ANTIMALARIAL POTENTIAL OF LEAF AND STEM BARK FRACTIONS OF METHANOL EXTRACT OF *DIOSPYROS MESPILIFORMIS* ON *PLASMODIUM BERGHEI* (NK 65) INFECTED MICE

BY

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(M.Sc/SCIE/5438/2011-2012)

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A THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL, AHMADU BELLO UNIVERSITY, ZARIA, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE (M.Sc) IN BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY
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ZARIA, NIGERIA

JANUART, 2016

Declaration

I declare that the work in this thesis entitled ``**Antimalarial potential of leaf and stem bark fractions ofmethanol extract of *Diospyros mespiliformis* on *Plasmodium berghei* (NK-65) infected mice.**'' has been carried out by me in the department of biochemistry under the supervision of Prof. H.C. Nzelibe and Mr. O.A. Owolabi. The information derived from the literature has been duly acknowledged in the text and list of references provided. No part of this thesis was previously presented for another degree or diploma at this or other institute.

OGUCHE MERCY	____________________	____________________
Name of Student	Signature	Date

CERTIFICATION

This thesis entitled **''ANTIMALARIAL POTENTIAL OF LEAF AND STEM BARK FRACTIONS OF METHANOL EXTRACT OF *DIOSPYROS MESPILIFORMIS* ON *PLASMODIUM BERGHEI* (NK 65) INFECTED MICE.''** by Oguche Mercy meets the regulation governing the award of Master of Science of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

Professor H.C. Nzelibe	__________________	__________________
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ABSTRACT

Antimalarial potential of various fractions of the methanolic extract of the leaf and stem bark of *Diospyros mespiliformis* on *Plasmodium berghei* (NK-65) infected mice and the inhibitory effect of the extracts on cysteine protease of the parasite were investigated. The oral median lethal dose (LD_{50}) of the leaf and stem bark extracts were determined to be 400 and 200mg/kg body weight respectively. At the end of the four (4) days suppressive test,the effect of the fractions of leaf and stem bark methanol extracts on parasitaemia suppressive activity were dose-dependent for various fractions and n- butanol fraction having the highest suppressive activity. The effect of the leaf methanol extract on Liver marker enzymes (Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT)) showed significant ($P<0.05$) increase in the n-butanol and aqueous fractions and significant ($P<0.05$) increase in the serum level of (Alkaline phosphatase (ALP)) of the infected group but the stem bark extract showed significant ($P<0.05$) increase in the infected group of all the fractions and significant ($P<0.05$) decrease in the infected but treated with low and high dose of the extract. Packed cell volume and Haemoglobin concentration showed significant ($P<0.05$) decrease in the infected group compared to normal control and infected treated group. The enzyme was purified to 12.25-fold and a yield of 35.69% on Sephadex G-50 column with a final specific activity of 47.30µmol/min. The inhibitory effect of the bioactive fractions of (leaf and stem bark), on the enzyme (cysteine protease) was dose dependent in which the leaf and stem bark extracts exhibit mixed non-competitive and competitive pattern of inhibition respectively. These compounds could serve as a good source of antidote for malaria and could as well guide the design of a novel drug without side effects.

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LIST OF ABBREVIATIONS AND SYMBOLS

ALT	-	Alanine aminotrasferase
AST	-	Aspartate aminotrasferase
ALP	-	Alkaline phosphatase
ANOVA	-	Analysis of variance
Hb	-	Heamoglobin concentration
PCV	-	Packed cell volume
%	-	Percentage
WHO	-	World Health Organisation
HIV	-	Human Immunodeficiency Virus
AIDS	-	Acquired Immunodeficiency Syndrome
RBC	-	Red blood cell
RNA	-	Ribonucleic acid
EC	-	Enzyme Commission
DNA	-	Deoxyribonucleic acid
ATP	-	Adenosine Triphosphate
PFK	-	Phoshofructo-1 kinase

SBTI - Soybean Trypsin Inhibition

SBBI - Soybean Bowman Brik Inhibition

PBS - Phosphate Buffer saline

AOAC - Association of Official Analytical Chemists

BAPNA - Benzyl Arginine Paranitroanilide

$_{P}H$ - Hydrogen ion concentration

EDTA - Ethylene diaminetetraacetic acid

PLANT-Pls - Plant inhibitor

H_2SO_4 - Tetraoxosulphate IV acid

KOH - Pottasium hydroxide

NaOH - Sodium hydroxide

GPT - Glutamic-pyruvic transaminase

GOT - Glutamic-oxalo transaminase

Hcl - Hydrochloric acid

BSA - Bovine Serum Albumin

CHAPTER ONE

1.1 INTRODUCTION

Malaria is a mosquito borne disease caused by the parasite *Plasmodium*. It is one of three globally important infectious diseases, including tuberculosis (TB) and HIV/AIDS (Coppi *et al*, 2006). It remains a major global health problem with 2.4 billion people living in areas at high risk of infection (Miura *et al*., 2009). The World Health Organization has estimated that in 2010, there were 216 million documented cases of malaria (WHO, 2010). Malaria results in mortality and morbidity around the world (Ku *et al.*, 2011) with an estimated 225 million malaria cases and 781,000 deaths being reported globally in 2009 (USAID, 2011).

