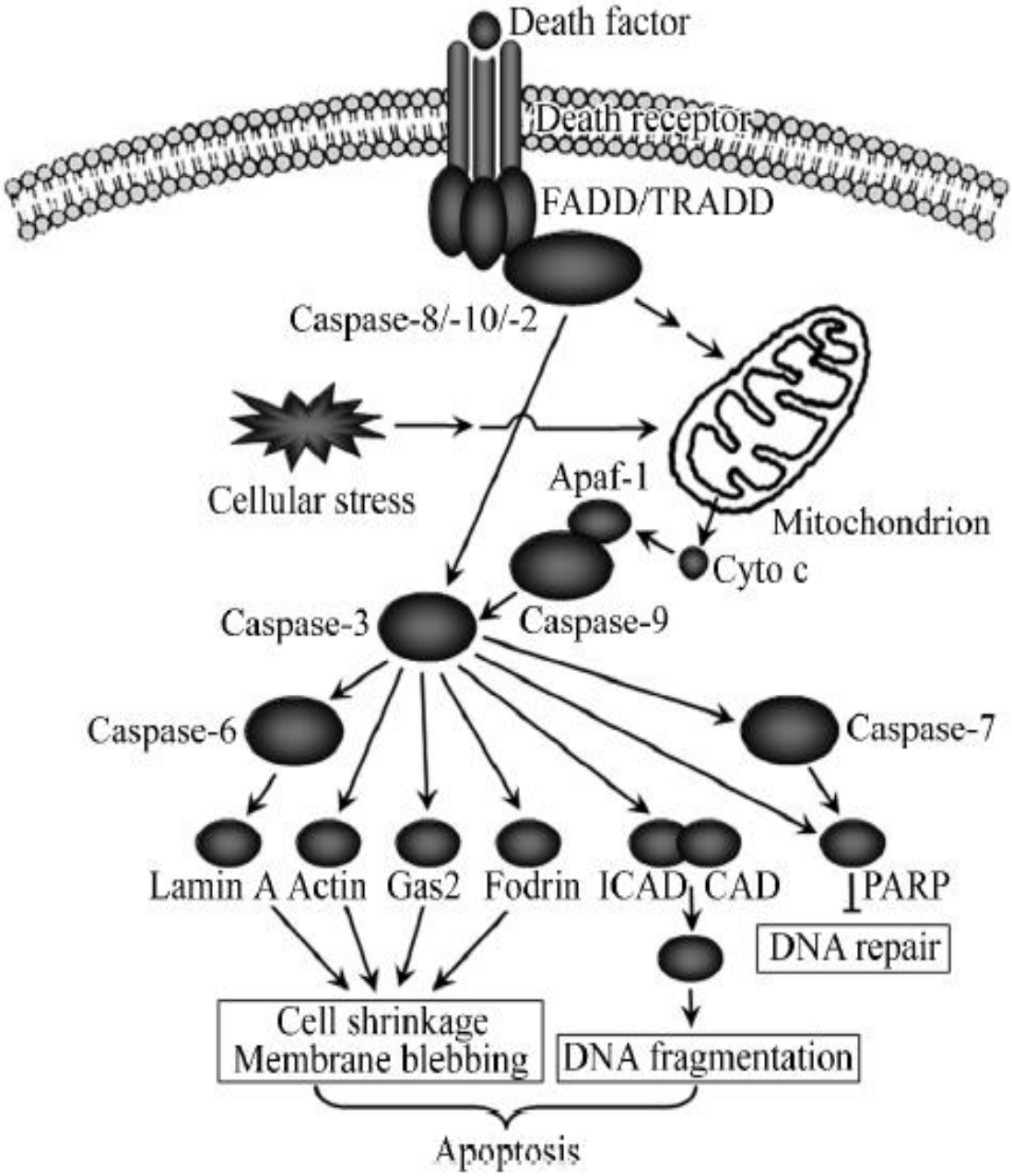


Figure 6: Downstream substrates of apoptosis executioner caspases (Hockenbery *et al*., 1993).

2.12.2 Extrinsic pathway of apoptosis

This pathway involves the engagement of death receptors, a member of tumor necrosis factor receptor (TNF-R) family andthrough the formation of death inducing signaling complex (DISC), results toactivation of caspases cascade, including caspase-8 and caspase-3 which in turn induce apoptosis. Apoptotic signals initiated by specific ligands like Fas ligand and TNF alpha are transmitted by death receptors (cell surface receptors). These receptors play animportant role by activating a caspase cascade within seconds of ligand binding and induction of apoptosis via this mechanism is usually veryrapid (Ashkenazi and Dixit, 1999). The binding of ligand leadsto a conformational change inthe intracellular domains of the receptors which shows the presence of a ‘death domain’ and allows the recruitment ofseveral apoptotic proteins tothe receptor. The protein complex formed is often referred toas the Death Inducing Signaling Complex (DISC). The final step in this process is the recruitment of caspase 8 tothe DISC which results in activation of caspase 8 and the inititation of apoptosis (Ashkenazi and Dixit, 1999).

2.12.3 Intrinsic/mitochondrial-dependent pathway of apoptosis

Mitochondria play a crucial role inthe regulation of cell death. They contain anti-apoptotic and pro-apoptotic proteins. The pro-apoptotic protein includes Apoptosis Inducing Factor (AIF), Smac/DIABLO and cytochrome C. These factors are released from the mitochondria following the formation of a pore inthe mitochondrial membrane called the Permeability Transition pore (PT pore). These pores are considered to form viathe action of the pro-apoptotic members of the bcl-2 family of proteins, which in turn are activated by apoptotic signals like growth factor deprivation, free radical damage or cell stress. Mitochondria also play a keyrole in amplifying the apoptotic signaling from the death receptors, with receptor recruited

caspase 8 activating the pro-apoptotic bcl-2 protein such as Bid (Algeciras-Schimnich *et al*., 2002).

2.12.4 The role of bcl-2 proteins

The bcl-2 proteins are a family of proteins involved in the response to apoptosis. These proteins such as Bad, Bax or Bid are pro-apoptotic while bcl-2 and bcl-XL are anti-apoptotic. The balance of pro- and anti-apoptotic bcl-2 proteins determines thesensitivity of cells to apoptotic stimuli. Cells becomemore sensitive to apoptosis when there is an excess of pro-apoptotic proteins and they tend to be more resistant whenthere is an excess of anti-apoptotic proteins. An excess of pro-apoptotic bcl-2 proteins at the surface of the mitochondria is considered to beimportant in the formation of the PT pore (Algeciras-Schimnich *et al*., 2002). The pro-apoptotic bcl-2 proteins areoften found in the cytosol where they act as sensors of stress or cellular damage and subsequently move to the surface of the mitochondria where the anti-apoptotic proteins arelocated following cellular stress. The interaction between pro- and anti-apoptotic proteins modifies thenormal function of the anti-apoptotic bcl-2 proteins and can lead to the formation of pores in the mitochondria resulting in the release of cytochrome C and other pro-apoptotic molecules fromthe intermembrane space. This in turn leads to the formation of the apoptosome and caspase cascadeactivation.

The release of cytochrome C from the mitochondria is important in theinduction of apoptotic process. Cytochrome C interact with a protein called Apaf-1 once it is released into the cytosol and its interaction leads tothe recruitment of pro-caspase 9 into a multi-protein complex with cytochrome C and Apaf-1 called the apoptosome. Formation of the apoptosome leads to activation of caspase 9 and theinduction of apoptosis (Algeciras-Schimnich *et al*., 2002).

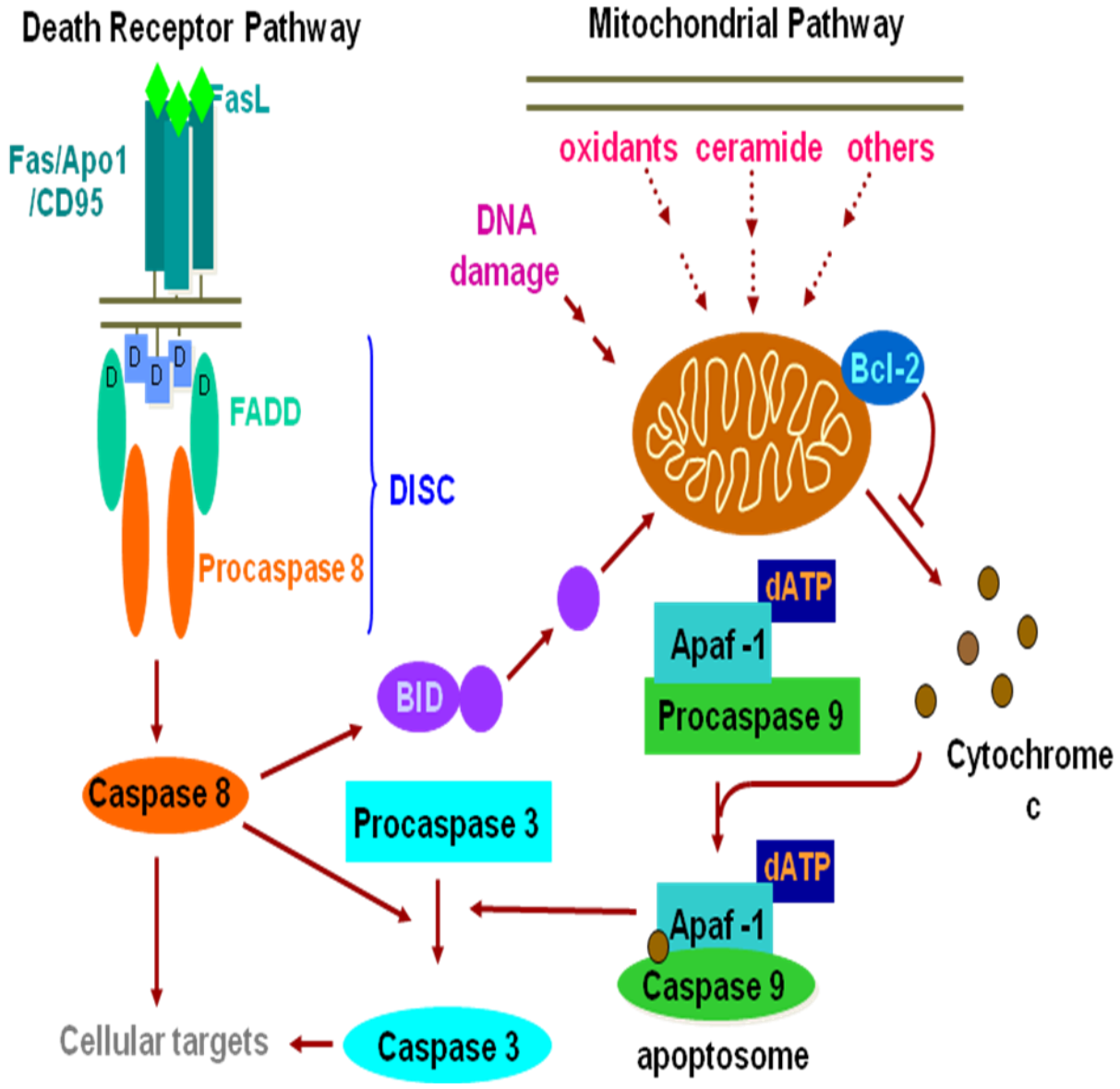


Figure 7: Apoptotic pathway involving mitochondria (Hengartner, 2000)

2.13 Mitochondrial membrane permeability transition pore

The sudden increase in the permeability of the mitochondrial membranes to molecules less than 1.5 KDaltons in molecular weight is regardedas mitochondrial permeability transition. Permeability transition occurs whenthere is opening of pores, a protein formed in the membranes of mitochondria undercertain pathological conditions. Induction of the permeability transition pore results to swelling of mitochondria and cell death which plays animportant role in some types of apoptosis.

2.13.1 Mitochondrial ca^{2+} overload and the PT pore

Mitochondrial Ca^{2+} overload is a major feature of cell injury. But overload *per se* is probablyinocuous. Isolated mitochondria can accumulate Ca^{2+} with impunity as long as exogenous adenine nucleotides are supplied and intramitochondrial pyridine nucleotides are maintained in a sufficiently reduced state (LeFurgey *et al*., 1988). In contrast, in theabsence of exogenous adenine nucleotides and in thepresence of high Pi or peroxides mitochondrial Ca^{2+} overload invariably leads to PT pore opening. PT pore opening wasfirst observed by Haworth and Hunter in the late 1970s. The osmotic behaviour of Ca^{2+}-plus-Pi-treated mitochondria suspended in poly (ethylene glycol) showed asharp cut-off in permeability at *M*r 1500, consistent with the induction of alarge pore of discrete size (Hunter and Haworth, 1979).

2.13.2 Permeability transition pore components

The opening in the mitochondria is the beginning of cell death. Some proteins identified with the permeability transition pore include, adenine nucleotide translocase (ANT), voltage-dependent anion channel (VDAC) and Cyclophylline D. Whenthere is Ca^{2+} overload coupled with oxidative stress, calcium binds toreceptors on the ANT causing depletion in adenine

nucleotide and increase in Pi concentration. Cyclophylline D plays animportant regulatory role in apoptosis. The binding of calciumattracts cyclophylline D binding on the ANT. Cyclophylline D exhibits a peptidyl cis-tras isomerise activity that changes the conformation of ANT leading to non-selective entry and exit into the mitochondria. This swells up the mitochondria and the cristea moves towards the outer mitochondria, bursting itscontent thereby releasing mitochondria proapoptotic factors into the cytosol such as cytochrome c, apoptosis activating factor 1 (Apaf-1) etc.

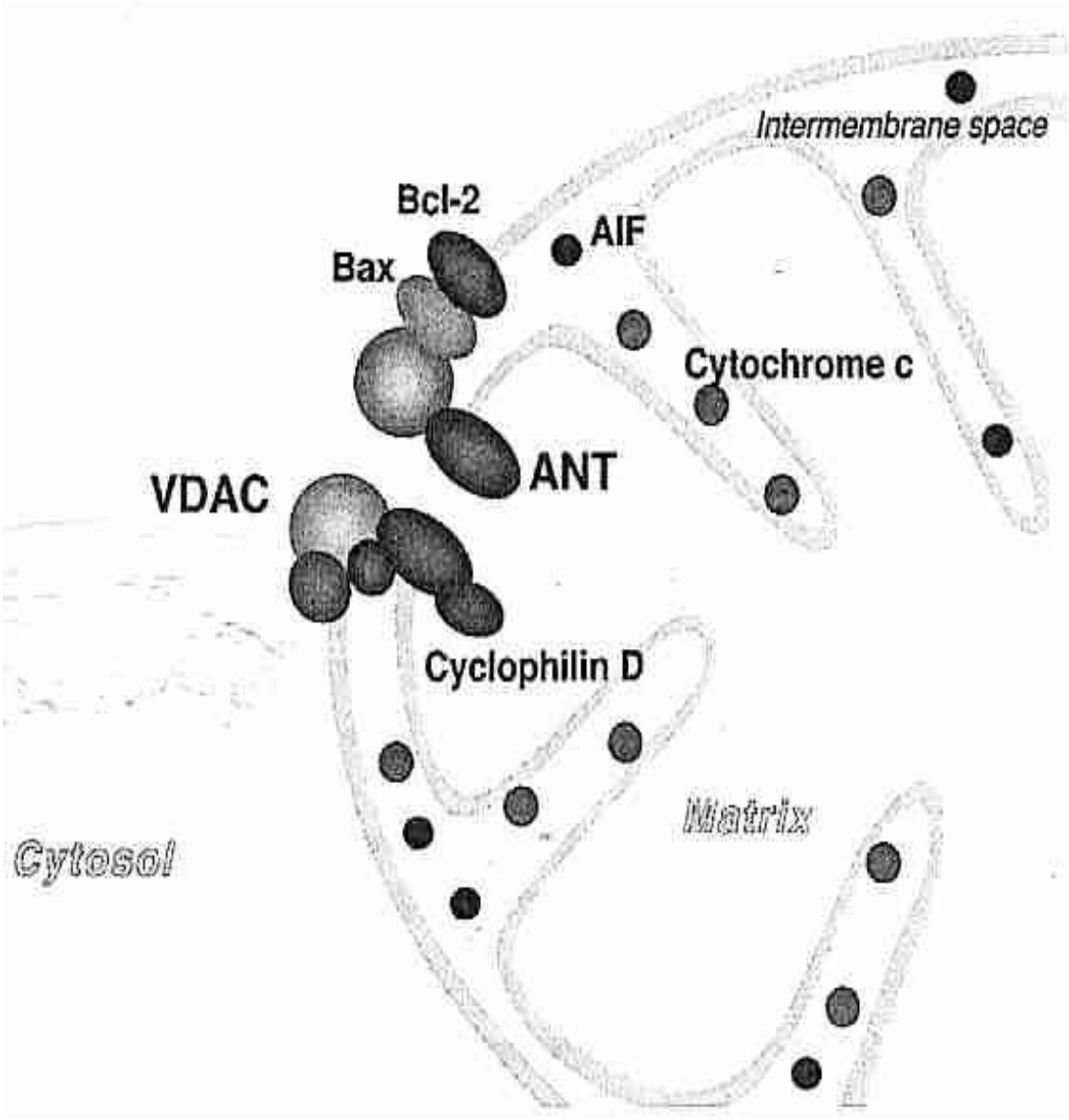


Figure 8: Components of the Mitochondrial Permeability Transition Pore (MPTP) (Hengartner, 2000).

2.14. Phytochemicals of Medicinal Plants

Phytochemicals are biologically active, naturally occurring chemical compounds found in plants. They provide health benefits for humans in addition to thoseascribed to micronutrients and macronutrients (Hasler and Blumberg, 1999). Various reports haveshown that bioactive phytochemicals or bionutrients found in medicinal plants play a significant role in preventing chronic diseases like cancer, diabetes and cardiovascular disease andare almost free from the side effects usually caused by synthetic chemicals (Fennell *et al*., 2004). Resistance against bacterial, fungal and pesticidal pathogens is associated to thepresence of these bioactive components in medicinal plants (Liu, 2004).

Phytochemicals that prevent against a variety of disease areclassified as dietary fiber, antioxidants, anticancer, immunity-potentiatingagents, pharmacological agents and detoxifying agents. Each class of these functional agents is made up ofseries of chemicals whichdiffers in potency. Examples of these plant chemicals are: flavonoids, cardiac glycosides, saponins, alkaloids, tannins, anthraquinones, sterols and triterpenes. A variety of drugs have been isolated from natural sources and many of these isolations were based on the traditional use of the agents (Rizvi *et al*., 2009).

2.14.1 Flavonoids

Flavonoids are the most studied phytochemical. They are water soluble polyphenolic molecules containing 15 carbon atoms and can bevisualized as two benzene rings attached together with a short three carbon chain. One of the carbons of theshort chain is always linkedto a carbon of one ofthe benzene rings, either directly or through anoxygen bridge, thereby forming a third middlering (Harnafi and Amrani, 2007). Flavonoids can be classified intofive major

subgroups, these include: flavones, flavonoids, flavanones, flavanols and anthocyanidins (Nijveldt *et al.*, 2001).

Flavonoids is composed of a wide range of substances thatplay essential role in protecting biological systemsagainst the harmful effects of oxidative processes on macromolecules, like DNA, lipids, carbohydrates and proteins (Atmani *et al*., 2009). Flavonoids identified from medicinal plants havebeen established to possess high antioxidant potential and also protect against free radical related diseases dueto their hydroxyl groups (Kris-Etherton *et al.*, 2002; Vaya *et al.*, 2003). They are reported topossess many useful properties such as anti-inflammatory activity, antimicrobial activity, oestrogenic activity, anti-allergic activity, antioxidant activity, cytotoxic antitumor activity and enzyme inhibition (Tapas *et al*., 2008).

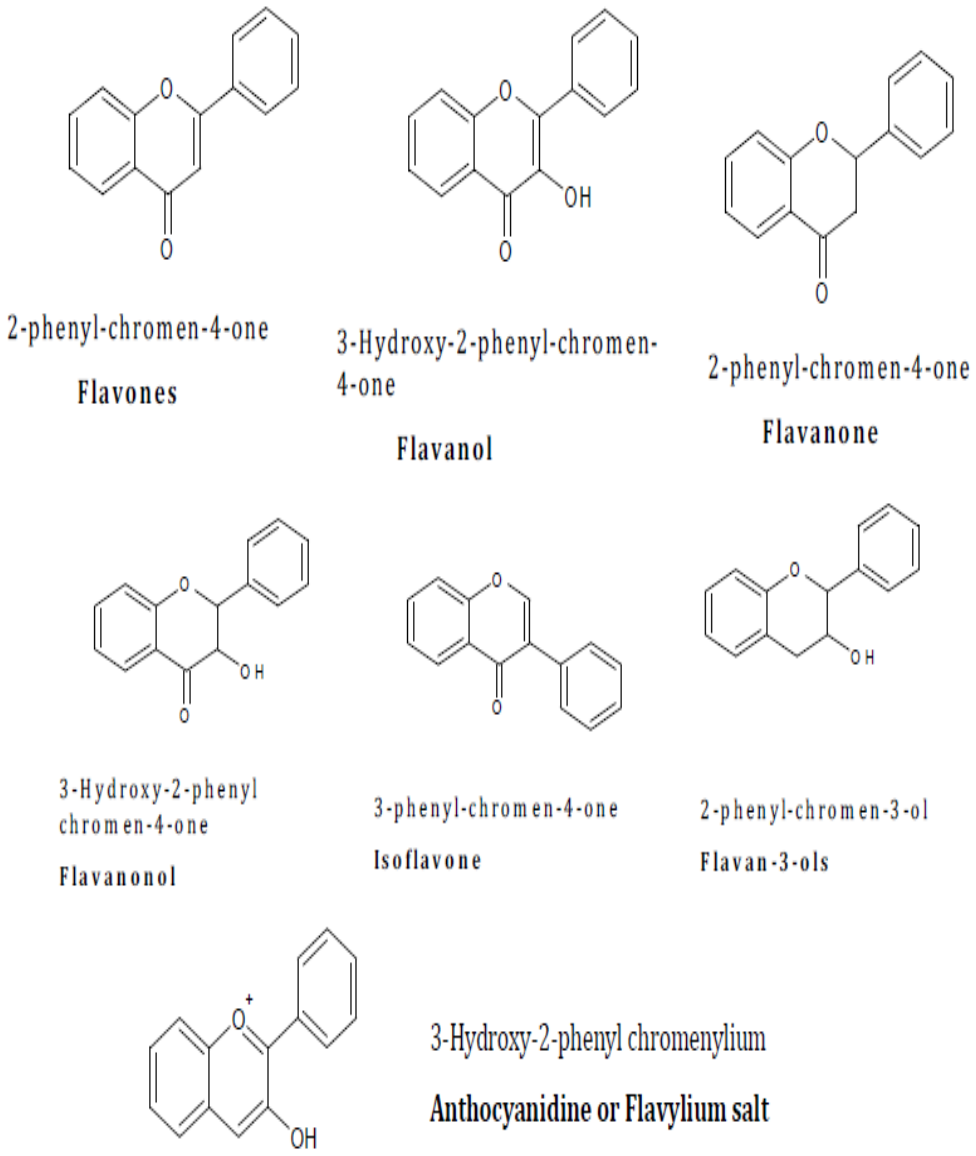


Figure 9: Chemical structures of some representative flavonoids (Harnafi and Amrani, 2007).

2.14.2 Saponins

They occur naturally as surface glycosides and arelargely produced by plants. They are alsoproduced by some bacteria and lower marine animals (Yoshiki *et al.*, 1998). The name saponin was derived from its ability to formstable soap-like foams in aqueoussolutions (Francis *et al.*, 2002). The structure of saponin consist of a sugar moiety (glucose, galactose, glucuronic acid, xylose, rhamnose or methyl pentose) which islinked via a glycosidic linkage and attached to a hydrophobic aglycone (sapogenin) whichmay be triterpenoid or steroid. The changes in the structure, position of the attachment and nature ofthe side chains of these moieties on the aglycone contribute to the complexity in the structure of saponin. There aretwo types of saponins which includes the steroid and triterpenoid saponins. The steroid saponins areoften found in medicinal plants (Fenwick *et al.*, 1991) while triterpenoid saponins arefound in many legumes such as soyabeans, beans, peas, lurcene, etc.

Both steroidal and triterpene aglycones have anumber of different substituents (H, COOH, CH_3) in which the sugars canbe attached tothe aglycone as one, two or threeside chains (Oleszek and Bialy, 2006). The biological importance of saponin hasbeen widely reported. Saponin exhibits antimicrobial activity and hasa lytic effect on erythrocyte membrane (Francis *et al*., 2002). The hypoglycemic effect hasbeen demonstrated on some plant isolates (Yoshikawa *et al.*, 2001). Saponin isolates havealso shown specificinhibition on thegrowth of cancer cells in-vitro (Marino *et a.l*, 1998), antifungal properties (Wang *et al.*, 2000) and antioxidant activities (Yoshiki *et al.*, 1998).

2.14.3 Tannins

Tannins are of two different types, condensed and hydrolysable tannins whichare polyphenolic compounds of plantsource (Mc Sweeney *et al.*, 2001). Hydrolysable tannins are gallic acid and ellagic acid esters. Hydrolysable tannins areprone to enzymatic and non-enzymatic hydrolysis than phenolic acids, and they arenormally soluble inwater. According to the products of hydrolysis, hydrolysable tannins arefurther classified into; gallotannins (which yield gallic acid and glucose) and ellagitannins (which yield ellagic acid and glucose) (Haslam, 1989).

Proanthocyanidins are polymers of flavan-3-ols linkedthrough an interflavan carbon bond that is not susceptible to hydrolysis. Proanthocyanidins are usually referred to ascondensed tannins. The nomenclature for proanthocyanidin is as aresult of oxidation reaction catalyzed by an acid which produces the red anthocyanidins uponheating proanthocyanidin in acidic alcohol solutions. The most common anthocyanidins produced are cyanidin and delphinidin from the corresponding proanthocyanidins, procyanidin and prodelphinidin (Reed, 1995). The antinutritive effects of tannins areassociated in their ability to combine withdietary proteins, polymers (such as cellulose, hemicelluloses and pectin) and minerals thusretarding their digestion. Tannins can also impair digestive processes by complexingwith enzymes and endogenous proteins (McSweeney *et al.*, 2001).

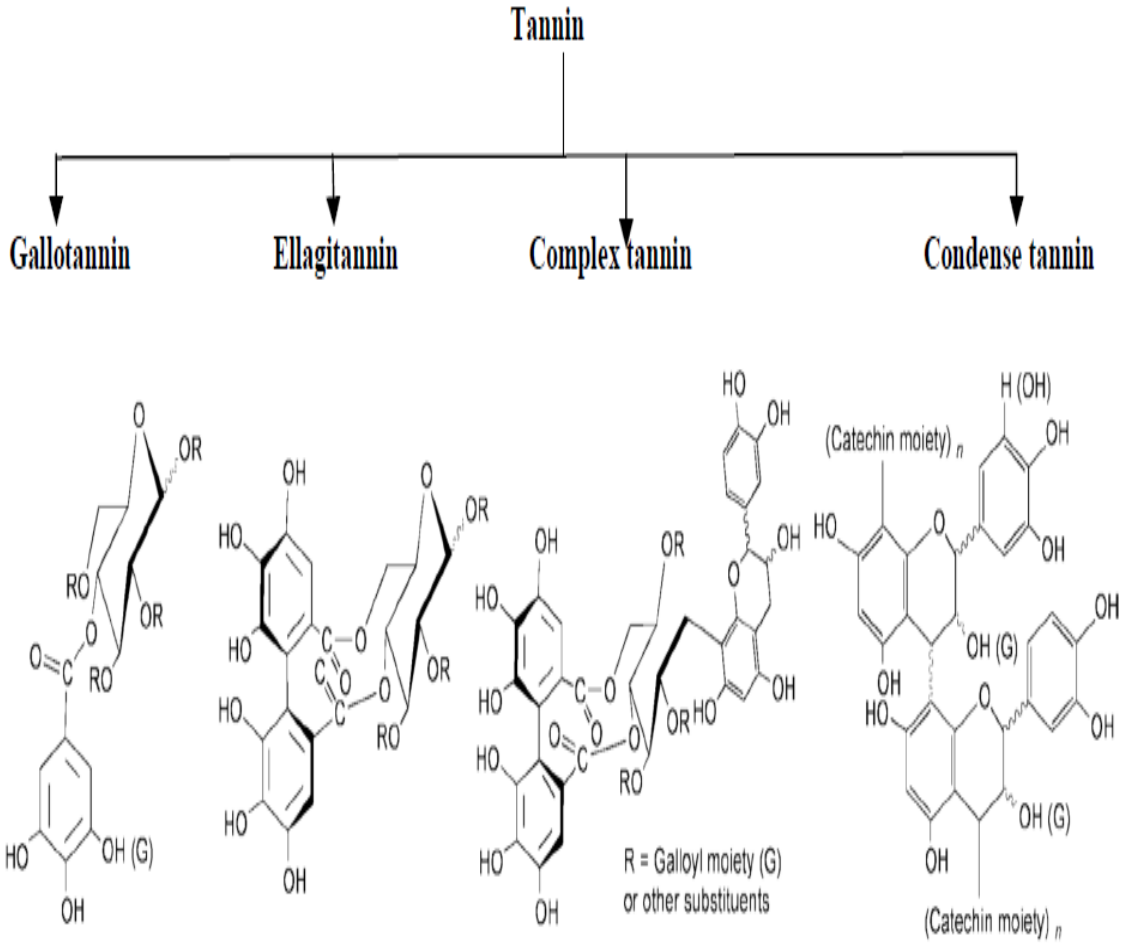


Figure 10: Classification of tannins (Mc Sweeney *et al.*, 2001).

2.14.4 Alkaloids

Alkaloids belong to a groupof complex nitrogen-containing compounds derived froma variety of sources, including microbes, marine organisms and plants, via complex biosynthetic pathways. Alkaloids are a structurally diverseclass of nitrogen containing compounds with over 12,000 structures elucidated from plants (Verpoorte and Memelink, 2002; Wink, 2003). Well known alkaloid compounds include purine alkaloids (caffeine and theobromine), benzylisoquinoline alkaloids (berberine and morphine), tropane alkaloids (cocaine and scopolamine) and monoterpenoid indole alkaloids (vinblastine, ajmaline). Alkaloids are classified based ontheir primary metabolite: purine alkaloids areproduced from adenine or guanine, isoquinoline alkaloids (IQA) aresynthesized from tyrosine, tropane alkaloids (TA) are produced from ornithine and monoterpenoid indole alkaloids (MIA) derived from tryptophan (Marasco and Schmidt-Dannert, 2007).

Alkaloids find use in the treatment of awide range ofailments and diseases, such as antimalarials and analgesics agents, anticancer agents, and in thetreatment of hypertension, parkinsonism andcentral nervous system disorders (Rathbone and Bruce, 2002). Some alkaloids havestimulant property as caffeine and nicotine. Morphine isused as the analgesic and quinine as the antimalarial drug (Rao *et al*., 1976).

i) Purine alkaloids

Caffeine (1,3,7-trimethylxanthine) is a purine alkaloid found in high concentrations in coffee and tea. It is a product of nucleic acid catabolism and synthesized through a series of three *S*-adenosyl-L-methionine (SAM) dependent methylation steps carried out by *N* methyltransferases.

ii) Benzylisoquinoline alkaloids

Benzylisoquinoline alkaloids (BIQA) are a large diverse class of compounds used as analgesics (morphine, codeine), antimicrobials (berberine, sanguinarine), and muscle relaxants (papaverine and (+)-tubocurarine) (Marasco and Schmidt-Dannert, 2007).

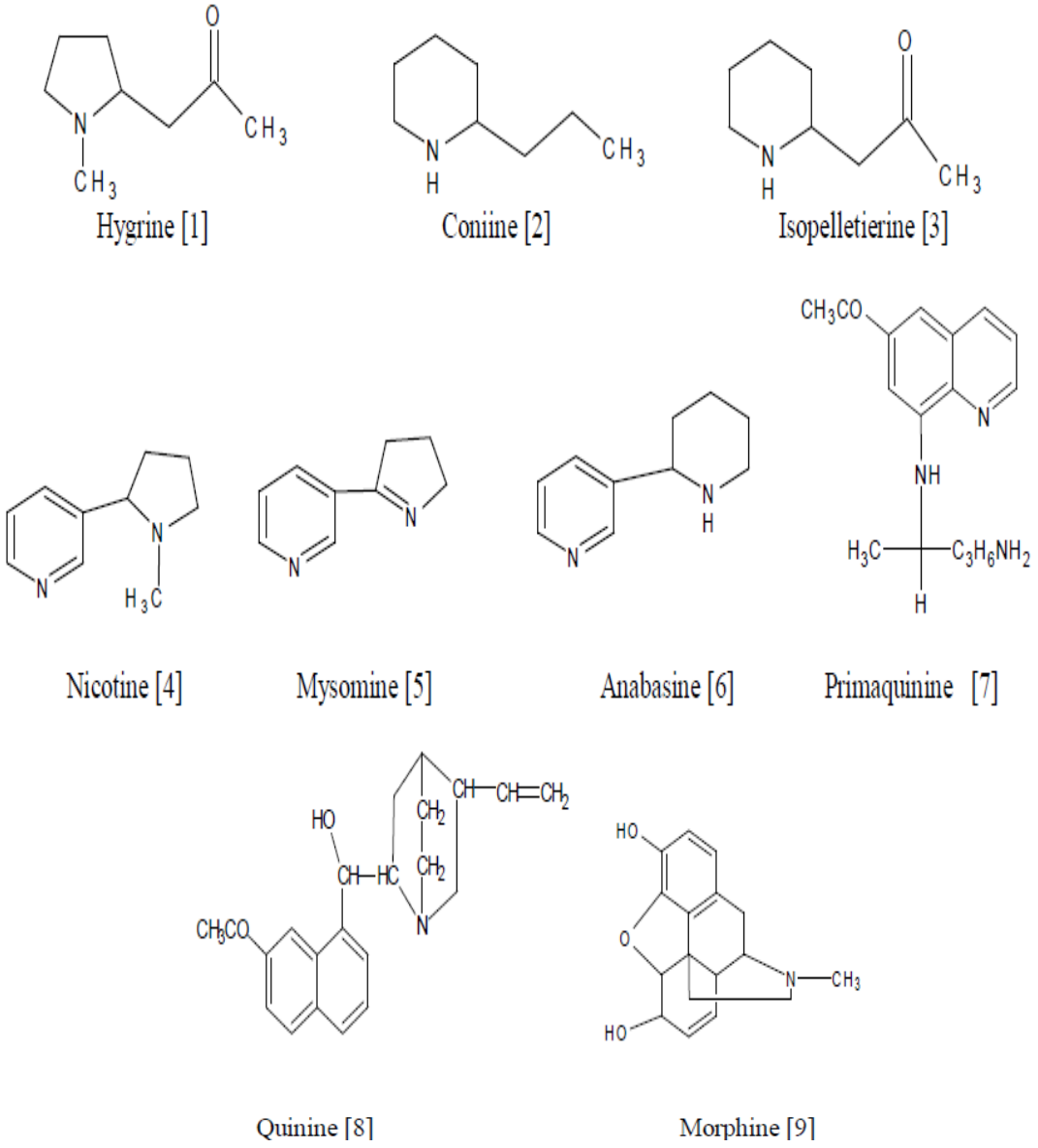


Figure 11: Structures of the important naturally occurring alkaloids (Molyneux *et al*., 1996)

2.14.5 Terpenes

Terpenes belong to a group of lipid-soluble compounds. Their structure includes one or more 5-carbon isoprene units, which aresynthesized ubiquitously by all organisms viatwo pathways, the mevalonate and deoxy-d-xylulose pathways (Rohmer, 1999). The classification of terpenoids isbased on the number of isopreneunits they contain. Isoprene, which itself is synthesized and released by plants, comprises one unit and isclassified as a hemiterpene; monoterpenes incorporate twoisoprene units, sesquiterpenes incorporate three units, diterpenes comprise four units, sesterpenes include fiveunits, triterpenes incorporate six units, and tetraterpenes has eightunits. Terpenoids havebeen reported to possessmedicinal properties such as antimalarial, anti-ulcer, anticarcinogenic, antimicrobial or diureticactivity (Langenheim, 1994; Dudareva *et al*., 2004).

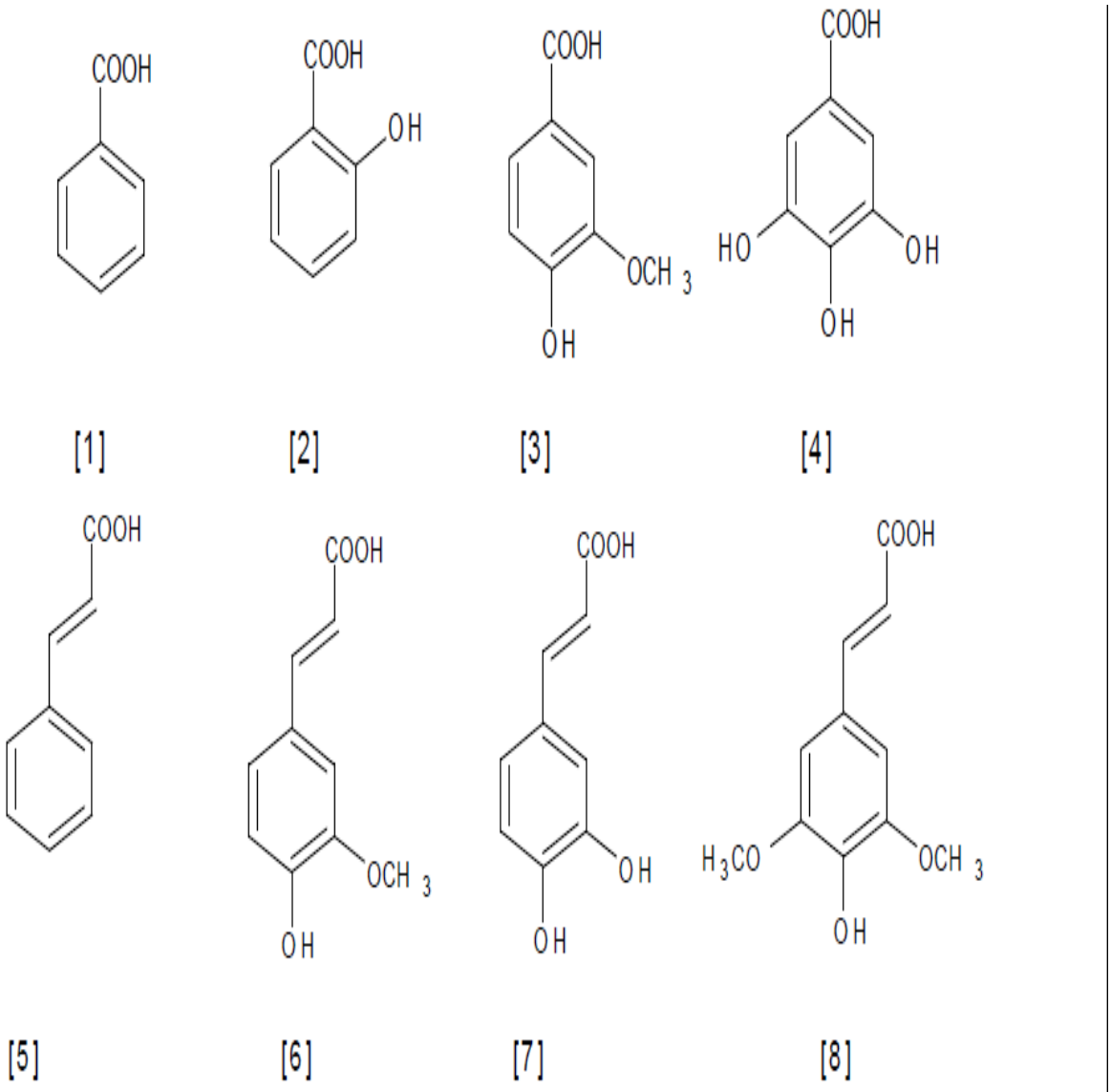
2.14.6 Cardiac glycosides

Chemically, glycosides contain acarbohydrate (glucose) and a non-carbohydrate part (aglycone or genin) which areusually alcohol, glycerol or phenol (Kar, 2007; Firn, 2010). Glycosides can be readily hydrolyzed intoits components with mineral acids orferments. The aglycone part of cardiac glycosides isa tetracyclic steroid having anunsaturated lactone ring of 5 or 6 members attached toit. Glycosides are classified according tothe type of sugar component, chemical nature of aglycone or pharmacological action. Cardiac glycosides are classified into two groups according to the lactone ring: the C-23 cardenolides with an α,β-unsaturated δ-γ-lactone (butenolide), and the C-24 bufadienolides with a di-unsaturated γ -lactone (pentadienolide). The sugar moiety isnormally attached viathe C-3 hydroxyl group orthe aglycone.

The majority of the saccharides found in cardiac glycosides arehighly specific. They are 2,6-dideoxyhexoses, such as D-digitoxose, D-diginose or L-oleandrose. These sugars givea positive reaction with the Keller-Killiani reagent. Cardiac glycosides have been usedas drugs for the management of cardiac insufficiency. An example is digitoxin from Digitalis, where the sugar moiety is linkedto the aglycone digitoxigenin via the C-3 hydroxyl group (De Padua *et al.*, 1999).

2.14.7 Phenolic acids

Phenolic compounds are group of secondary metabolites with awide range of pharmacological activities. Plant phenolic compounds differ in molecular structure, and are characterized by hydroxylated aromatic rings (Balasundram *et al*., 2006). The naturally occurring phenolic acids contain two different carbon frameworks whichare hydroxycinnamic and hydroxybenzoic structures. Phenolic acids havebeen studied majorly for their properties against oxidative damage leading tovarious degenerative diseases, like cardiovascular diseases, inflammation and cancer. Phenolic acids may be found in food plants as glycosides or esters with other natural compounds such as sterols, alcohols, glucosides and hydroxyfatty acids. Studies on bioavailability of phenolic acids emphasized boththe direct intake through food consumption and the indirect bioavailability deriving bygastric, intestinal and hepatic metabolism (Battisti *et al*., 2008). Phenolics acid hasdiverse biological activities, whichinclude antiulcer, anti-inflammatory, antioxidant22, cytotoxic andantitumor, antispasmodic, and antidepressant activities (Ghasemzadeh *et al*., 2010).



Hydroxybenzoic acid are Benzoic acid [1], Salicylic acid [2], Vailinilic acid [3], Gallic acid [4] and Hydroxycinnamic aid are Cinnamic acid [5], Ferulic acid [6], Sinapic acid [7] and Caffeic acid [8].

Figure 12: Structures of the important naturally occurring phenolic acids (Balasundram *et al.*, 2006).

2.15. Medicinal plants used for malaria treatment

Medicinal plants havebeen used to treat malaria for many years in various parts of the globe. The first antimalarial drug used was extracted from thebark ofthe Cinchona (Rubiaceae) species. As early as 1632, infusions of the plant bark were used forthe treatment of human malaria (Baird *et al*., 1996). Some years later, quinine was isolated and characterized thus becoming the oldest and mostimportant antimalarial drug (Saxena *et al*., 2003). An ancient medicinal plant, Artemisia annua was rediscovered in China in the seventies asan important source of the antimalarial artemisinin (Bruce-Chwatt, 1982; Klayman, 1985). Artemisinin-combination therapies (ACT) were formally accepted asfirst-line treatment of uncomplicated malaria in Nigeria from 2005 onwards (Mokuolu *et al*., 2007). However, the use of ACT is limited because of itshigh costs, limited production of artemisinin derivatives toGood Manufacturing Practices (GMP) standards and toxicity (Haynes, 2001; Malomo *et al*., 2001; Borstnik *et al.*, 2002; Adebayo and Malomo, 2002; Afonso *et al*., 2006; Boareto *et al*., 2008).

In Nigeria, numerous species of plant are used by some local people for medicinal purposes and many areused for malaria treatment across all ethnic and cultural groups inthe country. Examples of plant used for malaria treatment in Nigeria are: *Morinda lucida* (Rubiaceae) (Makinde *et al*., 1994; Awe and Makinde 1998; Tor-Anyiin *et al*., 2003; Avwioro *et al*., 2005); *Moringa oleifera* Lam. (Moringaceae) (Shuaibu *et al*., 2008); *Alstonia boonei* (Apocynaceae) (Odugbemi *et al*., 2007; Ajibesin *et al*.,2008; Majekodunmi *et al*., 2008); *Fagara zanthoxyloides* (Rutaceae) (Odebiyi and Sofowora, 1979; Kassim *et al*., 2005); *Carica papaya* (Caricaceae) (Tor-Anyiin *et al*., 2003; Ibe and Nwufo 2005); *Khaya senegalensis* (Egwim *et al*., 2002; Shuaibu *et al*., 2008); *Khaya grandifoliola* (Obih and Makinde, 1985; Agbedahunsi *et al*., 1998); *Spathodea campanulata* (Bignoniaceae) (Makinde *et al*., 1988); *Azadirachta indica*

(Meliaceae) (Iwu *et al*., 1986; Ehiagbonare 2007); *Nauclea latifolia* (Rubiaceae) (Phillipson and Wright, 1991; Ajaiyeoba *et al*., 2006; Ajibesin *et al*., 2008); *Quassia amara* (Ajaiyeoba *et al*., 1999); *Vernonia amygdalina* (Compositae) (Tor-Anyiin *et al*., 2003; Ehiagbonare, 2007); *Tithonia diversifolia* (Elufioye and Agbedahunsi 2004;. Ajaiyeoba *et al*., 2006); *Zingiber officinale* (Zingiberaceae) (Odugbemi *et al*., 2007); *Sida acuta* (Malvaceae) (Obute, 2005); *Phyllanthus amarus* (Euphorbiaceae) (Ajaiyeoba *et al*., 2003; Ajibesin *et al*., 2008); *Piliostigma thonningii* (Leguminosae-Caesalpinioideae) (Tor-Anyiin *et al*., 2003; Ajaiyeoba *et al*., 2006); *Mangifera indica* Linn. (Anacardiaceae) (Awe *et al*., 1998; Igoli *et al*., 2005; Ajaiyeoba *et al*., 2006); *Harungana madagascariensis* (Guttiferae) (Iwalewa *et al*., 2008); *Funtumia africana* (Apocynaceae) (Odugbemi *et al*., 2007) and *Enantia chlorantha* Oliv. (Annonaceae) (Agbaje and Onabanjo 1991; Ajaiyeoba *et al*., 2006).

These antimalarial plants areused mainly in form of monotherapy while feware taken together in combined therapies. An example is the multi-herbal extract referred toas 'Agbo-Iba' madeup of *Mangifera indica* leaf and bark, *Cajanus cajan* (pigeon pea) leaf, *Nauclea latifolia* leaf, *Euphorbia lateriflora* leaf, *Cymbopogon giganteus* leaf, *Uvaria chamae* bark and *Cassa alata* leaf (Nwabuisi, 2002). Another multi-herbal combination isthe mixture of *Anacardium occidentale* leaves, *Azadirachta indica* leaves, *Carica papaya* leaves, and *Cymbopogon citratus* leaves used in 'steam therapy', in whichthe patients are covered with a thick blanket and madeto inhale the vapour fromthe cooking pot (Nwabuisi, 2002).

2.16. *Adansonia digitata*

Adansonia digitata L. generally known asthe African baobab belongs tothe family of Bombacaceae. It is found inthe hot, dry savannahs of sub-Saharan Africa and isthe most widespread of the *Adansonia* species on the African continent. The Baobab treeis found in areas in Botswana, Namibia, Mozambique and South Africa, and it is localize in semi-arid and sub-humid regions of western Madagascar (Kamatou *et al*., 2011). The integrated taxonomic information system for *Adansonia digitata* is shown in Table 1.

Adansonia digitata is called by several local names in central Africa as 'bu- hibab' (fruit with several seeds), it is called 'Ose' in Yoruba and 'Kuka' in Hausa language. The English common names for the Baobab include dead-rat tree, monkey-bread tree, upside-down tree, bottle tree, lemonade tree and cream of tartar tree.

Adansonia digitata is usually known for itsexceptional girth and height. The tree can grow up to 28 meters in girth, 25 meters in height, and canlive for several hundred years.. In young trees the trunk isconical while inmature trees, it may be cylindrical or bottle shaped (Yusha'u *et al*., 2010). The Baobab tree was named *Adansonia* to commemorate the French surgeon Michel Adanson (1726-1806); the species name *digitata* meaning hand-like, isin reference to the shape of theleaves (Baum, 1995).

Table 1: The integrated taxonomic information system for *Adansonia digitata*.

Kingdom	*Plantae* – Plants
Subkingdom	*Tracheobionta* – Vascular plants
Superdivision	*Spermatophyta* – Seed plants
Division	*Magnoliophyta* – Flowering plants
Class	*Magnoliopsida* – Dicotyledons
Subclass	*Dilleniidae*
Order	*Malvales*
Family	*Bombacaceae* – Kapok-tree family
Genus	*Adansonia* L. – Adansonia
Species	*Adansonia digitata* L. – Baobab

(Adapted from National plant database, 2004)

Figure 13: *Adansonia digitata* leave.

The leaves of *Adansonia* are foliate and aresimple during the young stage. The flowers are white, showy and pendulous (Sidibe and Williams 2002). The bark issmooth, soft and fibrous and isreddish brown to grey in colour (Gebauer *et al*., 2002). The fruits are variable in appearance, but tend tobe ovoid and covered with velvety hairs; the pulp isdry and mealy, enclosed inside awoody pericarp (Sidibe and Williams 2002).

The root system of *A. digitata* extends more thanthe height of thetree, contributing to its capacity to survive indry climates. The range of the shallow root system enables thetrees to gather and store substantial amounts ofwater during the heavy rainfalls, which they utilize to photosynthesize when they areleafless (Sidibe and Williams 2002). The treeis named as: 'The small pharmacy or chemist tree' because every part of the African baobabtree (leaves, bark, fruits, seeds) hasreported tohave medicinal and nutritional properties and areused by humans for multiple purposes (Von Maydell, 1990; Van Wick and Gericke, 2000; Kamatou *et al*., 2011).

2.16.1. Traditional and Medicinal uses of *Adansonia digitata*

A. digitata havebeen shown to havenumerous health benefits which are related tothe presence of bioactive compounds (terpenes, saponins, tannins and many more) that are isolated from itsvarious parts like fruits and leaves (Ramadan *et al*., 1993). The dry pulp iseither eaten fresh or dissolved inmilk or water to make a refreshing drink (Sibibe and Williams 2002). The leaves, bark and fruit pulp havebeen used locally asanalgesics and immuno-stimulants in the treatment of diseases such as fever, diarrhoea, cough and dysentery (Vermaak *et al*., 2011).

The oil from the seeds isused as food, fuel, cosmetics and medicines inthe tropical treatment of various kinds of skinailments (Chivandi *et al*., 2008; Kamatou *et al*., 2011). The fruit is 6 to 8 inches or 15 to 20 centimetres long. It contains 50% more calcium thanspinach, it is

high in antioxidants, and has ten times thevitamin C of an orange (Sidibe *et al*., 1998; Gebauer *et al*., 2002). Fiber from the bark isused to make rope, baskets, cloth, musical instrument strings, and waterproof hats (Von maydell, 1990). Fresh baobab leaves iseaten as vegetable and is medicinally used totreat asthma, kidney and bladder disease, and several othermaladies (Von maydell, 1990).

2.16.2. Bioactivity studies of *Adansonia digitata*

Many researches havebeen conducted on different parts of the plant. Leaf infusions are used astreatment for kidney and bladder diseases, blood clearing and asthma diarrhea, fever, inflammation (van Wick and Gericke, 2000). Several scientific studies havebeen carried out on the plant such as onits anti-diarrheic properties (Tal-Dia *et al*., 1997); itsanti inflammatory, analgesic (pain killing) and antipyretic (temperature reducing) properties (Ramadan *et al*., 1994; Karumi et al., 2008); itsantimicrobial and antifungal activities (Le Grand, 1989) and itseffect against sickle cell anemia (Adesanya *et al*., 1988).

Tanko *et al*., 2008 reported thehypoglycemic activity of *A. digitata* stem bark extracton blood glucose levelsof streptozocin-induced diabetic rats. Masola *et al*., 2009 reported thatthe root and stem barks of baobab contain bioactive agent which isresponsible for itsantimicrobial activity. The bark of baobab hasbeen sold commercially in Europe for the treatmentof fever, particularly that causedby malaria (Brendler *et al*, 2003). Earlier studies by (Ajaiyeoba, 2005; Musila *et al*., 2013) showed that *Adansonia digitata* hassignificant antimalarial properties.

2.16.3. Bioactive Principles isolated from *Adansonia digitata*

Adansonia digitata hasbeen widely reported to contain many bioactive compounds which areresponsible for its diverse biological activities. Baobab fruit havebeen reported to contain detectable levels of α-carotene and lutein (Sena *et al.*, 1998). The alkaloid 'adansonin' isolated from the bark of *Adansonia digitata* isbelieved to be the active principle for treatment of malaria and otherfevers (Sidibe & Williams, 2002).

A variety of chemicals havebeen isolated and characterized from *A. digitata*. They belong to the classes of sterols, terpenoids, flavonoids, vitamins, amino acids, carbohydrates, and lipids. Friedelin, baurenol and lupeol were identified inthe bark of baobab. In addition, betulinic acid was isolated from thebark whereas theleaf produced taraxerone and acetate of lupeol and baurenol (Shukla *et al.*, 2001). The oil of baobab seeds hasbeen shown tocontain high amounts of linoleic and oleic acid aswell as α-linolenic and palmitic acid (Glew *et al.*, 1997; Ezeagu *et al.*, 1998).

Also, a new flavanol glycoside isolated from theroots was characterized as 3,7-dihydroxy-flavan-4-one-5-O-β-D-galactopyranosyl (1→4)-β-D-glucopyranoside (Chauhan *et al*., 1984). A flavanone glycoside isolated from theroots of *A. digitata* was characterized as 3,3,4-trihydroxy flavan-4-one-7-O-α-L-rhamnopyranoside. Quercetin-7-O-β-D-xylopyranoside was also isolated from theroots of this plant (Chauhan et al., 1987; Shukla et al., 2001). Proanthocyanidins as major compounds, viz., (-)-epicatechin, epicatechin-(4→β8)-epicatechin, epicatechin-(4→β6)-epicatechin, epicatechin-(2β→O→7, 4β→8)-epicatechin, and epicatechin-(4→β8)-epicatechin-(4→β8)- epicatechin hasbeen isolated and characterized from thepericarp of *Adansonia digitata* L. (Bombaceae) fruits (Abdelaaty, 2006).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and Reagents

Reduced glutathione (GSH), adenosine triphosphate (ATP), 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), ethylene glycol-bis (2-aminoethylether) tetraacetic acid (EGTA), quercetin, apigenin, cis-vaccenic acid, rutin and kaempferol, hydrogen peroxide (H_2O_2) and Thiobarbituric acid (TBA) were obtained from Sigma Chemical Company, USA. Sodium acetate, magnesium chloride ($MgCl_2$), trichloroacetic acid (TCA), ammonium molybdate, ferrous sulphate, potassium dichromate ($K_2Cr_2O_7$), glacial acetic acid, ethylenediamine tetraacetic acid (EDTA), sodium chloride (NaCl) were obtained from BDH Poole, U.K. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine, uric acid and urea assay kits were obtained from Randox Laboratories, U.K. All other chemicals were of analytical grade.

3.1.2 Plant Materials: Collection and Identification

The stem of *A. digitata* (Bombacaceae) was collected fromIdo-Ekiti, Ekiti State Nigeria. Identification and authentication ofthe plant werecarried out by Mr. M.T Soyewo atthe herbarium unit ofthe Forest Research Institute of Nigeria (FRIN) Ibadan, Oyo State, Nigeria where a voucher specimen number (FHI 109806) was deposited.

3.1.3 Animal handling

The albino mice weighing between 18 and 22g were obtained from the animalhouse, Institute of Advance Medical Research and Training (IAMRAT) College of Medicine, University of Ibadan, Nigeria. The animals wereacclimatized and housed in individual cages ina temperature and humidity controlled room, havinga 12 hours light and darkcycle. All the animals had free access to their respective feed and clean drinkingwater throughout the period ofthe experiment. All animal experiments were conducted according tothe guidelines of National Institute of Health (NIH publication, 1985) for laboratory animalcare and use. The workwas approved bythe University of Ilorin Ethical Review Committee with approval number UERC/ASN/2015/069.

3.2 Methods

3.2.1 Extraction of Plant Material

3.2.1.1 Preparation of plant extracts

The stem bark peels were air-dried at 25°C. The dried stembark was pulverized to powder using an electric blender andwas stored in a glass container. Pulverized stem bark was soaked insufficient volume of methanol for 72 hours at 25°C. It was repeatedly stirred aftereach 24 hours. The mixture was filtered after 72 hours and the filtrate was concentrated usingrotary evaporator at 40^0C. The concentrate was heated over awater bath which was stored ina refrigerator at 4°C.

3.2.1.2 Solvent partitioning

To 35g of the dried methanol extract, 500mL of distilled water was added toform aslury, which was transferred into aseparating funnel and washed repeatedly with chloroform until near exhaustion. The marc remaining was then washed with ethylacetate untilnear exhaustion. The aqueous fraction was filtered toremove plant fibers. The obtained fractions: aqueous fraction (AF), ethylacetate fraction (EF) and chloroform fraction (CF) were concentrated underpressure using rotary evaporator at 40°C. The concentrate was heated over awater bath which was stored ina refrigerator at 4°C.

3.2.2 Secondary metabolites and antioxidant activities of *A. digitata* stem bark extracts

3.2.2.1 Phytochemical Screening of *A. digitat*a stem bark extract

Phytochemical screening of the extract was doneusing standard phytochemical methods. It involves detection of alkaloids, flavonoids, terpenoids, saponins, tannins, anthraquinones, and cardiac glycosides (Trease and Evans, 1989; Sofowora, 1993).

3.2.2.2 Total Phenol Content

The total phenolic content of extract was determined using theFolin-Ciocalteu method as modified by Hong *et al*. (2001).

Procedure

0.01 mL of different concentrations of the extracts was rapidly mixedwith 0.1ml of Folin-Ciocalteu reagent and 0.3ml sodium carbonate (15%w/v) solution. This was left inthe dark for 30mins and the absorbance wasread at 760nm after 30mins on a spectrophotometer. Tannic acid was used as standard forthe calibration curve. The total phenol content wasexpressed as mg/g tannic acid equivalents (TAE) with the following equation: $C = (cV)/m$,

where C = total phenol contents, mg/g plant extract in TAE,

c = concentration of tannic acid obtained from calibration curve (mg/ml),

V = the volume of the sample solution (ml),

m = weight of the sample (g).

3.2.2.3 Total Flavonoid Content

The total flavonoid content was estimated according tothe method of Kumaran and Karunakaran (2007) with aslight modification.

Procedure

At different concentrations ofthe plant extract, 0.5ml of the extract and standard (quercetin) wereput in test tubes. 3.0ml of methanol and 0.1ml of 10% aluminum chloride solution were added intothe test tubes. 200ml of 1M potassium acetate solution was added to the mixtures and then diluted with 2.8ml of distilled water. This was incubated for 30 minat 25°C to complete reaction andthe absorbance was measured at 415nm using methanol as blank. Total flavonoid content of the extract wasexpressed as quercetin equivalents (QE) after calculation using the following equation: C = (cV)/m

where C = total flavonoid content, mg/g plant extract in QE, c = concentration of quercetin obtained from calibration curve (mg/ml), V= the volume of the sample solution (ml), m = weight of the sample (g).

3.2.2.4 Alkaloids

Alkaloid content was estimated using the method of Harborne (1973).

Procedure

To 5g of sample, 200 ml of 10% acetic acid inethanol was added and left for 4 h. The mixture was filtered followed by concentration ofthe extract on a water bath to about aquarter of the original volume. Ina drop wise manner, Conc. NH_4OH was added tothe extract until the precipitation was complete. The solution was left to settle. Then the precipitate was collected and washed with dilute NH_4OH followed byfilteration. The alkaloid contained inthe residue is allowed to dry and weighed. The percentage oftotal alkaloid content was calculated as:

Percentage of total alkaloids (%) = Weight of residue×100/Weight of sample

3.2.2.5 Tannins

Tannin content was estimated usingthe method of Van-Burden and Robinson (1981).

Procedure

To 500 mg of sample, 50 ml of distilled water wasadded followed by continouos shakenwith a mechanical shaker for 1 h. The resulting mixture was filtered into a 50 ml volumetric flask. This is followed by taken out 5 ml ofthe filtered solution into a test tube and then mixed it with 2 ml of 0.1 M $FeCl_3$ in 0.1 N HCl and 0.008 M potassium ferrocyanide and theabsorbance was readat 120 nm within 10 min.

3.2.2.6 Saponins

Saponin content was determined according to the method of (Obadoni and Ochuko, 2001)

Procedure

To 20 g of sample, 100 mlof 20% ethanol was added and the mixture heated ona hot water bath with constant stirring at about 55°C for 4 h. The mixture was filtered and then the

residue was extracted again withanother 200 ml of 20% ethanol. Atabout 90°C, the combination ofthe extract was reduced to 40 ml ona hot water bath. 20 ml diethyl ether was added to the concentrate and placed into a separating funnel with vigorous shaking. The aqueous layer was separated from the etherlayer which was discarded. In order to repeat the purification process, 60 ml of n-butanol was added. The combined n-butanol extracts werewashed twice with 10 ml of 5% aqueous NaCl and the remaining solution was heated in awater bath. After evaporation the samples were dried inoven, weighed and saponin content was calculated as percentage.

3.2.2.7 Quantification of phenolics by HPLC-DAD

Quantification of phenolics in *A. digitata* extracts (crude and partitioned fractions) were carried out using areverse phase HPLC-DAD. The analysis was performed under gradient conditions using C_{18} column. *A. digitata* extracts (crude and partitioned fractions) ata concentration of 15 mg/ml was injected by means ofa model SIL-20A Shimadzu Auto sampler. Separations were carried out using Phenomenex C_{18} column (4.6 mm x 250 mm x 5 μm particle size) according tothe method described by (Colpo et al., 2014) withslight modifications.

Quantifications were carriedout by integration ofthe peaks using the external standard method. The chromatography peaks were established by comparing its retention time with those ofreference standards and by DAD spectra (200 to 500 nm). All chromatography operations were carried outat ambient temperature and intriplicate as described by Boligon et al., 2014.

3.2.2.8 Identification of chemicals using gas chromatography/mass spectrophotometry

Identification of constituents of ethylacetate partitioned fraction of *A. digitata* using GC/MS. The GC/MS analysis wasperformed using Agilent Technologies GC/MS (Model

7890A) equipped with Agilent 19091 S-433HP-5MS 5% phenyl methyl silox column (30m x 250µm x film thickness 0.25µm). The analysis works onthe principle that a mixture will separate into individual substances when heated. Pure helium gas was used as a carrier gas ata flow rate of 1.5ml/minute. GC/MS analysis resulting in chromatogram was compared tocomplete library using data baseof National Institute of Standard and Technology (NIST).

3.2.3 Evaluation of *In Vitro* Antioxidant Potentials

3.2.3.1 Assay of DPPH Radical Scavenging Activity

DPPH radical scavenging activity of *A.digitata* extract was determined as described by Molyneux, 2004.

Principle

The relatively stable 2,2-dipheny-1-picrylhydrazyl ($DPPH^{\cdot}$) free radical isused for antioxidant activity measurement of lipid soluble compounds. It is known that afreshly prepared $DPPH^{\cdot}$ solution exhibits adeep purple colour with anabsorption maximum at 517 nm. This purple colour generally fades when anantioxidant molecule can quench $DPPH^{\cdot}$ (by providing hydrogen atoms orby electron donation, conceivably via afree radical attack on the $DPPH^{\cdot}$ molecule) and convert them to ableached product (i.e. 2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine), resulting in a decrease inabsorbance (Mishra *et al.*, 2012).

Procedure

1ml of 0.1mM DPPH was added to 1ml of various concentration ofthe extract in atest tube. Tannic acid was used asthe reference for comparison. The mixture was incubated for 30 mins in darkat room temperature and theabsorbance was read at 517 nm. The $DPPH^{\cdot}$ scavenging activity was calculated inpercentage as given below:

$$\% \text{ Inhibition} = 100 - \left[\frac{Abs_{ext} - Abs_{blank}}{Abs_{control}} \right] X\ 100$$

Where: Abs_{ext} = Absorbance with extract and DPPH

Abs_{blank} = Absorbance with extract without DPPH

$Abs_{control}$ = Absorbance without extract with DPPH

3.2.3.2 Assay of Reducing Potential

The Fe^{3+}- reducing power of the extract was determined by the method of Oyaizu (1986) with slight modification.

Principle

Radical chain reactions couldbe terminated when substances exhibiting high reducing tendencies donate electrons which canreact with free radicals converting them tomore stable products in the process. The principle of this assay therefore was based onthe reduction of Fe^{3+}/ferricyanide complex tothe ferrous form, and the Fe^{2+} was monitored by measuring the formation of Perl's Prussian blue at 700 nm (Chung *et al.*, 2002).

Procedure

0.5 ml of extract of different concentrations weremixed with 0.5 ml phosphate buffer (0.2 M, pH 6.6) and 0.5 ml potassium hexacyanoferrate (0.1%), followed by incubationat 50°C in a water bath for 20 min. After incubation, 0.5 ml of TCA (10%) wasadded to terminate the reaction and thereaction mixture was centrifuged at 3000 g for 5 min. The upper portion ofthe solution (1 ml) was mixed with 1 ml distilled water and 0.1 ml $FeCl_3$ solution (1%) was added. The reaction mixture was allowed tostay for 10 min at room temperature. The absorbance was

read at 700 nm against distilled water blank. Increase inabsorbance indicated greater reducing power.

3.2.3.3 Assay of Metal ion Chelation Activity

The ferrous ion chelating activity of extract was evaluated by astandard method (Minnoti and Aust, 1997) with slight modification.

Principle

It is based on distruption of o-phenanthroline-Fe^{2+} complex in the presence of chelating agent.

Procedure

Various final concentrations ofthe plant extract (150µl) were added to 900 µM ferrous sulfate solution. The reaction mixture wasincubated for 5 minat room temperature, before the addition of 78µl of 1,10-phenanthroline (0.25% w/v, aqueous). The absorbance was readat 510nm. Ethylene diamine tetra aceticacid (EDTA) was used as the standard. The iron (II) chelating activity was calculated as percentage inhibition ofthe red colouration resulting from the reaction of Fe^{2+} with phenantroline as follows:

$$\% \text{ Metal ion chelated} = \left[\frac{Abs_{ctrl} - Abs_{ext}}{Abs_{ctrl}} \right] X\ 100$$

Where: Abs_{ext} = Absorbance of solution with extract

Abs_{ctrl} = Absorbance of solution without extract but vehicle.

3.2.3.4 Hydroxyl Radical Scavenging Activity

This was assayed as described by (Jin *et al*., 1996).

Principle

The phenanthroline was employed to determine therate constant for reaction between antioxidants and hydroxyl radicals. Phenanthroline is degraded by hydroxyl radicals. The assay is thus based onthe quantification ofthe degradation product of Phenanthroline. Hydroxyl radical was generated by the Fe^{2+}-H_2O_2 system (the Fenton reaction).

Procedure

The hydroxyl radical was produced in amixture of 1.0ml of 0.75mM 1,10-phenanthroline, 2.0 ml of 0.2 M sodium phosphate buffer (pH 7.4), 1.0ml of 0.75mM $FeSO_4$ and 1.0ml of H_2O_2 (0.01%,v/v). Followed by addition of 1.0ml sample, the mixture was incubated at 37^oC for 30 min. The absorbance ofthe mixture was read at 536nm. Distilled water and mannitol were used asthe blank and positive control respectively. The OH^- scavenging activity was calculated by the equation:

Hydroxyl radical scavenging activity (%) = (Abs. sample – Abs. blank)/(Abs_0 Abs. blank) x 100

where Abs_0 is the absorbance of the distilled water instead of H_2O_2 and sample in the assay system.

3.2.4 Evaluation of antimalarial effect of *A. digitata* stem bark extracts

3.2.4.1 Investigation of the therapeutic effect of *A. digitata* on *P. berghei* infected mice

Therapeutic effects ofthe aqueous and methanolic extract of *Adansonia digitata* stem barkon established infection was estimated according tothe method of Ryle and Peters (1970).

Procedure

The micewere inoculated intraperitonially withan inocula size of 1x 10^7 of chloroquine susceptible strain of *P. berghei* infected erythrocytes and parasitemia wasconfirmed after 72

hours. The animals were grouped intosix containing eight mice each. They were treated forfive days withtwo differenct extract concentrations (200mg/kg body weight/day and 400mg/kg body weight/day). 5mg/kg body weight/day was given tothe chloroquine group and the control received 5% v/v tween 80 (the vehicle used todissolve the extract). Collection of blood was done daily from themice tails tomake thin films. The film was fixed in 100% methanol andair-dried before stainingwith Giemsa stain. Parasitemia wasexamined microscopically (using x 100 immersion oil objective). The packed cell volume (PCV) was estimated usingthe microhematocrit method. Percentage parasitemia and percentage clearance/chemosupression were estimated.

% Parasitemia= (Total number of parasitized cells/Total number of cell) x 100%

% Clearance/Chemosupression= [(Negative control parasitemia) – (Parasitamia with drug)]/Negative control parasitemia.

Adult albino mice weighing 20±2 g were divided into six groups (I-VI) of eight animals each.

Group I: Animals were orally administered the vehicle (5% v/v tween 80) only and served as the control.

Group II: Animals were orally administered 200mg/kg b.w aqueous extract of *A. digitata* stem bark (AEAD).

Group III: Animals were orally administered 400mg/kg b.w aqueous extract of *A. digitata* stem bark (AEAD).

Group IV: Animals were orally administered 200mg/kg b.w methanolic extract of *A. digitata* stem bark (MEAD).

Group V: Animals were orally administered 400mg/kg b.w methanolic extract of *A. digitata* stem bark (AEAD).

Group VI: Animals were orally administered 5mg/kg b.w chloroquine.

Five animals from each group were sacrificed 5 days after treatment toprepare the serum and tissues homogenates (liver, kidney and heart) which were used forvarious biochemical analysis. The remaining animals were monitored forpost infection.

3.2.4.2 Investigation of the Suppresive effect of *A. digitata* on *P. berghei* infected mice

The method of Peters (1967) was used to evaluate the suppressive effects of some fractions and extracts of *Adansonia digitata* stem bark.

Procedure

Albino mice weighing 20±2g were challengedby intraperitoneal injection with standard inoculum of *P. berghei* with 1x 10^7 infectederythrocytes. The animals were grouped intonine containing 5 mice each. They were treated forfive days withtwo differenct extract concentrations (200mg/kg body weight/day and 400mg/kg body weight/day) for each ofthe fraction and the crude extracts. 5mg/kg body weight/day was given tothe chloroquine group and Artemether-Lumefantrine group. Thecontrol received 5% v/v tween 80 (the vehicle used todissolve the extract). Parasitemia was examined microscopically and the PCV was determined onday of infection and day five bythe microhematocrit method. Percentage parasitemia and percentage clearance/chemosupression were calculated.

3.2.4.3 Investigation of the prophylactic effect of *A. digitata* on *P. berghei* infected mice

The method of Peters (1967) wasused to evaluate the Prophylatic effects ofsome fractions and extracts of *Adansonia digitata* stem bark.

Procedure

The animals were grouped intonine containing five mice each. They were pretreated forfive days withtwo different doses (200mg/kg body weight/day and 400mg/kg body weight/day) for both the fraction and the crude extracts. Chloroquine and Artemther-Lumefantrine groups werealso pretreated with 5mg/kg body weight/day, whilethe control received thevehicle (5% v/v tween 80) only. After fivedays the mice were challenged withan inoculums size of 1x 10^7 of chloroquine susceptible strain of *P. berghei* infected erythrocytes. Blood were collected fromthe mice tails andsmeared onto microscope slidesafter 72 hours to make thin film. Parasitemia and PCV were estimated onday of infection and dayfive by the microhematocrit method. Percentage parasitemia and percentage clearance/chemosupression were calculated.

Group I: Animals were orally administered the vehicle (5% v/v tween 80) only and served as the control.

Group II: Animals were orally administered 200mg/kg b.w crude methanolic extract of *A. digitata* stem bark (MEAD).

Group III: Animals were orally administered 400mg/kg b.w crude methanolic extract of *A. digitata* stem bark (MEAD).

Group IV: Animals were orally administered 200mg/kg b.w ethylacetate partitioned fraction of methanolic extract of *A. digitata* stem bark (EFAD).

Group V: Animals were orally administered 400mg/kg b.w ethylacetate partitioned fraction of methanolic extract of *A. digitata* stem bark (EFAD).

Group VI: Animals were orally administered 200mg/kg b.w chloroform partitioned fraction of methanolic extract of *A. digitata* stem bark (CFAD).

Group VII: Animals were orally administered 400mg/kg b.w chloroform partitioned fraction of methanolic extract of *A. digitata* stem bark (CFAD).

Group VIII: Animals were orally administered 5mg/kg b.w chloroquine.

Group IX: Animals were orally administered 5mg/kg b.w Artemeter-Lumefantrine.

3.2.5. Collection of Blood Samples and Preparation of Samples

Animals were anaesthetized by putting them injar containing cotton wool soaked in chloroform. They were allowed to go into unconscious state after which theywere brought out for sacrifice. The animals were sacrificed and blood was collect by cutting their jugular veins. Part of the blood was collected into EDTA coated bottles to prevent clotting, and preserved for heamatological analysis. The other part ofthe blood was collected in plain bottles and allowed to clot forthe preparation of serum. The clotted blood was centrifuged at 3000 rpm for 5 minutes according to Ogbu and Okechuckwu (2001). The clear supernatant (serum) wasthen separated from thepellet and kept frozen untill required (Weathrole, 1989).

3.2.6. Preparation of Tissue homogenates

The animals were quickly dissected and thetissues (liver and kidney) removed. They were cleanedof blood and weighed before keeping in ice-cold 0.25M sucrose solution. Tissue homogenates were prepared by suspending the tissue inice-cold 0.25M sucrose buffer solution

with pH 7.4 which gives afinal volume of five times dilution (Sakpere, 1981) and homogenized in atefflon homogenizer. The homogenates werelater centrifuged at 10000 *g* for 15 minutes at 4^{o}C andthe supernatants, were collected, stored under 4°C until needed forthe biochemical assays.

3.2.7 Measurement of Haematological Parameters

Haemoglobin concentration, PCV, RBC count, MCV, TWBC count, WBC differentials, and platelets count were determined using the automated blood analyser SYSMEX KX21 as described by Dacie and Lewis (1991).

Principle

SYSMEX KX21 is an automatic multi-parameter blood cell counter, which employ the differences in characteristics possessed by each of the blood components to distinguish them and estimate their numbers.

Procedure

Fifty microlitres of blood sample was injected into the equipment, the machine aspirates the sample and sends appropriate volumes into the red blood cell and white blood cell chambers where they are diluted with cell pack (whose active ingredients are 6.38g/dl NaCl, 0.2g/L sodium tetraborate, 1.0g/L boric acid and 0.2g/L EDTA) to required dilutions. In the red blood cell chamber, the red blood cells and platelets are counted on the basis of their lack of nucleus by the direct counting method but they are differentiated based on their sizes. As the red blood cells are counted, their sizes and other parameters are also determined and this information is digitized. With the aid of appropriate formulae, the packed cell volume is calculated. In the white blood cell chamber, a fixed volume of stromatolyser-WH solution (whose active ingredients are 8.5g/L

organic quaternary ammonium salt, 0.6g/L sodium chloride) is added automatically to the blood already diluted with appropriate volume of the cell pack to obtain a final dilution of 1: 500. The Stromatolyser-WH solution lyses the red blood cells and so the remaining cell stroma is at a level undetectable by the instrument.

At the same time, the white blood cell membrane is preserved and the white blood cells are stabilized at a level detectable by the instrument. They are then counted by the direct counting method. The white blood cells are also differentiated into lymphocytes, neutrophils and mixed cell population (comprising of monocytes, basophils and eosinophils) based on the sizes of their nuclei and are presented as percentage of the whole white blood cell count. The haemoglobin released during red blood cell lysis in the white blood cell chamber is converted to the red methaemoglobin. A portion of this diluted sample is transferred to the haemoglobin detector where the absorbance of the red pigment is measured to give blood haemoglobin level. MCV, MCH and MCHC were calculated from the values obtained for RBC, haemoglobin concentration and packed cell volume using standard formulae.

Calculation

$$\text{MCV (fl)} = \frac{\text{PCV X 1000}}{\text{RBC } (10^{12}/\text{l})}$$

$$\text{MCH (pg)} = \frac{\text{Hb (g/l)}}{\text{RBC } (10^{12}/\text{l})}$$

$$\text{MCHC (g/l)} = \frac{\text{Hb (g/l) X 100}}{\text{PCV}}$$

3.2.8 Markers of inflammation

3.2.8.1 C-Reactive Protein (CRP)

C-reactive protein was assayed for using ELISA kit.

Principle

ELISA kit uses Sandwich-ELISA as the method. The micro ELISA plate provided inthe kit hasbeen pre-coated with an antibody specific to Mouse CRP. Addition of substrate solution which include Rat CRP, biotinylated detection antibody and Avidin-Horseradish Peroxidase (HRP) conjugate forms enzyme-substrate reaction which gives ablue colorouration. Addition of sulphuric acid solution terminates the enzyme-substrate reaction and theblue colour changes to yellow. The intensity ofthis yellow colouration is measured at 450 nm. The OD value is proportional tothe concentration of Mouse CRP.

Procedure

To each well, 100µL of standard orsample were added and incubated for 90 minutes at 37^{o}C. The liquid were removed and then 100µL biotinylated detection antibody were addedand incubated for 1 hour at 37^{o}C. The resulting solution were aspirated and washed 3 times. After washing, 100µL HRP conjugate were added which also incubated for 30 minutes at 37^{o}C. Also, the resulting solution were aspirated and washed for 5 times. 90µL substrate reagent was added and incubated for 15 minutes at 37^{o}C. The solution wasread at 450nm immediately after addition of 50µL stop solution.

3.2.8.2 Tumor Necrosis Factor Alpha (TNF-α)

Tumor Necrosis Factor Alpha (TNF-α) was assayed for using ELISA kit.

Procedure

To each well, 100µL of standard or sample wereadded and incubated for 90 minutes at 37^{o}C. The liquid were removed and then 100µL biotinylated detection antibody were addedand incubated for 1 hour at 37^{o}C. The resulting solution were aspirated and washed 3 times. After washing, 100µL HRP conjugate were added which also incubated for 30 minutes at 37^{o}C. Also, the resulting solution were aspirated and washed for 5 times. 90µL substrate reagent was added and incubated for 15 minutes at 37^{o}C. The solution wasread at 450nm immediately after addition of 50µL stop solution.

3.2.9 Markers of Hepatic Damage

3.2.9.1 Alkaline Phosphatase (ALP) Activity

The method of Wright et al. (1972) was employed in this assay.

Principle

ALP activity was measured by determining the concentration of p-nitrophenol formed when ALP reacts with p-nitrophenyl phosphate.

p-Nitrophenylphosphate + H_2O $\xrightarrow{ALP}$ Phosphate + p-nitrophenol

P-Nitrophenyl phosphate (PNPP) is hydrolysed to p-nitrophenol and phosphoric acid at pH 10.1 byalkaline phosphatase. The p-nitrophenol confers yellowish colour on the reaction mixture and its intensity can be monitored by the spectrophotometer at 400nm to give a measure of the enzyme activity.

Procedure

2.2ml of 0.1M carbonate buffer (pH 10) was drawn into atest tube followed by addition of 0.1ml of 0.1M MgSO4.7H2O and 0.2ml enzyme source (serum or tissue homogenaste). The mixture was incubated in awater bath at 37^{o}C for 10 minutes. 0.5ml PNPP (19mM) was added and incubated again for 10 minutes at 37^{o}C in a water bath. 2.0ml of NaOH (2.0N) was then added to stop the reaction. A blank was prepared inthe same way but contain distilled water instead of enzyme source. It was thoroughly mixed and absorbance ofthe test read against the blank at 400nm using a spectrophotometer.

Calculation

Activity (nM/min/ml) = ΔA/min x 1000 x Vt x F

18.8 x Ve x L.

Where ΔA/ min = Change in absorbance/min

Vt = Total reaction volume (5ml)

F = Dilution factor

Ve = volume of enzyme source

L = Light path = 1cm

1000 = Factor introduced to enable enzyme activity be expressed in nm/min/ml.

18.8 = Extinction coefficient of para-nitrophenol.

Specific Activity = Activity (nmol/ mg protein/min). Protein concentration (mg/ml)

3.2.9.2 Assay of Alanine Aminotransferase (ALT) Activity

Alanine aminotransferase (ALT) activity was determined in serum and tissue homogenates based on the method of Reitman and Frankel (1957).

Principle

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

Alanine aminotransferase is measured by monitoring the concentration of pyruvate hydrazone formed with 2,4-dinitrophenylhydrazine (Reitman and Frankel, 1957). The enzyme alanine aminotransferase catalyzethe transfer of amino group from L-alanine to α- oxoglutarate to form L-glutamate and oxaloacetate. The oxaloacetate formed is unstable and is quantitatively decarboxylated to pyruvate which isthen complexed with 2,4-dinitrophenylhydrazine (DNPH) to produce anintensely coloured hydrazone on the addition of NaOH. This coloured complex absorbs radiation at 530-550nm.

Procedure

ALT buffered substrate (1.0ml) was incubated at 37^{o}C for 5 minutes followed bythe addition of 0.2ml enzyme source (serum or tissue homogenate). It was further incubated for 30 minutes at 37^{o}C after which 1.0ml DNPH (0.001M) was added. The mixture was leftat room temperature for 20 minutes after which 10.0ml of 0.4N NaOH was added tostop the reaction. A blank contain distilled water instead of enzyme source. Each tube was mixed and the absorbance of test sample read against the blank at 546nm after 5 minutes using a spectrophotometer. The pyruvate concentration corresponding tothe observed absorbance was read in the standard curve and this wasused to calculate the specific activity.

3.2.9.3 Assay of Aspartate Aminotransferase (AST) Activity

Aspartate aminotransferase (AST) activity was determined based on the method of Reitman and Frankel (1957).

Principle

AST catalyses the formation of oxaloacetate and glutamate from aspartate and α-oxoglutarate. The oxaloacetate produced is unstable and is decarboxylated to pyruvate using aniline citrate. The pyruvate produced is allowed to form anintensely coloured complex with dinitrophenyl hydrazine (DNPH) onthe addition of 0.4N Na0H, which canbe measured bya spectrophotometer.

Procedure

AST buffered substrate (1.0ml) was incubated at 37^{o}C for 5 minutes followed bythe addition of 0.2ml enzyme source (serum or tissue homogenate). It was further incubated for 60 minutes at 37^{o}C after which 2 drops of alinine citrate wasadded. It was mixed and allowed to stand for 5 minutes in a water bath followed by the addition of 1.0ml DNPH (0.001M). The mixture was left at room temperature for 20 minutes after which 10.0ml NaOH (0.4N) wasadded tostop the reaction. A blank contain distilled water instead of enzyme source. The content was mixed and the absorbance ofthe test read against the blank at 546nm after 5 minutes using a spectrophotometer. The pyruvate concentration corresponding tothe observed absorbance was read from the standard curve and this was used to calculate the specific activity.

3.2.9.4 Determination of Serum Total Protein

The protein concentration in the serum and tissue homogenate was estimated by the Biuret method of Gornall *et al*. (1949).

Principle

The biuret method depends on the principle that when a solution of protein is treated with Cu^{2+} in moderately alkaline medium, a purple coloured chelateis formed between Cu^{2+} and the peptide bonds of the protein. The intensity of the purple colour is proportional to the amount of protein present.

Procedure

Serum (1.0ml) sample was drawn into atest tube containing 4.0ml biuret reagent. Blank was also prepared which contain 1.0ml distilled water instead of serum. It was thoroughly mixed and allowed tostand for 30 minutes after which the absorbance of the sample wasread against the blank at 540nm using spectrophotometer. The absorbance obtained foreach sample was extrapolated from the protein standard curve to obtain the corresponding protein concentration.

Calculation:

Protein concentration (mg/ml) = protein conc. x dilution factor.

3.2.10 Renal Function Tests

3.2.10.1 Serum Creatinine Estimation

The method used for the determination of creatinine was described by Tietz et al. (1994).

Principle

Creatinine reacts with picrate ion in alkaline medium to produce a red-orange adduct whose intensity is directly proportional tothe concentration of creatinine and can be measured with the spectrophotometer at 510nm.

Procedure

Serum (0.2ml) was added to 2.0ml creatinine working reagent in a test tube labelled sample. 2.0ml of creatinine standard (2mg/dl) was also mixed with 2.0ml creatinine working

reagent in another test tube labeled standard. The mixture in each test tube was transferred into the cuvette and the absorbance read against a blank after 20 and 80 seconds at 510nm using a spectrophotometer.

Calculation:

$$\text{Creatinine (mg/dl)} = \frac{\text{(Absorbance 80 sec – absorbance 20 sec) sample}}{\text{(Absorbance 80 sec – absorbance 20 sec) standard}} \times \text{(conc. of std)}$$

3.2.10.2 Serum Urea Estimation

The concentration of urea in the serum was determined by the colorimetric Urease-Berthelot method.

Principle

Urea is hydrolysed to ammonia in the presence of urease in the serum. The ammonia is measured photometrically by Berthelot`s reaction.

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

$$NH_4^+ + \text{Salycilate} + NaClO \xrightarrow{\text{Nitroprusside}} \text{Indophenol}$$

Procedure

The test tube contained 10 µl of serum, standard urea or distilled water (blank) and 100 µl of reagent 1 (116 mM EDTA, 6 mM sodium nitroprusside, 1 g/l urease). The mixture was gentlymixed and incubated at 37°C for 10 min. Thereafter, 1 ml each of reagent 2 (120 mM phenol diluted with distilled water, 1:6) and reagent 3 (27 mM sodium hypochlorite diluted with distilled water, 1:34 and 0.14 N NaOH) wereadded and further incubated for 15 min 37°C. The absorbance wastaken at 580nm against the blank.

Calculation:

$$\text{Urea conc (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Concentration of standard (50 mg/dl)}$$

3.2.10.3 Serum Uric acid Estimation

Concentration of uric acid in the serum was determined based on the method of Reitman and Frankel (1957).

Principle

An enzyme known as uricase converts uric acid to allantoin and hydrogen peroxide and also under the catalytic influence of peroxidase, oxidizes 3,5-dichloro-2-hydrobenzenesulfonic acid and 4-aminophenazone to a red-violet quinoneimine compound.

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

$$2H_2O_2 + \text{3,5-dichloro-2-hydrobenzenesulfonic} + \text{4-aminophenazone} \xrightarrow{\text{Peroxidase}}$$

N-(antipyryl)-3-chloro-5-sulfonate-p-benzo-quinoneimine

Procedure

The test tube contained 10 µl of serum, standard uric acid or distilled water (blank) and 500 µl ofreagent. The mixture was gently mixed and incubated at 37°C for 5 min. Thereafter, the absorbance was measuredat 546nm within 30 min against the blank.

Calculation:

$$\text{Urea conc (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Concentration of standard (mg/dl)}$$

3.2.11 Electrolyte Status

3.2.11.1 Estimation of Calcium Level

Cacium level was determined by the method of Tietz, (1986) using commercially available kits (Teco Diagnostics, CA).

Principle

Calcium reacts with cresolphthalein complexone in 8-hydroxyquinoline to form a colored complex (purple color) that absorbs at 570 nm (550 – 580 nm). The intensity of the color is proportional tothe calcium concentration. Color intensifiers and astabilizer are present to minimize interference by other metallic ions.

Calcium + O-Cresolphthalein Complexone $\xrightarrow[\text{Medium}]{\text{Alkaline}}$

Calcium - Cresolphthalein Complexone Complex (purple color)

Procedure

Equal volumes of Calcium Color Reagent A (O-Cresolphthalein Complexone 0.14mM, 8-Hydroxyquinoline 13mM) and Calcium Buffer B (Diethylamide 363mM, Potassium Cyanide 2mM), was mixed and allowed tostand for ten (10) minutes at room temperature before use, this will serveas the working reagent. Exactly 1.0 ml of the working reagent was pipette intothe test tubes and 0.02 ml of the sample was added to the tubes and thoroughly mixed. This was allowed tostand for at least 60 seconds before it is read on the spectrophotometer at 570 nm.

Calculations

$$\frac{\text{Abs. of sample}}{\text{Abs. of standard}} \times \text{Concentration of Standard (mg/dl)}$$

3.2.11.2 Estimation of Magnesium Level

Magnesium level was estimated by the method of Henry (1974) using commercially available kits (Teco Diagnostics, CA).

Principle

Magnesium forms a colored complex with calmagite in alkaline medium to produce a red complex thatis measured spectrophotometrically at 530 nm. The color produced is proportional to the magnesium concentration.

Procedure

The working reagent is prepared by mixing 10 volumes ofcolor reagent (Calmagite 0.006% w/v; stabilizer 2.0% w/v; surfactant 0.03% w/v) with one (1) volume of buffer reagent (2-Ethylaminoethanol 6.0 w/v; potassium cyanide 0.10% w/v, EGTA 1.18 mM) in a disposable plastic container. 1.0 ml of working reagent was dispensed toeach tube. Then 0.01 ml (10 µl) sample was added to its respective tube and mixed gently. This was incubated for five (5) minutes at 25°C, after incubation, absorbance reading was takenat 530 nm, zero spectrophotometer withthe reagent blank. The standard (2 mEq/L magnesium lodate, tetrahydrate) was also treated in the same manner as the samples.

Calculations

$$\frac{\text{Abs. of sample}}{\text{Abs. of standard}} \times \text{Concentration of Standard (2 mg/dl)} = \text{Magnessium (mg/dl)}$$

3.2.11.3 Estimation of Potassium Level

Potassium level was determined by the method of Henry (1974) using commercially available kits (Teco Diagnostics, CA).

Principle

The amount of potassium is determined byusing sodium tetraphenylboron in a specifically prepared mixture to produce a colloidal suspension. The turbidity of which is proportional to potassium concentration in the range of 2 - 7 mEq/L.

Procedure

Precisely 1.0 ml of Potassium reagent (Sodium Tetraphenylboron 2.1 mM) was dispensed to each tube. Then 0.01 ml (10 µl) sample was added toits respective tube and mixed gently. This was incubated for three (3) minutes at room temperature, after incubation, absorbance reading was taken at 500 nm, zero spectrophotometer withthe reagent blank. The standard reagent equivalent to 4 mEq/L was also treated in the same manner as the samples.

Calculations:

$$\frac{\text{Abs. of Unknown}}{\text{Abs. of Standard}} \times \text{Concentration of Standard (mEq/L)} = \text{Potassium Concentration (mEq/L)}$$

3.2.11.4 Estimation of Sodium Level

Sodium level was determined by the method described by Tietz, (1986) using commercially available kits (Teco Diagnostics, CA).

Principle

Sodium is precipitated as triple salt, sodium magnesium uranyl acetate. Excess uranium reacts with ferrocyanide to produce a chromophore whose absorbance is inversely proportional to concentration of sodium in the sample.

Procedure

A 1.0 ml aliquot of filtrate reagent (uranyl acetate 2.1 mM and Magnesium Acetate 20 mM in ethyl alcohol) was pipetted into all tubes. 50 µl of sample was then added to all tubes respectively and distilled water to the blank. Continuous and vigorous shaking of all tubes was maintained for 3 minutes. Then all the tubes were centrifuged at high speed (1500g) for 10 minutes and the supernatant was carefully tested, taking care not to disturb the protein precipitate. Filterates from the above supernatant were put in a label test tubes corresponding to the filtrate tubes. 1.0 ml acid reagent (diluted acetic acid) was pipetted to all tubes. Addition of 50µl of supernatant to respective tubes was done and mixed gently. 50 µl of color reagent was added to all tubes and mixed. The spectrophotometer was calibrated to zero with distilled water and the absorbance was read at 550 nm. The standard reagent (Sodium Chloride solution: 150 mEq/L of sodium) was also treated in the same manner as the samples.

Calculations:

$$\frac{(\text{Abs. of Blank} - \text{Abs. of Sample}) \times \text{Conc. of Standard (mEq/L)}}{(\text{Abs. of Blank} - \text{Abs. of Standard})} = \text{Potassium Conc. (mEq/L)}$$

3.2.11.5 Estimation of Chloride Level

Chloride level was determined by the method of Henry (1974) using commercially available kits (Teco Diagnostics, CA).

Principle

Chloride ions form a soluble, non-ionized compound with mercuric ions and will displace thiocyanate ions from non-ionized mercuric thiocyanate. The released thiocyanate ions react with ferric ions to form a colour complex that absorbs light at 480nm. The intensity of the colour produced is directly proportional to the chloride concentration.

$Hg(SCN)_2 + 2Cl^- \longrightarrow HgCl_2 + 2SCN^-$

$3SCN^- + Fe^{3+} \longrightarrow 4Fe(SCN)_3$ red complex

Procedure

Briefly 1.5 ml of chloride reagent (0.058 mM Mercuric nitrate, 1.75 mM Merrcuric thiocyanate, 0.74 mM Mercuric chloride and 22.3 mM Ferric nitrate with non reactive ingredients and stabilizers in dilute acid and ethanol) was dispensed to each tube. Then 0.01 ml (10 µl) sample was added to its respective tube and mixed gently. This was incubated for 5 minutes at 37^0C, after incubation, absorbance reading was taken at 480 nm. The standard reagent equivalent to 100 mEq/L was also treated in the same manner as the samples.

Calculations

$$\frac{\text{Abs. of Unknown}}{\text{Abs. of Standard}} \times \text{Concentration of Standard (mEq/L)} = \text{Chloride Concentration (mEq/L)}$$

3.2.12 Antioxidant Status Determination

3.2.12.1 Superoxide Dismutase (SOD) Activity

The activity profile of SOD in the tissues homogenates was determined by the method of Sun and Zigma (1978).

Principle

It is based on the ability of superoxide dismutase to inhibit the autoxidation of epinephrine at alkaline pH. Superoxide (O^{-}_{2}) radical generated by the xanthine oxidase reaction caused the oxidation of epinephrine to adrenochrome. The adrenochrome produced per O_2^- increased with increasing pH and also increased with increasing concentration of epinephrine.

Procedure

The reaction mixture (3ml) contained 2.95ml 0.05M sodium carbonate buffer pH 10.2, 0.02ml of homogenate and 0.03ml of 2mM epinephrine in 0.005N HCl wasused to initiate the reaction. The reference cuvette contained 2.95ml buffer, 0.003ml of substrate (epinephrine) and 0.02ml of distilled water. The absorbance was readat regular interval of 1 min for 5 min at 480nm. $\varepsilon = 4020 M^{-1}cm^{-1}$ (Zou et al., 1986).

Activity of SOD = $\Delta A/min \times TV/\varepsilon \times SV$

Where ΔA = change in absorbance,

TV = total volume,

SV = sample volume,

ε = molar extinction.

3.2.12.2 Catalase Activity

The catalase activity in the tissues homogenates was determined by the method of Sinha (1972).

Procedure

The reaction mixture (1.5ml) contained 1.0ml of 0.01M phosphate buffer (pH 7.0), 0.1ml of tissue homogenate and 0.4ml of 0.02M H_2O_2. Addition of 2.0ml of dichromate-acetic reagent (5% potassium dichromate and glacial acetic acid weremixed in 1:3 ratio) wasused to stopped the reaction and absorbance was readat regular interval of 1 min for 5 min at 620nm against the blank.

$\varepsilon = 40M^{-1}cm^{-1}$

The catalase activity will be obtained from the expression:

$CAT = \Delta A/min \times TV/\varepsilon \times SV$

Where ΔA = change in absorbance,

TV = total volume,

SV = sample volume,

ε = molar extinction

3.2.12.3 Estimation of Reduced Glutathione (GSH) Level

The method of Sedlak and Lindsay (1968) was followed in estimating the level of reduced glutathione (GSH).

Principle

This is based on the development of a relatively stable (yellow) colour when 5′,5′ – dithiobis - (2-nitrobenzoic acid, DTNB) (Ellman's reagent) is added to sulfhydryl compounds.

Procedure

To 5ml of the sample, 1ml of 10% TCA was added and centrifuge at 3000rpm. 5ml of supernatant wastreated with 0.5ml of Ellmans reagent (5.5¹-dithiobis (2-nitrobenzoic acid) (DTNB) in 100ml of 0.1% sodium nitrate) and 3.0ml of phosphate buffer (0.2M, pH 8.0). The absorbance was readat 412nm against the blank.

Concentration of GSH = $\Delta A \times TV/\varepsilon \times SV$

$\varepsilon = 1.34 \times 10^{4} M^{-1} cm^{-1}$

where ΔA = change in absorbance,

TV = total volume,

SV = sample volume,

ε = molar extinction.

3.2.12.4 Assessment of Lipid Peroxidation

Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) according to the method of Varshney and Kale (1990).

Principle

Under acidic condition, malondialdehyde (MDA) produced from the peroxidation of fatty acid membranes and food products react with the chromogenic reagent, 2-thiobarbituric acid (TBA) to yield a pink coloured complex with maximum absorbance at 532nm and fluorescence at 553nm. The pink chromophore is readily extractable into organic solvents such as butanol.