Blood parasite protozoa are causative agents in some of the major tropical animal diseases for human and animals. Some examples include *Plasmodium* for malaria. *Plasmodium* infects more than 300 million people and results in the death of more than one million people annually. It has been estimated that nearly half of the earth's population is at the risk for contracting this deadly illness. Malaria begins with a bite from an infected female Anopheles mosquito, which introduces the protists via its saliva into the circulatory system, and ultimately to the liver where they mature and reproduce. The disease causes symptoms that typically include fever and headache, which in severe cases can progress to coma or death. Malaria is widespread in tropical and subtropical regions, in a broad band around the equator, including much of Sub-Saharan Africa, Asia, and the Americas (Trampuz *et al*,2003).

Human malaria is caused by five different species of the protozoan parasite Plasmodium: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale Plasmodium knowlesi and Plasmodium malariae,* all of which are transmitted by the female Anopheles mosquito (Hardman *et al,* 2001) .Although all the five species of malaria parasite can infect and cause illness, only the malaria caused by *Plasmodium falciparum* is known to be potentially life threatening in humans. Infection with *P.falciparum* is therefore a medical emergency (Martin *et al* 2004).The severity of *P.falciparum* infections is due to high *percentage* of red blood cells (RBCs) that are infected by this particular *Plasmodium* (Bruxvoort *et al,* 2014). In extreme infections, up to 80% of RBCs can be parasitized and destroyed. This massive red cell destruction has two primary effects: (i) severe anaemia and (ii) Clogging of the circulation to vital organs, particularly the brain and kidneys. The circulatory clogging occurs because the infected RBCs produce sticky projections that bind the cells of the walls of the small blood vessels (capillaries) and to other RBCs, forming obstructing clumps of cells called rosettes. Also, chemicals called cytokines are released, causing fever, malaise and other signs of inflammation (Kain *et al,* 1998). In endemic areas, it is estimated that about 1% of patients, mostly children and pregnant women, with *P. falciparum*infection die of the disease. Plasmodium berghei was first described by Vincke *et al* (1948) and *Plasmodium berghei*NK-65 strain was isolated from Anopheles dureni millecampsi in 1964.*Plasmodium berghei*, a rodent parasite, a valuable model organism for the investigation of human malaria as the organisms are similar in most essential aspects of morphology, physiology and life cycle of these parasites is simple and safe (Laar *et al*, 2015).*Plasmodium berghei's* genetic map has been completed, bringing hope that further understanding of all malarias will be possible, and that elements that cause berghei to be noninfectious to humans can be brought into play to deal with other malarias (Janse *et al*., 2006).

All the *Plasmodium* species causing malaria in humans are transmitted by mosquito species of the genus *Anopheles*. Genus of mosquito include; *Aedes, Culex, Culiseta, Mansonia*and*Theobaldia* which transmit malaria but not to humans (Wersndorfer *et al*,1999).

Despite the substantial progress made in the treatment of parasitic diseases, malaria remains a significant therapeutic challenge especially because of the wide spread resistance of malaria parasites to currently available anti-malarial agents, the resistance of the mosquito vectors to currently available insecticides, the limited success in the development of malarial vaccines and the debilitating adverse reactions of conventional anti-malarial drugs. These have stimulated the search for new pharmacologically active agents that can overcome these barriers (Pretorius *et al*, 2013). There is a long standing tradition for the use of phytomedicines for the treatment of malaria. The plant kingdom remains a major target in the search of lead compounds and new drugs to treat this debilitating parasitic disease. Quinine isolated from *Cinchona* and quinghaosu from *Artemisiaannua* L. for instance illustrates the potential value of herbal medicines for development of antimalarial drugs. A major advance in the search for effective treatment for drug-resistant malaria came with the discovery of artemisinin and its derivatives. Severe malaria is treated with intravenous or intramuscularquinine or, since the mid-2000s, the artemisinin derivative artesunate, which is superior to quinine in both children and adults and is given in combination with a second anti-malarial such as mefloquine(Pretorius *et al*, 2013). Resistance has been developed to several antimalarial drugs; for example, chloroquine-resistant *P. falciparum* has spread to most malarial endemic areas, and emerging resistance to artemisinin has become a problem in some parts of Southeast Asia. Artemisinin drugs, which originated from the Chinese herb qing hao (*Artemisia annua*), belong to a unique class of compounds, the sesquiterpene lactone endoperoxides. The parent compound of this class is artemisinin (quinghaosu), whereas dihydroartemisinin (DHA), artesunate, artemether, and β-artemether are the most common derivatives of artemisinin; DHA is the main bioactive metabolite of all artemisinin derivatives (artesunate, artemether, β-arteether, *etc.*), and is also available as a drug itself (Bruxvoort *et al*, 2014). The absence of efficient vaccine against these diseases, the absence or the high toxicity of the few drugs against America and Africa and the emergence of chemoresistance against plasmodium falciparum emphasize the necessity to produce new antiparasitic strategies.