Procedure

An aliquot of 0.4 ml of the tissue homogenate was mixed with 1.6 ml of Tris-KCl buffer to which 0.5 ml of 30% TCA wasadded. Then 0.5 ml of 0.75% TBA was added and placed in a water bath for 45 minutes at 80^{o}C. This was then cooled in ice and centrifuged at 3000 *g*. The clear supernatant wascollected and absorbance measured against ablank containing distilled water at 532 nm. Lipid peroxidation in units/mg protein or gram tissue was computed with a molar extinction coefficient of 1.56 x 10^5 $M^{-1}cm^{-1}$.

$$\text{MDA (units/mg protein)} = \frac{\text{Absorbance x volume of mixture}}{E_{532nm} \text{ x volume of sample x mg protein}}$$

3.2.13 Histopathological Examination

Hematoxylin-Eosin staining technique was employed. The staining method involves application ofhemalum; a complex formed from aluminium ions and oxidized hematoxylin which colorsnuclei of cells. Materials colored blue by hemalum areoften said to be basophilic. The nuclear staining followed by counterstaining with an aqueous or alcoholic solution of eosin Y, colors eosinophilic and other structures invarious shades of red, pink and orange. The

histopathological assessment and photomicrography of the prepared slides wasdone using an Olympus light Microscope with attached Kodak digital camera.

3.2.14 *In Vitro* Biological Assays

3.2.14.1 *In Vitro* antimalarial effects of Ethylacetate partitioned fraction of *A. digitata* stem back extract.

During proteolysis of hemoglobin in the parasite food vacuole where hemoglobin is enzymatically digested, large quantities of toxic heme are released which are rapidly converted to highly insoluble and relatively unreactive microcrystalline dimer called hemozoin. Chloroquine and related drugs have been demonstrated to inhibit synthetic hemozoin (β-hematin) formation.

Procedure

Antimalarial activity of ethylacetate partition fraction of *Adansonia digitata* stem bark extract was evaluated bythe method explained by (Afshar *et al*., 2011) with some modifications. Different concentrations (0.02–0.32 mg/mL in DMSO) ofthe extracts and pure compounds were prepared and added tothe reaction mixture which contains 3 mM of hematin, 10.0 mM oleic acid, and 1 M HCl. The volume ofthe resulting mixture was adjusted to 1.0 mL using sodium acetate buffer, pH 5, and incubated overnightat 37°C. Chloroquine diphosphate and Artesunate were used as apositive control. After incubation, samples were centrifuged at 14000 rpm for 10 min. The hemozoin pellets werewashed repeatedly and incubated at 37°C for 15 min withconstant shaking in 2.5% (w/v) SDS in phosphate buffered saline. Then, the final wash wasdone using 0.1 M sodium bicarbonate until aclear supernatant is observed which is usually achieved in 3–8 washes. The supernatant was discarded after the final wash and the pellets were dissolved in 1.0

mL of 0.1 M NaOH. Hemozoin content was determined bymeasuring the absorbance at 400nm. The results were expressed as % inhibition (*I*%) of heme crystallization using the following equation:

$I\% = [(AN-AS)/AN]\times 100$

where, AN = absorbance of control and AS = absorbance of samples.

3.2.14.2 Effects of some phenolics quantified from Ethylacetate partitioned fraction of *A. digitata* stem back extract on mitochondrial membrane permeability transition pore.

3.2.14.2.1 Isolation of Liver Mitochondria

Low ionic strength mitochondria were isolated according to the method described by Johnson & Lardy (1967).

Procedure

The animals were sacrificed by cervical dislocation, dissected and the liver was immediately excised and trimmed to remove excess tissue. The liver was washed several times in Buffer C, until a clear wash was obtained, then weighed and minced with a pair of scissors. A 10% suspension was prepared by homogenizing the liver in a Teflon-glass cup homogenizer. The whole process was carried out on ice to preserve the integrity of the mitochondria. The suspended tissue (liver) in Buffer C was implored into a refrigerated MSE centrifuge, where the nuclear fraction and cell debris were sedimented by low speed centrifugation at 2,300rpm for 5 minutes. The supernatant was re-centrifuged at the same speed and time to remove unbroken cells. The supernatant thus obtained was centrifuged at 13,000rpm for 10 minute to sediment the

mitochondria. The brown mitochondria pellet obtained after the supernatant was discarded was washed by re-suspending in Buffer D and centrifuged at 12,000rpm for 10 minutes. This washing stage was done twice. The mitochondria were immediately suspended in a solution of ice-cold MSH Buffer (Mannitol, Sucrose, HEPES-KOH, pH 7.4), then dispensed in Eppendorf tubes in aliquot and placed on ice for immediate use.

3.2.14.2.2 Mitochondria Protein Determination

Mitochondria protein content was estimated by the procedure of Lowry *et al.* (1951) using Bovine Serum Albumin (BSA) as standard.

Principle

Lowry *et al.* (1951) found that the pre-treatment of the protein sample with alkaline copper markedly increased the colour developed during the reduction reaction of phosphor-18-molybdictungstic reagent. In their assay medium, they also added a mixture of Na_2CO_3 and NaOH to buffer the pH around 10 to neutralize the Phosphoric acid produced by the degradation of the phosphormolybdictungstic complex at alkaline pH.

The Folin-Cioclateau test is sensitive: samples containing as little as 5mg of protein can be readily analysed. The reaction of protein in solution with Folin reagent occurs in 2 steps, which lead to the final colour in protein.

1) Reaction with Cu in alkalinemedium: Cu^{2+} + Protein $\longrightarrow$ Cu^{2+}---Protein

2) Reduction of the phosphomolybdictungstic reagent by the Cu-treated protein

Procedure

3ml of Reagent D was added to protein samples mixed and left at room temperature for 10 minutes. 0.3ml of Reagent E was added after 10 minutes. Quickly, the mixture was shaken vigorously and left for another 30 minutes after which the absorbance was read at 750nm wavelength on Camspec M105 spectrophotometer. The results obtained were used to plot a standard protein curve.

Table 2: Protocol for Protein Estimation (Lowry *et al*., 1951)

Test tubes in duplicates	1	2	3	4
Standard BSA (µl) solution	-	20	60	100
Distilled H_2O (µl)	1000	980	940	900
Reagent D (µl)	3000	3000	3000	3000
Reagent E (µl)	300	300	300	300

3.2.14.2.3 Mitochondria Swelling Assay

Mitochondria that have accumulated Ca^{2+} can be induced to undergo a permeability transition. The inner membrane becomes non-selectively permeable to small (1500 Da) solution. Mitochondria swelling were assessed according to the method of Lapidus and Sokolove, 1993.

Principle

The principle behind this method is that when the mitochondria swell, their refractive index changes and thus less light is passed across the cuvette which results in a decrease in the

light absorbance measured with a spectrophotometer. In a means to avoid any complications that changes in the redox state of respiratory chain components might cause, the wavelength of the incident light should be at the isobetic point from the cytochromes (540nm) as used in several studies on isolated mitochondria.

Procedure

Mitochondria swelling were determined according to the method of Lapidus and Sokolove (1993). Mitochondria (0.4mg/ml) were pre-incubated in the presence of 200mM Rotenone for 3.5 minutes, prior to the addition of 5mM Sodium succinate. Assay for mitochondrial swelling in the absence of a triggering agent (Ca^{2+}) involved preincubation of the mitochondria in the presence of MSH buffer (swelling buffer), 0.8µM rotenone for $3^1/_2$minutes after which 5mM succinate was added to energize the reaction. Assay for mitochondrial swelling in the presence of a triggering agent (Ca^{2+}) involved preincubation of the mitochondria in the presence of MSH buffer (swelling buffer), 0.8mM rotenone for 3 minutes after which Ca^{2+} was added. Thirty (30) seconds later Succinate was added to energize the reaction. Assay for mitochondrial swelling inhibition in the presence of Spermine involved preincubation of mitochondria in the presence of MSH buffer, 0.8mM rotenone and 1mM Spermine for 3minutes after which Ca^{2+}, the triggering agent was added then 30 seconds later, 5mM Succinate was added to energize the reaction. Absorbance was taken at a wavelength of 540nm in a Camspec M105 spectrophotometer every 30 seconds for 12 minutes. Swelling was measured as decrease in absorbance within the time space of 12 minutes. The temperature was maintained at 37^0C and swelling rate quantified as an A540/min/mg.

Table 3: Protocol for Mitochondria Swelling Medium Assay at A540nm

Sample	Buffer (µl)	Water (µl)	Rotenone (µl)	Spermine (µl)	Mitochodria (µl)	$CaCl_2$ (µl)	Succinate (µl)
Blank	2500	-	-	-	-	-	-
No Triggering Agent	2200	Varied	10	62.5	Intactness volume	-	50
Triggering Agent	2200	Varied	10	62.5	Intactness volume	25	50
Inhibitor	2200	Varied	10	62.5	Intactness volume	25	50

Note: *Reading was taken at 540nm*

NTA: Buffer ⟶ Water ⟶ Rotenone ⟶ Mitochondria $\xrightarrow{3.5min}$ Succinate

TA: Buffer ⟶ Water ⟶ Rotenone ⟶ Mitochondria $\xrightarrow{3min}$ $CaCl_2$ $\xrightarrow{0.5min}$ Succinate

I: Buffer ⟶ Water ⟶ Rotenone ⟶ Spermine ⟶ Mitochondria $\xrightarrow{3min}$ $CaCl_2$ $\xrightarrow{0.5min}$ Succinate

In vitro: Buffer ⟶ Water ⟶ Rotenone ⟶ Extract ⟶ Mitochondria $\xrightarrow{3min}$ $CaCl_2$ ⟶ Succinate

3.2.14.3 Assessment of Mitochondria ATPase

3.2.14.3.1 Isolation of Mitochondrial for ATPase Activity

The animals were sacrificed by cervical dislocation, dissected and the liver was immediately excised and trimmed to remove excess tissue. The liver was washed several times in sucrose, until a clear wash was obtained, then weighed and minced with a pair of scissors. A 10% suspension was prepared by homogenizing the liver in a Teflon-glass cup homogenizer. The whole process was carried out on ice to preserve the integrity of the mitochondria. The suspended tissue (liver) in sucrose was implored into a refrigerated MSE centrifuge, where the nuclear fraction and cell debris were sedimented by low speed centrifugation at 2300rpm for 5 minutes. The supernatant was re-centrifuged at the same speed and time to remove unbroken cells. The supernatant thus obtained was centrifuged at 13000rpm (10,000g) for 10 minutes to sediment the mitochondria. The brown mitochondria pellet obtained after the supernatant was discarded, was washed and re-suspended in sucrose and re-centrifuged at 12000rpm (9000g) for 10 minutes. This washing stage was done twice. The mitochondria were immediately suspended in a solution of ice-cold of very little sucrose and then dispensed in Eppendorf tubes in aliquot and placed on ice for immediate use. Protein determination was carried out by Lowry et al (1951).

3.2.14.3.2 Determination of Inorganic Phosphate

This was performed according to the method described by Ronner et al. (1977) modified by Bewaji (2004).

Principle

The principle is that molybdic acid in the presence of inorganic phosphate gives a yellow coloured compound which is reduced to give a blue coloured compound. Ascorbic acid can be used as the reducing agent and the intensity of the colour thus formed is directly proportional to the concentration of inorganic phosphate. 1mM Na_2Po_4 was used for the standard curve for the determination of inorganic phosphate liberated.

Procedure

Different concentrations of 1mM Na_2PO_4 were used for the standard curve as below in the protocol. To the 5ml of de-proteinizised supernatant in a test tube, 1000µl of 1.25% Ammonium Molybdate and 1000µl of 2% freshly prepared solution of Ascorbic acid were added. After through by mixing with gentle shaking, the tube was allowed to stand for 30 minutes. The absorbance was read at 660nm wavelength on Camspec M105 Spectrophotometer against distilled water as blank. The graph of standard PO_4 was obtained by plotting absorbance against phosphate concentration.

Table 4: Protocol of Phosphate curve

TT	d.H_2O (ml)	1mM Na_2PO_4 (µl)	1.25%NH_4Molybdate (ml)	9%Ascorbate (ml)
1	1000	-	1	1
2	980	20	1	1
3	960	40	1	1
4	940	60	1	1
5	920	80	1	1
6	900	100	1	1

3.2.14.3.3 Determination of mitochondria ATPase activity

Adenosine triphosphatase activity was determined by a modification of the method of Lardy and Wellman (1953).

Principle

The mitochondria which is the site of oxidative phosphorylation is also the site for ATP synthesis via the f_1f_0 (ATP Synthase). Coupling of electron transport chain with oxidative phosphorylation is a key step for ATP (which is a nucleotide) production. ATP synthase in an intact mitochondria synthezises ATP but if not well coupled hydrolyzes ATP resulting in the release of inorganic phosphate.

Procedure

Each reaction vessel contained 65mM Tris-HCl buffer at pH7.4, 0.5mM KCl, 1mM ATP and 0.25mM Sucrose in a total reaction volume of 2mls. Distilled H_2O or test compound was added accordingly. The reaction which was started by the addition of the mitochondrial fraction was allowed to proceed for 30 minutes with constant shaking at 37^oC. Mitochondria were put in all the tubes at 30 seconds interval except the tube containing ATP only. 1ml of sodium dodecyl sulfate (SDS) was put in the zero time test tube after the addition of the mitochondria and the reaction in the other reaction tubes was stopped by the addition of 1ml of sodium dodecyl sulfate (SDS) after which 1000µl ammonium molybdate was put into all the test tubes. L-ascorbate was then added to the test tubes at 30 seconds interval for 20 minutes, after the 20^{th} minute the absorbance reading was taken at 660nm.

Table 5: Protocol for mitochondrial ATPase activity

Test tube	0.25mM Sucrose (µl)	0.5mM KCl (µl)	65mM Tris (µl)	H_20 (µl)	Extract (µl)	1mM ATP (µl)	Mito (µl)	Uncoupler (µl)
Uncoupler	250	200	1300	137	-	40	23	50
Mito only	250	200	1300	227	-	-	23	-
ATP only	250	200	1300	210	-	40	-	-
Mit+ATP	250	200	1300	187	-	40	23	-
10 µl	250	200	1300	177	10	40	23	-
30 µl	250	200	1300	157	30	40	23	-
50 µl	250	200	1300	137	50	40	23	-
70 µl	250	200	1300	117	70	40	23	-
Zero tube	250	200	1300	187	-	40	23	-

3.2.17 Molecular Docking Studies

This study deals with the evaluation of inhibitory activity of secondary metabolites of *Adansonia digitata* on malaria-associated protein using *in silico* docking studies. Ca ATPase (2KNE) and Ca transporter CAX (4K1C) were downloaded from Protein Databank (http://www.rcsb.org). Apigenin, quercetin and cis-vaccenic acid were used as the ligand and they were downloaded from zinc.docking.org. The various ligands and water molecules were deleted from the original protein. The ligands were re-docked into the active site of each protein as a method of revalidation. A flexible Cis-Vaccenic, Apigenin and Quercetin structures were prepared in a pdbqt file with appropriate torsion numbers. Molecular Docking was carried out using Autodock vina (Trott and Olson, 2010). The Root Mean Square Deviation (RMSD) and

Affinity Energy (Trott and Olson, 2010) were used in selecting the best interaction proses. The protein-ligand interactions were identified using Discovery Studio Visualizer (version 16).

3.2.16 Statistical Analysis

Results calculated from replicate data were expressed as means±standard error of/deviation from means (SEM/SD). With the exception of data on mitochondrial ROS production which was analyzed using two-way ANOVA followed by Bonferroni post test, one-way analysis of variance wasused for data analysis with Newman-Keuls comparison of means. The level of significance was set at $p < 0.05$. Statistical Analysis, Graphing, curve fitting, and IC_{50} were performed using Graph Pad Prism (ver.5.0a).

CHAPTER FOUR

4.0 RESULTS

4.1 Secondary metabolites and antioxidant activities of *A. digitata* stem bark extracts

4.1.1 Phytochemical Screening of *A. digitata* stem bark extract

The summary of the phytochemicals inthe aqueous and methanol extract of *Adansonia digitata* stem bark is presented in Table 6. The results revealed the presence of alkaloids, saponins, tannins, terpenoids, cardiac glycosides and flavonoids, due to positive reactions.

4.1.2 Quantitative phytochemical composition of *Adansonia digitata* stem bark extract

The total phenolic and flavonoid, alkaloid, saponin and tannin contents of aqueous and methanolic extract of Adansonia digitata stem bark are presented in Table 7.

Table 6: Phytochemical screening of aqueous and methanolic extract of *Adansonia digitata* stem bark.

Phytochemicals	Aqueous Extract	Methanol Extract
Alkaloids	+	+
Saponin	+	+
Tannin	+	+
Steroids	-	-
Phlobatannin	-	-
Anthraquinone	-	-
Terpenoids	+	+
Cardiac glycosides	+	+
Flavonoids	+	+

KEY: + = Presence of constituent; - = Absence of constituent

Table 7: Quantitative phytochemical composition of aqueous and methanolic extract of *Adansonia digitata* stem bark in antioxidant properties

Activity/Sample	Aqueous Extract	Methanol Extract
Total Phenol (mg/g TAE)	1.00 ± 0.00^{b}	2.15 ± 0.01^{a}
Total Flavonoid (mg/g QE)	4.37 ± 0.00^{a}	4.68 ± 0.00^{a}
Alkaloid (mg/100g GAE)	9.68 ± 0.6^{a}	9.84 ± 0.8^{a}
Tannin (mg/100g GAE)	10.20 ± 31.20^{a}	10.35 ± 31.25^{a}
Saponins (mg/100g GAE)	13.00 ± 5.0^{a}	12.30 ± 6.5^{b}

TAE = Tannic acid equivalent, QE = Quercetin equivalent and GAE = Gallic acid equivalent

4.2 High performance liquid chromatography (HPLC) analysis of phenolics

4.2.1 Phenolic Contents of aqueous and methanolic stem bark extract of *Adansonia digitata*

Table 8 shows the phenolics and flavonoids composition of aqueous and methanolic stem extract of *Adansonia digitata*.

HPLC analysis showed thepresence of flavonoids (rutin, quercitrin, kaempferol, luteolin and apigenin), and phenolics acids (gallic, chlorogenic and caffeic acids) in the aqueous and methanolic extract of *Adansonia digitata* stem bark). The chromatogram showed thepresence of the gallic acid (retention time, t_R = 9.83 min, peak 1), chlorogenic acid (t_R = 21.39 min, peak 2), caffeic acid (t_R = 27.48 min, peak 3), rutin (t_R = 40.15 min, peak 4), quercitrin (t_R = 45.06 min, peak 5), kaempferol (t_R = 54.63 min, peak 6), luteolin (t_R = 68.37 min, peak 7) and apigenin (64.11 min, peak 8).

4.2.2 Phenolic Contents of partitioned fraction of crude methanolic extract of *Adansonia digitata* stem bark.

Table 9 shows the phenolics and flavonoids composition of partitioned fraction of crude methanolic extract of *Adansonia digitata* stem bark.

HPLC fingerprinting of methanolic extract of *A. digitata* partitioned fractions revealed the presence of thegallic acid (retention time t_R = 11.87 min, peak 1), chlorogenic acid (t_R = 21.09 min, peak 2), caffeic acid (t_R = 23.49 min, peak 3), rutin (t_R = 33.71 min, peak 4), quercetin (t_R = 41.93 min, peak 5) and luteolin (t_R = 51.78 min, peak 6).

Table 8: Phenolic acids and flavonoid composition of crude extract of *Adansonia digitata* stem bark using HPLC.

Compounds	**Aqueous (mg/g)**	**Methanolic (mg/g)**
Gallic acid	0.63 ± 0.01^{a}	2.29 ± 0.03^{a}
Chlorogenic acid	0.67 ± 0.01^{a}	0.73 ± 0.01^{b}
Caffeic acid	1.84 ± 0.03^{b}	0.25 ± 0.02^{c}
Rutin	0.59 ± 0.02^{a}	1.87 ± 0.01^{d}
Quercitrin	2.38 ± 0.01^{c}	1.85 ± 0.03^{d}
Kaempferol	2.41 ± 0.03^{c}	3.69 ± 0.01^{e}
Luteolin	1.87 ± 0.01^{b}	4.05 ± 0.01^{f}
Apigenin	0.60 ± 0.01^{a}	1.81 ± 0.02^{d}

Results are mean ± standard deviations

Table 9: Phenolic acids and flavonoid composition of partitioned fraction of crude methanolic extract of *Adansonia digitata* stem bark using HPLC

Compounds	**MEAD (mg/g)**	**EFAD (mg/g)**	**CFAD (mg/g)**
Gallic acid	1.51 ± 0.01^{a}	2.35 ± 0.02^{a}	0.06 ± 0.01^{a}
Chlorogenic acid	2.24 ± 0.02^{b}	4.86 ± 0.03^{b}	0.09 ± 0.04^{a}
Caffeic acid	1.49 ± 0.01^{a}	2.29 ± 0.01^{a}	0.17 ± 0.02^{b}
Rutin	5.18 ± 0.01^{c}	1.07 ± 0.01^{c}	-
Quercetin	4.97 ± 0.04^{d}	6.19 ± 0.02^{d}	-
Luteolin	0.36 ± 0.01^{e}	4.54 ± 0.01^{e}	-

MEAD = Methanolic extract of *A. digitata*, EFAD = Ethyl acetate partitioned fraction of *A. digitata* and CFAD = Chloroform partitioned fraction of *A. digitata*

4.3 Chemical profile of ethylacetate partitioned fraction of crude methanolic extract of *Adansonia digitata* stem bark using gas chromatography/mass spectrophotometry

The compounds identified from the ethylacetate fraction of crude methanolic extract are shown in Table 10.

Table 10: Compounds identified from Ethylacetate fraction of *Adansonia digitata* extract using gas chromatography/mass spectrophotometry (GC/MS)

Peak	**RT (min)**	**% of Total Area**	**Compound**
1.	37.364	1.99	Bicyclo [3.1.1] Heptane
2.	38.582	7.19	n-Hexadecanoic acid
3.	38.731	9.90	n-Hexadecanoic acid
4.	39.682	1.84	Phytol
5.	39.760	14.25	Cis-13,16-Docosadienoic acid, methyl ester
6.	39.902	45.69	Cis-vaccenic acid
7.	40.011	11.04	Octadecanoic acid
8.	40.161	5.59	2-Propanone, 1-(3,5,5-trimethyl-2-cyclohexen-1-ylidene
9.	42.015	2.51	Phthalic acid, hept-4-yl-Isohexeyl ester

4.4 *In Vitro* Antioxidant Potentials

4.4.1 DPPH Radical Scavenging Activity of *Adansonia digitata* stem bark Extract

The ability of *A. digitata* stem bark extract to scavenge the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical ascompared to the reference tannic acid is shown in Fig. 14. The extract exhibited concentration-dependent decrease in DPPH radical. MEAD showed ahigh radical scavenging ability with the lowest IC_{50} value of 14.62 µg/ml.

4.4.2 Reducing Property of *Adansonia digitata* stem bark Extract

The results obtained from the screening of the reductive potential of AEAD and MEAD is shown in Fig. 15. The reduction of Fe^{3+} to Fe^{2+} in the presence of AEAD and MEAD was employed to determine the reductive capability of the extract. AEAD showed concentration dependent reducing property with thehighest absorbance of 0.564 at 400 µg/ml when compared to the absorbance of MEAD, 0.407 at 400µg/ml. AEAD exhibited higher reducing effect than the MEAD. The difference inthe reducing power of AEAD and MEAD is significant ($P < 0.05$).

4.4.3 Metal Chelating Property of *Adansonia digitata* stem bark Extract

The results of Fe (II) chelating activity of *Adansonia digitata* stem bark extract is presented in Fig. 16. The standard, EDTA exhibited anexcellent ferrous ion-chelating capacity at all concentration when compared to the extracts. Among the extract, MEAD had the highest ferrous ion-chelating ability withthe lowest IC_{50} value of 28.95 when compared to AEAD with IC_{50} value of 38.75. The ferrous ion-chelating ability of the extracts are concentration dependent manner.

4.4.4 Hydroxyl radical-scavenging Property of *Adansonia digitata* stem bark Extract

The results of Hydroxyl radicals activity of *Adansonia digitata* stem bark extract is presented in Fig. 17. AEAD showed higher hydroxyl radical scavenging ability than MEAD. The difference in OH radical scavenging in the two extracts issignificant statistically ($p < 0.05$). The hydroxyl radical scavenging capability of AEAD was comparable to mannitol atall concentrations.

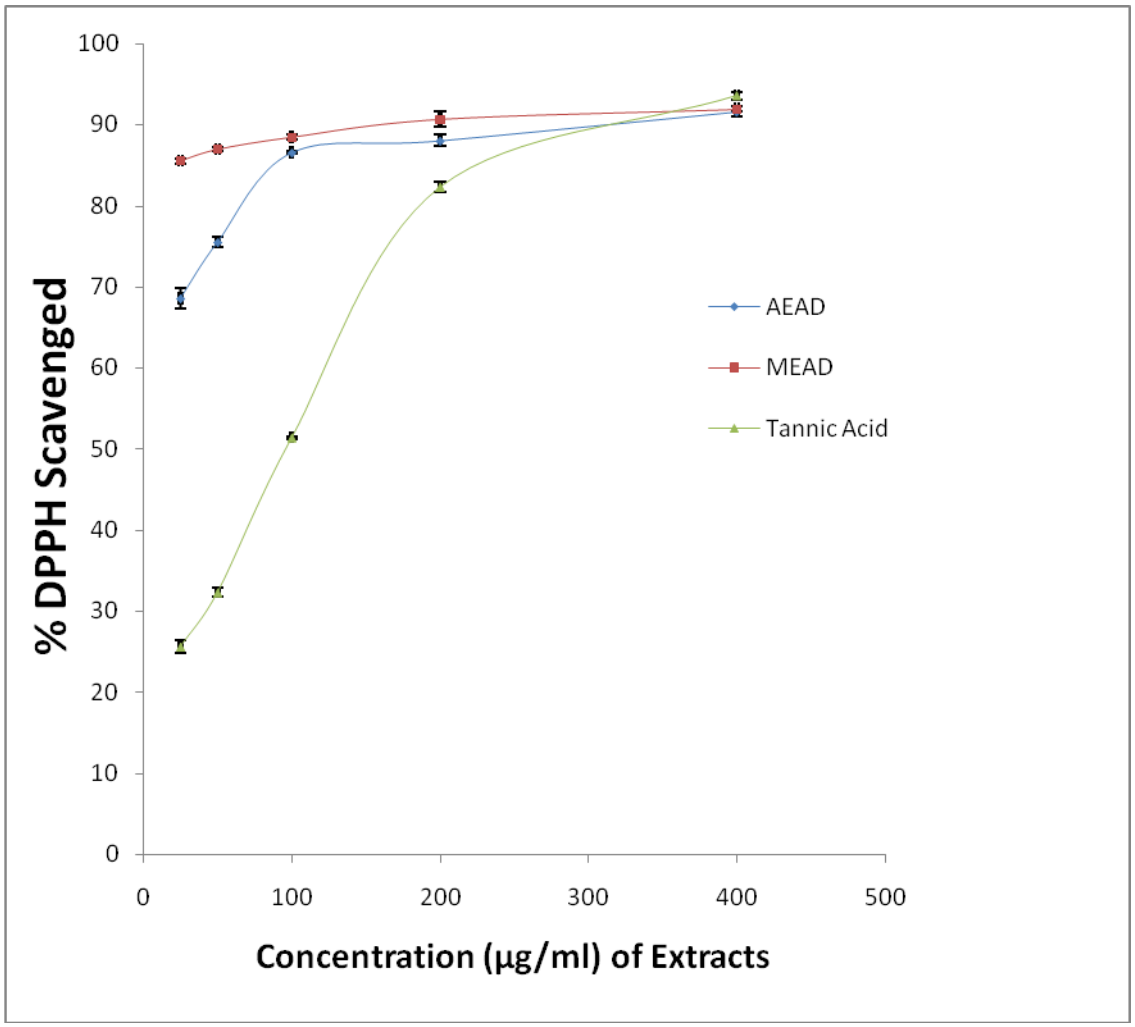


Figure 14: DPPH free radical-scavenging activities of aqueous and methanolic extracts of *Adansonia digitata* stem.

[†]The results are mean ±SEM of triplicate measurements.

[**]P<0.05 vs 0 µg/ml; [***]P<0.01 vs 0 µg/ml

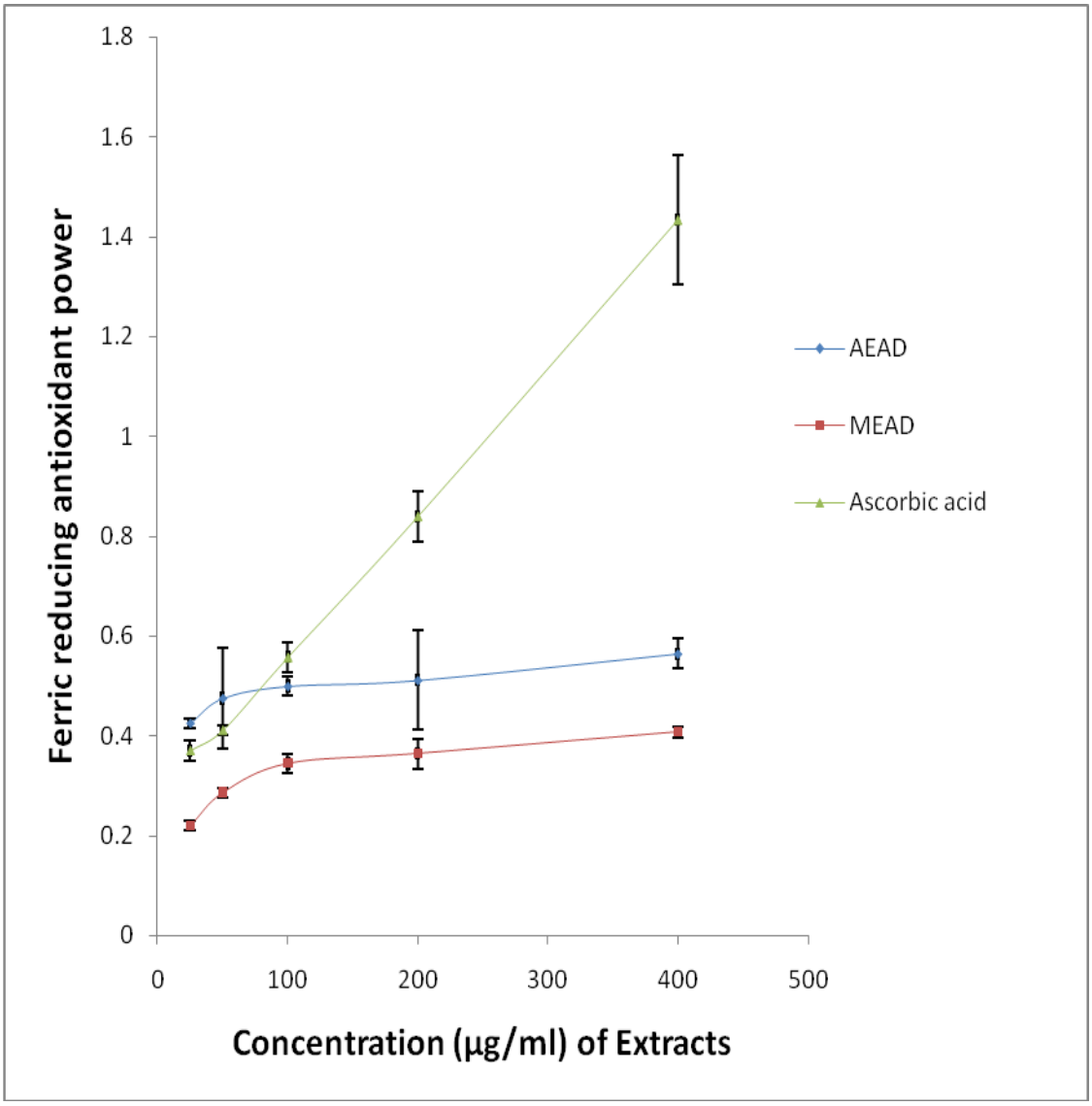


Figure 15: Ferric-reducing antioxidant power of aqueous and methanolic extracts of Adansonia digitata stem.

†The results are mean ±SEM of tiplicate measurements.

**P<0.05 vs 0 µg/ml; ***P<0.01 vs 0 µg/ml

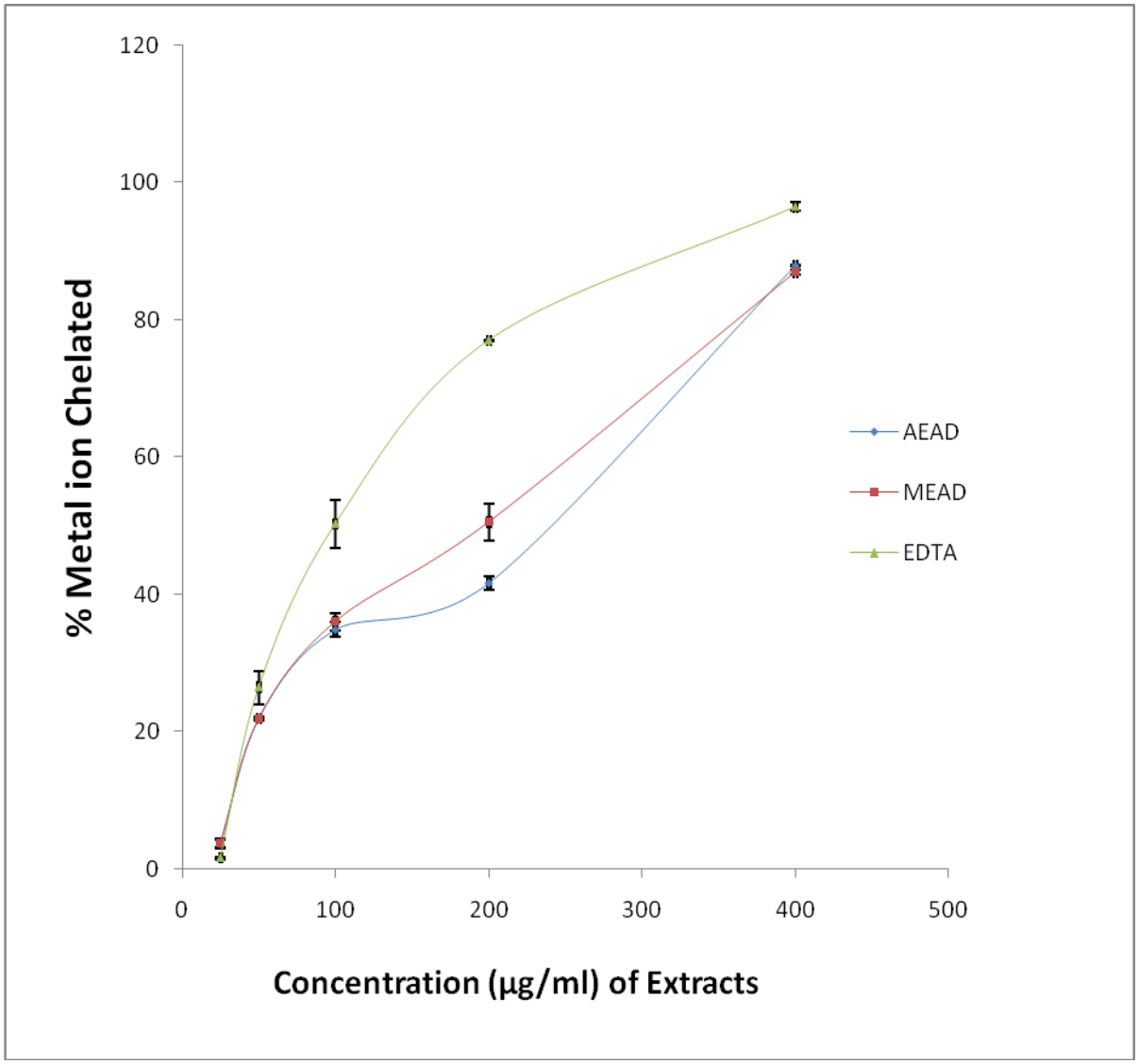


Figure 16: Ferrous ion-chelating activities of aqueous and methanolic extracts of *Adansonia digitata* stem.

†The results are mean ±SEM of triplicate measurements.

**P<0.05 vs 0 µg/ml; ***P<0.01 vs 0 µg/ml

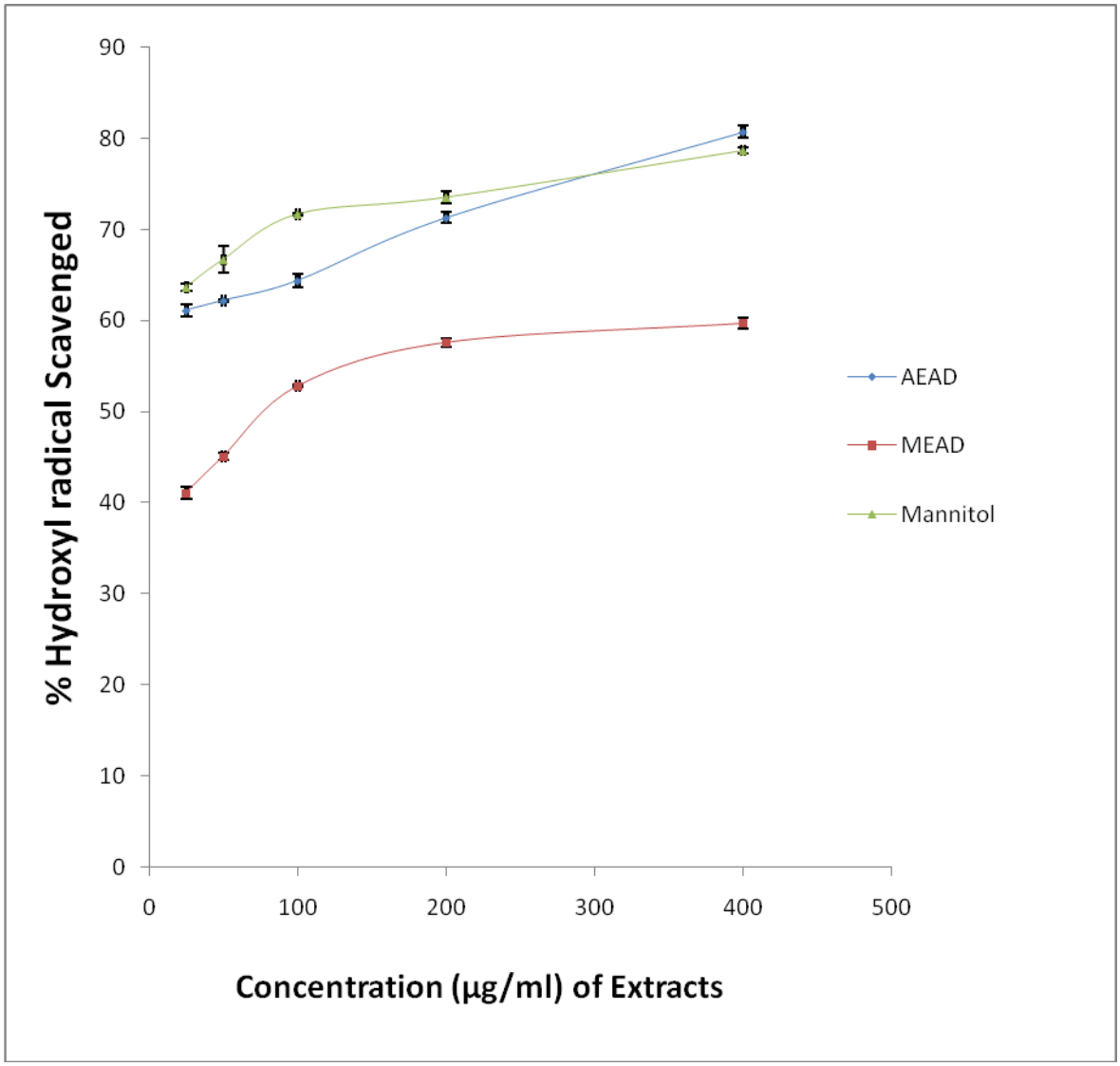


Figure 17: Hydroxyl radical-scavenging activities of aqueous and methanolic extracts of Adansonia digitata stem.

†The results are mean ±SEM of triplicate measurements.

**P<0.05 vs 0 µg/ml; ***P<0.01 vs 0 µg/ml

4.5 In vivo antimalarial activities of *A. digitata* stem bark extracts

4.5.1 Therapeutic effect of *A. digitata* on *P. berghei* infected mice

In Fig. 18, a daily rise in parasitemia levels in the control group and significant reduction inthe extract treated groups was noticed during the study. Parasitemia level at 400mg/kg body weight/day was significantly ($P< 0.05$) lowered when compared to 200mg/kg body weight/day. There was asignificant ($P< 0.05$) dose dependent reduction in parasitemia levels inthe two doses inrelation to control group. Chloroquine showed highest chemosupression/clearance and zero parasitemia was observed onthe third dayof treatment which was maintained throughout the experiment (Fig. 19).

In Fig. 20, the packed cell volume (PCV) was reduced significantly (20.00 ± 0.00) inthe control. There was improvement in the PCV of the extract treated groups with chloroquine group having the highest value (44.67 ± 2.60). Increase inmean survival time was observed inall the groups that received the extract relative to control (Fig. 21).

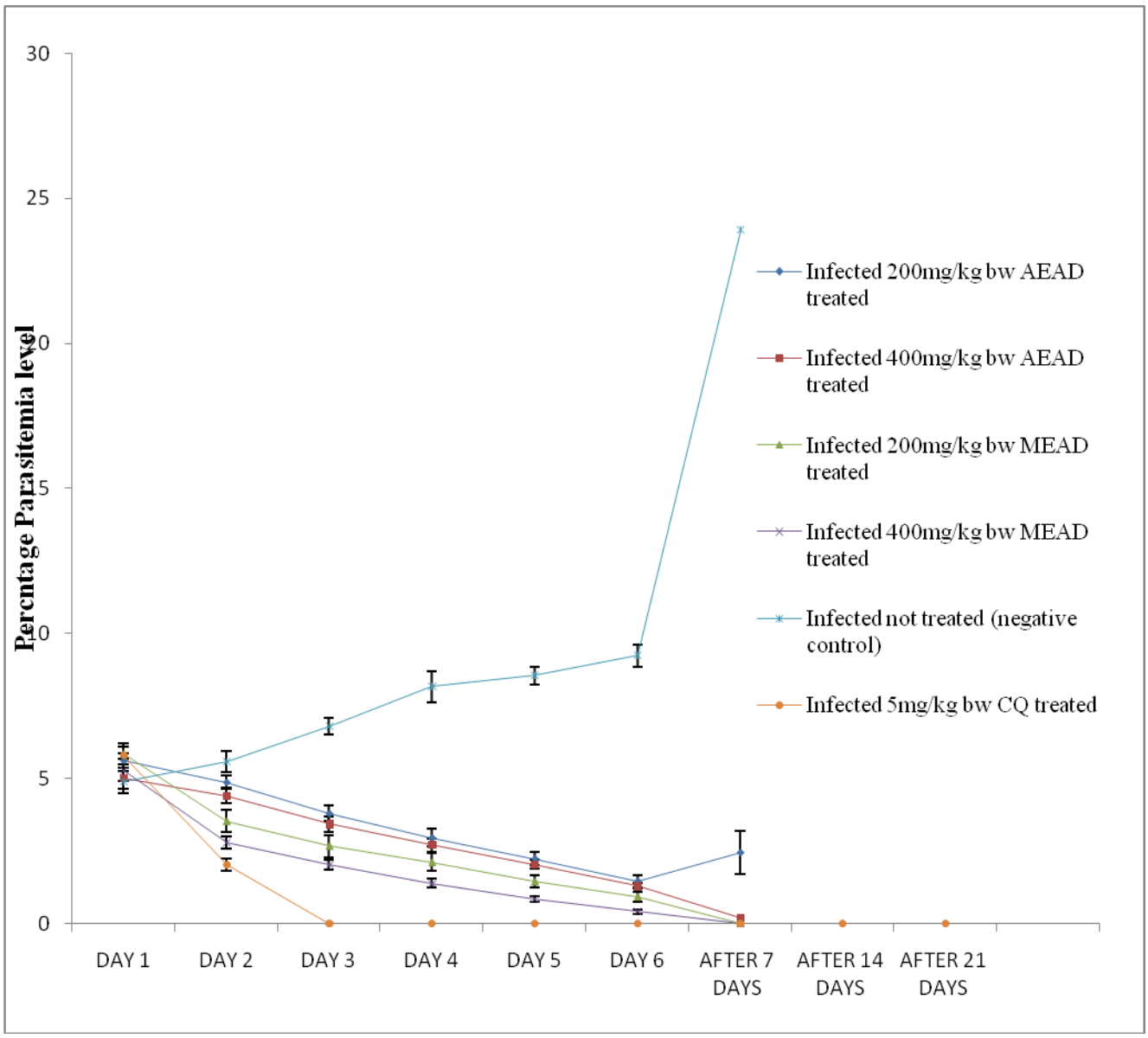


Figure 18: Percentage parasitemia in *P. berghei*- infected mice treated with extract of *Adansonia digitata* stem bark.

Results are mean of 8 determinations ± SEM

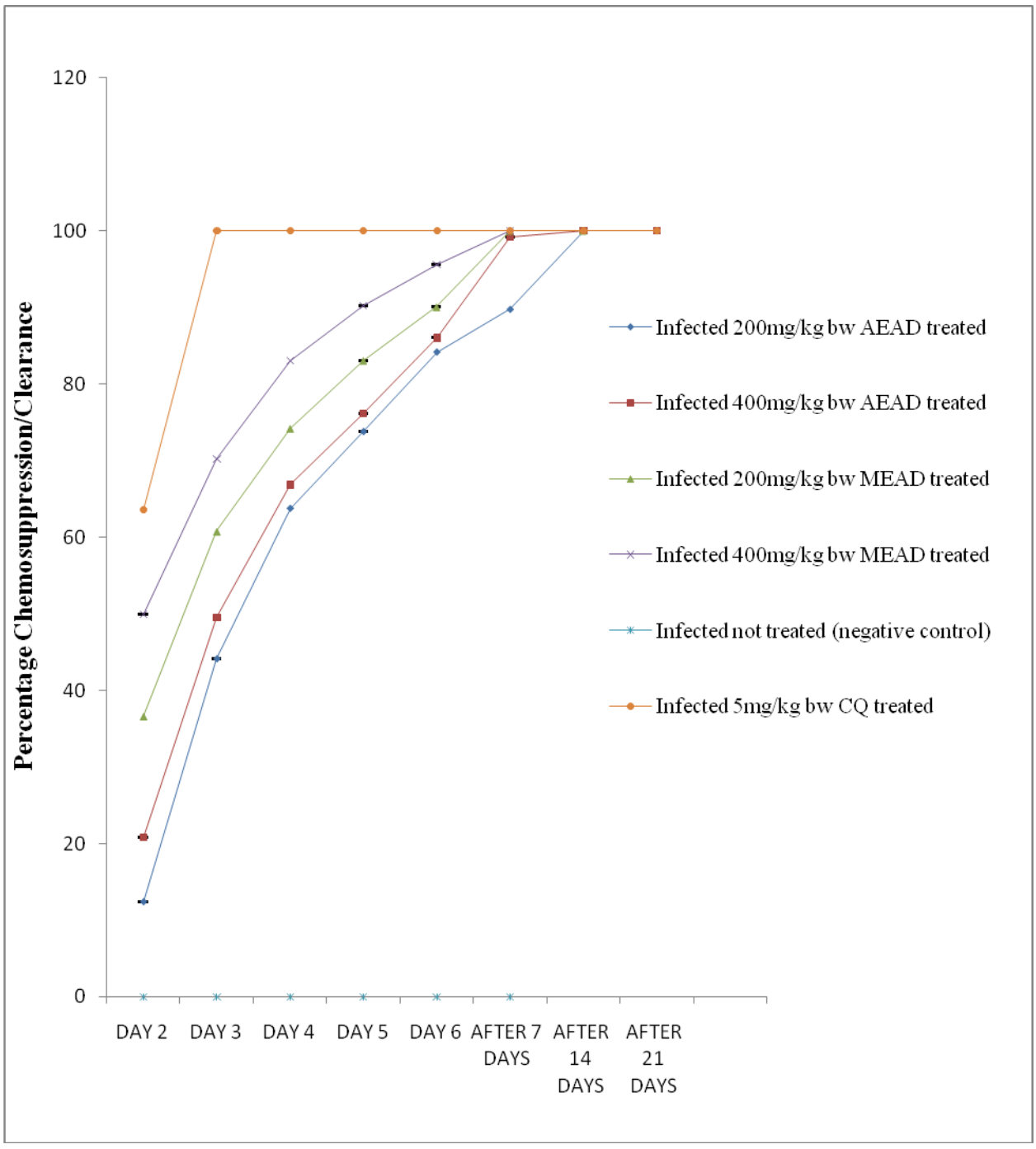


Figure 19: Percentage clearance/chemosupression in *P. berghei*- infected mice treated with extract of *Adansonia digitata* stem bark.

Results mean of 8 determinations ± SEM.

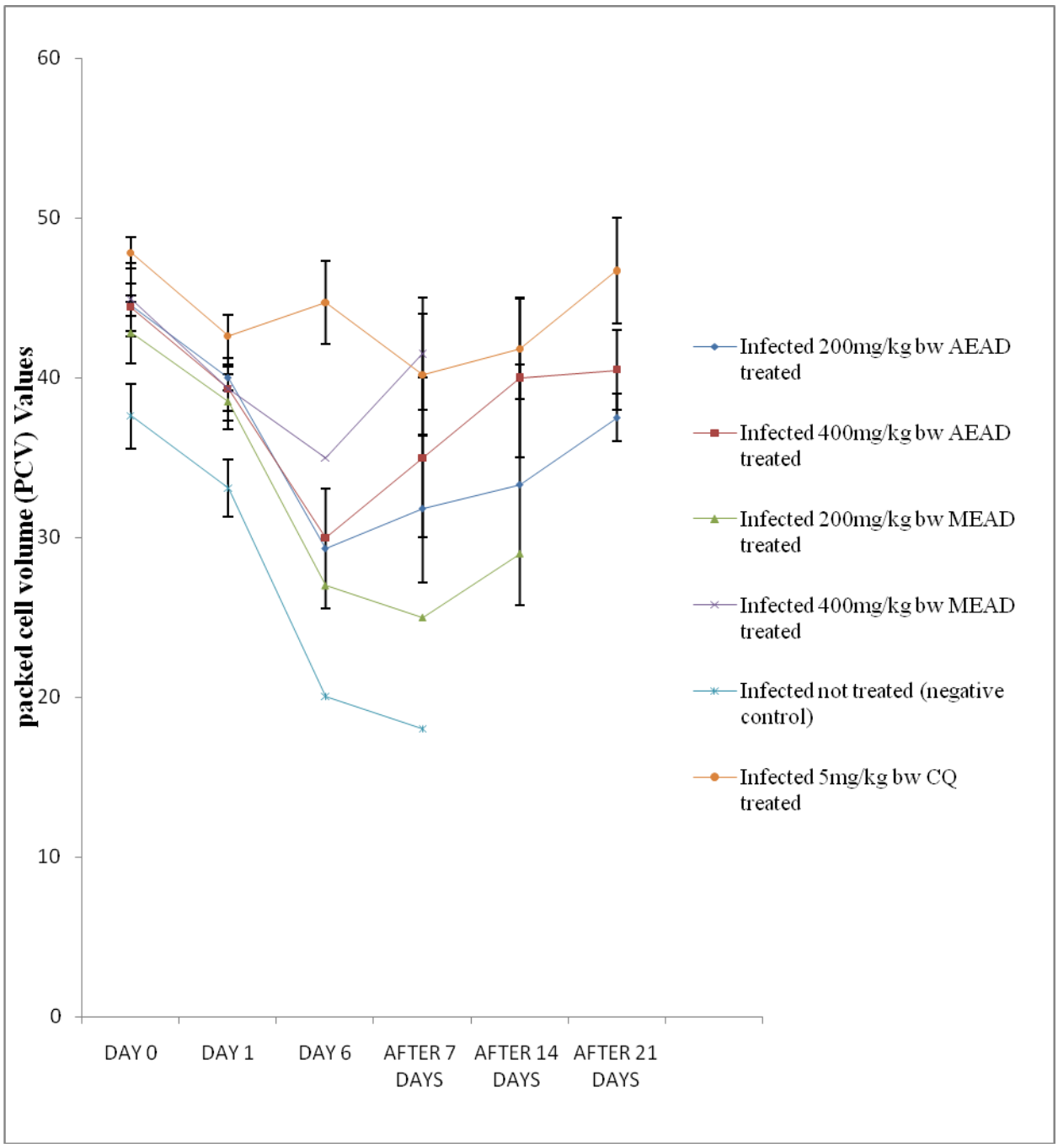


Figure 20: Packed cell volume of *P. berghei*- infected mice treated with extract of *Adansonia digitata* stem bark.

Results are mean of 8 determinations ± SEM.

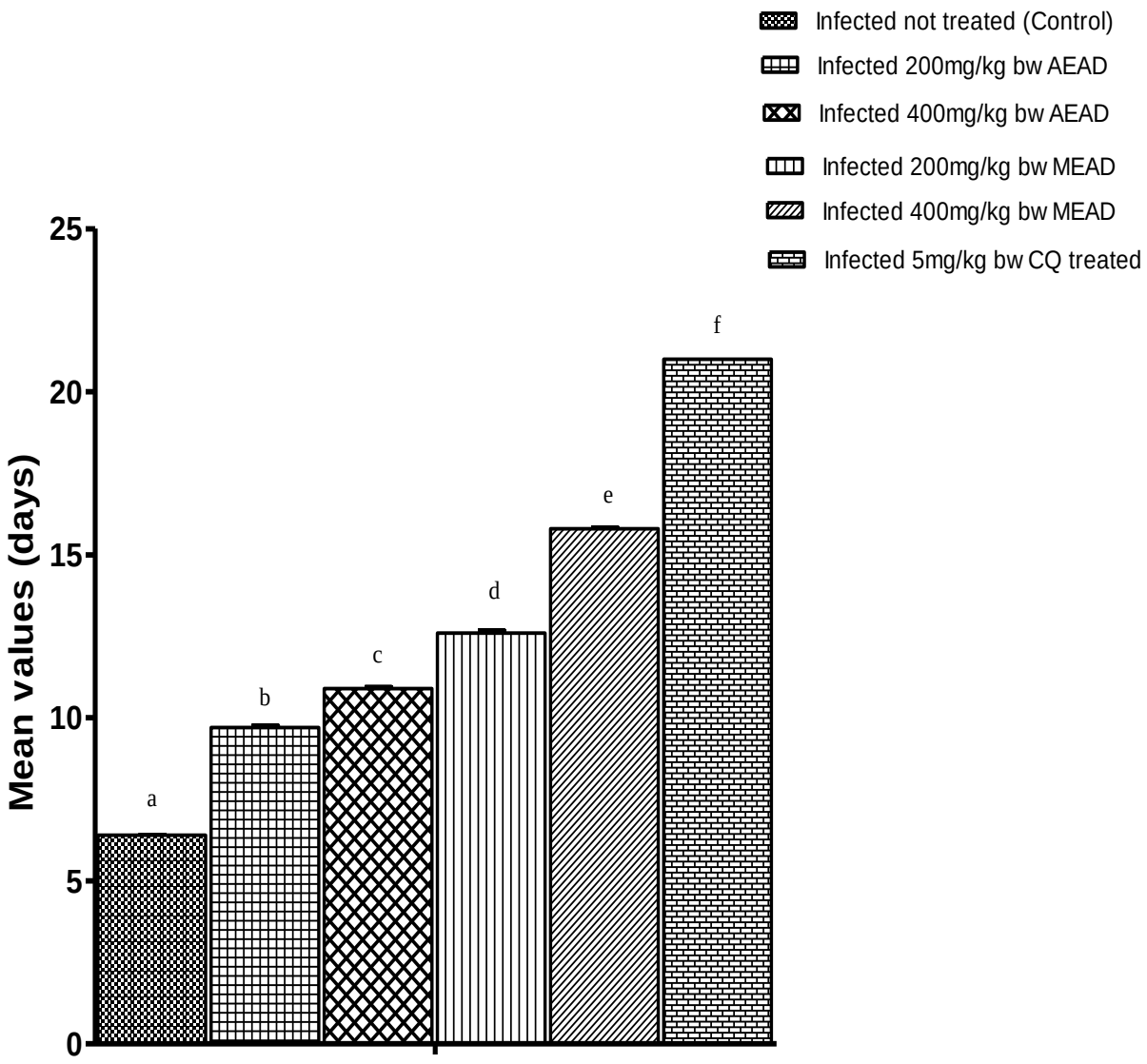


Figure 21: Mean survival time of *P. berghei*- infected mice treated with extract of *Adansonia digitata* stem bark.

Results mean of 8 determinations ± SEM.

4.5.2 Suppressive effect of *A. digitata* on *P. berghei* infected mice

Table 11 showed the effect of some fractions and extract of *Adansonia digitata* stem bark on early infection. There was adose dependent reduction inpercentage parasitemia in both the fractions (chloroform and ethylacetate) and the extract (aqueous and methanol). The parasitemia level in chloroquine and artemether-lumefantrine groups were significantly lowered than thefractions and extract treated groups. The fractions and extract treated group onday 5 at 400mg/kg body weight for AQ, ME, EF, and CF caused chemosupression of 72.98 %, 80.28%, 81.66% and 70.41% respectively. The reference drug, AL and CQ caused 100% and 100% chemosuppressive respectively. Artemether-lumefantrine was found to be more effective than chloroquine. The packed cell volume (PCV) inthe control and treatment group is presented in Fig. 22. There was an improvement in PCV in a dose dependent manner.

Table 11: **Suppressive effects of *Adansonia digitata* stem bark extract and fractions on *P. berghei*-infected mice**

DAYS		1		3		5	
		%P	**%C**	**%P**	**%C**	**%P**	**%C**
Control	5%v/v	3.57 ± 0.07[a]	-	4.23 ± 0.12[a]	-	5.07 ± 0.12[a]	-
ME	200mg	3.10 ± 0.21[a]	13.17 ± 0.00[d]	2.70 ± 0.35[a]	36.17 ± 0.00[d]	1.70 ± 0.12	66.47 ± 0.00[d]
	400mg	2.43 ± 0.28[ab]	31.93 ± 0.00[b]	1.70 ± 0.15	59.81 ± 0.00[b]	1.00 ± 0.12	80.28 ± 0.00[b]
EF	200mg	3.00 ± 0.21[a]	15.97 ± 0.00[d]	2.43 ± 0.29[c]	42.55± 0.00[c]	1.63 ± 0.18	67.85 ± 0.00[d]
	400mg	2.40 ± 0.25[ab]	32.77 ± 0.00[b]	1.50 ± 0.15[#]	64.54 ± 0.00[b]	0.93 ± 0.19[#]	81.66 ± 0.00[b]
CF	200mg	3.07 ± 0.23[a]	14.01 ± 0.00[d]	2.63 ± 0.32[a]	37.83± 0.00[d]	1.67 ± 0.15	67.06 ± 0.00[d]
	400mg	2.77 ± 0.27[ab]	22.41 ± 0.00[c]	2.30 ± 0.15	45.63 ± 0.00[c]	1.50 ± 0.15	70.41 ± 0.00[cd]
AL	5mg	1.07 ± 0.15[b]	70.31 ± 0.00[a]	0.00 ± 0.00	100 ± 0.00[a]	0.00 ±0.00	100 ±0.00[a]
CQ	5mg	1.13 ± 0.12[b]	68.35 ± 0.00[a]	0.00 ± 0.00	100 ± 0.00[a]	0.00 ±0.00	100 ±0.00[a]

200mg=200mg/kg body weight dose; 400mg= 400mg/kg body weight dose; 5mg= 5mg/kg body weight dose; 5%v/v= 5%v/v of tween-80; %P= percentage parasitemia; %C= percentage clearance, ME= Methanolic crude extract, EF= Ethylacetate fraction, CF= Chloroform fraction, AL= Artemeter-lumifantrine, CQ= Chloroquine.

Each value is a mean of several determinations ± SE after five days of exposure to treatment

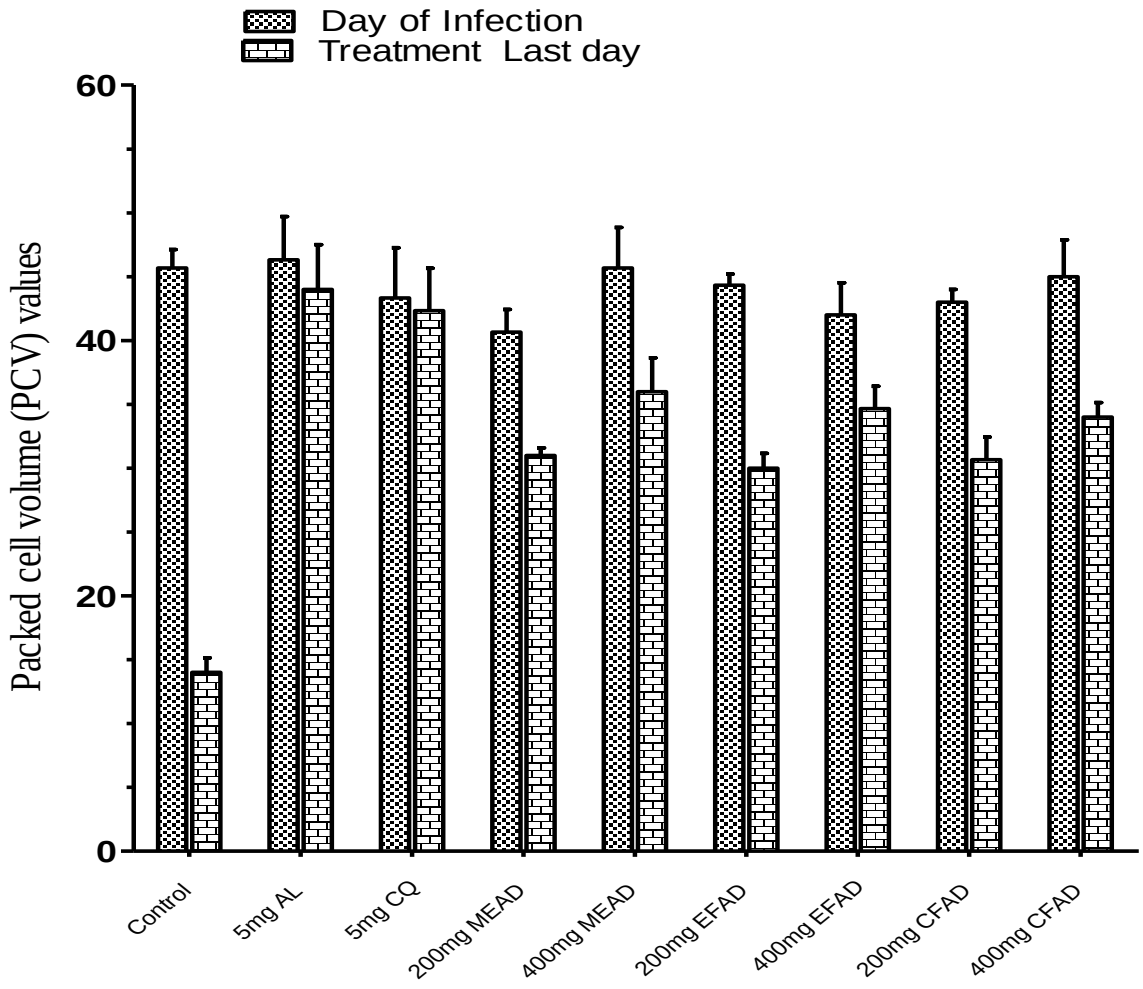


Figure 22: Packed Cell Volume (PCV in percentage) of the suppressive treated groups

Each value is a mean of several determinations ± SE after five days of exposure to treatment

4.5.3 Prophylactic effect of *A. digitata* on *P. berghei* infected mice

Table 12 shows the prophylactic effect of fractions and extract of *Adansonia digitata* stem bark on *P. berghei* infected mice. There was adelay in parasitemia in all the treatment groups relative to untreated control. The percentage chemosupression of AL and CQ on day 1 weresignificantly ($P< 0.05$) higher than both the 200mg and 400mg dose ofthe fraction and extract. As the experiment progressed, the activity of EF though lowered than the AL and CQ became significantly ($P< 0.05$) higher than CF, ME and AQ treated groups. This was noticeable both in percentage parasitemia and percentage chemosupression. Onday five at 400mg/kg body weight dose, AQ, ME, EF, and CF caused chemosupression of 61.48%, 68.31%, 74.76% and 62.05% respectively while the standard drug AL and CQ caused 100% and 100% chemosupression respectively. AL was observed to cause a delay in parasitemia than CQ.

Figure 23 shows the packed cell volume of the prophylactic groups. The PCV also improves in dose dependent manner. EF had the highest PCV value and differed insignificantly from other treated groups.

Table 12: **Prophylactic effects of *Adansonia digitata* stem bark extract and fractions on *P. berghei*-infected mice**

DAYS		1		3		5	
		%P	**%C**	**%P**	**%C**	**%P**	**%C**
Control	5%v/v	3.75 ± 0.28^{a}	-	4.00 ± 0.07^{a}	-	5.27 ± 0.19^{a}	-
ME	200mg	3.30 ± 0.16^{a}	12.00 ± 0.00^{c}	2.52 ± 0.35^{b}	37.00 ± 0.00^{c}	2.07 ± 0.19^{b}	60.72 ± 0.00^{c}
	400mg	2.40 ± 0.51^{ab}	36.00 ± 0.00^{d}	1.80 ± 0.15^{bc}	55.00 ± 0.00^{bc}	1.67 ± 0.59^{bc}	68.31 ± 0.00^{bc}
EF	200mg	3.18 ± 0.10^{a}	15.20 ± 0.00^{c}	2.25 ± 0.47^{b}	43.75 ± 0.00^{c}	1.77 ± 0.29^{bc}	66.22 ± 0.00^{c}
	400mg	2.12 ± 0.35^{ab}	43.47 ± 0.00^{c}	1.50 ± 0.25^{bc}	62.50 ± 0.00^{b}	1.33 ± 0.17^{c}	74.76 ± 0.00^{b}
CF	200mg	3.24 ± 0.17^{a}	13.60 ± 0.00^{c}	2.28 ± 0.21^{b}	43.00 ± 0.00^{c}	1.87 ± 0.39^{bc}	64.52 ± 0.00^{c}
	400mg	2.87 ± 0.50^{ab}	25.33 ± 0.00^{c}	2.28 ± 0.15^{b}	43.00 ± 0.00^{c}	2.00 ± 0.25^{b}	62.05 ± 0.00^{c}
AL	5mg	0.68 ± 0.07^{b}	81.87 ± 0.00^{a}	0.00 ± 0.00	100 ± 0.00^{a}	0.00 ± 0.00	100 ± 0.00^{a}
CQ	5mg	1.00 ± 0.12^{b}	73.33 ± 0.00^{b}	0.00 ± 0.00	100 ± 0.00^{a}	0.00 ± 0.00	100 ± 0.00^{a}

200mg=200mg/kg body weight dose; 400mg= 400mg/kg body weight dose; 5mg= 5mg/kg body weight dose; 5%v/v= 5%v/v of tween-80; %P= percentage parasitemia; %C= percentage clearance, ME= Methanolic crude extract, EF= Ethylacetate fraction, CF= Chloroform fraction, AL= Artemeter-lumifantrine, CQ= Chloroquine.

Each value is a mean of several determinations ± SE after five days of exposure to treatment.

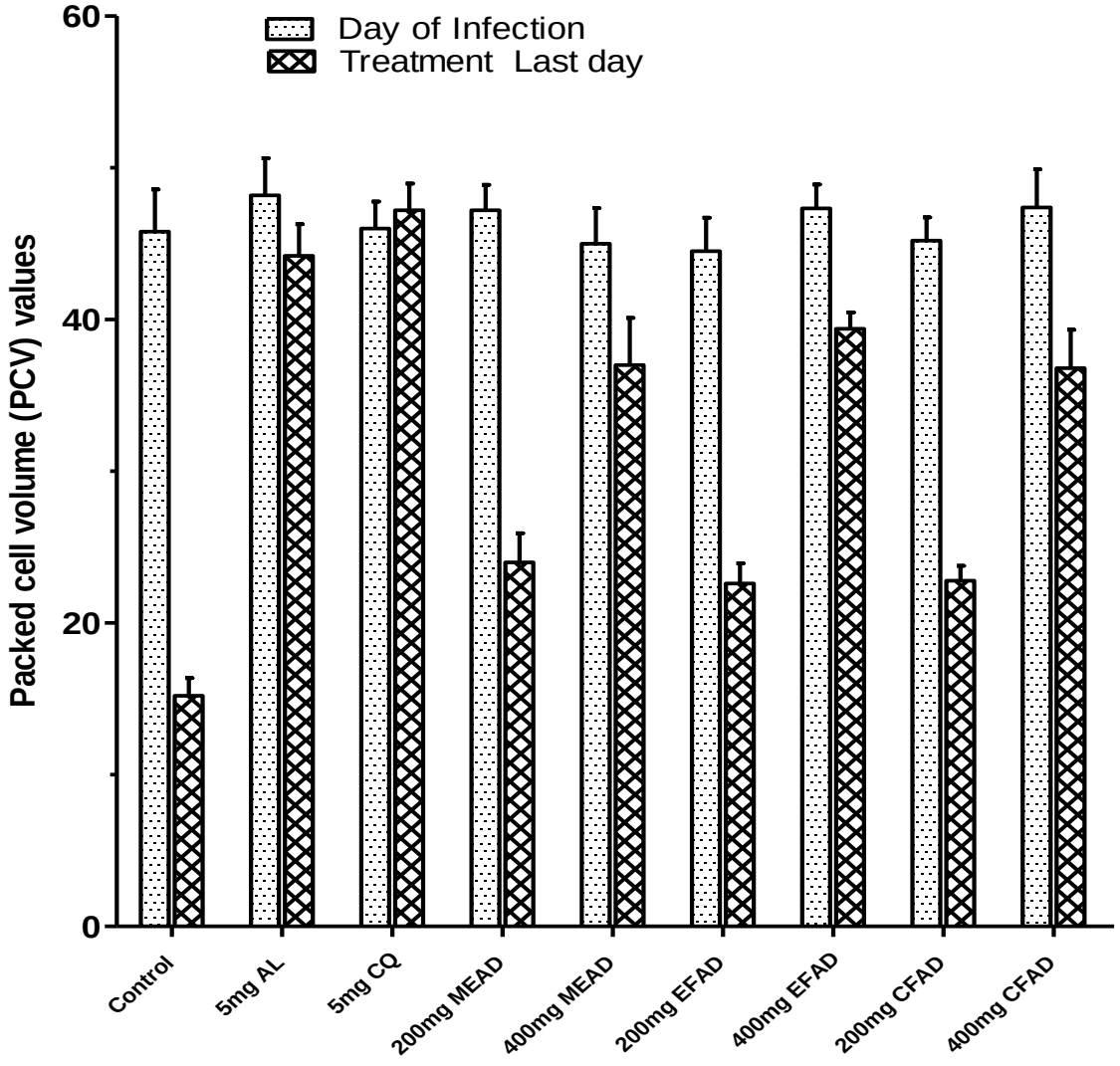


Figure 23: Packed Cell Volume (PCV in percentage) of the prophylactic treated groups

Each value is a mean of several determinations ± SE after five days of exposure to treatment

4.6 Haematological Parameters

4.6.1 Haemoglobin concentration

Figure 24 shows the pattern of haemoglobin concentration of animals administered with *Adansonia digitata stem bark* extract. Administration of the extract increased the haemoglobin concentration significantly ($P<0.05$) when compared to the control. The standard drug chloroquine had the highest heamoglobin concentration. Administration of *Adansonia digitata* stem bark extract reduced the total white blood cell count (Fig. 25) and platelet count (Fig. 26).

4.6.2 Red blood cell differentials

Percent neutrophil, lymphocyte and monocyte are shown in Fig. 27 while the effect of administration of *A. digitata* on mean corpuscular heamoglobin (MCH), mean corpuscular heamoglobin concentration (MCHC) and mean cell volume (MCV) are presented in Fig. 28.

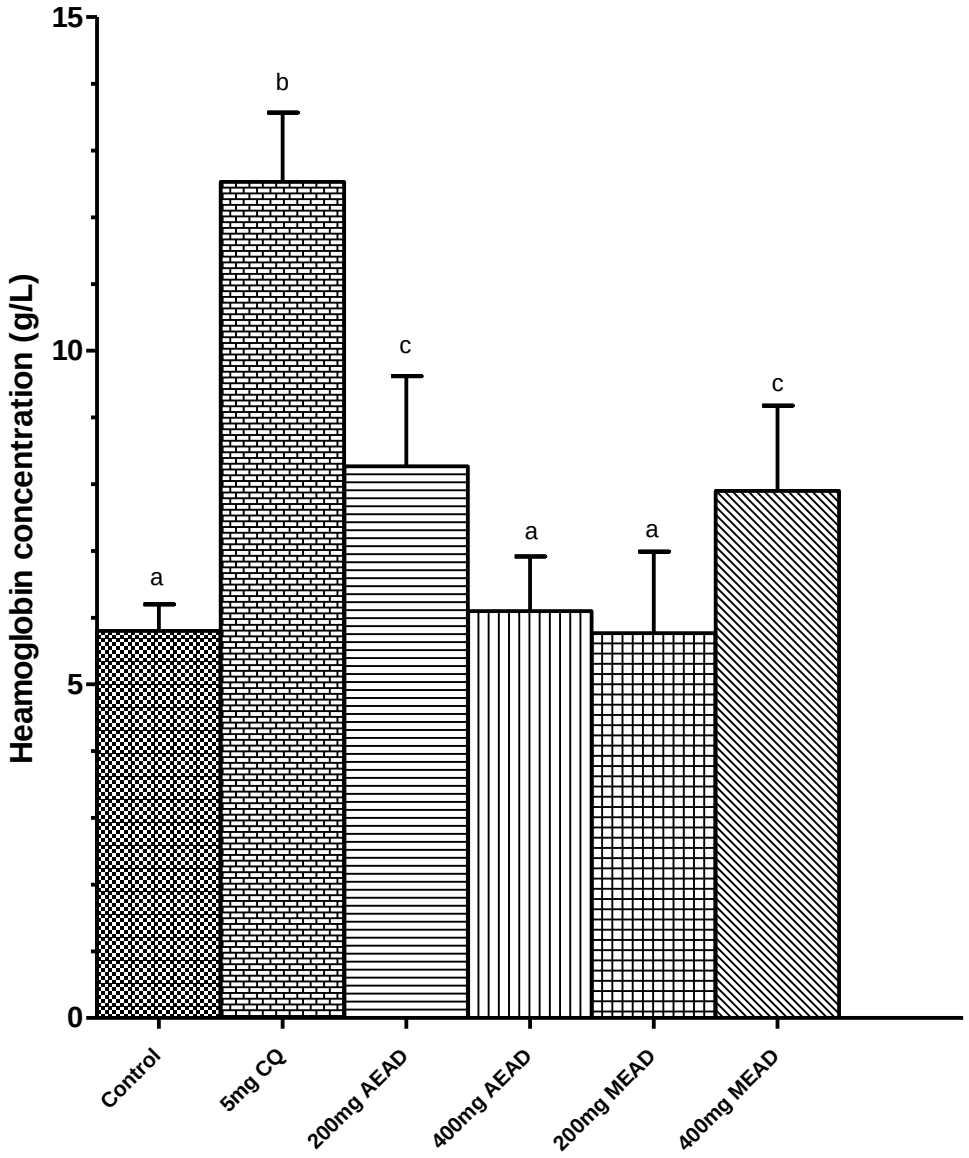


Figure 24: Haemoglobin concentration after oral administration of *Adansonia digitata stem bark* extract

Each value is a mean of several determinations ± SE after five days of exposure to treatment

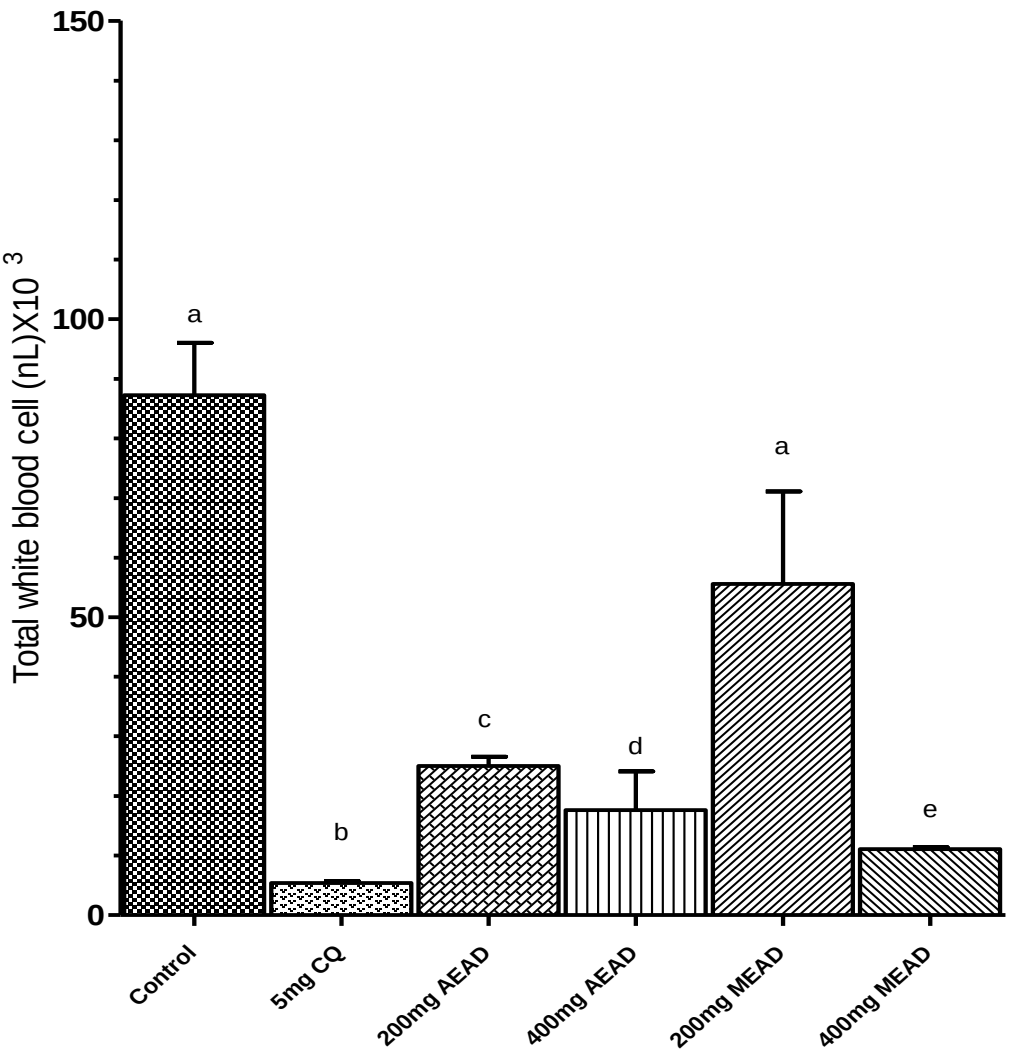


Figure 25: Total white blood cell count after oral administration of *Adansonia digitata stem bark* extract

Each value is a mean of several determinations ± SE after five days of exposure to treatment

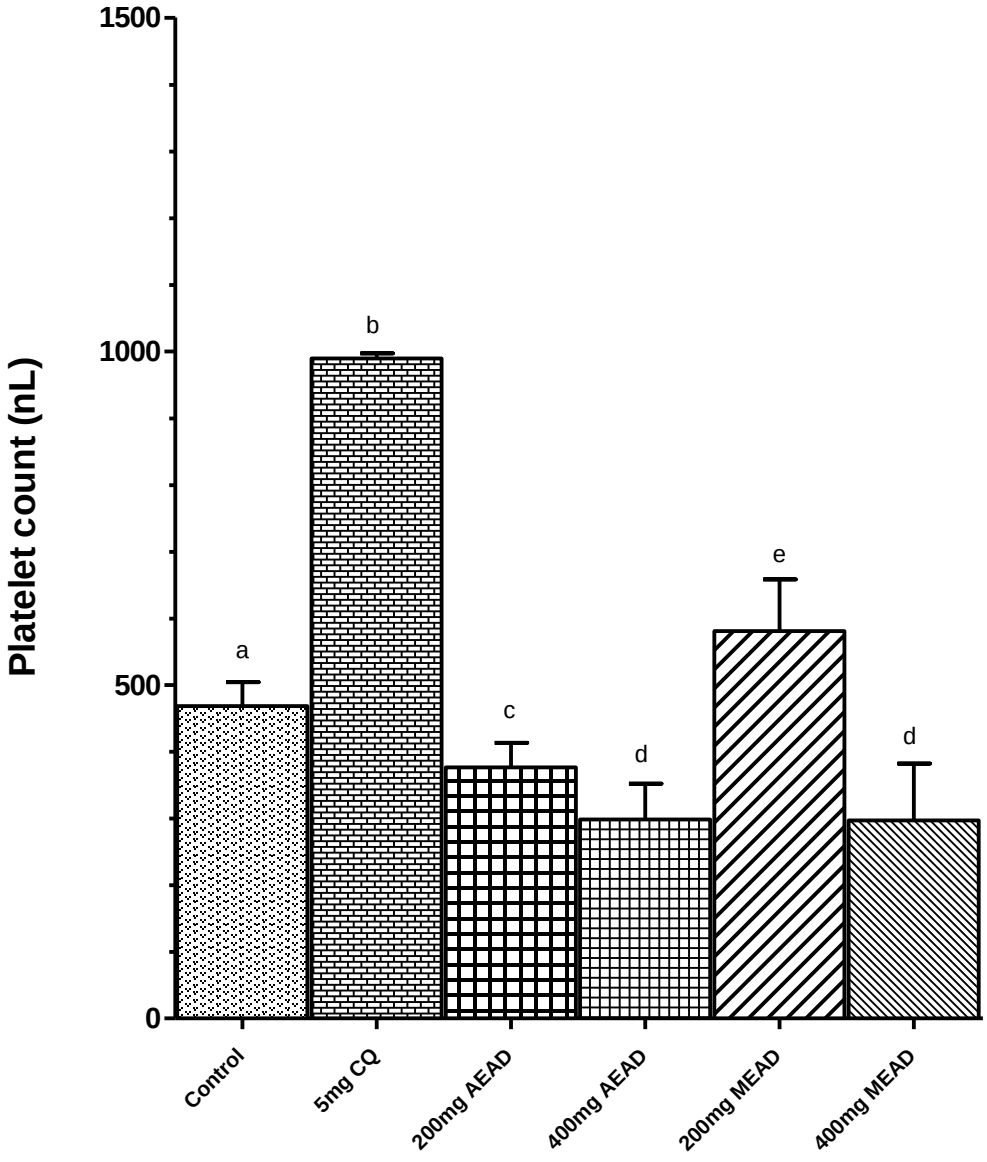


Figure 26: Platelet count after oral administration of *Adansonia digitata stem bark* extract

Each value is a mean of several determinations ± SE after five days of exposure to treatment

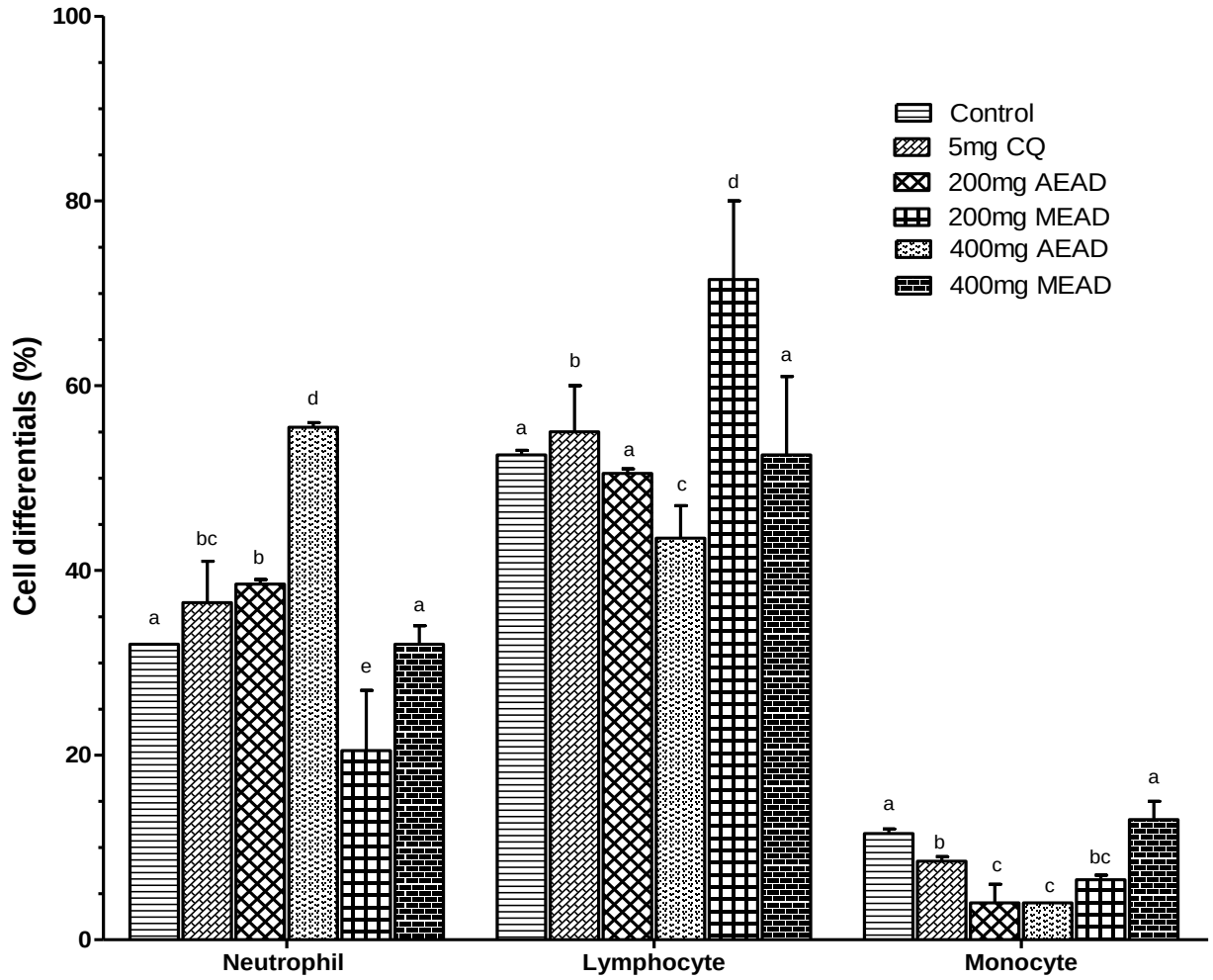


Figure 27: Percent Neutrophil, Lymphocyte and Monocyte after oral administration of *Adansonia digitata stem bark* extract

Each value is a mean of several determinations ± SE after five days of exposure to treatment

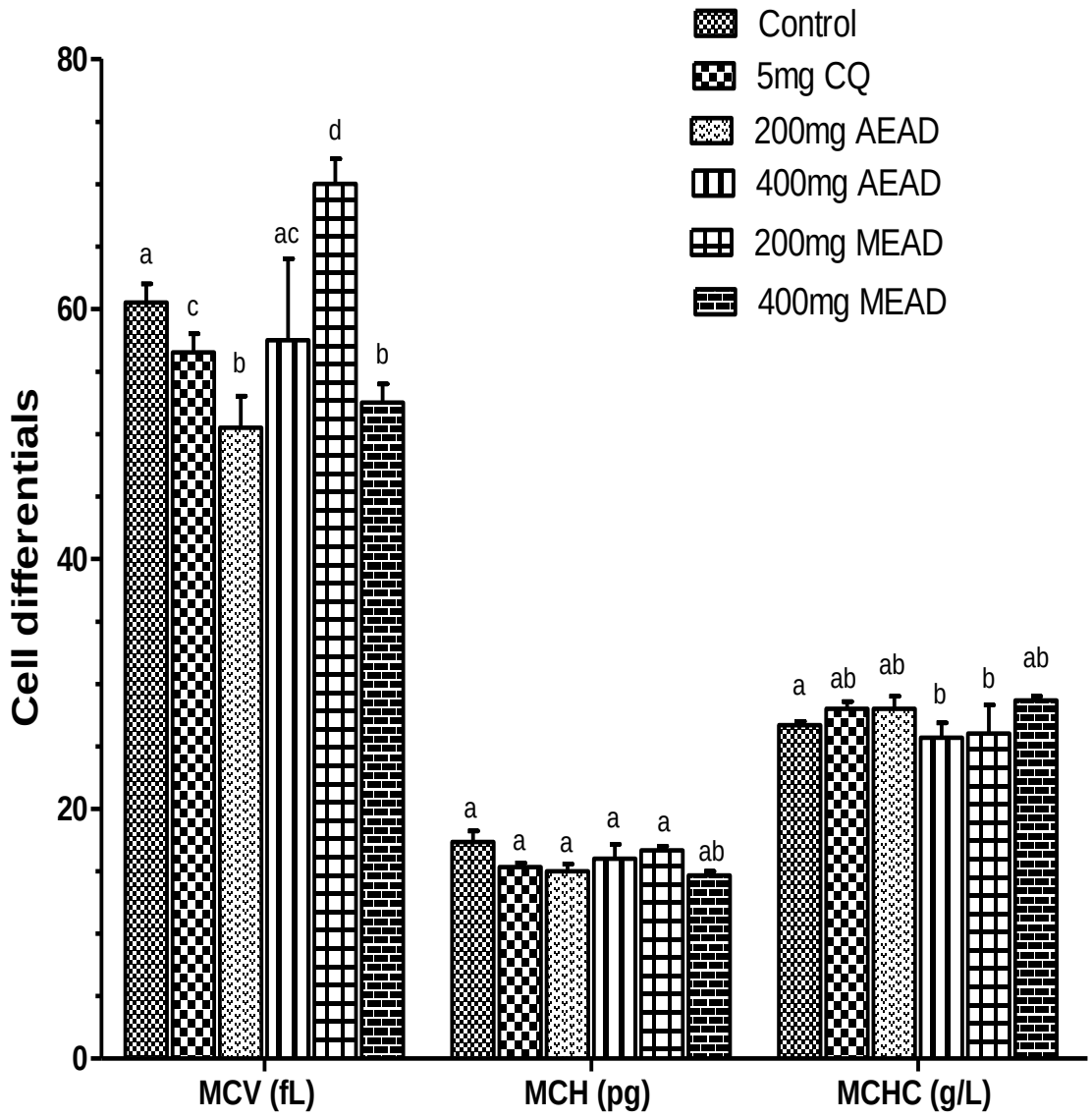


Figure 28: Red blood differentials after oral administration of *Adansonia digitata stem bark* extract

Each value is a mean of several determinations ± SE after five days of exposure to treatment

4.7 Effect of oral administration of *A. digitata* extract on markers of inflammation in *P. berghei* infected mice.

4.7.1 C-Reactive Protein (CRP)

The serum C-reactive protein (CRP) concentrations of mice infected with *P. berghei* and the group treated with *A. digitata* stem bark extract is presented in Fig. 29. The CRP concentration ofthe control increased significantly ($P<0.05$ when compared with the group treated with the extract and chloroquine and the baseline.

4.7.2 Tumor Necrotic Factor-α (TNF-α)

Figure 30 depicts the concentration of tumor necrotic factor-alpha (TNF-α) of mice infected with *P. berghei* andthe group treated with *A. digitata* stem bark extract. The serum concentrations of TNF-α was significantly ($P<0.05$) lowered inthe extract and chloroquine treated groups when compared to the control.

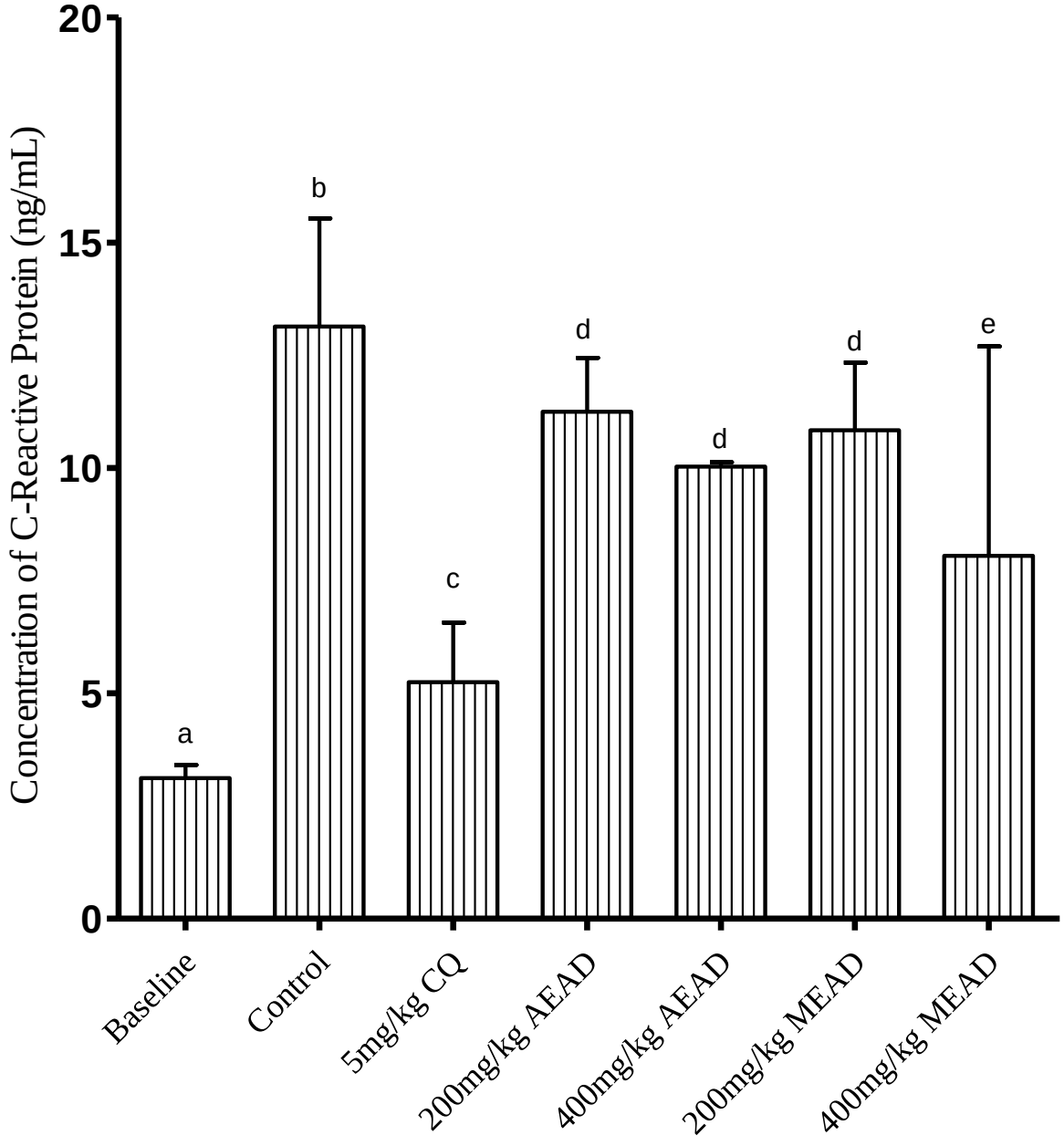


Figure 29: Serum concentration of C-reactive protein in *P. berghei* infected and treated mice. Results are mean ± SEM.

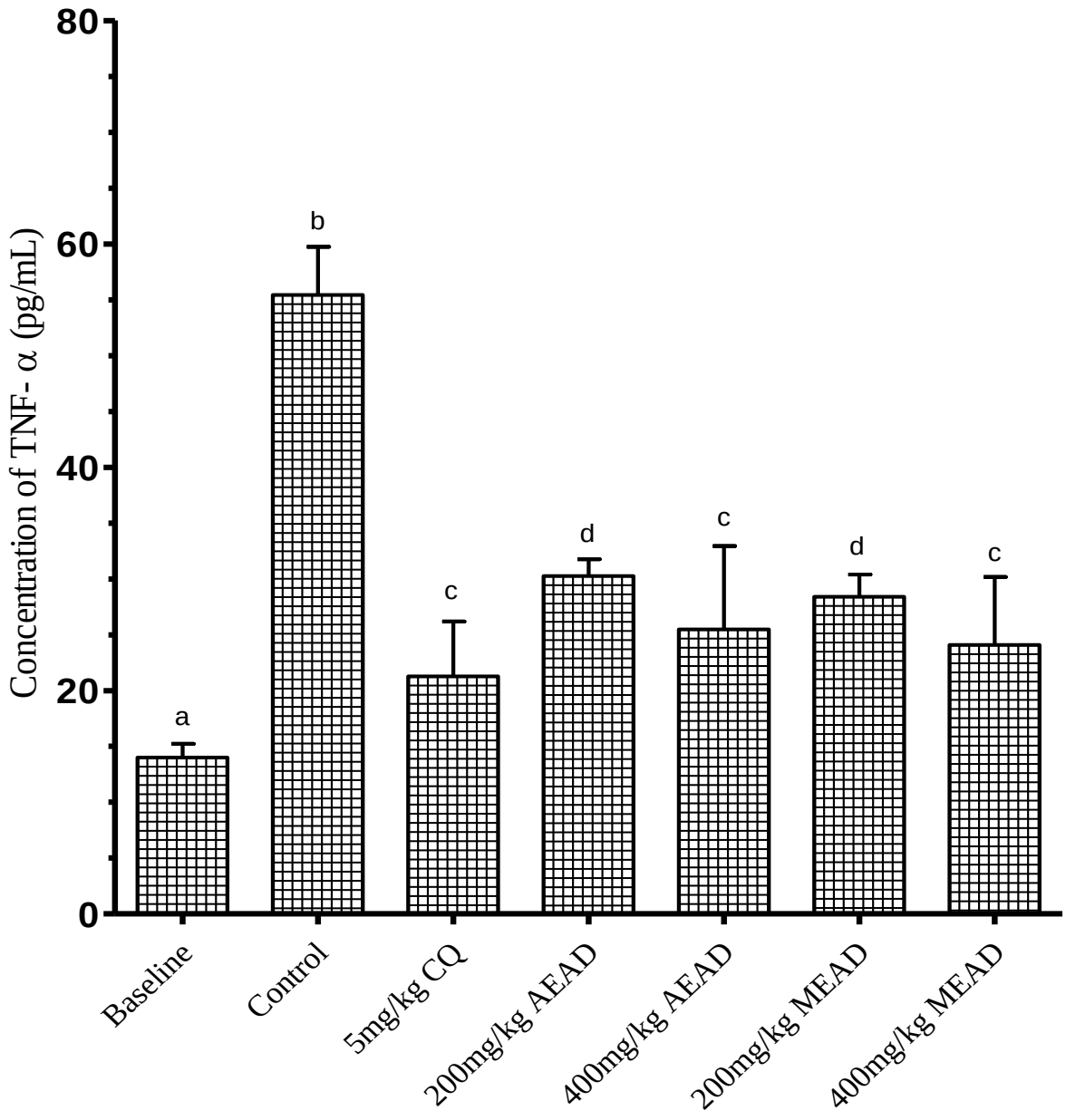


Figure 30: Serum concentration of tumor necrotic factor-alpha (TNF-α) in *P. berghei* infected and treated mice.

Results are mean ± SEM.

4.8 Effect of oral administration of *Adansonia digitata* extract on Hepatic Markers Enzymes.

4.8.1 Alkaline Phosphatase (ALP)

The alkaline phosphatase activity in the serum and tissues of animals administered with *Adansonia digitata* stem bark extract is presented in fig. 31. The ALP activity of the control is significantly ($p<0.05$) higher in both the serum and tissues when compared with the animals administered with the extract and chloroquine.

4.8.2 Alanine Aminotransferase (ALT)

The alanine aminotransferase activity in the serum and tissues of animals administered with *Adansonia digitata* stem bark extract is presented in fig. 32. The ALT activity of the control is significantly ($p<0.05$) lower in both the serum and tissues when compared with the animals administered with the extract. The chloroquine treated group had the highest ALT activity.

4.8.3 Alanine Aminotransferase (AST)

The aspartate aminotransferase activity in the serum and tissues of animals administered with *Adansonia digitata* stem bark extract is presented in fig. 33. The AST activity of the control is significantly ($p<0.05$) lower in the serum and tissues when compared with the animals administered with the extract. The chloroquine treated group also had the highest AST activity.

4.8.4 Total protein

The result of total protein in the tissues of the control and animals administered with the extract of Adansonia digitata stem bark is presented in fig. 34. The total protein content in all the tissues increased significantly ($p<0.05$) when compared with the control.

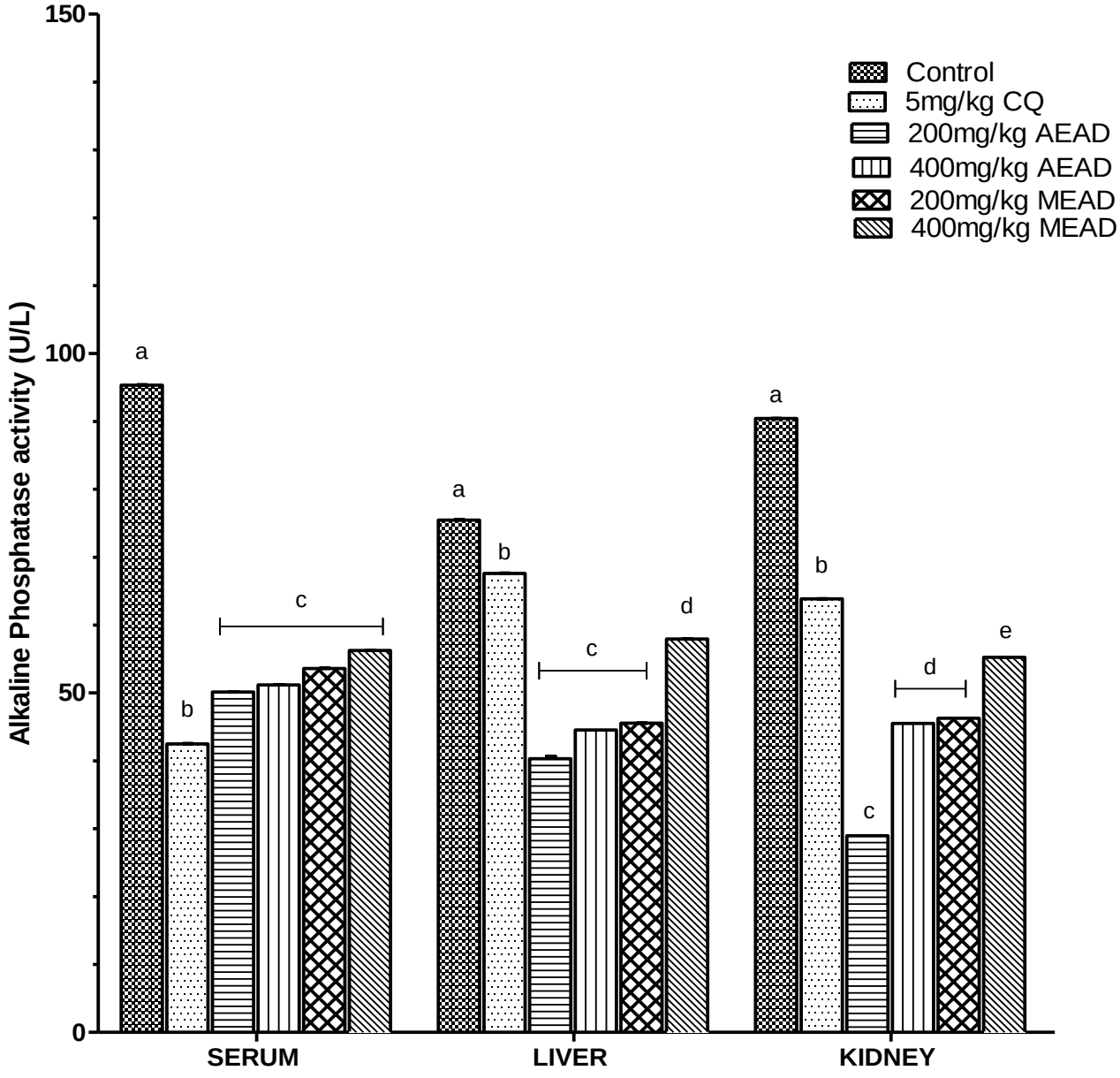


Figure 31: Alkaline Phosphatase activity after treatment with extracts of *Adansonia digitata stem bark*

Results are mean ± SEM.

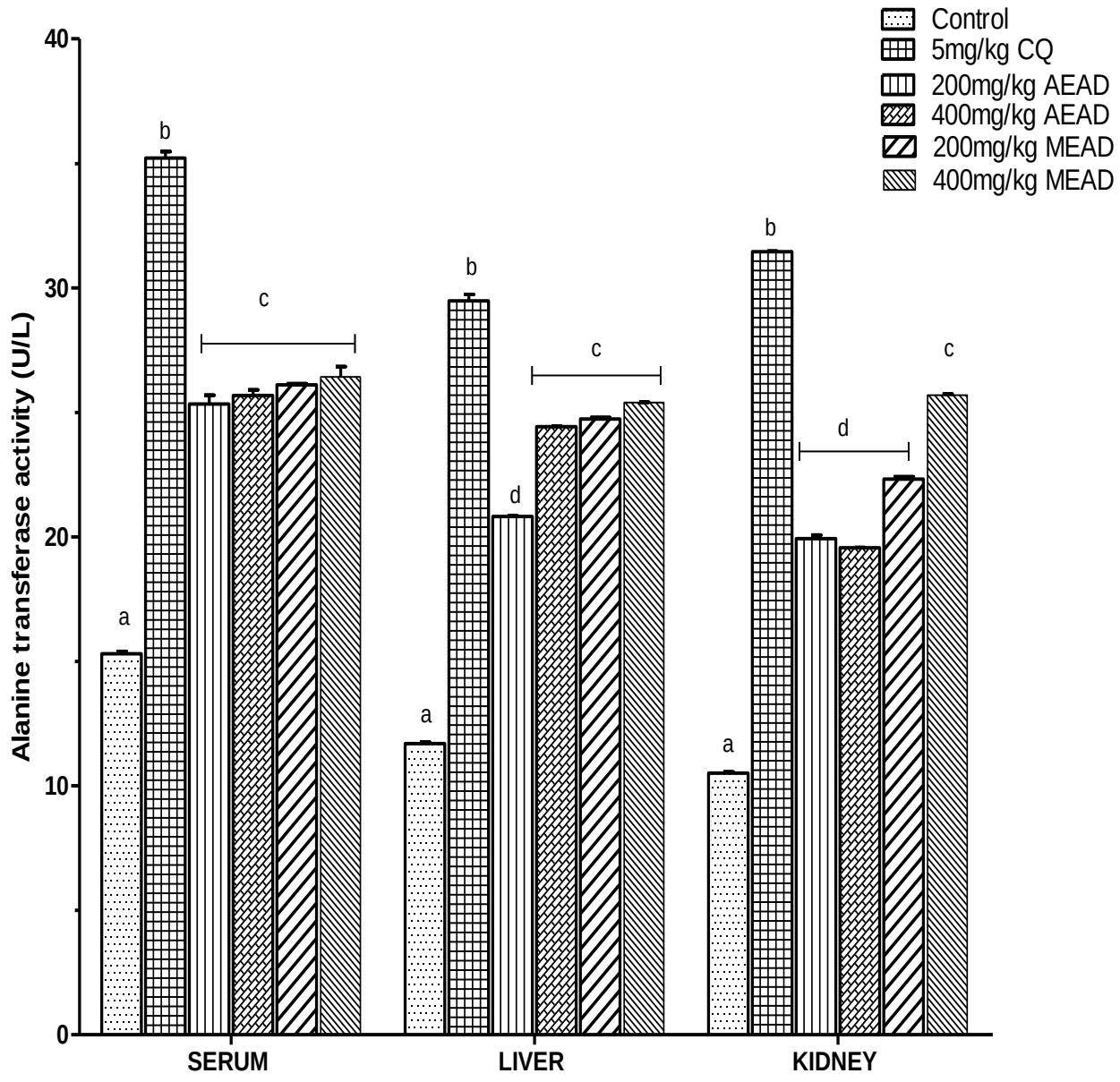


Figure 32: Alanine aminotransferase activity after treatment with extracts of *Adansonia digitata stem bark*

Results are mean ± SEM.

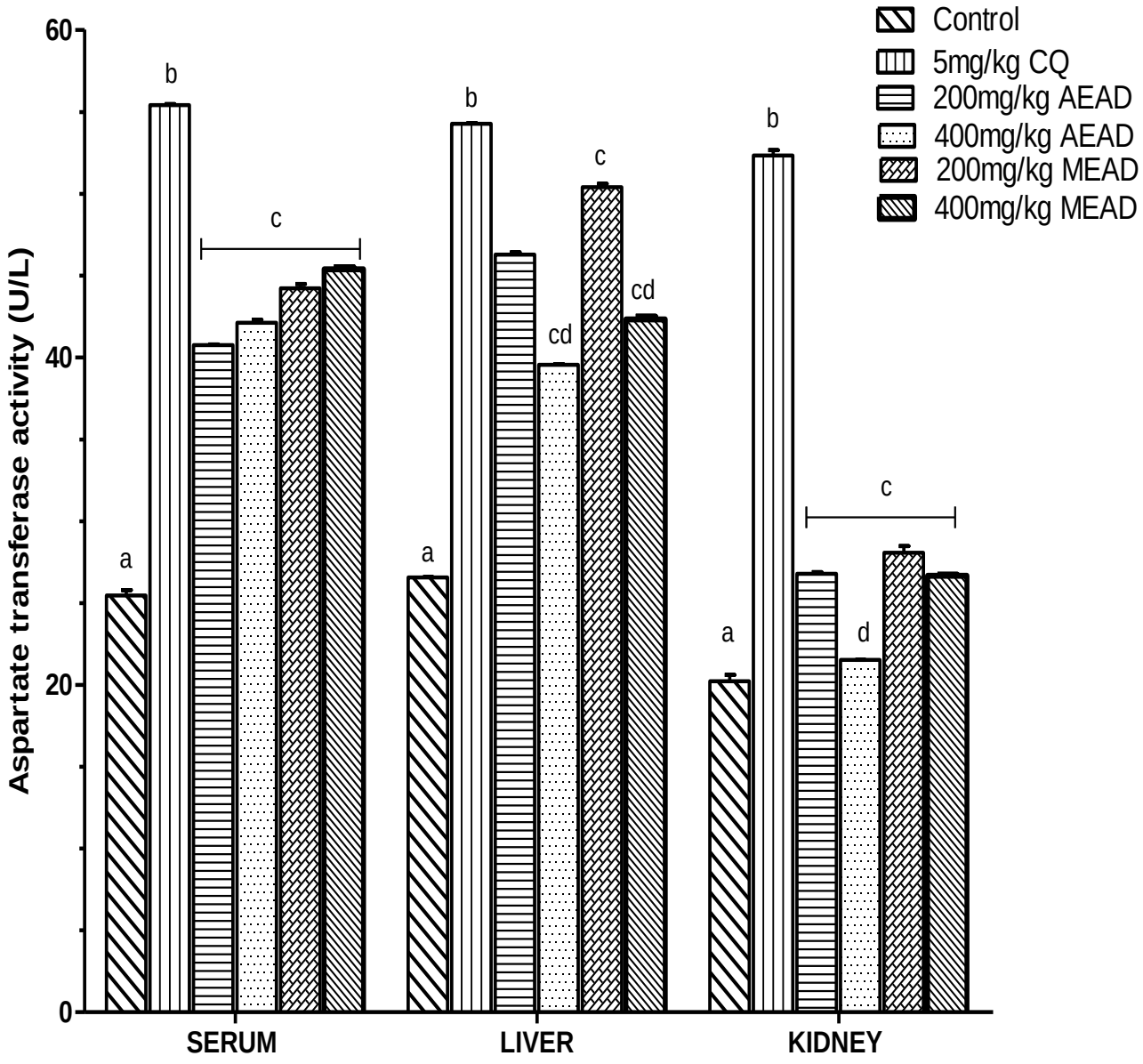


Figure 33: Aspartate aminotransferase activity after treatment with extracts of *Adansonia digitata stem bark*

Results are mean ± SEM.

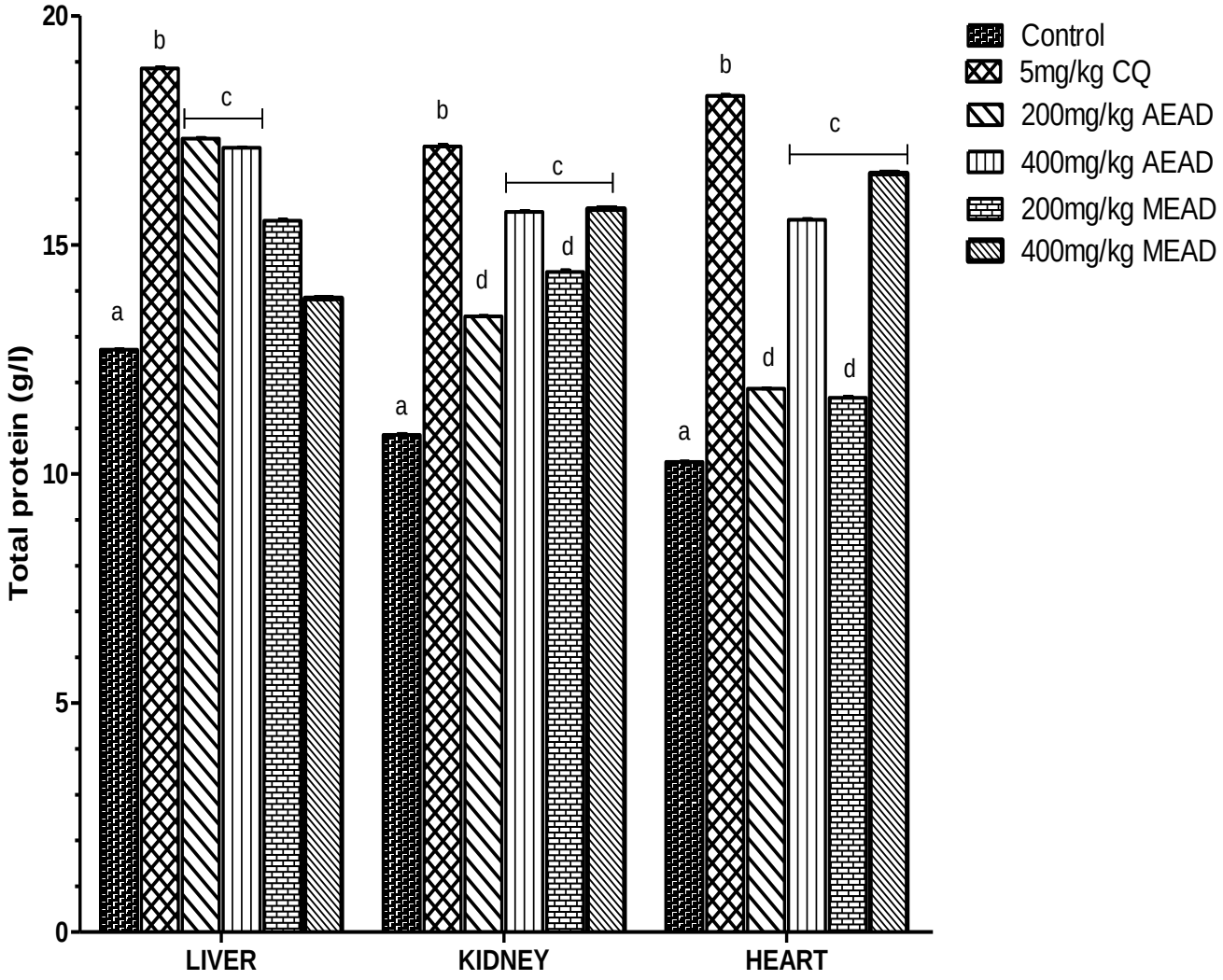


Figure 34: Total protein after treatment with extracts of *Adansonia digitata stem bark*

Results are mean ± SEM.

4.9 Effect of oral administration of *Adansonia digitata* extract on Renal Markers

The serum creatinine, urea and uric acid concentration following treatment with extract of *Adansonia digitata* is presented in Fig. 35 to Fig. 37. The creatinine concentration of the untreated control is significantly ($P<0.05$) higher than the groups treated with the extract while the urea and uric acid concentration of the control was lowered significantly ($P<0.05$) when compared with the extract treated groups.

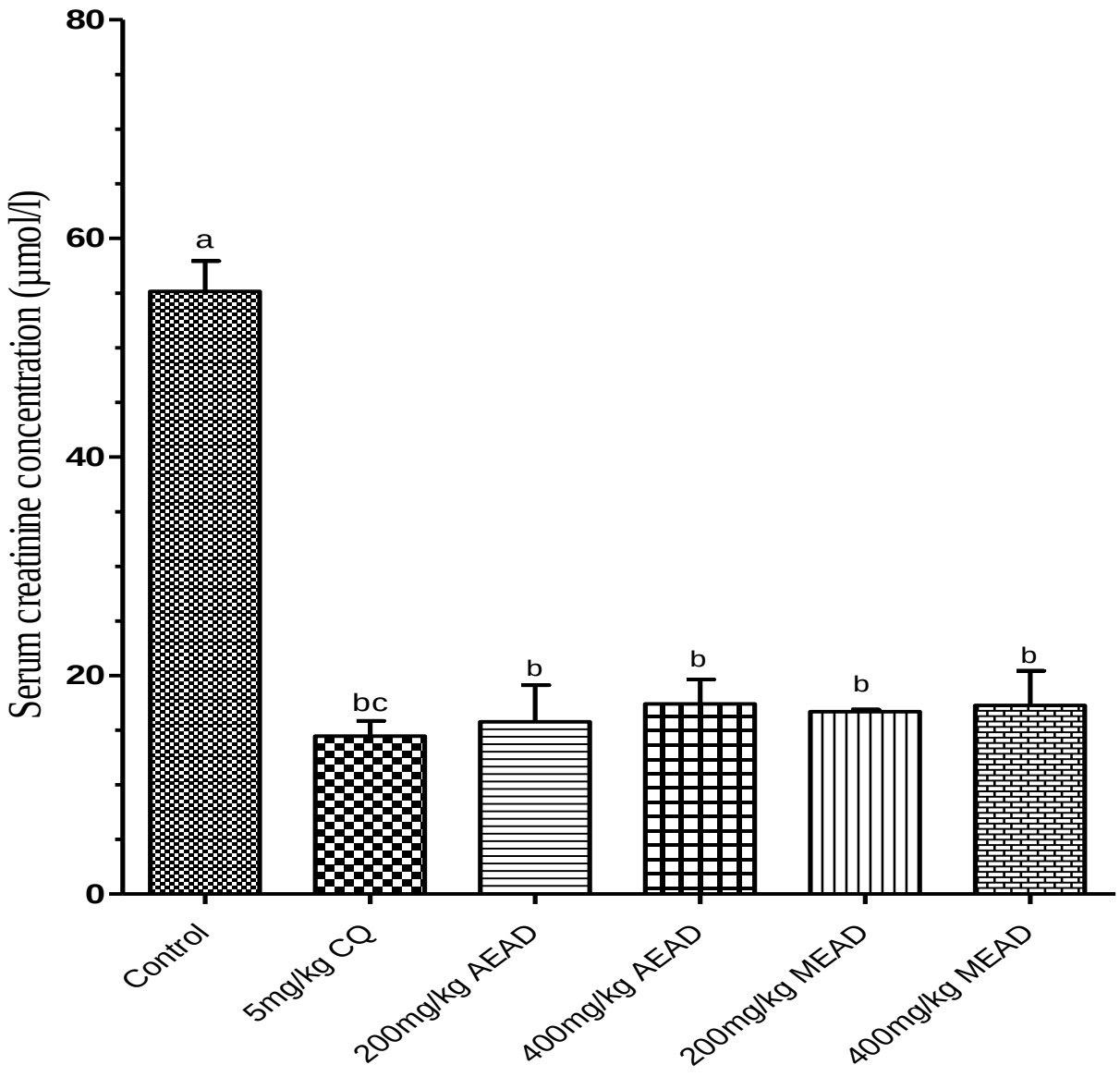


Figure 35: Creatinine concentration after treatment with extracts of *Adansonia digitata stem bark*

Results are mean ± SEM.

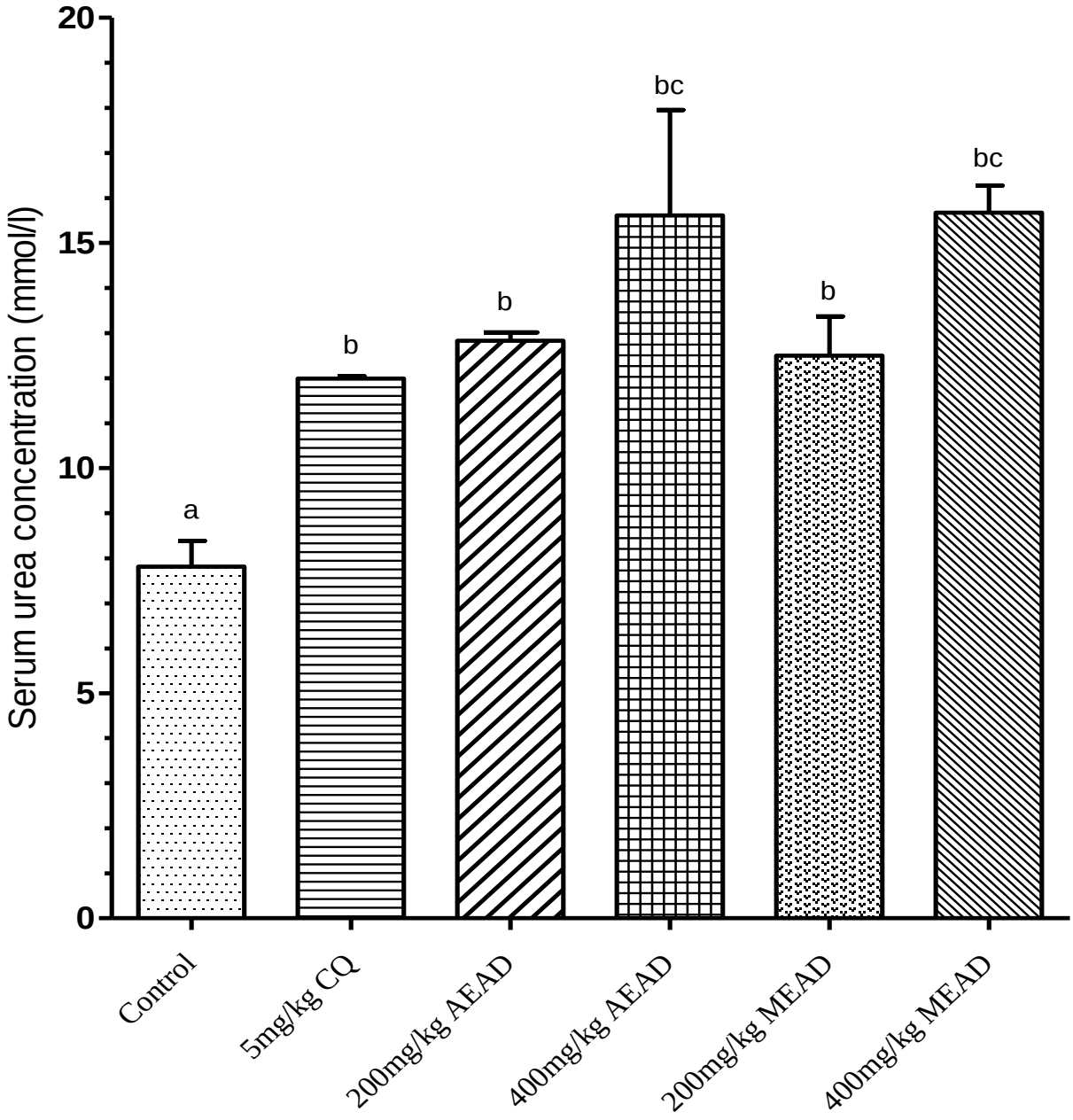


Figure 36: Urea concentration after treatment with extracts of *Adansonia digitata stem bark*

Results are mean ± SEM.

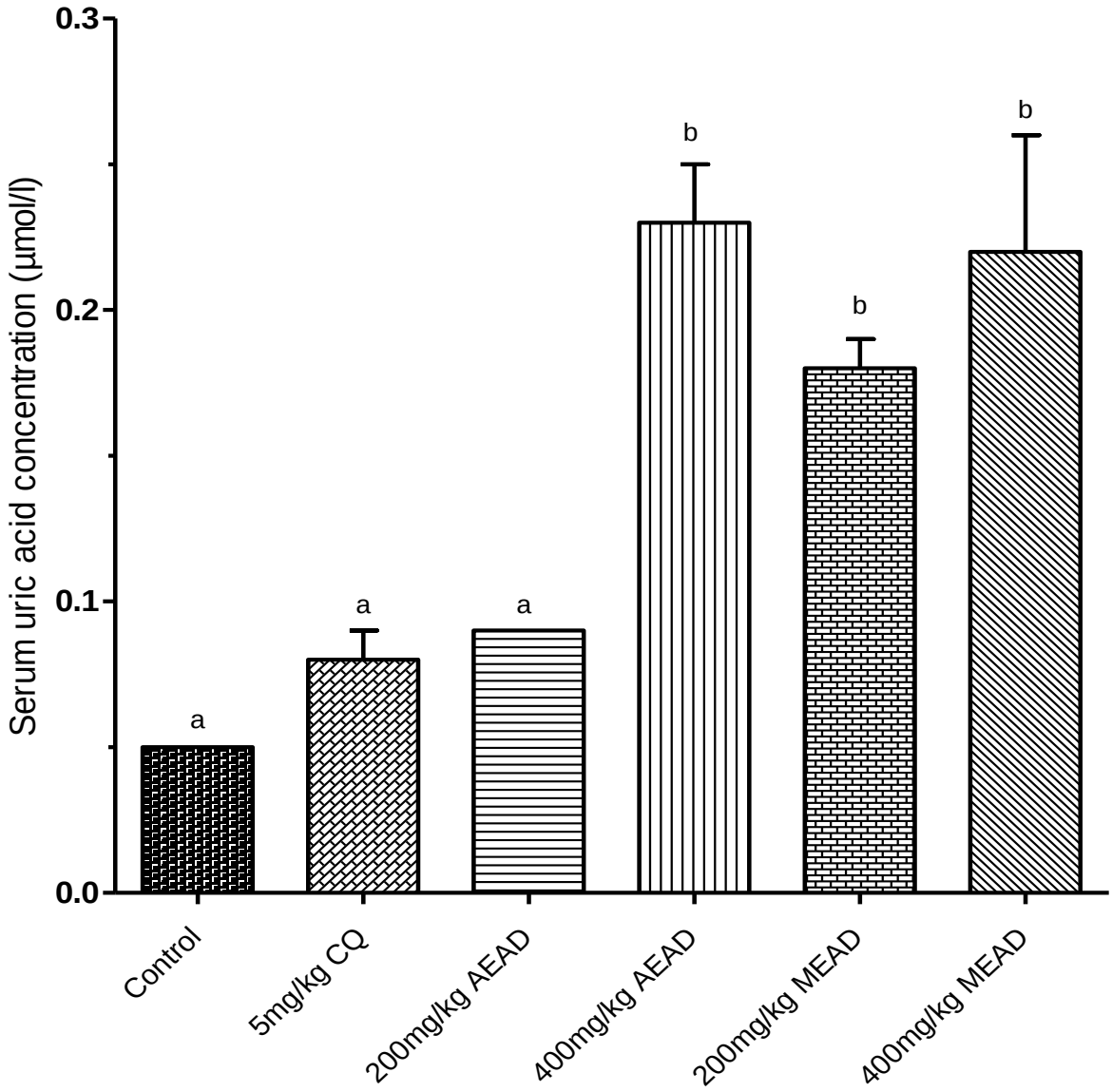


Figure 37: Uric acid concentration after treatment with extracts of *Adansonia digitata stem bark*

Results are mean ± SEM.

4.10 Effect of oral administration of *Adansonia digitata* extract on Serum Electrolyte

Figure 38 to Fig. 42 depicts the results of oral administration of *Adansonia digitata* on serum electrolytes (Na^+, k^+, ca^{2+}, Mg^{2+} and Cl^-). The Na^+, ca^{2+} and Cl^- concentration of the control significantly ($p < 0.05$) increased when compared with the animals administered with the extract and chloroquine. The k^+ concentration of the control though higher was insignificantly different from the extract treated groups. However, Mg^{2+} concentration in the control was significantly ($p < 0.05$) lowered when compared with the extract treated group.

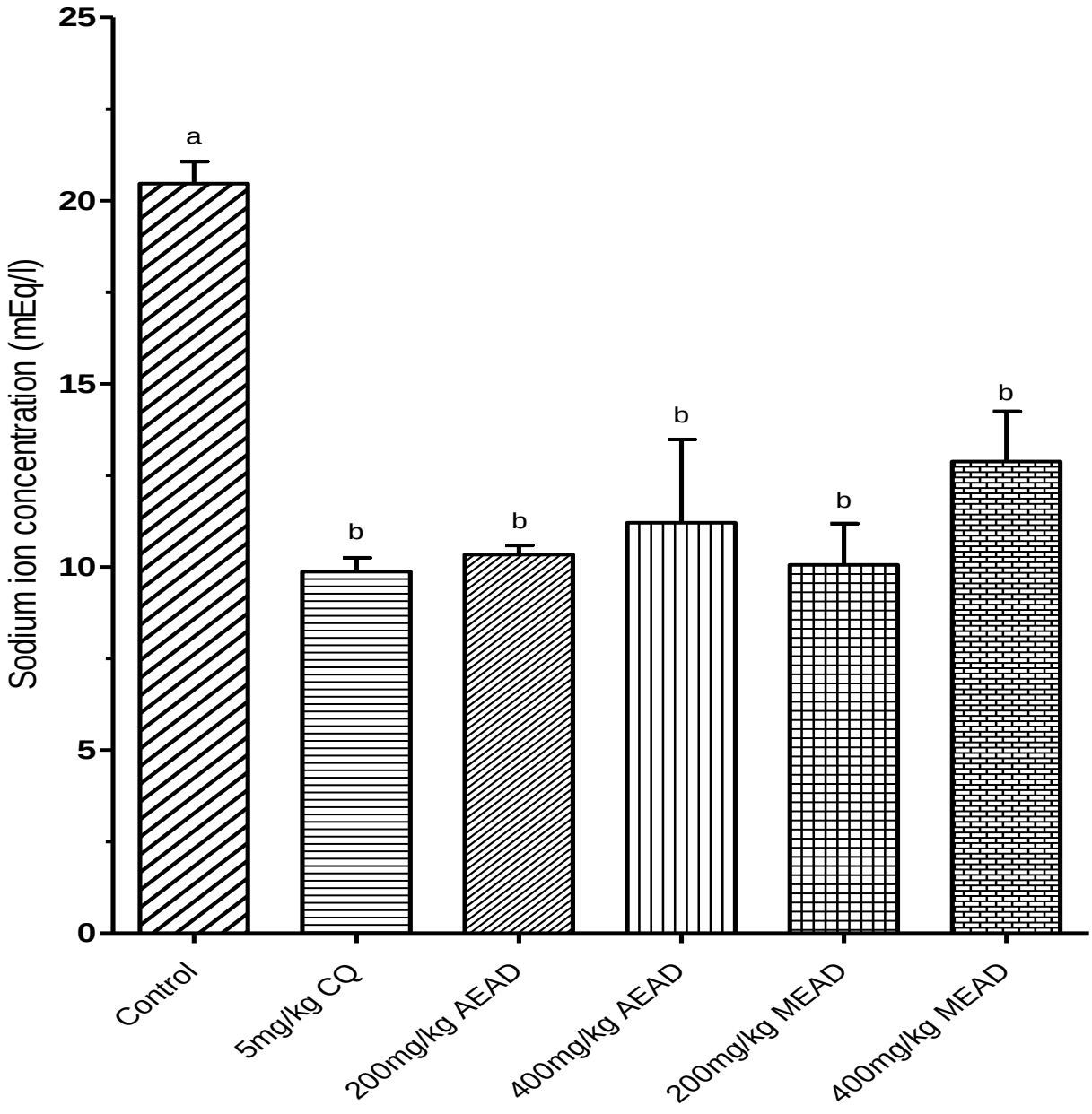


Figure 38: Sodium Ion Concentration of animals administered with extracts of *Adansonia digitata stem bark*

Results are mean ± SEM.

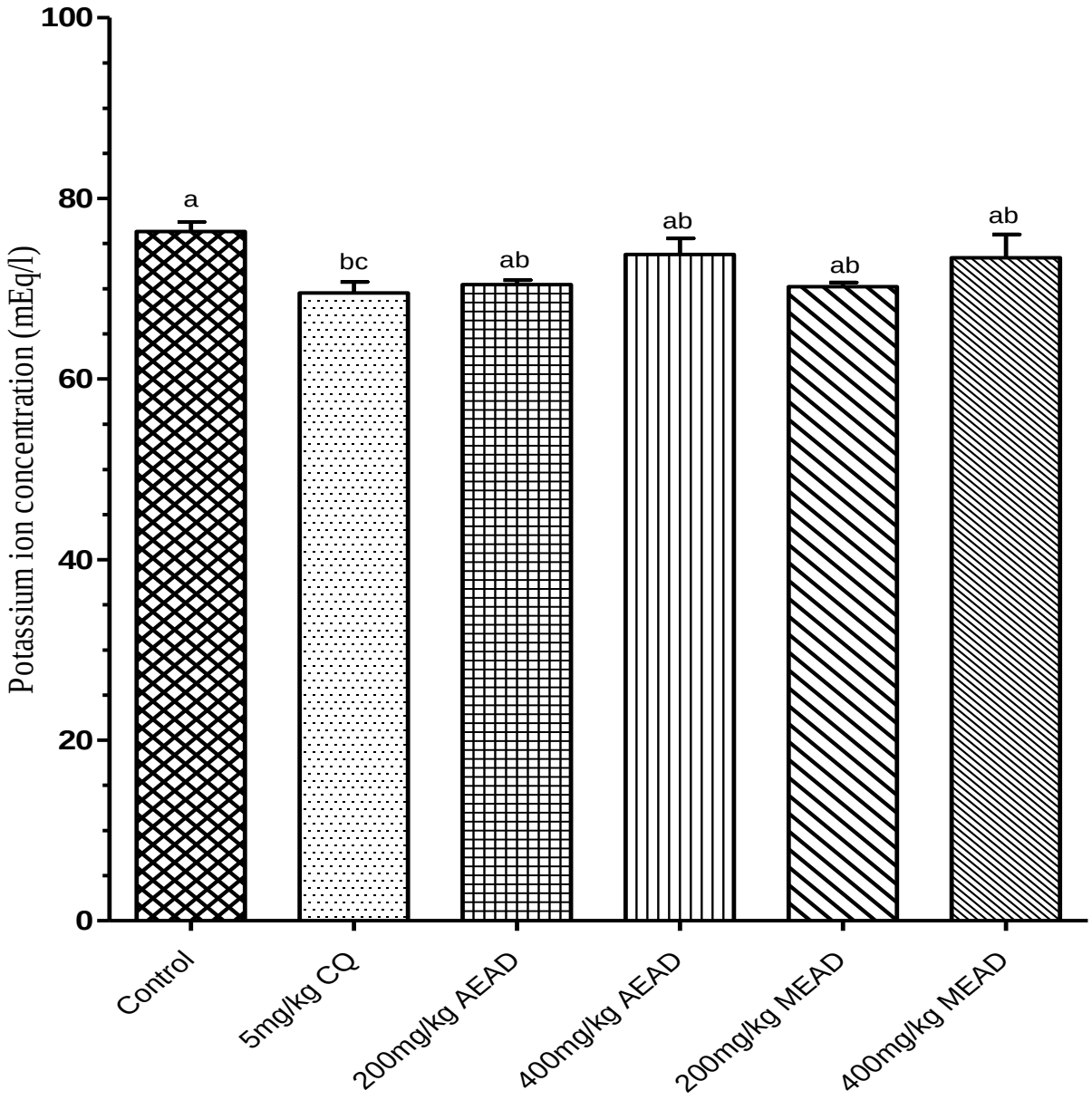


Figure 39: Potassium Ion Concentration of animals administered with extracts of *Adansonia digitata stem bark*

Results are mean ± SEM.

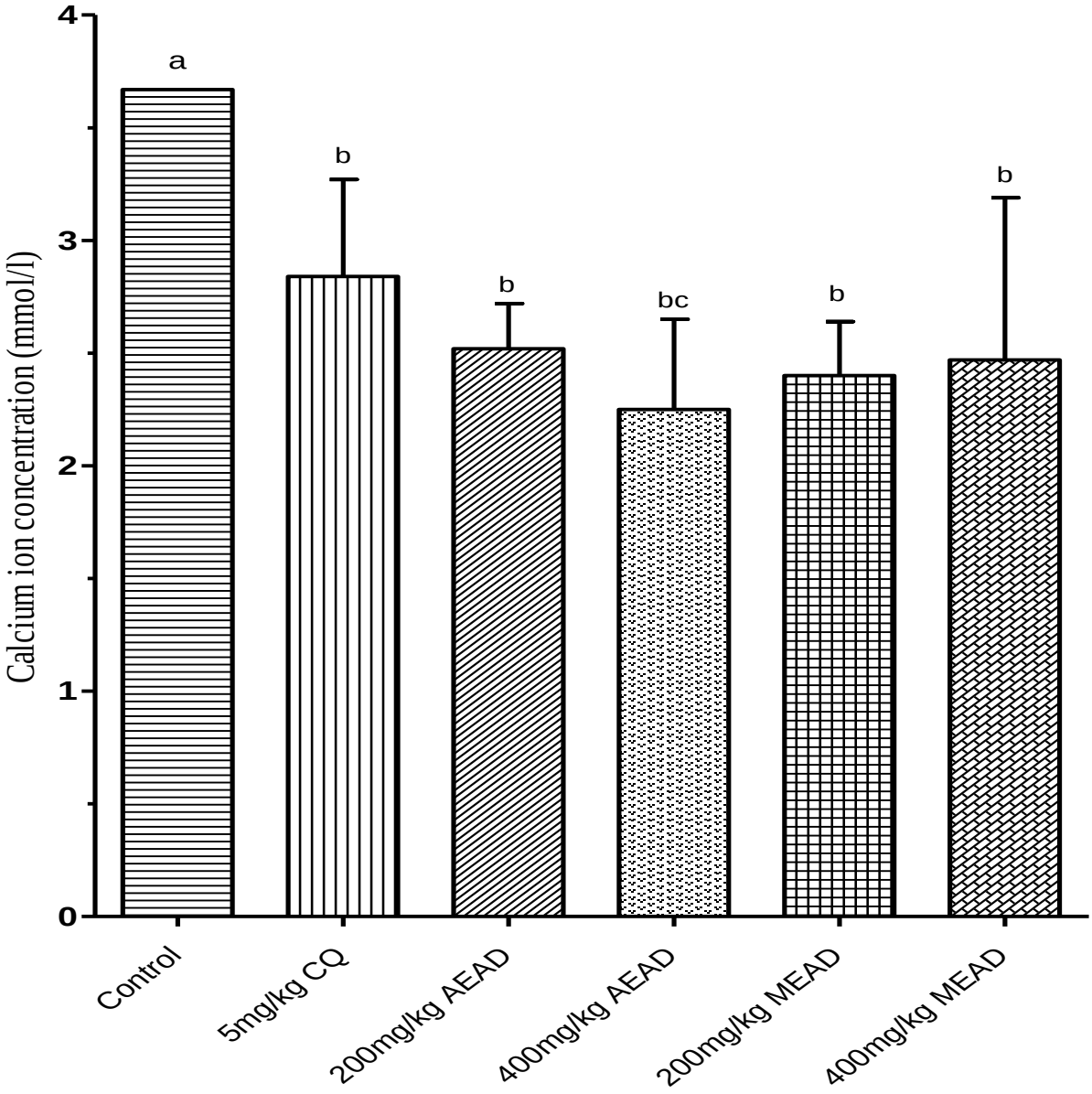


Figure 40: Calcium Ion Concentration of animals administered with extracts of *Adansonia digitata stem bark*

Results are mean ± SEM.

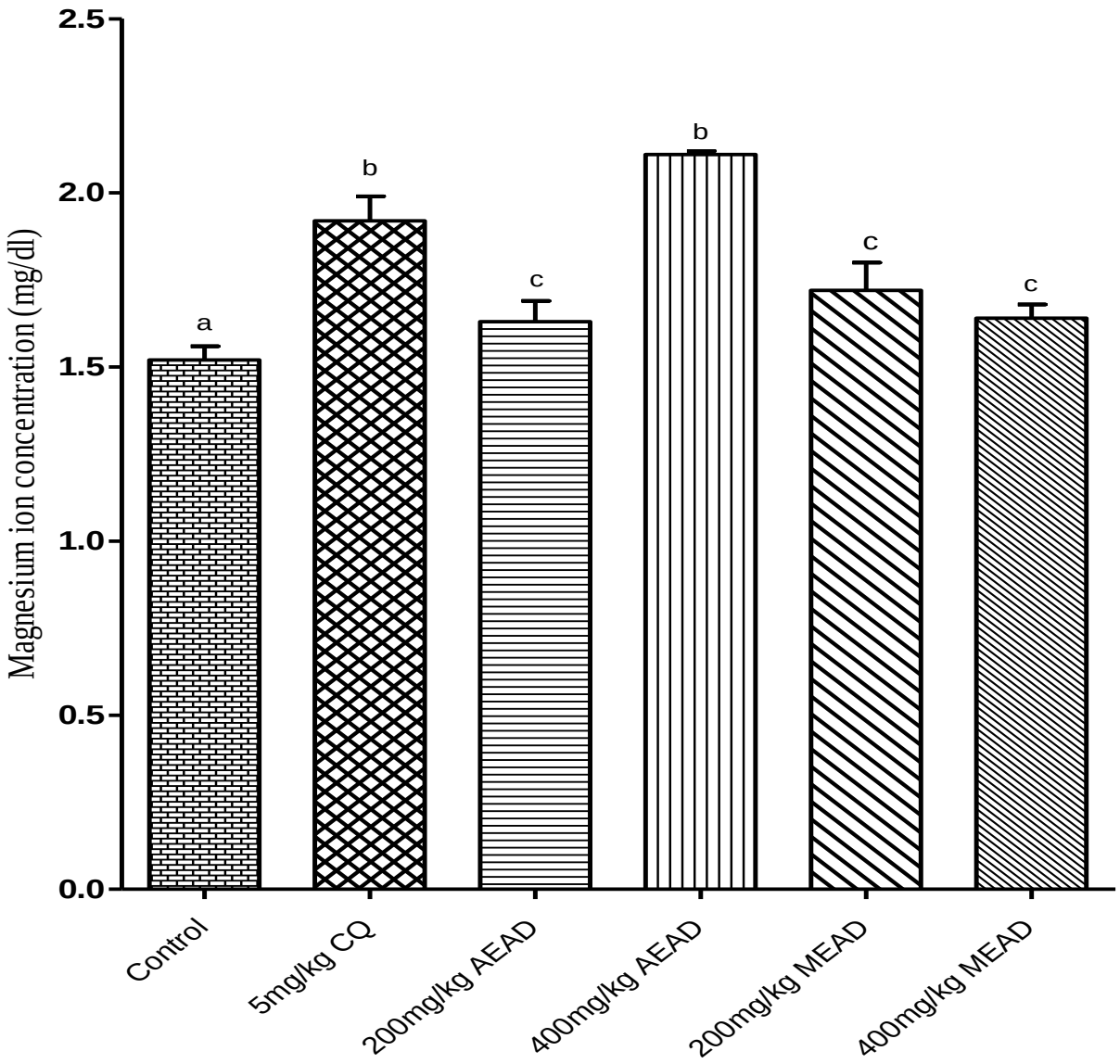


Figure 41: Magnesium Ion Concentration of animals administered with extracts of *Adansonia digitata stem bark*

Results are mean ± SEM.

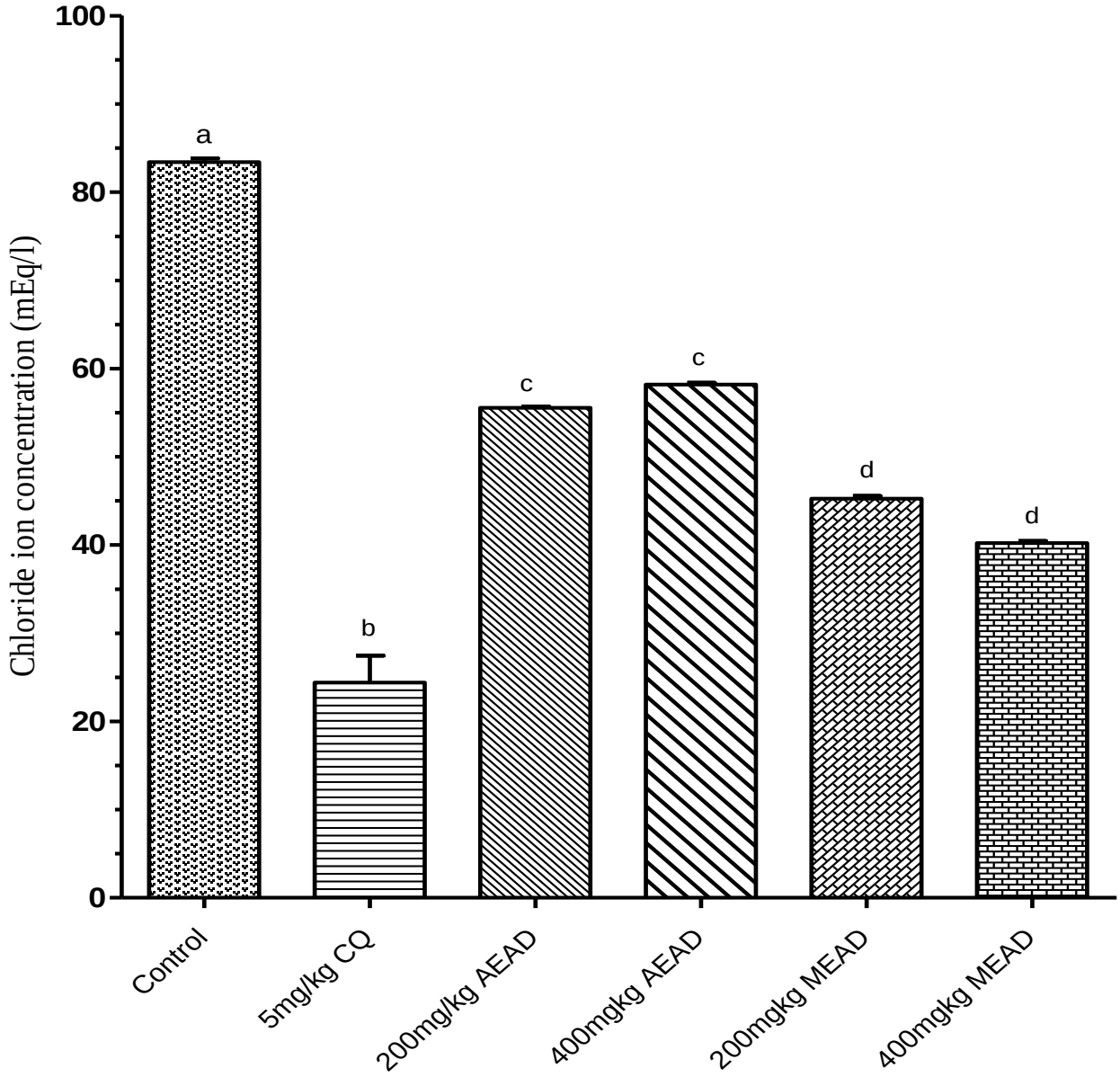


Figure 42: Chloride Ion Concentration of animals administered with extracts of *Adansonia digitata stem bark*

Results are mean ± SEM.

4.11 Effect of oral administration of *Adansonia digitata* extract on endogenous antioxidant system in mice

The malondialdehyde level of mice infected with *P. berghei* andthe groups treated with *Adansonia digitata* stem bark extract is presented in fig. 43. The malondialdehyde in the control increased significantly ($P<0.05$) in all the tissues (liver, kidney and heart) when compared with the group treated with the extract and chloroquine had the least malondialdehyde level.

Figure 44 to 46 depicts thestatus of the antioxidant enzymes after administration of *Adansonia digitata* stem bark extract. All the antioxidant enzyme status was higher significantly in the extract treated groups compared to the control inall the tissues (liver, kidney and heart). Chloroquine treated group had the highest status followed by the group treated with 400 mg/kg body weight methanolic extract.

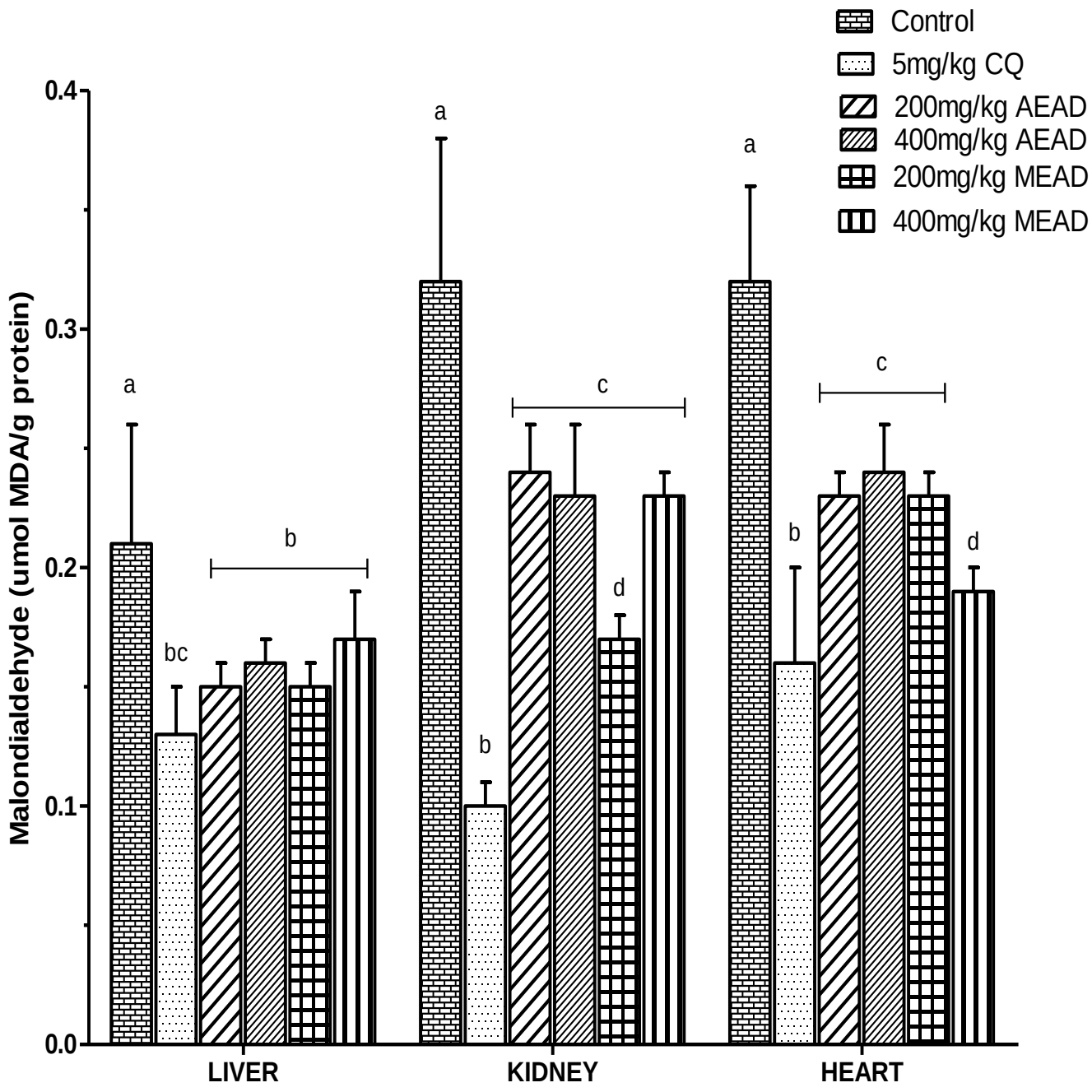


Figure 43: Malondialdehyde level after administration of Adansonia digitata stem bark extract

Results are mean ± SEM.

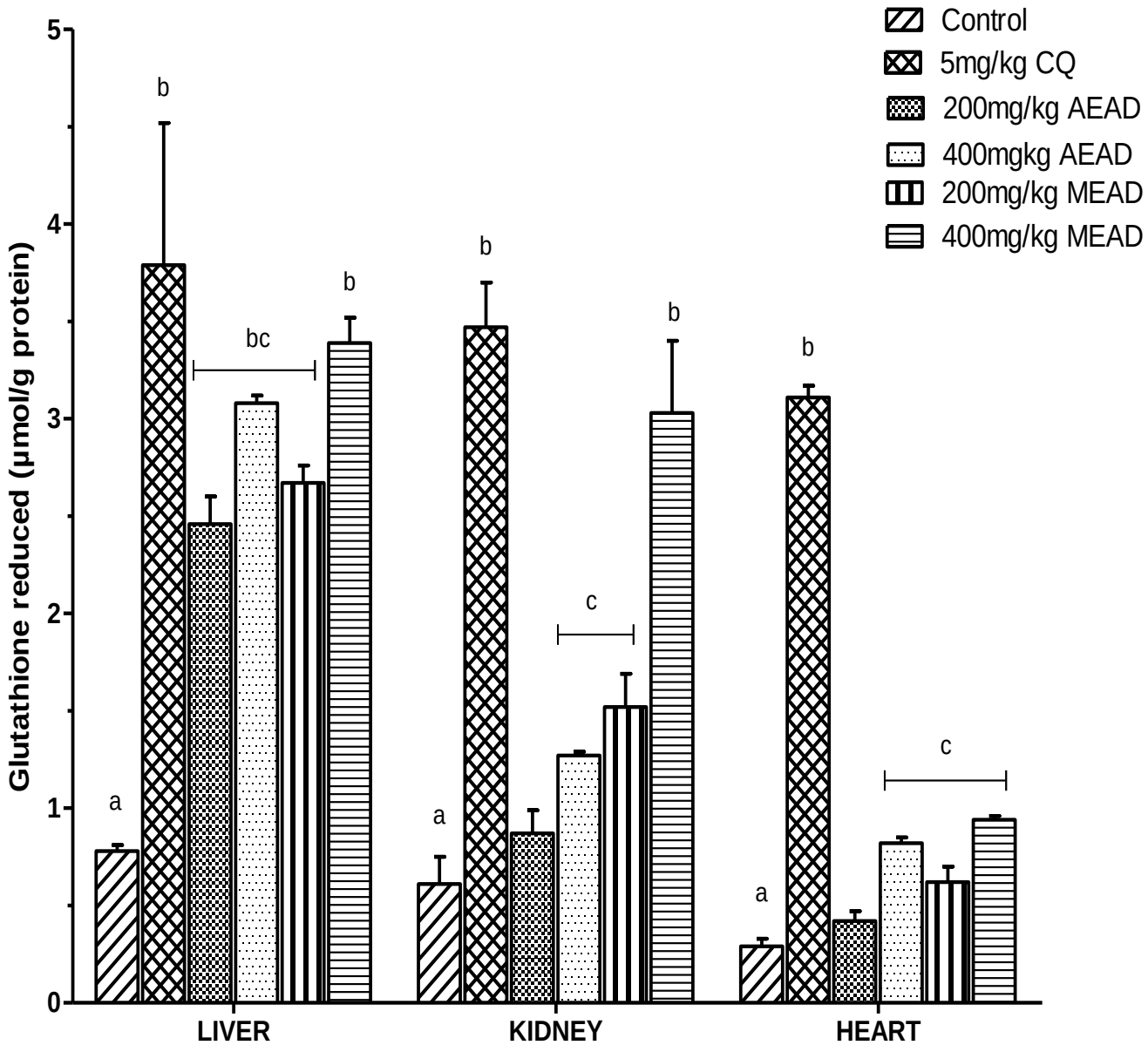


Figure 44: Reduced glutathione after administration of Adansonia digitata stem bark extract

Results are mean ± SEM.

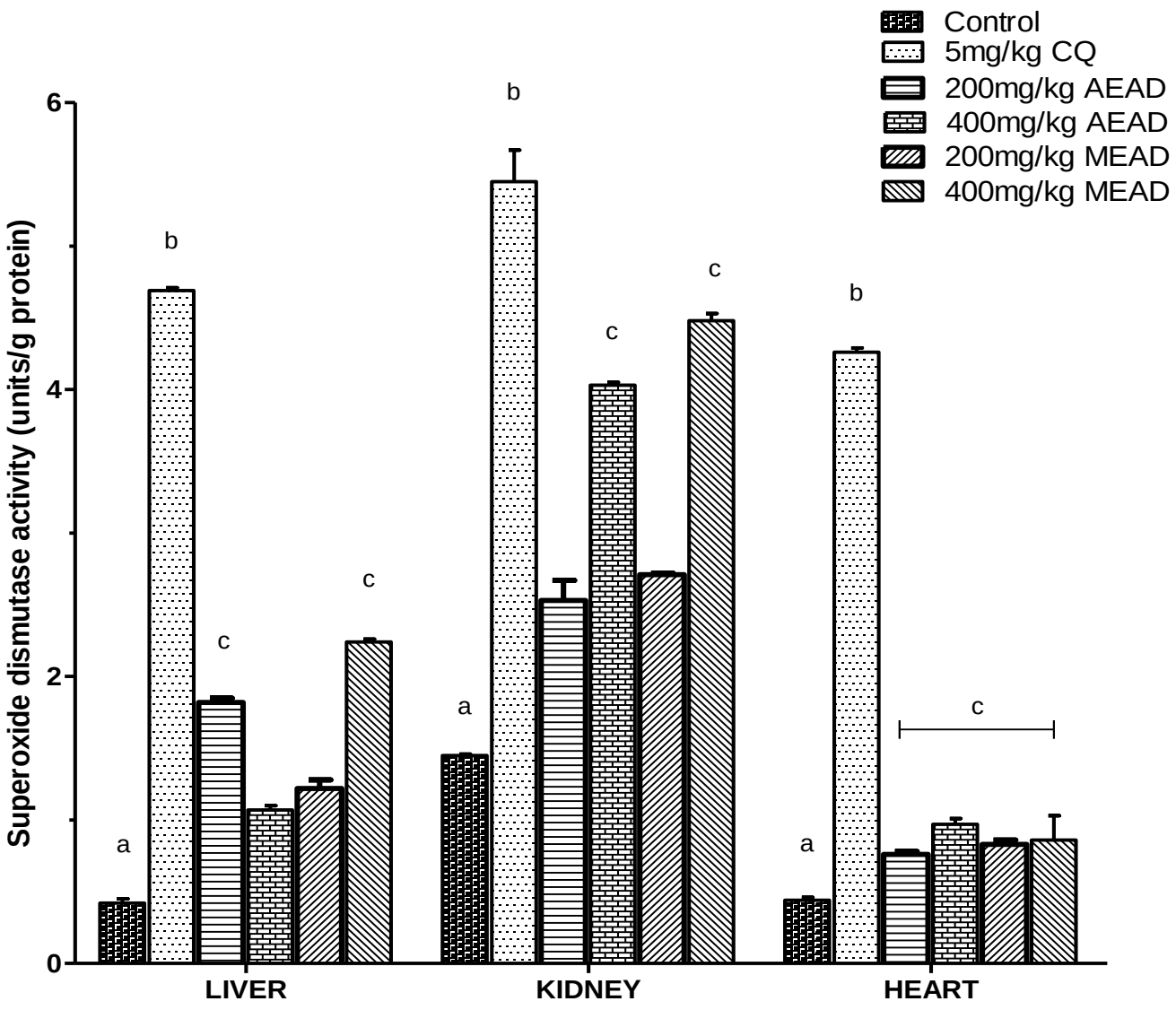


Figure 45: Superoxide dismutase activity after administration of Adansonia digitata stem bark extract

Results are mean ± SEM.

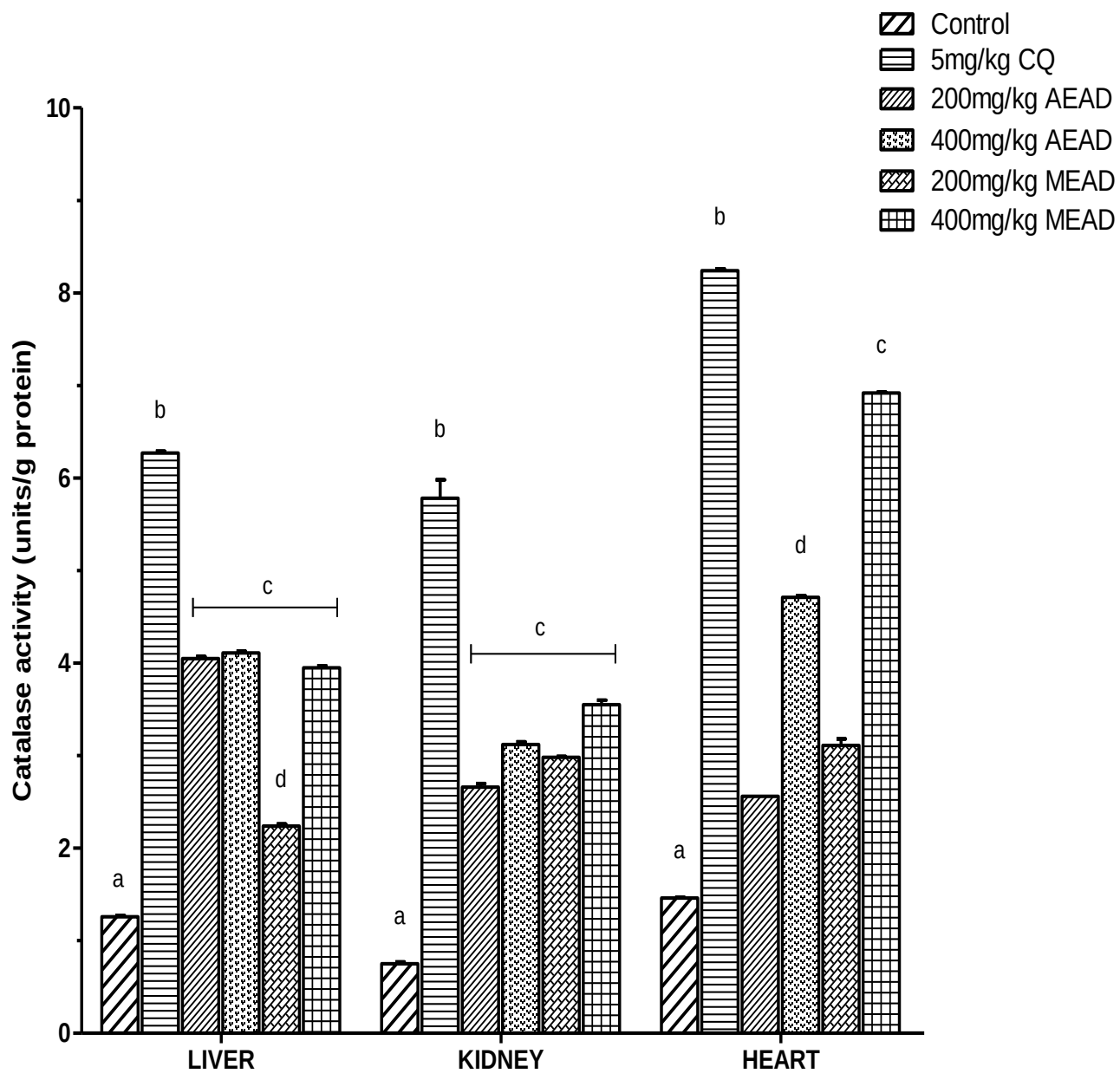


Figure 46: Catalase activity after administration of Adansonia digitata stem bark extract

Results are mean ± SEM.

4.12 Histopathological examination

The photomicrograph of liver, kidney and heart section of mice infected with *P. berghei* and treated with *Adansonia digitata* extracts are shown in plates 1-3.

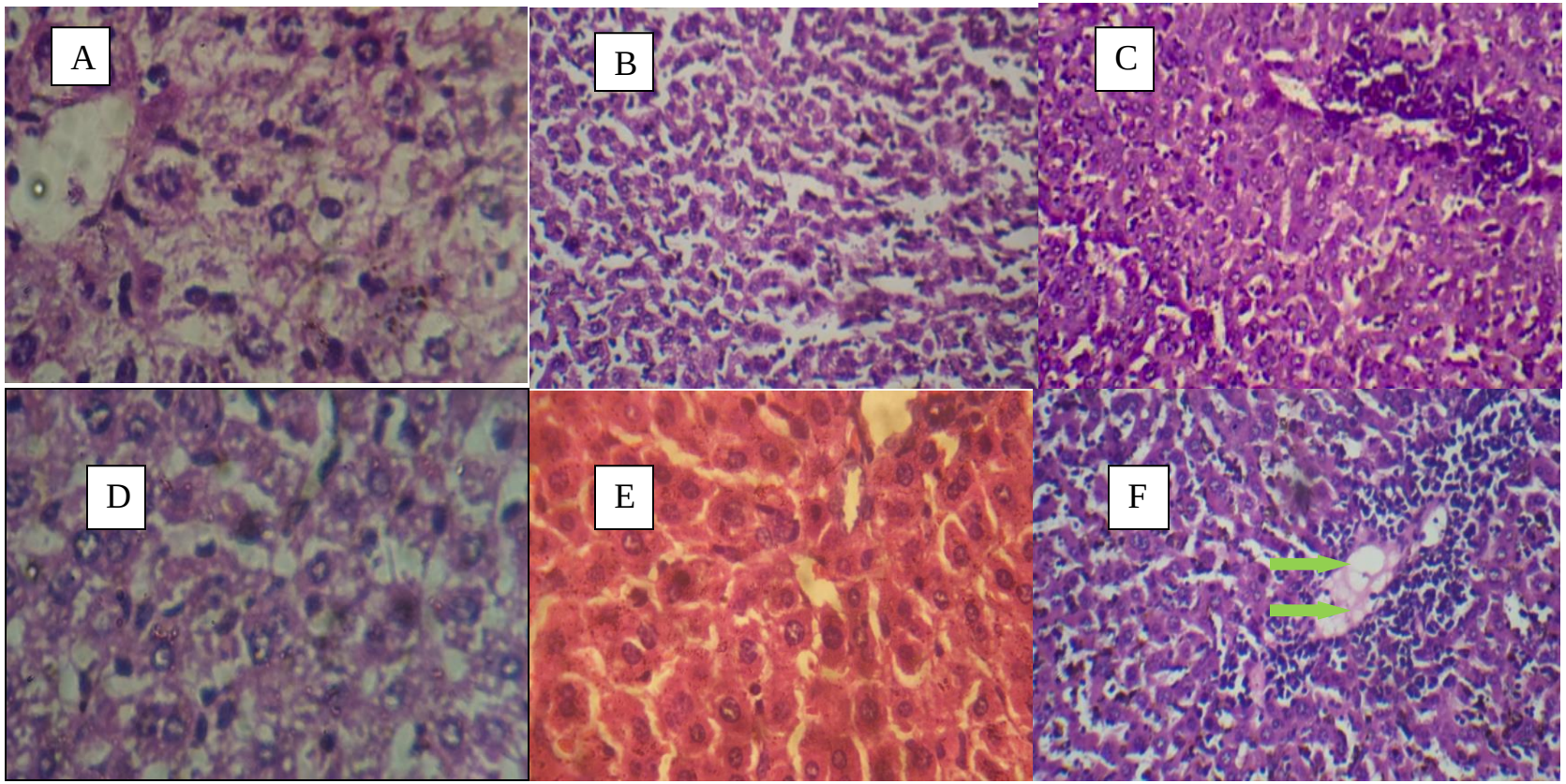


Plate 1: Photomicropgraph of liver section of mice infected and treated with *A. digitata* extract. 200mg/kg AEAD (A); 400mg/kg AEAD (B); 200mg/kg MEAD (C); 400mg/kg MEAD (D); 5mg/kg CQ (E) and Control (F). Hematoxylin-Eosin staining x 400 Magnification. Green arrow showed that there was a very severe periportal cellular infiltration by mononuclear cell with a diffuse hemosiderosis in the liver of the control (untreated) mice.

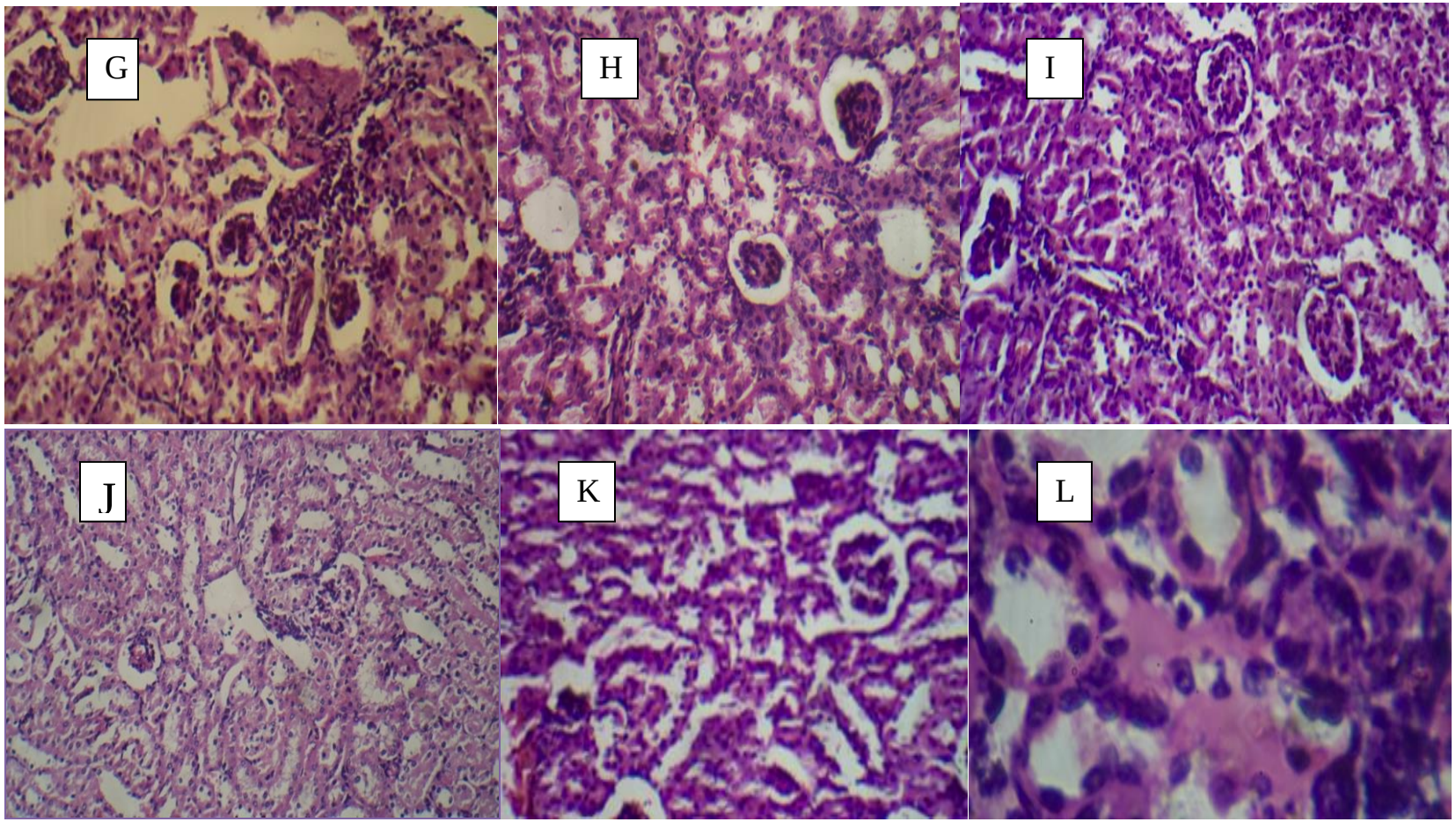


Plate 2: Photomicropgraph of kidney section of mice infected and treated with *A. digitata* extract. 200mg/kg AEAD (A); 400mg/kg AEAD (B); 200mg/kg MEAD (C); 400mg/kg MEAD (D); 5mg/kg CQ (E) and Control (F). Hematoxylin-Eosin staining x 400 Magnification.

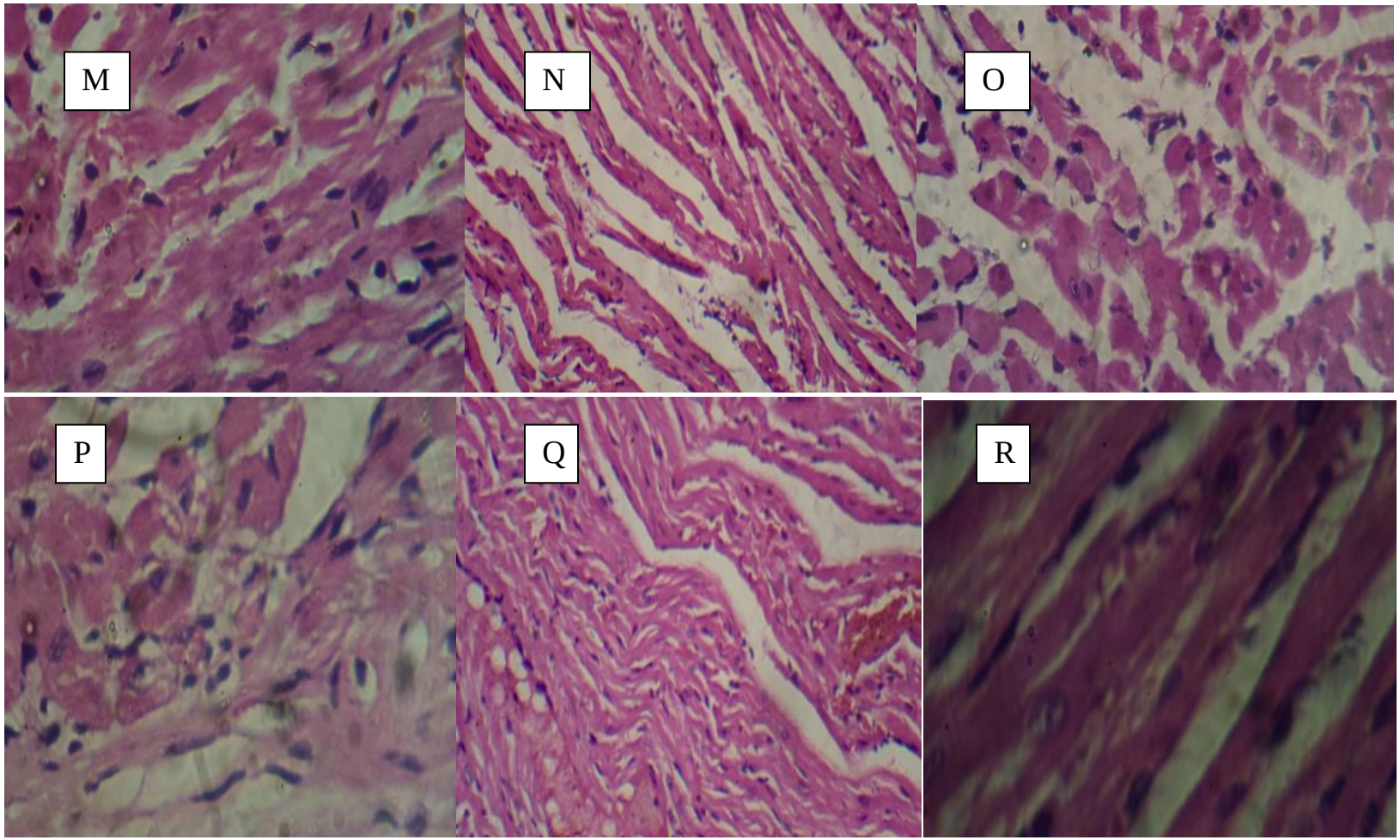


Plate 3: Photomicropgraph of heart section of mice infected and treated with *A. digitata* extract. 200mg/kg AEAD (A); 400mg/kg AEAD (B); 200mg/kg MEAD (C); 400mg/kg MEAD (D); 5mg/kg CQ (E) and Control (F). Hematoxylin-Eosin staining x 400 Magnification.

4.13 Effects of Ethylacetate partitioned fraction of *A. digitata* and some phenolics on β-hematin (synthetic hemozoin) formation.

The results from the cell free *β*-hematin formation assay of ethylacetate partitioned fraction of *A. digitata* stem bark extract and some phenolics are shown in Table 13. The inhibition of *β*-hematin formation is presented as percentage (% I). Artesunate and chloroquine were used as positive control with the most potent antimalarial activity and the extracts solvent (DMSO) was used as negative control without any antimalarial activity. The phenolics and the extract had considerable antimalarial activity especially apigenin which showed the most potent activity when compared to the standard antimalarial compounds. The IC_{50} values of ethylacetate extract fraction, quercetin, apigenin and cis-vaccenic acid are 1.167, 1.24, 1.094 and 1.298 respectively while that of artesunate and chloroquine are 0.952 and 0.864 respectively (Table 14).

Table 13: Percentage inhibition of β-hematin formation

Conc. mg/ml	Artesunate	Chloroquine	EtoAC Fraction	Quercetin	Apigenin	Cis-vaccenic acid
0.02	28.57 ± 0.06[a]	6.27 ± 0.01[c]	4.88 ± 0.00[c]	5.23 ±0.00[c]	12.54 ± 0.01[b]	1.05 ± 0.01[d]
0.04	53.31 ± 0.02[a]	40.77 ± 0.01[a]	5.92 ± 0.00[c]	9.06 ±0.00[c]	22.65 ± 0.04[b]	6.62 ± 0.02[c]
0.08	54.01 ± 0.01[a]	46.69 ± 0.01[a]	17.77 ± 0.05[b]	25.09 ±0.05[b]	23.34 ± 0.03[b]	7.32 ± 0.00[c]
0.16	54.70 ± 0.01[a]	47.74 ± 0.01[a]	21.25 ± 0.04[c]	25.44 ±0.05[c]	38.33 ± 0.01[b]	25.08 ± 0.05[c]
0.32	55.75 ± 0.01[a]	48.08 ± 0.01[a]	22.30 ± 0.04[c]	25.78 ±0.05[c]	42.50 ± 0.01[b]	28.22 ± 0.05[c]

The results are mean ±SEM of three determinations.

Table 14: The IC_{50} values of ethylacetate extract fraction and the phenolics

Compound/Extract	IC_{50} (mg/ml)
Artesunate	0.952
Chloroquine	0.864
EtoAC Fraction	1.167
Quercetin	1.124
Apigenin	1.094
Cis-vaccenic acid	1.298

4.14 Effects of Ethylacetate partitioned fraction of *A. digitata* and some phenolics on mitochondrial membrane permeability transition

4.14.1 Induction of the opening of mitochondrial membrane permeability transition pore by Ca^{2+} and inhibition by Spermine in normal rat liver mitochondria.

Figure 47 depicts the result obtained from the assessment of calcium-induced permeability transition in normal rat liver. The result shows a drastic reduction in absorbance at 540nm in the presence of exogenous Ca^{2+}. Ca^{2+} significantly induced the opening of mitochondria pore (0.639±0.024) when compared with the control (mitochondria in the absence of calcium: 0.058±0.002). The percentage inhibition of mitochondrial swelling by spermine was 52.46%.

4.14.2 Effect of Ethylacetate partitioned fraction of *A. digitata* and the standard drug Artesunate on mitochondrial membrane permeability transition pore in normal rat liver mitochondria.

Figure 48 to 51 shows the effect of the extract and the standard drug, artesunate on MMPT in the absence and presence of exogenous ca^{2+}. In the absence of ca^{2+}, the extract and artesunate inhibited the opening of the pore in a concentration dependent manner. Highest inhibition was observed at 25µg/ml. However, in the presence of exogenous ca^{2+}, the extract and artesunate induced the opening of mitochondrial membrane permeability transition pore in a concentration dependent mammer with the lowest concentration 5µg/ml having the highest inductive effect. The percentage induction of the extract and artesunate at 5µg/ml is 88.76% and 90.71% respectively. The change in absorbance at 540 nm of the effect of *A. digitata* in the presence of exogenous calcium is shown in Table 15 and the extent of induction of rat liver mitochondrial membrane permeability transition pore opening by artesunate and Ethylacetate fraction of *.A digitata* extract is shown in Figure 52.

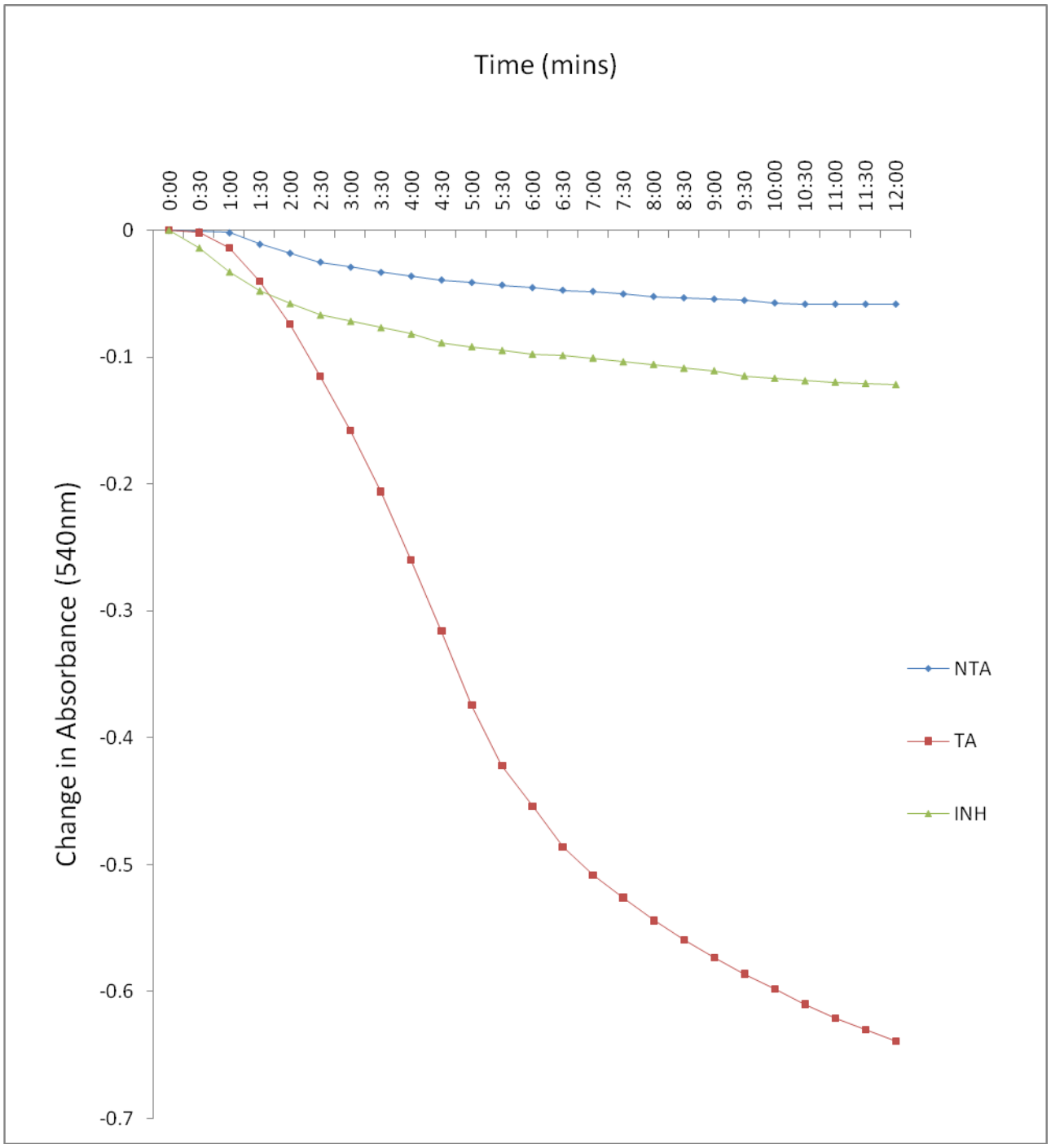


Figure 47: Calcium induced opening of normal rat liver mitochondrial membrane permeability transition pore and the reversal effect of spermine. NTA= No Triggering Agent, TA= Triggering Agent, INH= Inhibitor (Spermine)

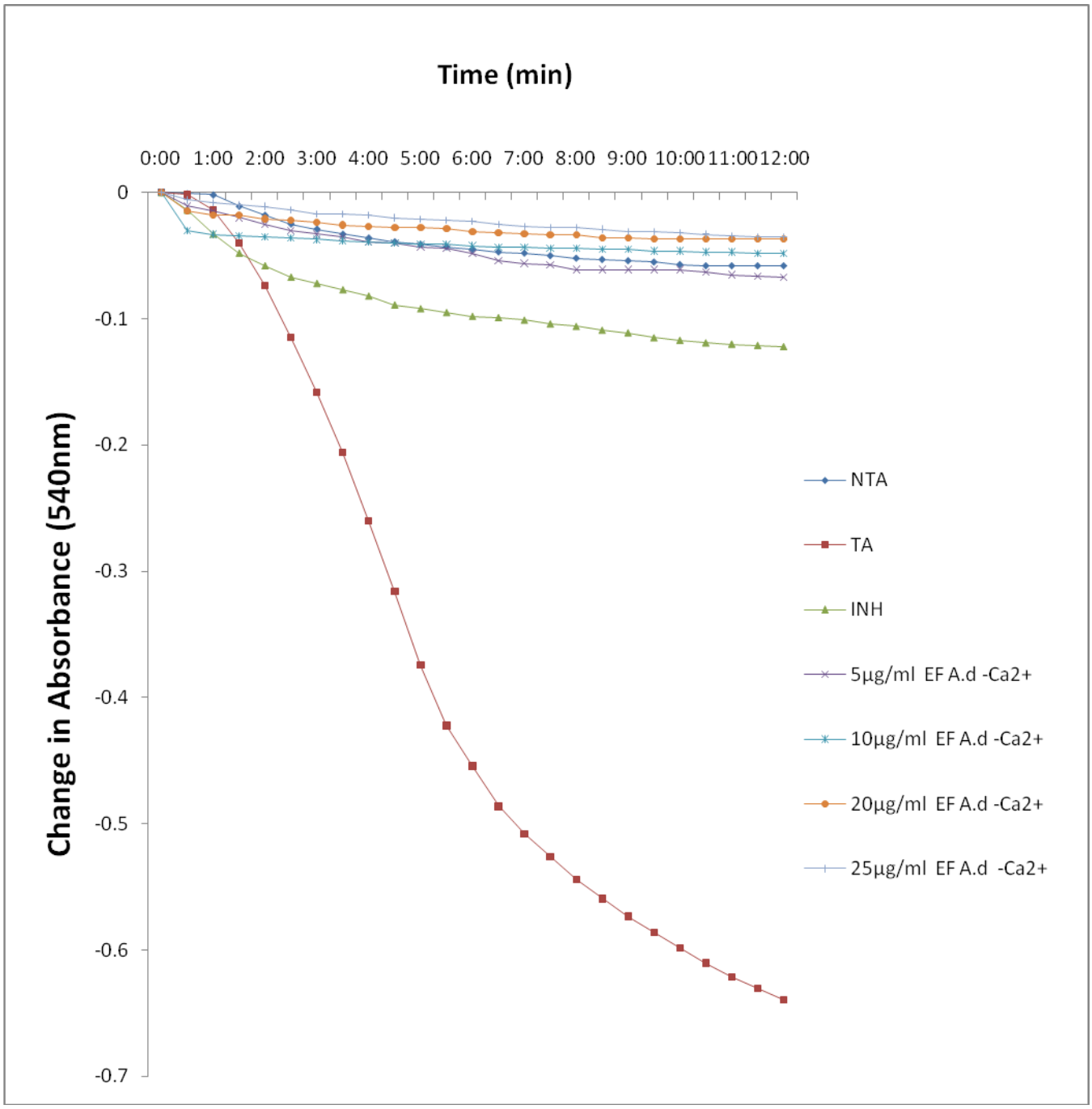


Figure 48: Varying concentrations of ethylacetate partitioned fraction of *Adansonia digitata* the stem bark on rat liver mitochondrial membrane permeability transition pore in the absence of exogenous calcium.

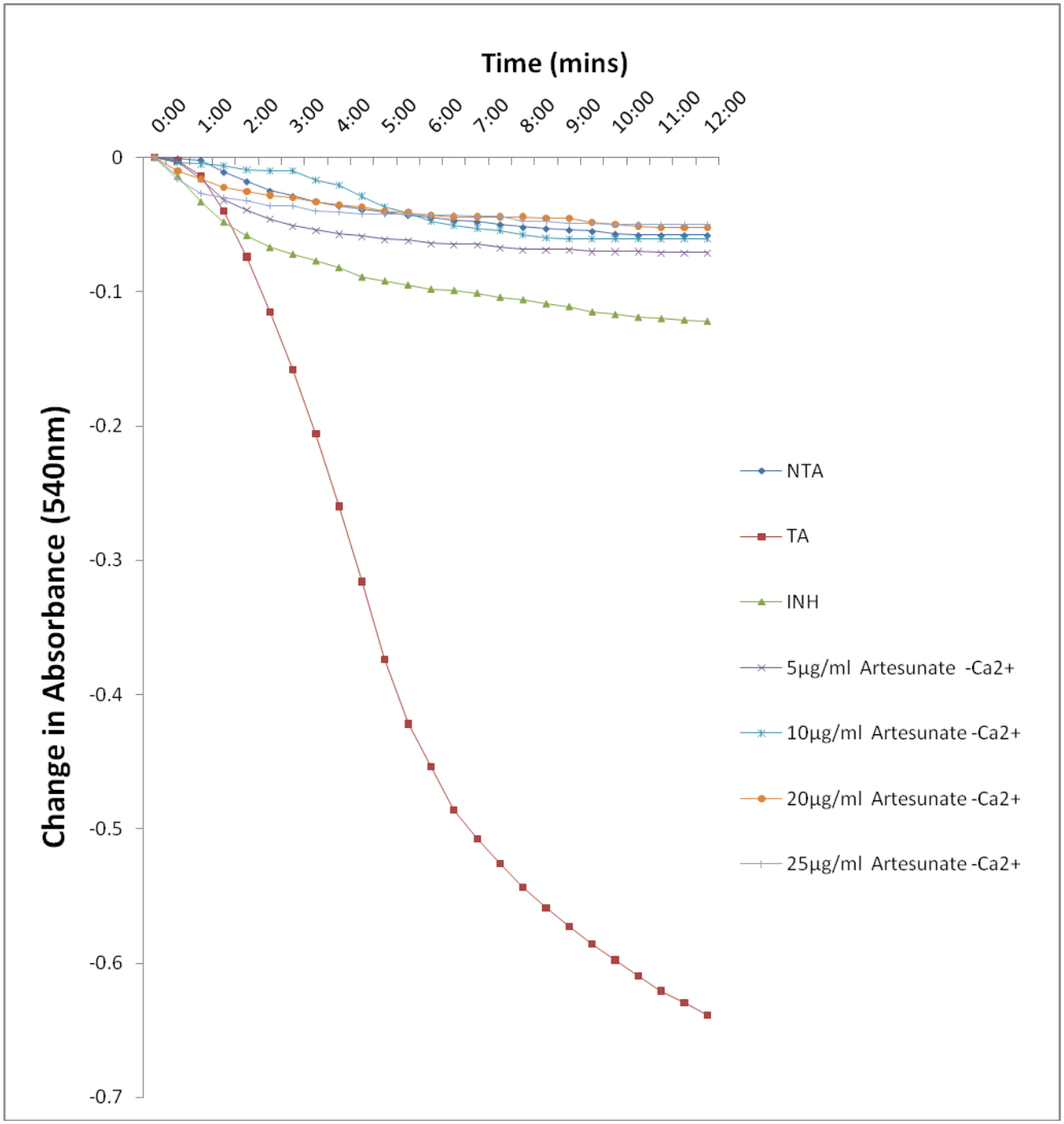


Figure 49: Varying concentrations of artesunate on rat liver mitochondrial membrane permeability transition pore in the absence of exogenous calcium

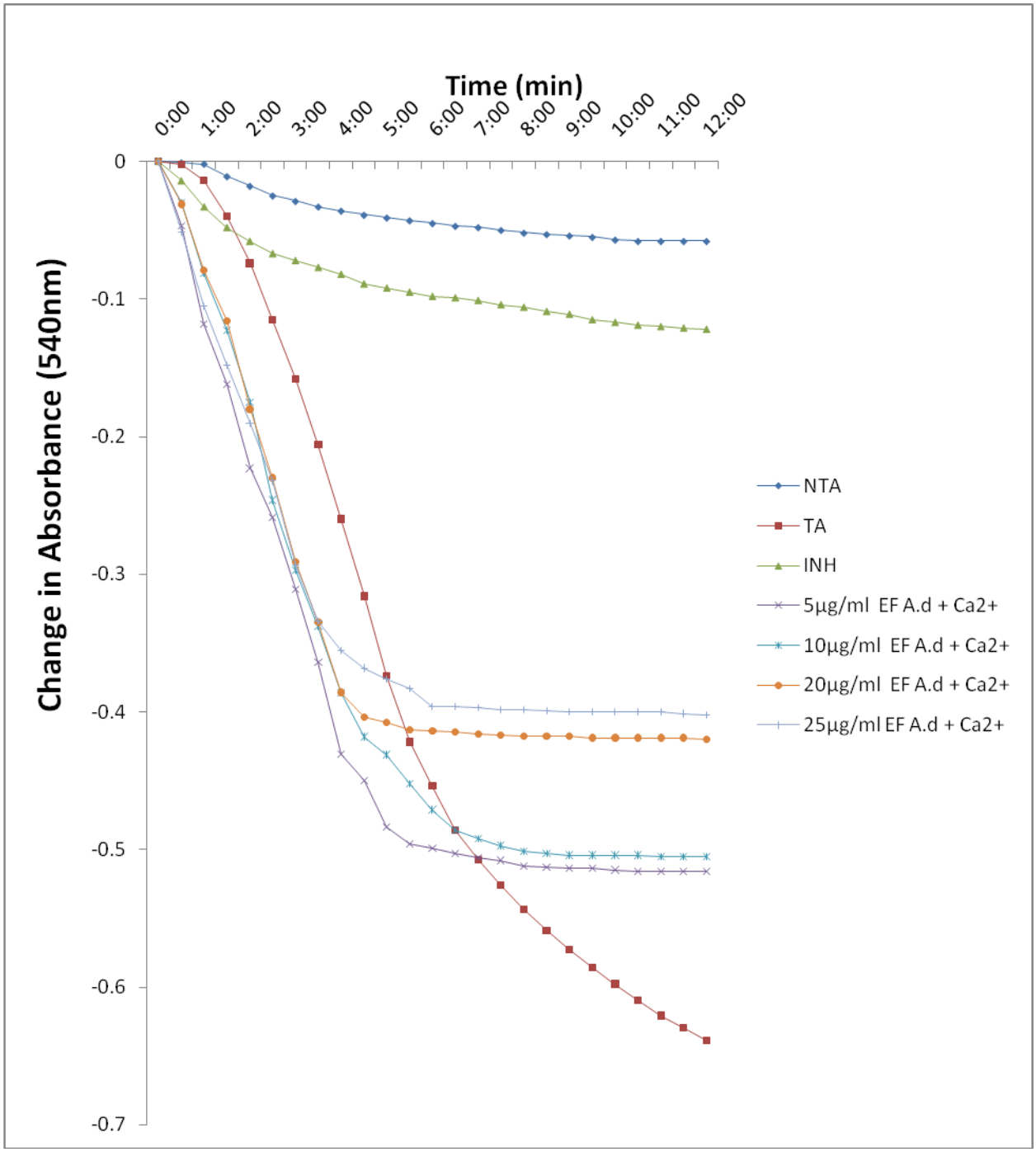


Figure 50: Varying concentrations of ethyl acetate partitioned fraction of *Adansonia digitata* stem bark extract on rat liver mitochondrial membrane permeability transition pore in the presence of exogenous calcium.

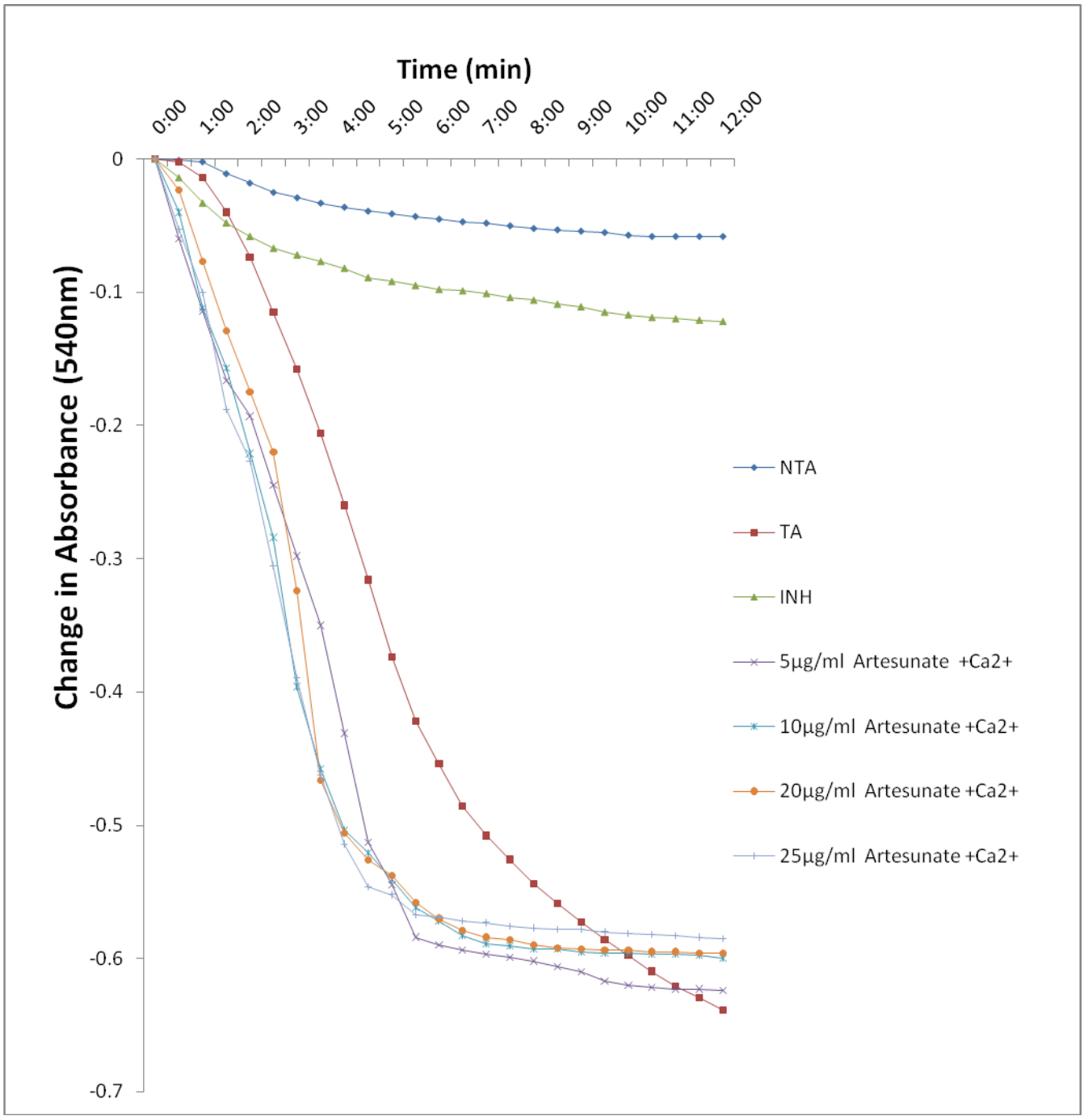


Figure 51: Varying concentrations of artesunate on rat liver mitochondrial membrane permeability transition pore in the presence of exogenous calcium.

Table 15: Change in absorbance at 540nm of the effect of Adansonia digitata in the presence of exogenous calcium

Groups	Change in Absorbance	Induction fold	% Induction
No triggering agent ($-Ca^{2+}$)	0.058 ± 0.002	1.0	-
Triggering agent ($+Ca^{2+}$)	0.639 ± 0.024	11.0	90.92
5µg/ml Artesunate	0.624 ± 0.025	10.8	90.71
5µg/ml EF of *A.d*	0.516 ± 0.005	8.9	88.76
10µg/ml Artesunate	0.600 ± 0.002	10.3	90.33
10µg/ml EF of *A.d*	0.505 ± 0.003	8.7	88.51
20µg/ml Artesunate	0.596 ± 0.002	10.3	90.27
20µg/ml EF of *A.d*	0.420 ± 0.007	7.2	86.19
25µg/ml Artesunate	0.585 ± 0.032	10.1	90.09
25µg/ml EF of *A.d*	0.402 ± 0.006	6.9	85.57

Results are mean ± SEM.

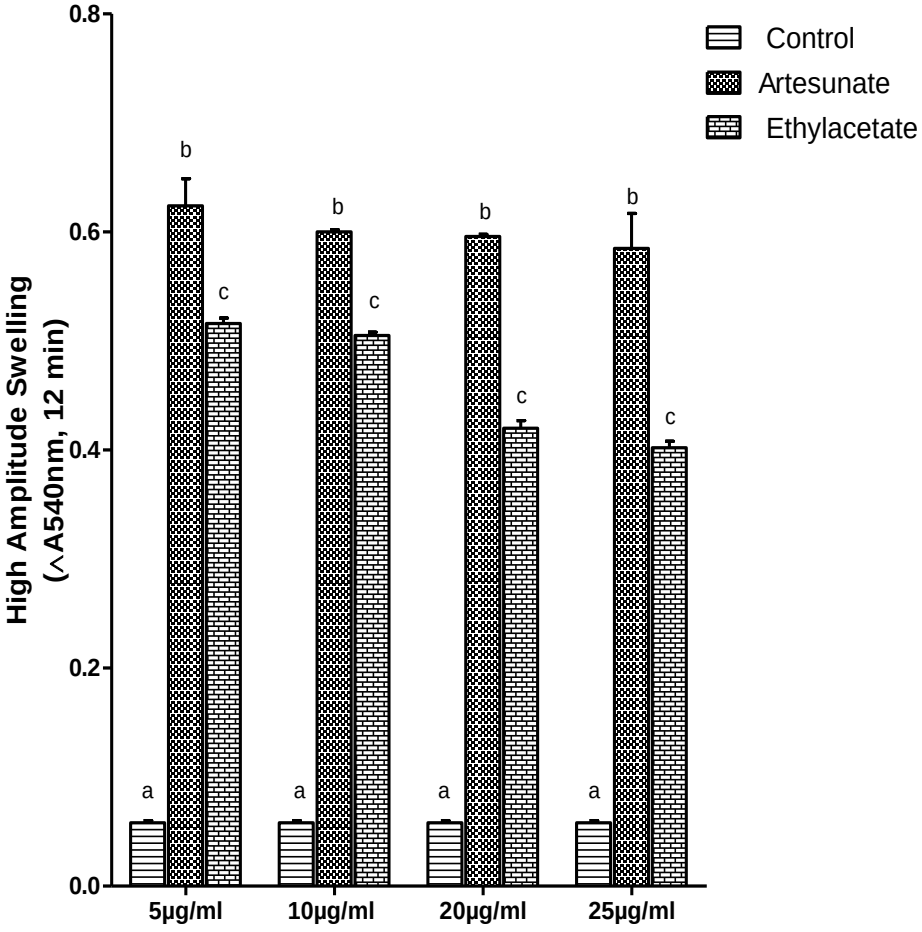


Figure 52: Comparison of the extents of induction of rat liver mitochondrial membrane permeability transition pore opening by artesunate and Ethylacetate fraction of *Adansonia digitata* extract.

4.14.3 Effect of Quercetin and Apigenin on mitochondrial membrane permeability transition pore in normal rat liver mitochondria.

Figure 53 and 54 shows the effect of the quercetin on MMPT in the presence and absence of exogenous ca^{2+}. Quercetin inhibited the opening of the pore in a concentration dependent manner in the absence of ca^{2+}. In the presence of exogenous ca^{2+}, quercetin also induced the opening of mitochondrial membrane permeability transition pore in a concentration dependent mammer with the lowest concentration 5µg/ml having the highest inductive effect.

Figure 55 and 56 shows the effect of the apigenin on MMPT in the presence and absence of exogenous ca^{2+}. In the absence of exogenous ca^{2+}, apigenin significantly ($P<0.05$) induced the opening of mitochondrial membrane permeability transition pore in concentration dependent manner. The inductive effect of apigenin is higher than that caused by ca^{2+} only. The percentage induction of apigenin at 80µg/ml is 91.82% while that of ca^{2+} is 88.28%. Also, in the presence of ca^{2+}, apigenin further induced the opening of the pore significantly ($P<0.05$) in concentration depependent manner when compared with the calcium only.

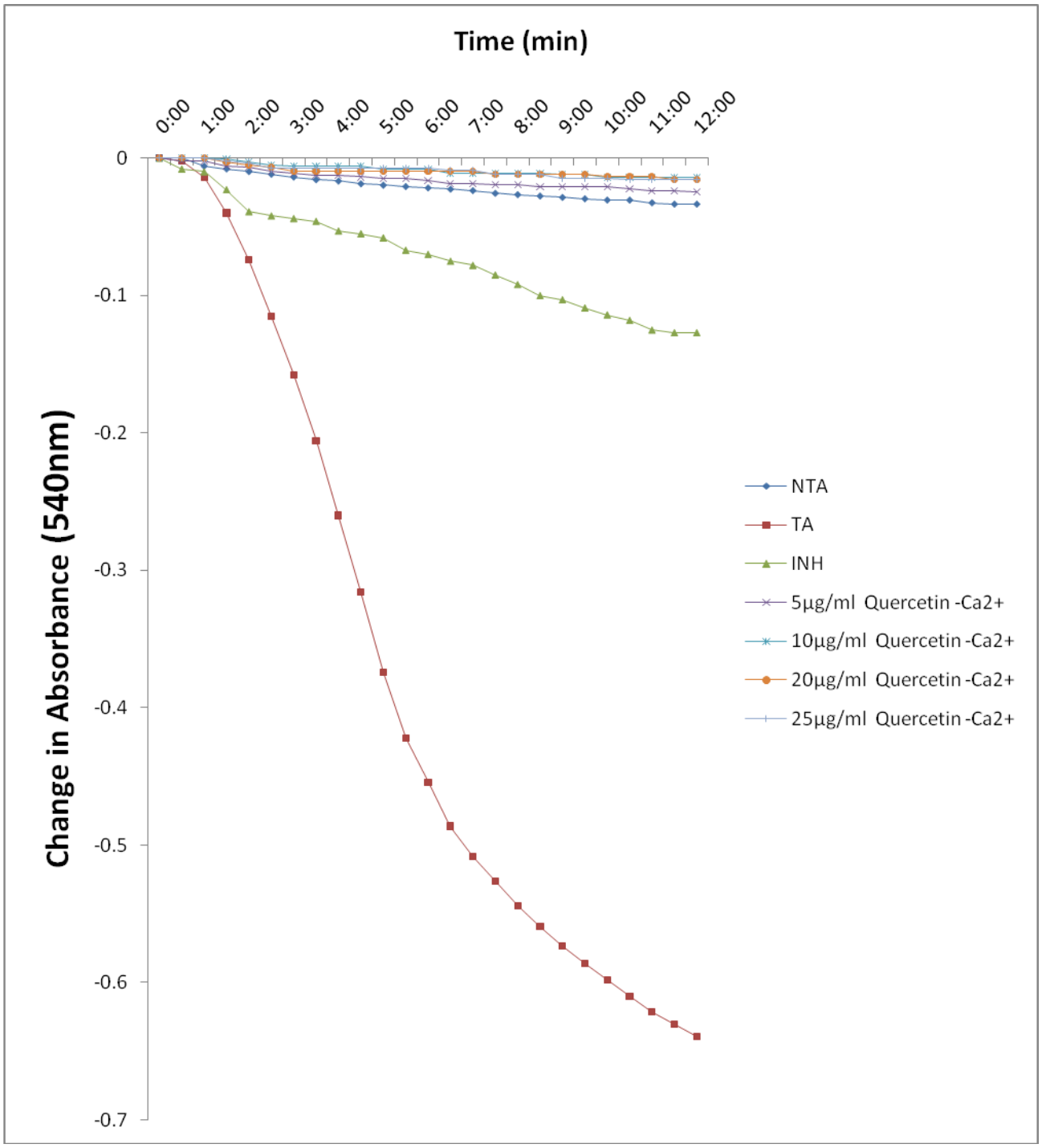


Figure 53: Varying concentrations of quercetin on rat liver mitochondrial membrane permeability transition pore in the absence of exogenous calcium

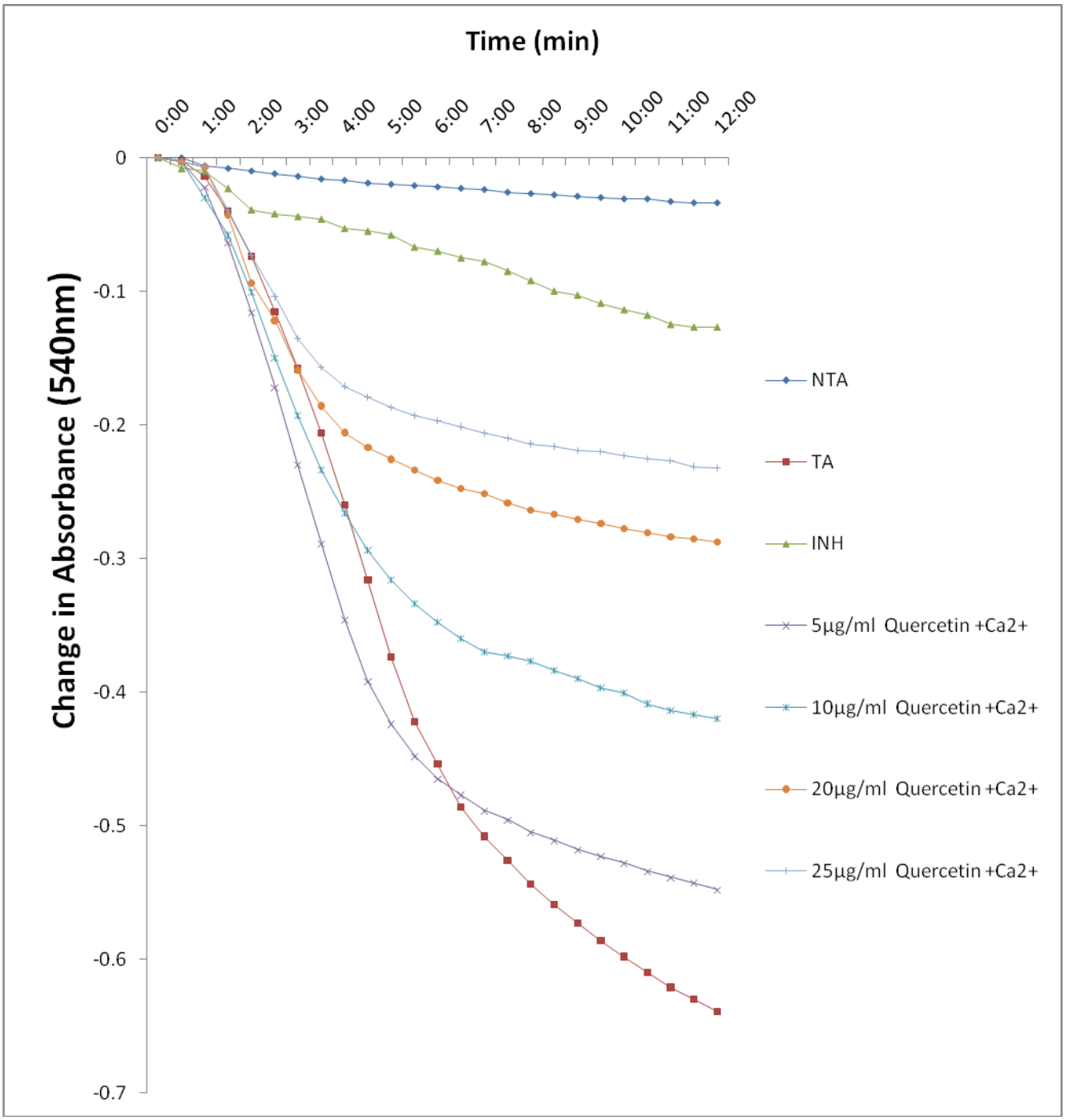


Figure 54: Varying concentrations of quercetin on rat liver mitochondrial membrane permeability transition pore in the presence of exogenous calcium.

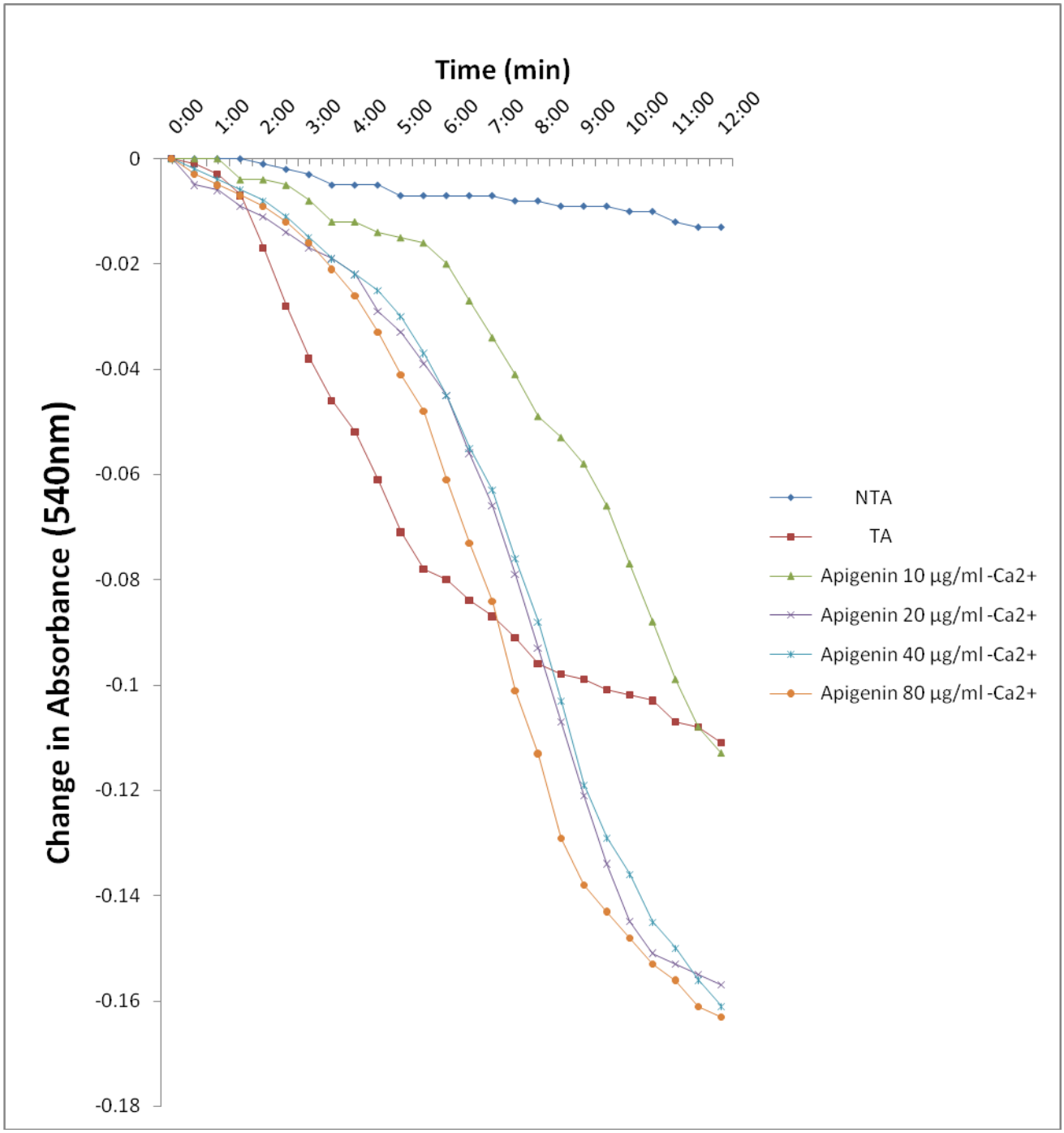


Figure 55: Varying concentrations of apigenin on rat liver mitochondrial membrane permeability transition pore in the absence of exogenous calcium

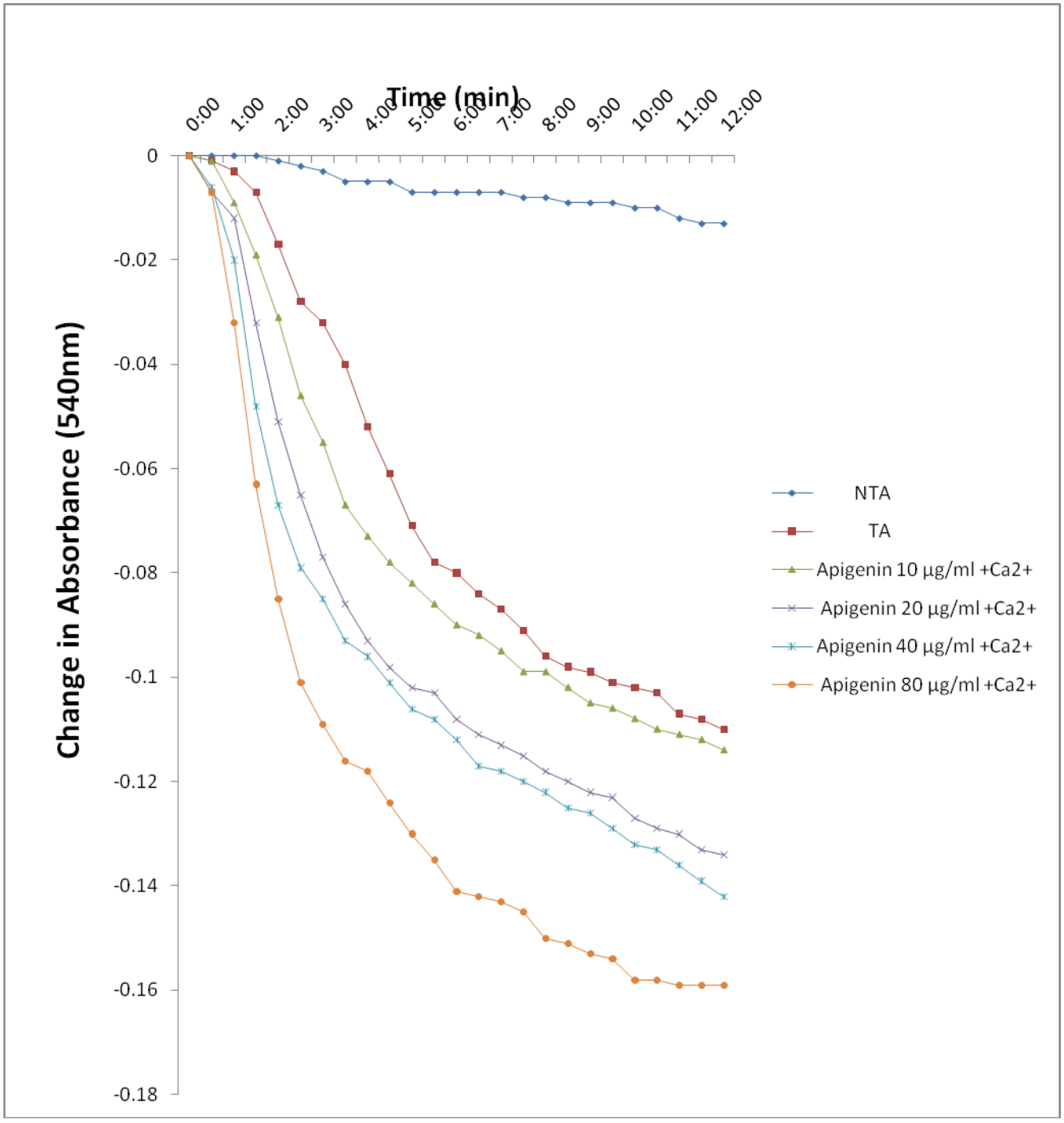


Figure 56: Varying concentrations of apigenin on rat liver mitochondrial membrane permeability transition pore in the presence of exogenous calcium.

4.15 Effects of Ethylacetate partitioned fraction of *A. digitata* on mitochondrial ATPase.

Figure 57 shows the effect of the extract on mitochondrial ATPase. The ATPase activity of the extract increased significantly ($P<0.05$) in concentration dependent manner. At lower concentration of the extract, the mitochondria was intact but at higher concentration 80µg/ml, the mitochondria was uncoupled. The uncoupler (2,4-dinitrophenol) significantly ($P<0.05$) uncouples the mitochondria when compared with the control and the extract.

Figure 4.45 shows the effect of the extract on mitochondrial lipid peroxidation. The extract significantly ($P<0.05$) inhibited mitochondrial lipid peroxidation in concentration dependent manner.

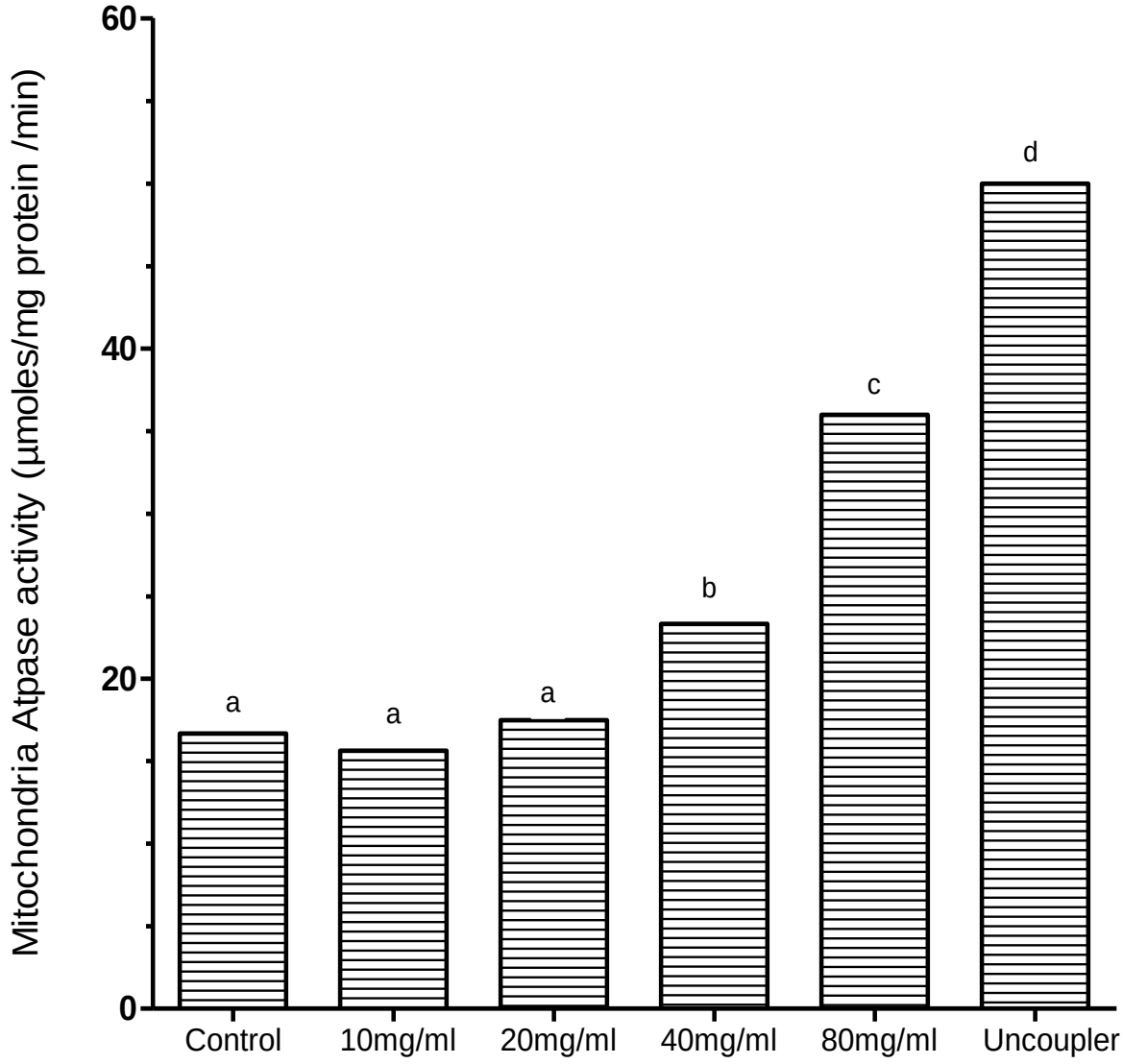


Figure 57: Effect of ethyl acetate partitioned fraction of *A. digitata* stem bark extract on m**itochondrial ATPase**

Results are mean ± SEM.

4.16 Molecular docking

The major chemical constituent present in the ethylacetate partitioned-fraction of *A. digitata* stem bark extracts were involved in docking study using AutoDock 4.2, based on the Lamarckian principle. The results revealed that all the selected compounds in the *A. digitata* showed binding energy ranging from -4.6 kcal/mol to -7.1 kcal/mol. Out of three chemical constituents, apigenin and quercetin has the highest docking score along with the highest number of hydrogen bonds formed while the cis-vaccenic acid has the least docking score with the least hydrogen bonds. The interaction of these constituents with the Ca^{2+}ATPase (2KNE) and Ca^{2+} transporter CAX (4K1C) is presented in Table 16.

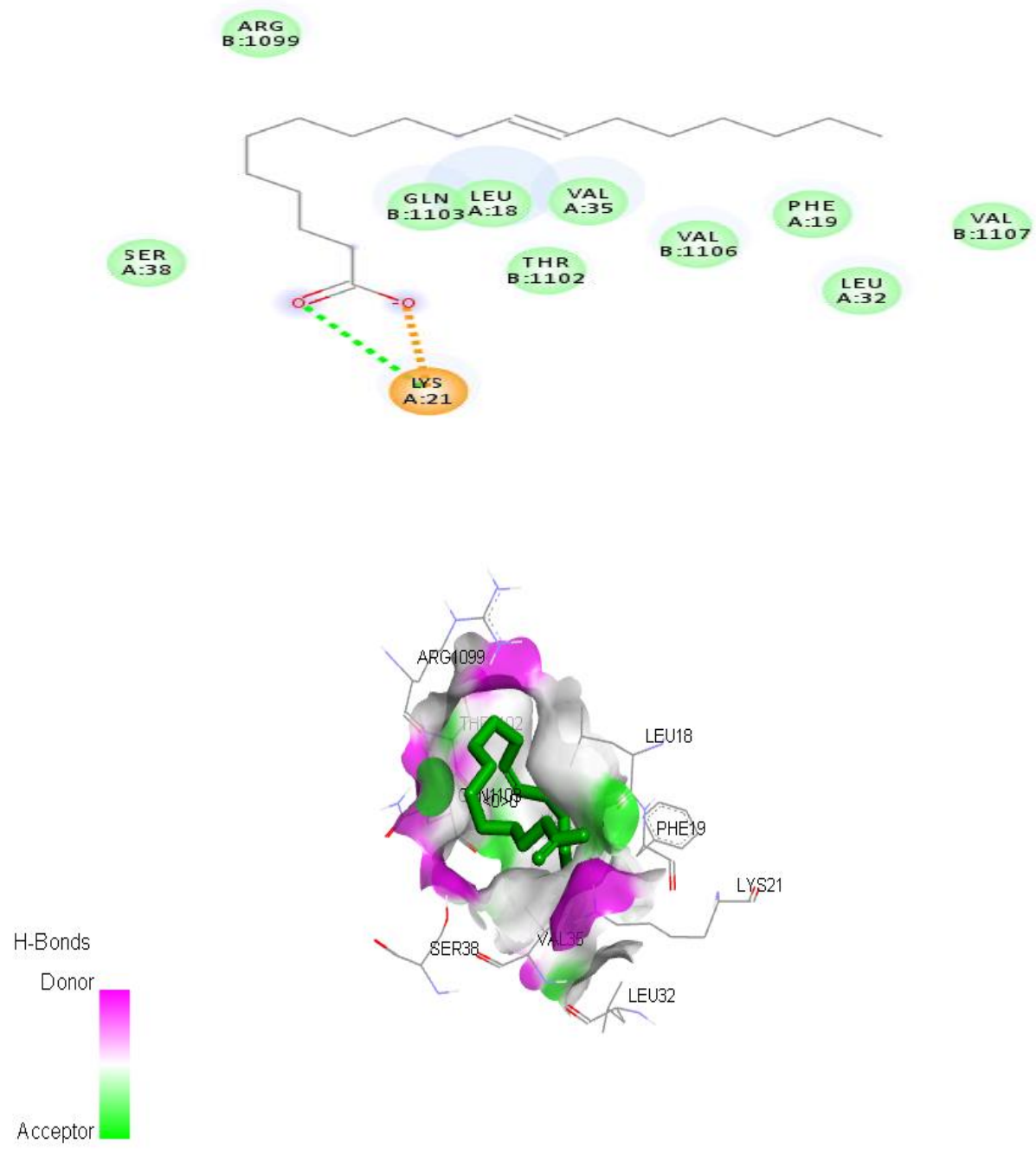


Figure 58: Ca^{2+}ATPase (2KNE) – Cis-vaccenic acid interaction

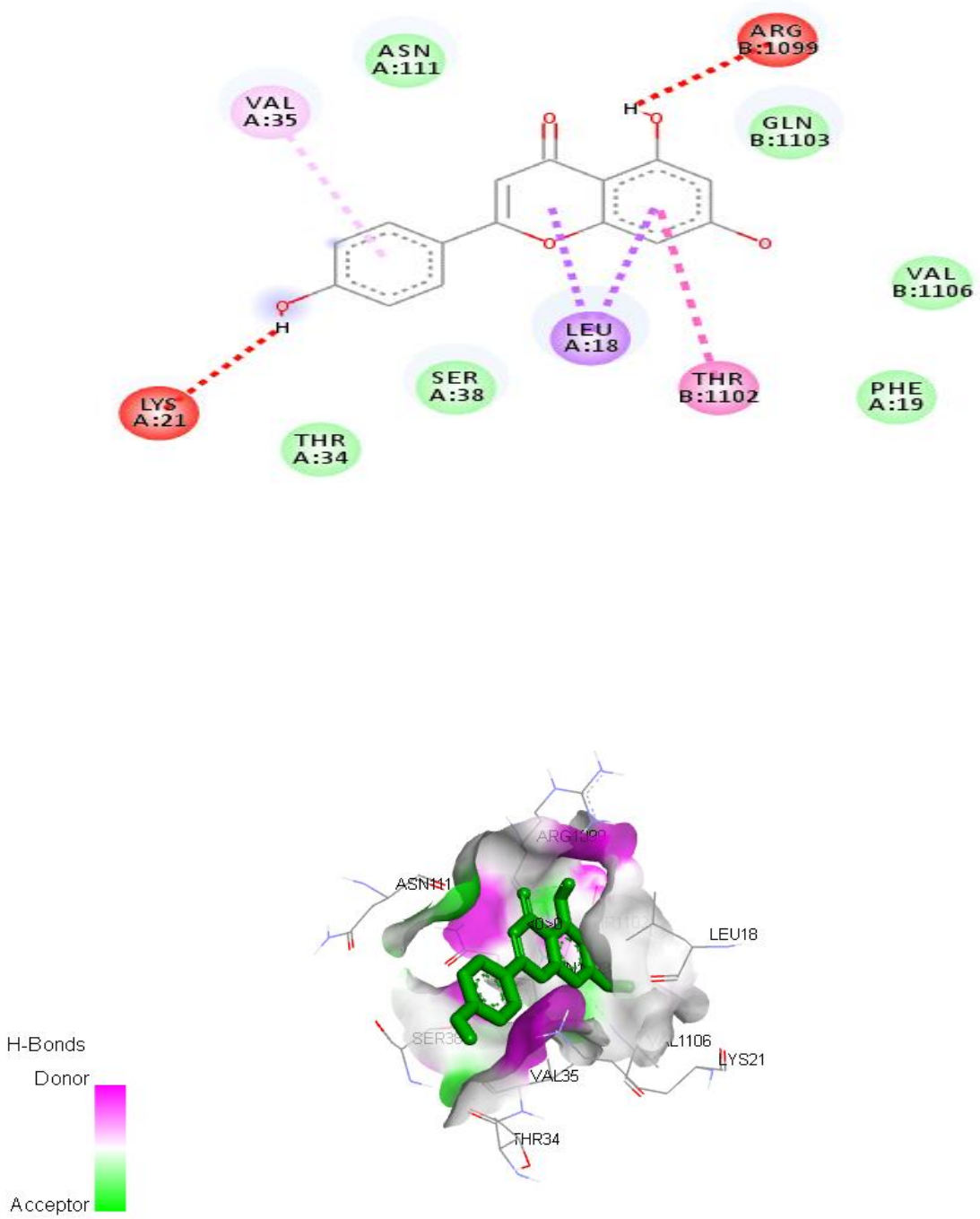


Figure 59: Ca^{2+}ATPase (2KNE) – Apigenin interaction

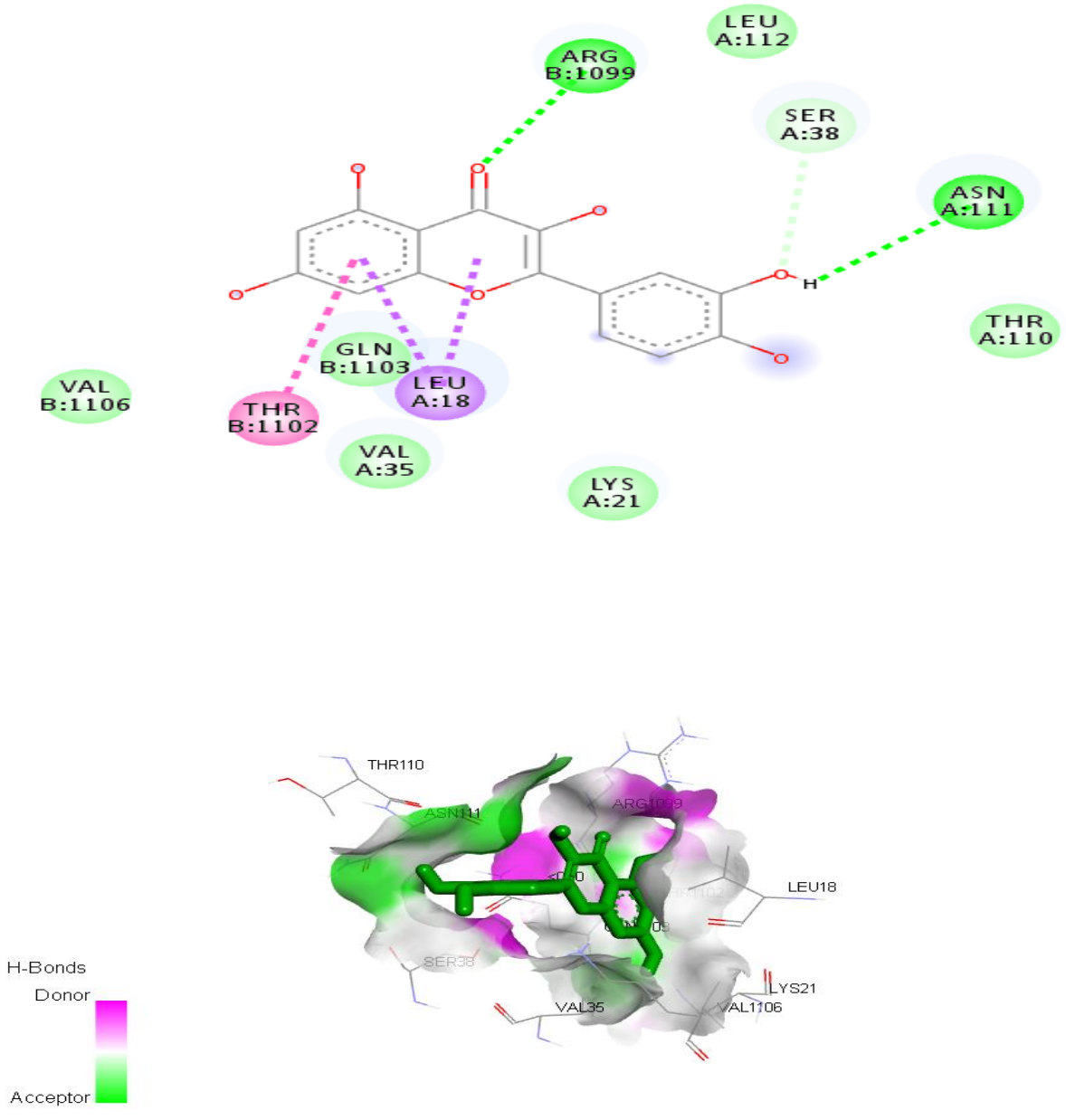


Figure 60: Ca^{2+}ATPase (2KNE) – Quercetin interaction

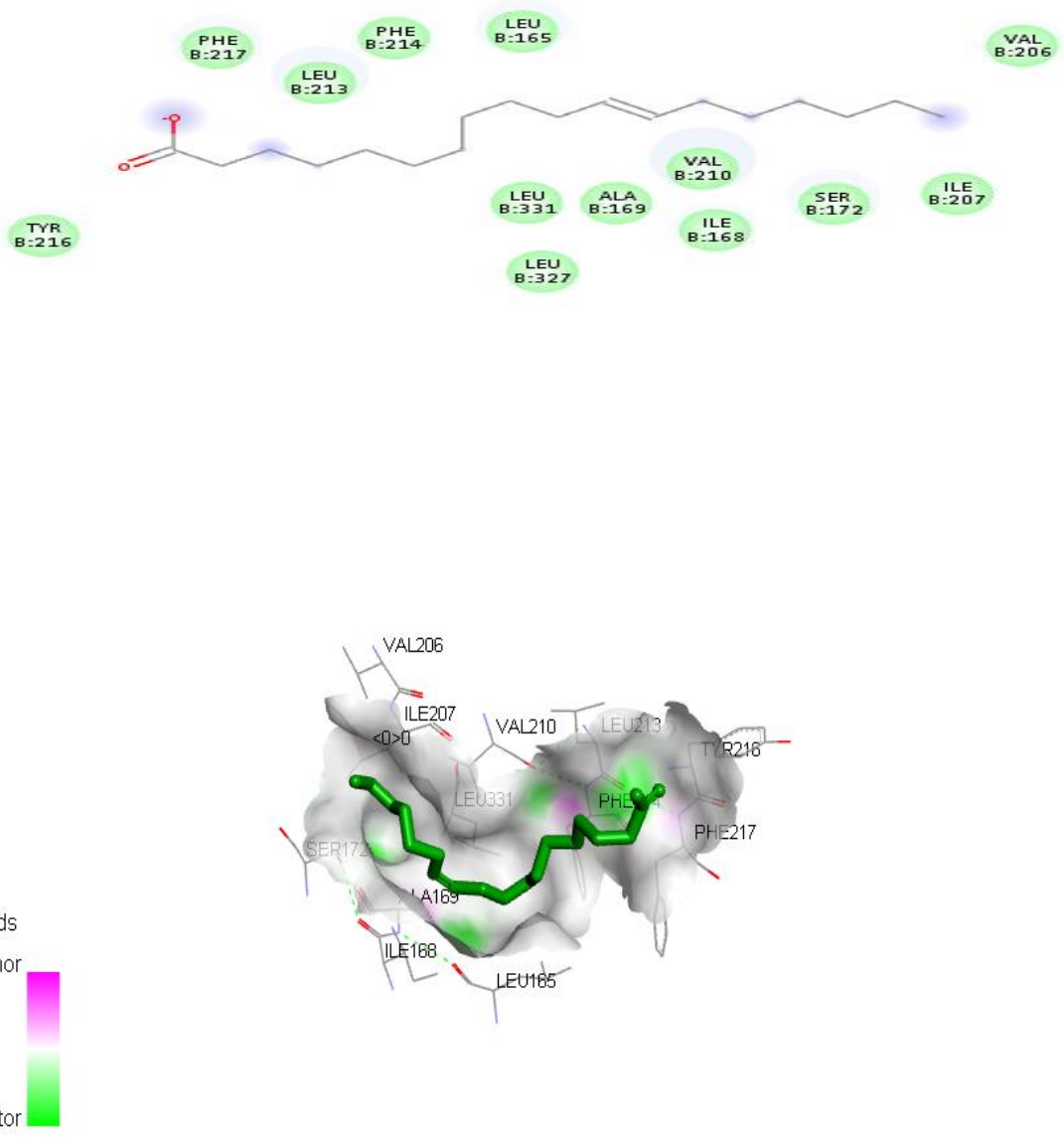


Figure 61: Ca^{2+} transporter CAX (4K1C) – Cis-vaccenic acid interaction

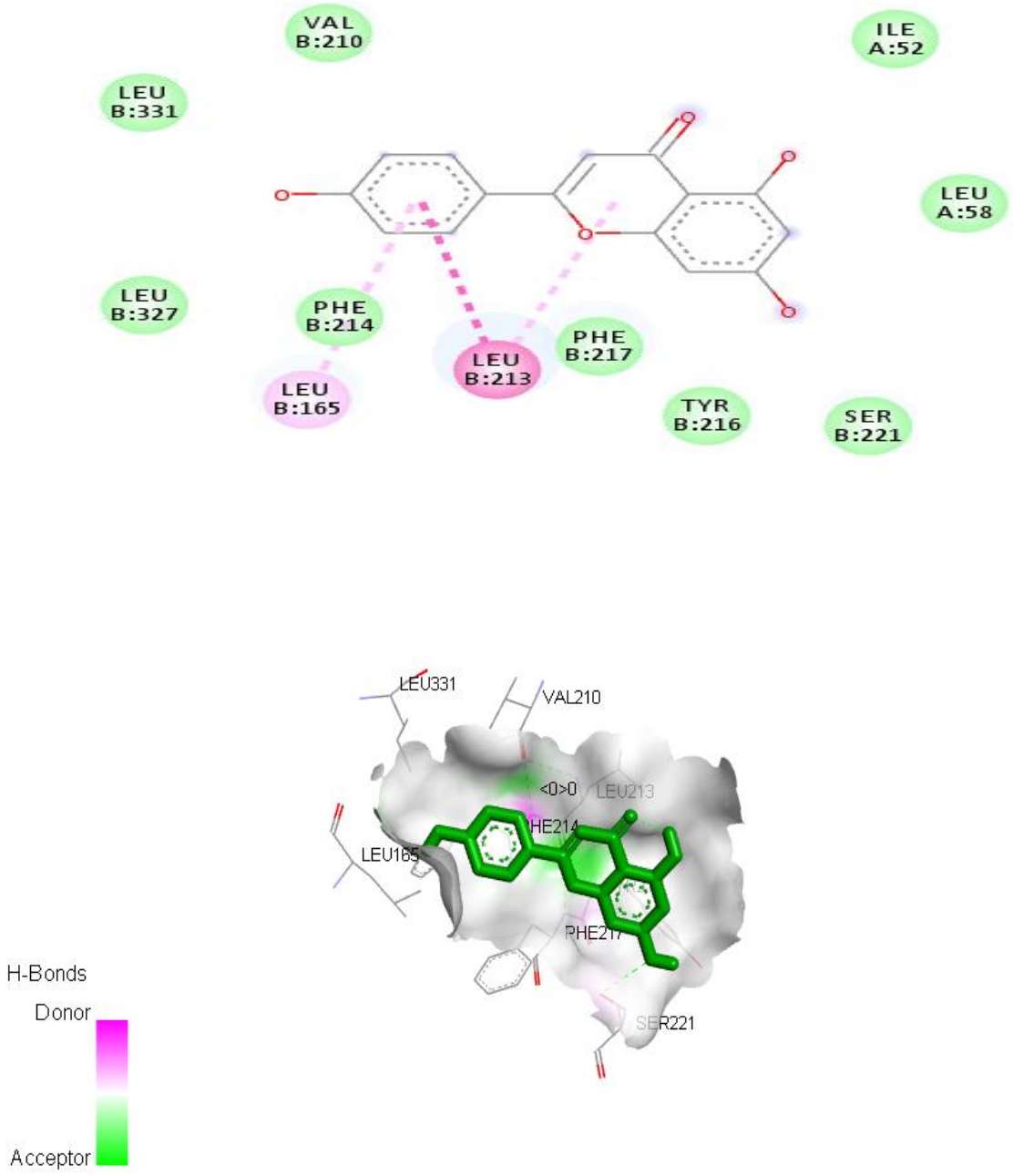


Figure 62: Ca^{2+} transporter CAX (4K1C) – Apigenin interaction

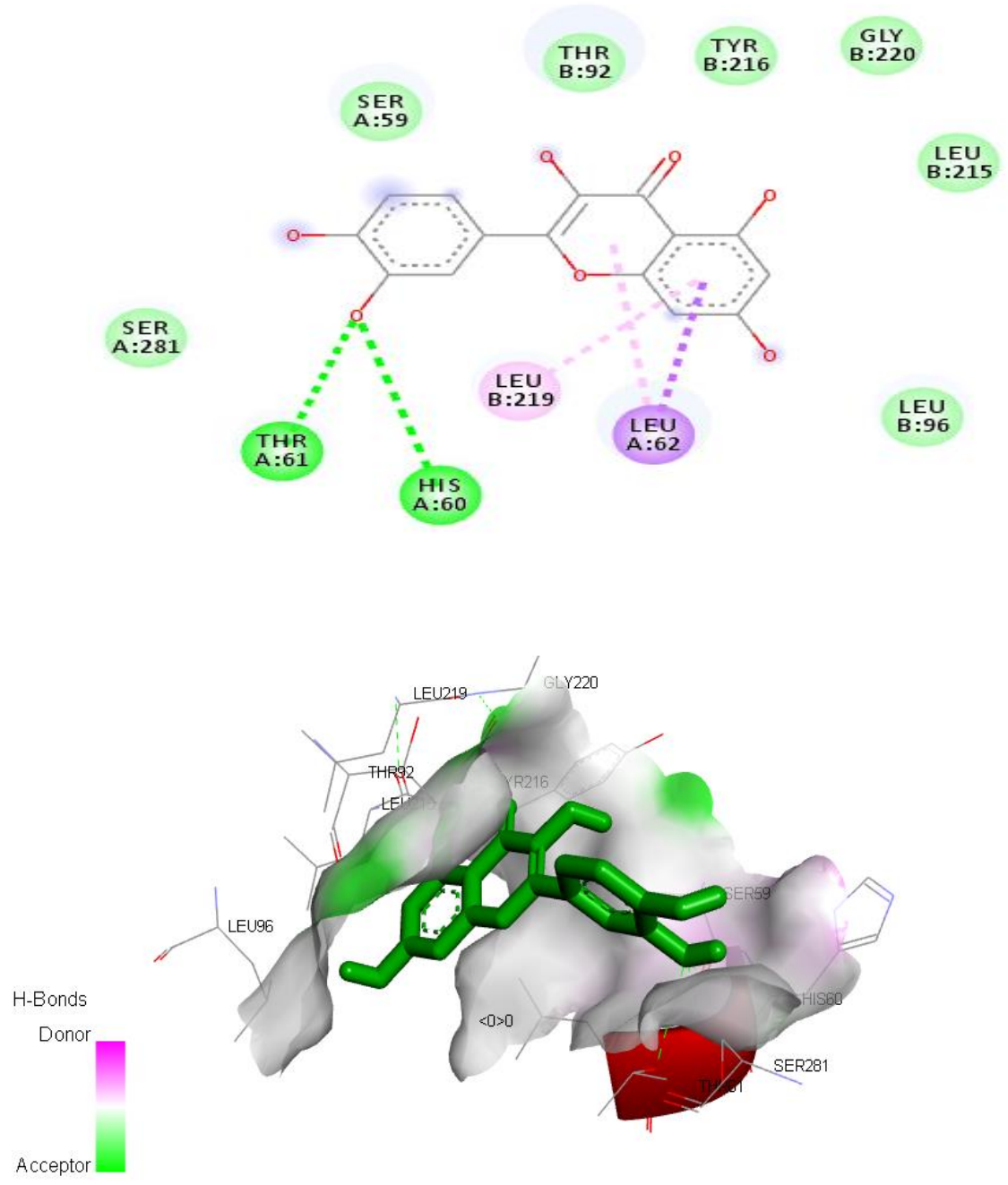


Figure 63: Ca^{2+} transporter CAX (4K1C) – Quercetin

Table 16: Summary of binding affinities and ligand-amino acid interactions

S/N	Protein	Compound	Binding affinities (Kcal/mol)	Ligand-Amino acid interactions
1.	Calcium ATPase SERCA	Cis-Vaccenic acid	-5.7	Lys21
		Apigenin	-6.6	Lys21, Leu18, Thr1102, Arg1099, Val35
		Quercetin	-6.5	Thr1102, Leu18, Asn111, Ser38, Arg1099
2.	CAX	Cis-Vaccenic acid	-4.6	-
		Apigenin	-7.1	Leu213, Leu165
		Quercetin	-6.8	Thr61, His60, Leu219, Leu62

CHAPTER FIVE

5.0 DISCUSSION

5.1 Identification of Compounds

Medicinal plants contain biologically active phytochemicals which have protective and therapeutic properties. These phytochemicals arefound as secondary plant metabolites that are useful for humanity. The phytochemical analysis of the aqueous (AEAD) and methanolic extract (MEAD) of *A. digitata* stem bark extracts showed the presence of alkaloids, saponins, tannins, terpenoids, cardiac glycosides and flavonoids. This is a confirmation of earlier work by Fasola and Iyamah (2014). The total phenolic content with tannic acid equivalent of MEAD was significantly higher than the AEAD. However, the flavonoid content of MEAD with quercetin equivalent washigher but not significantly different from the AEAD. Many of the antioxidants and therapeutic actions of phytochemicals are thought tobe associated with their biologically active polyphenol components, such as flavonoids and phenolic acids, which has powerful antioxidant activities (Pandey and Rizvi, 2009). The alkaloid, tannin and saponin content of MEAD and AEAD with gallic acid equivalent were not significantly different from each other, though they were higher in methanolic extract.

High performance liquid chromatography (HPLC) finger printing is a tool for estimating the phytohemicals in plant samples. It is a technique employed to identify, separate, quantify and purify individual components of amixture. It gives both quantitative and qualitative information and makes possible the screening of samples forthe presence ofnew compounds. The HPLC fingerprint of both the AEAD and MEAD showed thepresence of flavonoids (rutin, quercitrin, kaempferol, luteolin and apigenin) and phenolics (gallic, chlorogenic and caffeic acids). The amount of caffeic acid, quercitrin, kaempferol and luteolin werehigher in AEAD with quercitrin and kaempferol being the most abundant. In the MEAD, the amount of gallic acid, rutin,

quercitrin, kaempferol, luteolin and apigenin were higher with kaempferol and luteolin being the most abundant.

Moreso, the HPLC fingerprint of the ethylacetate partitioned fraction of crude methanolic extract of *A. digitata* stem bark (EFAD) showed the presence of phenolics (gallic, chlorogenic and caffeic acids) and flavonoids (rutin, quercetin and luteolin). In the EFAD, gallic acid, chlorogenic acid, caffeic acid, quercetin and luteolin constituted the bulk of phenolics and with quercetin being the most abundant. The amount of quercetin is statistically higher when compared to other flavonoids present in the EFAD. This, however supports the claim that quercetin is a well known flavonoid with strong antioxidant property capable of efficiently scavenging lipid free radicals and hydroxyl radicals and has also been reported to reduce oxidative stress (Palmeira, 2007).

The gas chromatography/mass spectrometry analysis of EFAD showed the presence of nine (9) compounds which include: Bicyclo (3.1.1) Heptane (Peak 1), n-Hexadecanoic acid (Peak 2), n-Hexadecanoic acid (Peak 3), Phytol (Peak 4), Cis-13-16-Docosadienoic acid (Peak 5), Cis-Vaccenic acid (Peak 6), Octadecanoic acid (Peak 7), 2-Propanone (Peak 8) and Phthalic acid (Peak 9). From the results, Cis-Vaccenic was found to be the most abundant with the highest peak and percentage of total area.

5.2 Antimalarial Activity

It hasbeen reported that single drug are less potent against malaria infection. Effective combined therapy includes artemisinin derivatives (David *et al*., 2004; Taylor and White, 2004; Winter *et al*., 2006). However, theuse of combined therapy is limited because of inaccessibility and high cost. Plants are generally believed tobe probable candidates and alternative sources of new drugs (Zirihi *et al*., 2005).

In the *in vitro* antimalarial studies, EFAD exhibited a considerable antiplasmodial activity by inhibiting the formation of β-hematin. The phenolics, quercetin and apigenin and the Cis-vaccenic acid from the ethylacetate partitioned fraction (EFAD) identified from the gas chromatography/mass spectrometer also had considerable antiplasmodial activity. The percentage inhibition of β-hematin formation by apigenin was comparable to the standard drug chloroquine and artesunate. Artesunate was observed tohave higher percentage inhibition of β-hematin formation but its IC_{50} was higher than chloroquine. This could be as a result of the mechanism of action of artesunate which has been attributed to the generation of free radicals (Efferth *et al*., 2003) through the cleavage of its endoperoxide bridge leading to free radicals and oxidative stress (Meshnick *et al*., 1993; Posner *et al*., 1993) which may not be through inhibition of hemozoin polymerization. The mechanism of action of chloroquine against *Plasmodium* parasite hasbeen reported and established to be through inhibition of hemozoin polymerization. The result of this study is in line with other previous work reported. Chloroquine had the highest antiplasmodial activity with the lowest IC_{50} value when compared to artesunate, other phenolics and the extract.

5.3 Antioxidant Activity

5.3.1 *In Vitro* Antioxidant Activity

DPPH (1,1-Diphenyl-2-Picryl-Hydrazyl) isa free radical compound that has been used widely to determine the free radical-scavenging ability of various samples. DPPH significantly decreases upon exposure to proton radical scavengers (Yamaguchi *et al*., 1998). From the result of this study, MEAD showed ahigh radical scavenging ability with the lowest IC_{50} value of 14.62. This showed that the MEAD was abetter scavenger of DPPH radical. The radical scavenging ability ofthe extracts is concentration dependent and is comparable tothe standard, tannic acid at 400µg/ml.

The mechanism of ion-chelating activity isthe ability to chelate transition metals which can promote the Fenton reaction. From the findings of this study, EDTA hadan excellent ferrous ion-chelating capacity atall concentrations when compared tothe extracts. Among the extract, MEAD had the highest ferrous ion-chelating ability with the lowest IC_{50} value of 28.95 when compared to AEAD IC_{50} value of 38.75. The difference in IC_{50} values of both the AEAD and MEAD are statistically significant ($p < 0.05$). Thetwo extracts exhibited ferrous ion-chelating ability in a concentration dependent manner.

Hydroxyl radicals are reactive oxygen species that initiate peroxidation oflipid membranes. The hydroxyl radical ($^{\bullet}OH$) is one ofthe most damaging free radicals in the body and canbe important mediator of damage to cell structures, nucleic acids, lipids and proteins (Valko *et al*., 2007). AEAD showed higher hydroxyl radical scavenging ability than MEAD. The difference in OH radical scavenging inthe two extracts is significant ($p < 0.05$). The hydroxyl radical scavenging capability of AEAD was comparable to mannitol atall concentrations.

The reduction of Fe^{3+} to Fe^{2+} inthe presence of AEAD and MEAD was employed to determine the reductive capability ofthe extract. AEAD showed concentration dependent reducing property with the highest absorbance of 0.564 at 400µg/ml. The reducing power of a compound is mainly dueto the availability of hydrogen atoms that could be donated to afree radical and convert it to amore stable product. Through this, the chain reaction initiated bythe free radical could be effectively terminated. The reducing ability of compound may serve asa significant indicator of its potential antioxidant activity (Reichenbach *et al*., 2005). From the result, AEAD exhibited higher reducing effect thanthe MEAD. The difference inthe reducing power of AEAD and MEAD is significant ($P < 0.05$). The transformation of Iron III to Iron II in the presence of either the extract orthe standard (ascorbic acid) is ameasure of reducing

capability (Halliwell, 1991). Increased absorbance ofthe reaction mixture correlates with greater reducing power.

5.3.2 *In Vivo* Antioxidant Activity

Reactive oxygen species (ROS) are generated inmany aerobic cellular metabolic processes. They include, but not limited to, species suchas superoxide and hydrogen peroxide which react with various intracellular targets, including lipids, proteins and DNA (Cerutti, 1985). Increased levels of ROS arecytotoxic, while lower levels are necessary forthe regulation of several key physiological mechanisms including cell differentiation (Allen and Balon, 1989), apoptosis (Hockenbery *et al*., 1993), cell proliferation (Shibanuma *et al*., 1988) and regulation of redox-sensitive signal transduction pathways (Lo *et al*., 1996). Increased levels of ROS can result in ROS-induced damage including chromosomal aberrations, mutation, carcinogenesis and cell death (Cerutti, 1985).

From the results of this study, Malondialdehyde (MDA) levels of infected untreated (control) were significantly ($P<0.05$) higher inthe liver, kidney and heart when compared to the groups treated withthe extract and chloroquine. Increase in MDA level of control could be attributed to production of ROS as aresult of malarial infection. The result is also substantiated with the histopathological examinations which showed that there was avery severe periportal cellular infiltration by mononuclear cell, with adiffuse hemosiderosis in the liver of the control animals. Among the treatment groups, the MDA levels inthe chloroquine treated group were insignificantly lower than the extract treated groups inthe liver but significantly ($P<0.05$) lower inthe kidney and heart. The levels of MDA inboth the AEAD and MEAD atthe two doses were insignificant from each other inthe liver, but significantly ($P<0.05$) different in the kidney and heart.

The endogenous antioxidant enzymes function by lowering theeffect of oxygen free radicals. Cell is made up of alarge number of antioxidants thatrepair or prevent damage caused by reactive oxygen species and also controls the redox-sensitive signaling pathways. From the results, the reduced glutathione levels inthe treatment groups increased significantly when compared tothe control in all the tissues. The group treated with 400mg/kg body weight dose of MEAD as comparable with the group that received chloroquine both in the liver and kidney.

Superoxide dismutase (SOD) converts superoxide into hydrogen peroxide and molecular oxygen, while the catalase and peroxidases convert hydrogen peroxide into water. The netresult is that thetwo potentially harmful species, superoxide and hydrogen peroxide are converted to water. Administration of *A. digitata* stem bark extracts significantly ($P<0.05$) raised SOD activity inall the tissues compared to control. The lower activity of SOD observed in the control could be dueto increased ROS production. Among the treated group SOD activity of chloroquine was significantly higher than extract treated groups in all the tissues (liver, kidney and heart). The SOD activity of the group treated with 400mg/kg body weight dose of MEAD was significantly higher than the group treated with 400mg/kg body weight dose of AEAD but insignificantly different from 200mg/kg body weight dose AEAD in the liver. However, in the kidney, the SOD activity are dosage dependent with the MEAD higher than AEAD, in the heart, the SOD activity of both MEAD and AEAD are not significantly different from each other but significantly higher than the control.

More so, the catalase activity ofthe treatment groups increased significantly compared to control. Chloroquine treated group hadthe highest catalase activity in all tissues. The catalase activities ofthe extract treated group inthe liver were insignificantly different from each other except the group that received 200mg/kg MEAD. In the kidney, the catalase activities ofthe extrat treated groups are not significantly different. In the heart, catalase activities are dose dependent with MEAD higher than AEAD treated groups at the two dosages.

5.4 Haematological Parameters

The haemoglobin (Hb) concentration of the control group was significantly lower than the treatment group. The significant decreased in Hb concentration of the control suggest a state of mild anaemia. Anaemia in acute malaria is due to increase in hemolysis and decrease in the rate of production of redblood cells, increased destruction of parasitized red blood cells and removal of both parasitized and unparasitized red blood cells (Antonio and Atul, 2000). Other factors contributing to anaemia in malaria include increased redcell deformability, splenic phagocytosis and/or pooling (Angus *et al*., 1999).

From the result of this study, aninverse relationship exists between packed cellvolume (PCV) and density of malaria which isin agreement with previous work by Guinovart *et al*., (2008). The PCV value obtained in the result showed an improvement over therapeutic treatment with the extract of *A. digitata* stem bark. Further purification of the putative ethylacetate fraction of *A. digitata* could make it apromising candidate both in the antiplasmodial activity and abetter PCV booster in malarial treatment. The density and/or proliferation rateof parasites is analoguos tothe state of multicellular organisms. The decrease of parasitemia density and the increase in PCV reflect the development of naturally acquired immunity against malaria. The mean PCV difference between treated and untreated animals suggests that malaria is agreater contributor to anaemia (Aponte *et al*., 2007).

The white blood cell (WBC) count of infected untreated animals was significantly higher than the extract and chloroquine treated groups. Significant increase in the total WBC count of the untreated group could be as aresult of increased production in order to fight infection. Moreso, high WBC count may result in production of injurious cytokines that may cause tissue damage (Charache *et al*., 1996). However, the decrease in total WBC by the extracts suggests that it may be immunosuppressive. The reduction in WBC could be due to reduced production of

white blood cells, redistribution of white blood cells from peripheral blood into the tissues or rapid destruction of white blood cell (Guyton and Hall, 1996).

Platelet abnormalities in malaria represent both qualitative and quantitative change. The observed significant reduction in platelet count in both the 200mg/kg and 400mg/kg body weight dose of AEAD and 400mg/kg body weight dose of MEAD when compared to control is an indication of their in vivo antimalarial potential. The association of platelet count and malaria infection has previously been described (Gerardin *et al*., 2002; Maina *et al*., 2010). The significant increase in platelet count of chloroquine treated group suggests its positive action on the production of thrombopoietin, which has been reported to be a primary regulator of platelet production. Platelets havebeen shown to mediate clumping of *P. falciparum* infected erythrocytes (Pain *et al*., 2001).

Neutrophil and lymphocyte counts were the most important leukocyte changes associated with malaria infection. The result of this study showed a significant reduction in percentage neutrophil (neutropenia) in the control when compared to the extract treated groups. This could be as a result of decreased level of production of neutrophil or increased removal from the system by macrophages and also parasitic infection. The observation from this study agrees with the work of Chen *et al*., (2005).

The decreased in percentage lymphocyte count was observed in untreated (control) group when compared to chloroquine MEAD at 200mg/kg dose. The observed decrease may be due to redistribution of lymphocytes with sequestration in the spleen (Erhart *et al*., 2004).

Increase in percentage monocytes count was observed in the control group when compared to the treatment groups. The increase is usually associated with infectious protozoa diseases. Mononuclear cells, which are activated by plasmodium during malaria infection, produce inflammatory cytokines liketumor necrosis factor (TNF) which stimulate the hepatic

synthesis of acute phase inflammatory proteins including C-reactive protein (CRP) which increase during malaria infection (Wickramasinghe and Abdalla, 2000).

From the result of this study, there was a significant increase in the mean cell volume (MCV) and mean corpuscular haemoglobin (MCH) of the control group compared to the extract treated groups. However, there was a reduction in the mean corpuscular haemoglobin concentration (MCHC) of the control group when compared to the treatment groups. The increase in MCV and MCHC are synonymous with red blood cell dehydration (Brugana and Tosteson, 1987). There is no evidence that relate higher level of MCV, MCH and MCHC and pathogenesis of malaria infection but it hasbeen suggested that people with α-thalassaemia protected malaria infection by a direct interaction between the parasite and the altered thalassaemia erythrocyte, resulting in reduced parasite load (Nagel and Roth, 1989). Most studies showed that α-thalassaemia homozygotes havebetter protection against severe malaria anaemia compared to heterozygotes (Williams *et al*., 2005; May *et al*., 2007).

5.5 Inflammation markers

The role of CRP in defence against infection in humans was related toits ability to bind to phosphorylcholine in membrane of microorganisms (Ballou and Kushner, 1992). CRP is a marker of mobidity and mortality inmalaria. It correlates closely with other complications in malaria and canbe used to predict severity. Inflammation is animportant part of acute phase response during infection. CRP isan acute phase protein that is involved in the activation of complement, acceleration of phagocytosis and detoxification ofsubstances related from the damaged tissues. From the result of this study, the serum concentrations of CRP inthe control animals increased significantly when compared to the baseline (uninfected mice) and the extract treated groups. When the inflammation or tissue destruction is resolved, CRP levels fall, making it a useful marker for monitoring disease severity (Young *et al*., 1991).

The observation from this study agrees withthe previous work by Paul *et al*., (2012). A significant relationship exists between serum CRP concentration and percentage parasitemia in the control group. The significant increase of CRP concentrations is anindication of inflammatory reaction during malaria (Hurt *et al*., 1994; Mansor *et al*., 1997). CRP is secreted so as tofight against *Plasmodium* parasite invasion (Volanakis, 2001).

Tumor necrosis factor (TNF) is a member of a family of structurally related cytokines that signal through specific cell-surface receptors that also form astructurally related family of proteins (Ware, 2003). TNF plays animportant rolein various immune and inflammatory processes, including cellular activation, survival and proliferation, as well as cell death by necrosis and apoptosis. Pro-inflammatory cytokines, especially TNF-α havebeen linked with severe malaria disease and are still vital for the initial control of parasitemia in human. The result of this study showed significant increase in serum concentrations of TNF-α in the control (untreated) mice when compared to the baseline (uninfected) mice and the groups treated with the extract of *A. digitata* stem bark. The mean serum concentrations of TNF-α in this study is however in consonant with the work reported by Luty *et al*., (2000). Elevated serum concentrations of TNF-α havebeen reported during malaria and highTNF-α concentrations correlate strongly with increasing severity of disease (Shaffer *et al*., 1991).

5.6 Liver Function

Serum contains enzymes, water, proteins, glucose, amino acids, lipids salts, antigen, hormones, antibodies and urea. Small alteration in tissue composition could significantly affect serum metabolites and enzyme levels. Measurement of theseenzymes is therefore valuable in clinical diagnosis, providing important information about the extent and nature of pathological damage to any tissue. From theresult of this study, significant ($p<0.05$) increase inalkaline phosphatase (ALP) activity of control group wasobserved in serum, liver and kidney. The control

(untreated) mice had significant ($p<0.05$) increase in serum ALP activity when compared to the groups that receive the extract. The tissue ALP activity in the control was also observed tobe lower than that of the Serum. The host liver is reported tobe affected in the early stage of *falciparum* malaria which results in significant modification in host hepatocyte morphology and physiology. The observed increase in serum ALP activity asobserved from the result is an indication of significant modification in the hepatocytes membrane leading to leakage of this enzyme out of the liver cells.

An increase in serum of these intracellular enzymes isan indication of cell damage. This is supported with the histopathological results which showed that there was avery severe periportal cellular infiltration by mononuclear cell, with adiffuse hemosiderosis inthe liver of the control (untreated) mice. The levels of parasitemia in the control show adirect relationship with the mean liver enzyme activity.

However, the alanine aminotransferase (ALT) activity ofthe control mice was significantly lowered in the serum, liver and kidney when compared to the groups that receive the extract. Among the treatment groups, chloroquine had the highest ALT activity both in the serum, liver and kidney. The ALT activity in the extract treated groups increased in dose dependent manner and the increase were observed significant.

Also, in the same manner, with ALT, the aspartate aminotransferase (AST) activity of the infected untreated (control) mice reduced significantly in the serum, liver and kidney when compared tothe extract treated groups. Chloroquine treated group also hadthe highest AST activity in the serum, liver and kidney when compared tothe groups that received the extract.

The total protein content of the infected untreated mice was also found tobe significantly lowered in the tissues when compared to the treated groups. The group that received chloroquine also hadthe highest total protein content which was significant when compared to other groups that received the extract.

5.7 Renal Function

The mechanism of renal failure in *falciparum* malaria is not known. Several hypotheses including mechanical obstruction by infected erythrocytes, fluid loss due to multiple mechanisms and alterations in the renal microcirculation etc. have been proposed (Eiam-Ong and Sitprija, 1998; Barsoum, 2000). From this study, the mean concentration of serum creatinine in parasitized untreated mice was significantly ($p<0.05$) higher than the chloroquine and extract treated groups. The differences in the serum creatinine concentrations of the extract treated groups were not significant ($p>0.05$). The result of this study is line with the work of Prakash *et al*., (2003) and Bagshaw *et al*., (2008) who reported that parasite density correlated positively with serum creatinine concentrations. Increase in serum or plasma creatinine concentration could be due to ineffective filtering ability of the kidney which could to renal function impairment. The result suggests compromise in the normal functioning of the kidney by the *P. berghei* malarial infection. This observation with experimental mice agrees with earlier findings in human patients infected with *P. falciparum* (Ekeanyanwu and Ogu, 2010).

The mean concentration of serum urea in the control was significantly lowered than the extract treated groups. The mean serum uric acid concentration in the control group was also observed to be lowered but not significantly different from the groups treated with chloroquine and 200mg/kg body weight AEAD. This significant reduction may be attributed tothe fact that plasma urea level is affected by a number of non-kidney related factors such as dehydration, food intake and tissue catabolism (WHO, 2000). Renal involvement in malaria istypically associated with severe disease, shock and electrolyte and hemodynamic disturbances (English *et al*., 1996; Maitland *et al*., 2003).

The result of this study is inline with the work of Sheiban, (1999) who reported that the mortality associated with severe renal failure commonly occurs inyounger children and those

with elevated creatinine levels and reduce urine output. Alterations in electrolyte metabolism are an important biomarker of malaria-associated disturbance in mineral homeostasis. Correction of fluid volume and electrolyte deficits hasbeen the standard care for any critically ill patients including those with severe malaria infection. This is because acidamia, hypokalemia, hypocalcemia and hyponatremia exacerbate myocardial dysfunction and increase the risk of arrhythmias (Khilnami, 1992; Kumar *et al*., 2001). Several studies havereported that hyponatremia is associated with malaria. Mild and severe hyponatremia havebeen described in *P. falciparum* malaria infection (Jasani *et al*., 2012; Das *et al*., 2014). This may be dueto hypovolemia following vomiting and diarrhea and decrease oral fluid and food intake typical of malaria infection. However, the result of this study is in contrast with the work of Jasani *et al*., (2012) and Das *et al*., (2014). There was significant ($p<0.05$) increase in sodium ion (Na^+) level in the parasitized untreated (control) mice when compared to the extract treated groups.

Reduced potassium ion (K^+) levels have alsobeen reported in malaria infection (Ikekpeazu *et al*., 2010; Jasani *et al*., 2012). Reduction in K^+ levels hasbeen attributed to loss of host potassium content during the course of malaria infection. Metabolic alkalosis has also been identified as a complication of malaria wherein K^+ is transferred from extracellular fluid (ECF) to the cell. Also, the result of this study is in contrast with earlier study by Ikekpeazu *et al*., (2010) and Jasani *et al*., (2012). There was significant ($p<0.05$) increase in the K^+ level in control mice when compared to the extract treated groups. However, the result is in consonant with the work of Maitland *et al*., (2005) who reported that severe hyperkalemia is associated with complicated malaria and enhanced mortality.

Increase serum calcium ion (Ca^{2+}) level was observed in the control mice compared to those treated with the extract. The increase in Ca^{2+} level in the control was not significantly different from the extract treated groups. Intra-erythrocytic calcium levels havebeen reported to be substantially increased in parasitized red blood cells (Krishna and Ng, 1989). From the

observation of hypercalcemia in this study, therefore suggest that increase in serum calcium levels may result from the intracellular release of calcium secondary to the predictable erythrocyte lysis dueto malaria infection. According to Maitland *et al*., (2005), metabolic acidosis, which is one of the complications of severe malaria leads to reabsorption of calcium from the bone leading to high serum calcium levels. In contrast to the findings of this study, hypocalcemia has been demonstrated in severe malaria infection (Singh and Singh, 2012).

Significant reduction in serum magnesium ion (Mg^{2+}) was observed in the control compared to the treated groups. This finding is consistent with previous findings (English *et al*., 1996). Diarrhea and vomiting which are the features of malaria infection hasbeen described as the common cause of loss of water and electrolytes (Miller *et al*., 2002).

Also the result of this study showed significant ($p<0.05$) increase in serum chloride ion (Cl^-) in the control mice compared to the group treated with the extract. This observation contradict the earlier report by Akpan *et al*., (2011) who reported low level of serum chloride in malaria infection.

5.8 Mitochondrial Membrane Permeability Transition

Permeability transition (PT), occur as a result of the opening of a voltage-dependent, high conductance channel regarded as the permeability transition pore located in the inner mitochondrial membrane. It is a sudden increase of inner mitochondrial permeability to solutes with molecular mass up to 1.5KDa. PT is implicated in apoptosis or necrosis as animportant event in the control of cell death or survival (Zoratti and Zabo, 1995; Ling *et al*., 2010).

Mitochondrial membrane permeability transition pore (MMPT) hasemerged as a promising target for diverse pharmacological interventions due tothe release of cytochrome c upon the opening of the pore which leads toinduction of apoptosis. Recent report suggest that generation of reactive oxygen species (ROS) and associated oxidative stress play crucial role in the development of systemic complication in malaria (Pabon *et al*., 2003). It hasbeen reported

that malaria infection decreases the levels of antioxidant enzymes and other antioxidants such as catalase, glutathione peroxidase, superoxide dismutase, albumin, reduced glutathione (GSH), ascorbate and plasma tocopherol (Clark et al., 1989).

The observation in the previous studies isin agreement with the findings of this study. From the results, *Plasmodium berghei* infection in mice induces oxidative stress in the liver, as evidenced from decreased cellular GSH, catalase and SOD concentrations or activities and increased lipid peroxidation. The decrease of these enzymes concentrations or activities correlated well with the degree of parasitemia inthe infected untreated mice. Lipid peroxidation in the liver of infected untreated mice also increased with thedegree of parasitemia. The concentrations of C-reative protein (CRP) and tumor necrosis factor-alpha (TNF-α) also increased significantly in the parasitized untreated mice, indicating an inflammatory reactions due to production of ROS and free radicals during malaria infection.

Reactive oxygen species, in addition to imparting oxidative insult by damaging structural and functional components of cellular systems, could also induce apoptosis (Sarafian and Bredesen, 1994; Czaja, 2002). Apoptosis, also regarded as programmed cell death is associated intimately with both physiology aswell as pathology in variety of cellular systems (Zimmermann *et al*., 2001). The programme cell may be either caspases-dependent or caspases-independent (Cande *et al*., 2002). The caspases-dependent apoptosis is mainly mediated through two distinct pathways, viz: the death receptor pathway (extrinsic) and the mitochondrial pathway (intrinsic). According to Simo *et al*., (2000) and Czaja, (2002), generation of ROS and associated oxidative stress hasbeen found to activate mitochondrial pathway of apoptosis. The key factor responsible for mitochondrial permeability transition pore opening is mitochondrial calcium overload especially when accompanied or coupled by free radicals and oxidative stress, adenine nucleotide depletion, elevated phosphate concentrations, mitochondrial depolarization, release of

intra-mitochondrial solutes, large amplitude swelling and outer membrane rupture (Crompton, 1999).

The results showed that calcium ions induced the opening of MMPT pore significantly ($P<0.05$) in rat liver mitochondria, while spermine inhibited calcium-induced opening of MMPT pore. Spermine hasbeen shown toprotect mitochondria from damage and thatthis protective effect reflects inhibition ofthe inner membrane permeability transition (Lapidus and Sokolove, 1993). Toninello *et al*., (1999) also showed that spermine blocks the collapse of membrane potential and release of Mg^{2+}, adenine nucleotides and Ca^{2+} observed when mitochondria are exposed to damaging concentrations of inorganic phosphate and Ca^{2+}. Ethylacetate extract fraction of *A. digitata* stem bark (EFAD) and artesunate (standard drug) inhibited the pore opening in the absence of calcium. However, in the presence of calcium, EFAD and artesunate significantly ($P<0.05$) induced the opening ofthe pore in a concentration dependent manner (88.76%, 88.51%, 86.19% and 85.57%0 respectively for EFAD and (90.71%, 90.33%, 90.27% and 90.09%) respectively for artesunate. The lowest concentration 5µg/ml was observed tohave the highest inductive effect in both the artesunate and EFAD which means with increase in dose, there was adecrease MMPT pore opening. This suggests that the cytotoxic property of the extract could be concentration dependent.

It was reported by earlier studies thatthe antimalarial activity of artemisinin and its derivatives depend on the cleavage of an endoperoxide bridge leading tothe production of carbon-centered free radicals and oxidative stress (Meshnick *et al*., 1999; Posner *et al*., 1993; Anyasor *et al*., 2009). Artemisinin could also react with iron or heme to generate free radicals (Wang *et al*., 2010). Also, Efferth *et al*., (2003) reported that the potent antimalarial activity of artesunate is attributed toits ability to generate free radicals. Interestingly, free radical and oxidative stress hasbeen implicated in mitochondrial membrane permeability transition pore opening (Danial and Korsmeyer, 2004). The opening of this pore is the point of noreturn for

apoptosis. Apoptosis, which is an important event in cell development, immune system, homeostasis and cell termination, is an appropriate defence system for the host to block infection by intracellular pathogen at avery early stage. It is highly specific and does not provoke inflammatory responses (Blaho, 2004).

The observation from this study suggest that ethylacetate extract fraction of *A. digitata* stem bark (EFAD) may contain certain bioactive compound or chemical group that influence mitochondria membrane permeability by causing the ROS-mediated opening of the pore. This property could be useful in pathological conditions that require an increased rate of apoptosis. More so, with the effect of EFAD on mitochondrial membrane permeability transition, the opening of the and subsequent induction of apoptosis could be one of its mechanism of action against malaria infection in addition to its considerable inhibition of β-hematin formation (hemozoin) as observed in the in vitro cell free study.

In the same manner, quercetin inhibited the opening of the pore in the absence of Ca^{2+} but significantly induced the opening of MMPT pore in the presence of Ca^{2+} in aconcentration dependent manner with the lowest concentration also having thehighest inductive effect. However, the effect of apigenin on MMPT was observed tobe different from the EFAD and quercetin. In the absence of exogenous Ca^{2+}, apigenin significantly induced the opening of MMPT pore in aconcentration dependent manner. It was observed thatthe inductive effect of apigenin was significantly higher than that caused by Ca^{2+} only. The percentage induction of apigenin at 80μg/ml is 91.82% while that of Ca^{2+} is 88.28%. apigenin further induced the opening of the pore significantly in the presence of Ca^{2+} in a concentration dependent manner when compared to the Ca^{2+} only.

5.8.1 Mitochondrial ATPase

Mitochondrion which is regarded as the site of oxidative phosphorylation is also reported as the site of ATP synthesis via f_1f_0 ATP synthase. ATP synthase (F-type ATPase) is a molecular machine that uses proton motive force to pump protons downhill the gradient. The chemiosmotic coupling hypothesis explains that the electron transport chain and oxidative phosphorylation are coupled by a proton gradient across the inner mitochondrial membrane. Intact mitochondrion synthesizes ATP but if not intact hydrolyzes ATP resulting in the release of ADP and inorganic phosphate. It hasbeen reported that under certain conditions, protons can re-enter the mitochondrial matrix without contributing to ATP synthesis. This process is regarded as proton leak or mitochondria uncoupling which is due to the facilitated diffusion of protons into the matrix. The process results in the unutilized potential energy of the proton electrochemical gradient being released as heat (Taylor *et al*., 2003).

From the results of this study, the electron transport chain uncoupler, 2,4-dinitrophenol significantly uncoupled the mitochondria when compared tothe ethylacetate extract fraction of *A. digitata* (EFAD) and control. The ATPase activity of EFAD increased significantly in a concentration dependent manner. The mitochondrion was significantly uncoupled at higher concentration of the extract. The induction of mitochondrial ATPase activity by the extract is a confirmation of its inductive effects in the opening of MMPT pore.

5.9. Molecular docking

Protein–ligand docking is used to predict the position and orientation of a ligand when it is bound to a protein receptor or enzyme. Pharmaceutical research employs docking techniques for a variety of purposes, mostly in the virtual screening of large databases of available chemicals in order to select likely drug candidates. In order to understand the mechanism of ligand binding and to identify potent calcium transporter inhibitors, a study involving molecular

docking and virtual screening was performed. Out of three chemical constituents, apigenin and quercetin has the highest docking score along with the highest number of hydrogen bonds formed while the cis-vaccenic acid has the least docking score with the least hydrogen bonds. Analysis of the results of the Autodock software shows that apigenin, quercetin and cis-vaccenic acid have a considerable binding affinity with the calcium transporters.

CONCLUSION

Based on the findings of this study, a significant relationship was obtained between phenolic content and antioxidant activity of *Adansonia digitata* indicating that phenolic compounds contribute significantly to antioxidant activity of the plant. Administration of *Adansonia digitata* stem bark extract after established infection reduced parasite progression. Consequently, a reduction in serum c-reactive protein and tumor necrosis factor-α occurred. The results showed that *Adansonia digitata* stem bark extract offers protection from malarial infection by reducing tissue peroxidation and increasing endogenous antioxidant status. The stem bark extract and fractions of *Adansonia digitata* has antimalarial property which can be explored for use against malarial infection. The antimalarial activity in *Adansonia digitata* stem bark was due to cis-vaccenic acid, quercetin and apigenin (4',5',7-trihydroxyflavone, 5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one). These bioactive compounds from *Adansonia digitata* influence mitochondria membrane permeability transition by inducing cell death through mitochondria-mediated pathway of apoptosis and also increased the activity of mitochondrial ATPase. The results from this study therefore suggest that the extract of *Adansonia digitata* could be a potential source of natural antioxidant that could be of great importance for the treatment of malaria.

SUMMARY OF RESULTS

i. AEAD and MEAD contained tannins, alkaloid, saponins, cardiac glycosides, flavonoids and terpenoids with saponins (13.00mg/100g GAE and 12.30mg/100g GAE) being the highest respectively.

ii. Parasitemia significantly ($P<0.05$) increased the serum concentrations of CRP, TNF-α, serum and tissue ALP, MDA, WBC, creatinine, monocyte whereas haemoglobin, protein were reduced.

iii. Parasitemia-treatment related changes in biomolecules were significantly reversed with most profound activity at 400 mg/kg BW MEAD.

iv. MEAD ameliorated/restored *Plasmodium berghei*-induced haematological, liver and kidney function indices, malondialdehyde and histoarchitectural changes in the animals.

v. Inhibition of β-hematin formation by ethylacetate solvent-partitioned fraction, phenolics; quercetin and apigenin and cis-vaccenic acid from the EFAD were significantly lower than chloroquine.

vi. Ethylacetate solvent-partitioned fraction and the phenolics; quercetin and apigenin significantly induced the opening of mitochondrial membrane permeability transition pore in the presence of calcium and also increased the activity of mitochondrial ATPase.

CONTRIBUTIONS TO KNOWLEDGE

i. The bioactive compounds; cis-vaccenic acid, quercetin and apigenin (4',5',7 trihydroxyflavone, 5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) were identified from *Adansonia digitata* stem bark extracts. These bioactive compounds from *Adansonia digitata* influence mitochondria membrane permeability transition by inducing cell death through mitochondria-mediated pathway of apoptosis.

ii. The inductive effects of *Adansonia digitata* stem bark extract on mitochondrial membrane permeability transition pore suggest that it may prove useful in situations where increased rate of apoptosis is required for chemotherapy.

iii. Opening of MMPT pore and induction of apoptotic process could likely be one of the mechanisms of action of antimalarial compound.

iv. The bioactive principles may be formulated as antimalarial drug.

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APPENDICES

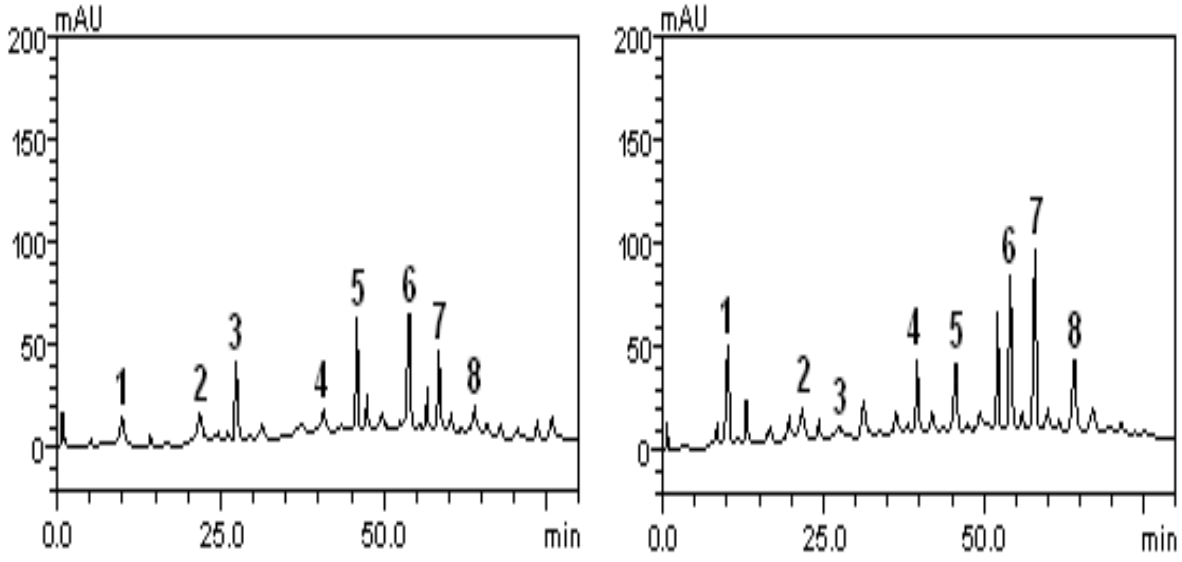

Figure A[†]: Representative high performance liquid chromatography profile of crude aqueous (AEAD) and methanolic (MEAD) extract from *Adansonia digitata* stem bark at a detection UV of 325nm.

[†] Gallic acid (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3), rutin (peak 4), quercitrin (peak 5), kaempferol (peak 6), luteolin (peak 7) and apigenin (peak 8).

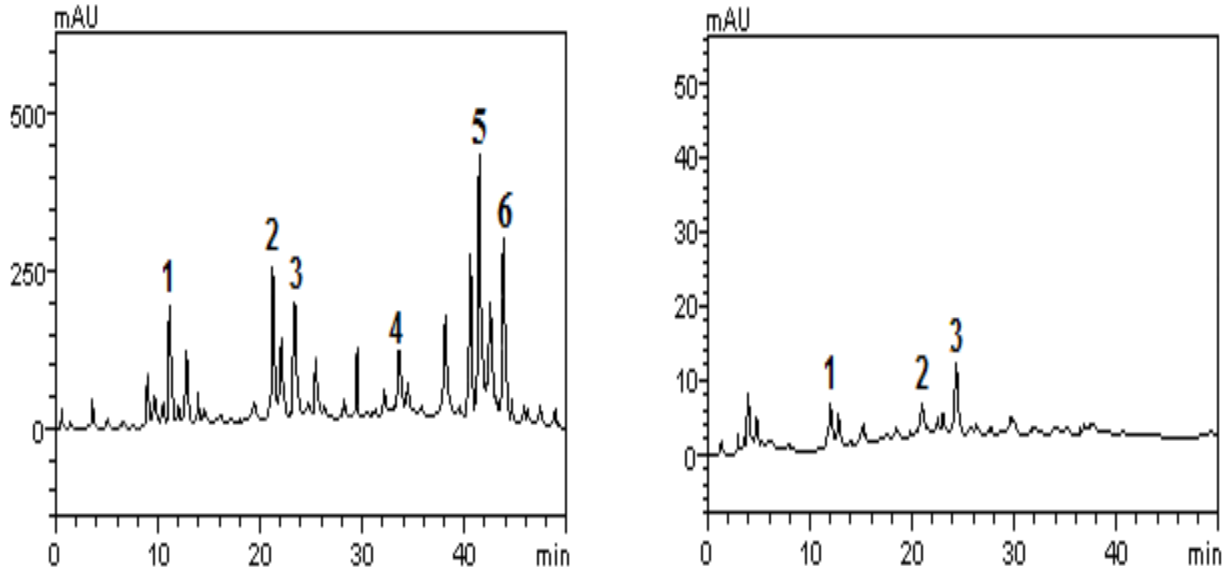

Figure B[†]: Representative high performance liquid chromatography profile of ethylacetate partitioned fraction (EFAD) and chloroform partitioned fraction (CFAD) from *Adansonia digitata* stem bark at a detection UV of 325nm.

[†]Gallic acid (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3), rutin (peak 4), quercetin (peak 5) and luteolin (peak 6).

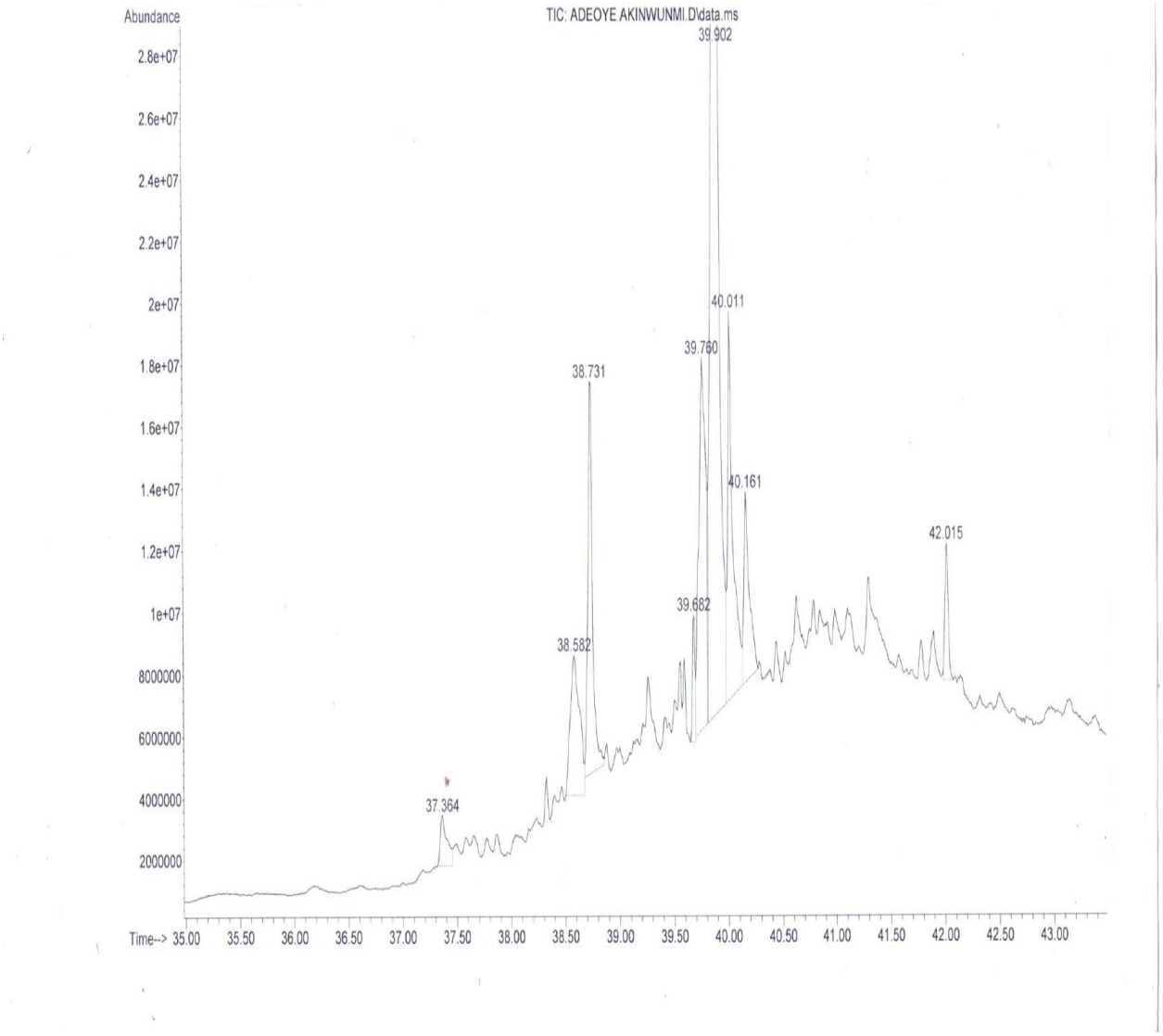

Figure C[†]: Gas chromatography mass spectrometry profile of ethylacetate partitioned extract fraction (EFAD) from *Adansonia digitata* stem bark.

Bicyclo (3.1.1) Heptane (Peak 1), n-Hexadecanoic acid (Peak 2), n-Hexadecanoic acid (Peak 3), Phytol (Peak 4), Cis-13-16-Docosadienoic acid (Peak 5), Cis-Vaccenic acid (Peak 6), Octadecanoic acid (Peak 7), 2-Propanone (Peak 8) and Phthalic acid (Peak 9).