Plasmodium falciparum has developed resistance to nearly all antimalarial drugs in current use, although the geographic distribution of resistance to any one particular drug varies greatly. In particular, Southeast Asia has a highly variable distribution of falciparum drug resistance; some areas have a high prevalence of complete resistance to multiple drugs, while elsewhere there is a spectrum of sensitivity to various drugs. The change in the malaria burden trend is a result of the interventions (such as, mechanical forms of protection such as the use of insecticide treated nets, administration of combination drug therapy regimens such as Arthemisin Combination Therapy's (ACTs) (MoHSS, 2009) and Indoor residual spraying) implemented by the malaria campaign 'Wipe out Malaria', established by the Ministry of Health and Social services (MoHSS) (WHO, 2010).

Diospyros mespiliformis, also known as African Ebony is a large deciduoustree found mostly in the savannas of Africa. Jackals are fond of the fruits; hence the common names. Mature trees have dark gray fissured bark. An adult tree reaches an average of 4 to 6 metres in height, though occasionally trees reach 25 metres. The foliage is dense and dark green with elliptical leaves, which are often eaten by grazing animals such as elephants and buffalo. The tree flowers in the rainy season; the flowers are imperfect, with genders on separate trees, and are cream-colored. The female tree bears fruit in the dry season and these are eaten by many wild animals; they are oval-shaped, yellow and about 20-30mm in diameter. When the fruits ripen they turn purple. The ethnopharmacological uses of different organ of *Diospyros* *mespiliformis*have been reported e.g. leaf decoctions are used against fever, whooping cough and wounds (Adzu *et al*, 2009). Pulvirised roots are taken to treat jaundice and root concoction as antihelmintic to ease childbirth and to treat bronchial diseases, prepared leaves are extremely applied to treat fever, pneumonia, conjunctivities and serve as antiseptic to wounds. *Diospyros mespiliformis* posses valuable medicinal properties and has been studied for various activities like ant-diabetic, antihelmintic, analgesic and anti-inflammatory activities. With the knowledge about this plant, it is necessary to subject this plant to detailed scientific studies, to determine its antimalarial potential and its efficacy in the treatment of malaria.

Cysteine proteases are so-named due to the function of a catalytic cysteine, which mediates protein hydrolysis via nucleophilic attack on the carbonyl carbon of a susceptible peptide bond. Cysteine protease plays important roles in the life cycles of malaria parasites. Cysteine protease inhibitors block haemoglobin hydrolysis and development in *Plasmodium falciparum*, suggesting that the cysteine proteases are appropriate therapeutic targets (Barrett *et al*, 2001).The degradation of heamoglobin is initiated by one of the two aspartic protease (plasmepsin I) that are located in the food vacuole while the second aspartic protease (plasmepsin II) prefer denatured globin as a substrate (Gluzman *et al*, 2002) Additionally, cyseine protease activation has been identified in heamoglobin degradation (Rosenthal *et al*, 1994) This protease called falcipain degrade, denature and renature heamoglobin invitro..Its substrate specificity,$_{P}$H optimum and inhibitor sensitivity indicate that it is papain family, cytseine protease (Rosenthal *et al*,1989).Inhibitor of papain family cysteine have also been shown to block erythrocyte invasion (Wasserman *et al*,1990) block heamoglobin hydrolysis and prevent parasite development (Rosenthal,1993) and cured mice infected with parasite.

Evidence indicate that, cysteine proteases play essential roles in malaria parasites accumulates, an obvious consideration is the inhibition of these enzymes to treat malaria. A number of older studies have supported this concept, with the demonstration that cysteine protease inhibitors have potent *in vitro* and *in vivo* antimalarial effects (Rosenthal, 2001) Drug development has shown that, protease are drug-able target and protease inhibitor are now licensed or in clinical development, to treat diabetes, thrombosis, osteoporosis, hypertension, cancer and infectious diseases (Bailly *et al*,2004).

Current limitation of vaccine and vector control as well as increasing resistance of malaria parasite for existing drug, necessitated the continous need for the search of new antimalarial agent and target. Potential target for chemotherapy include malaria parasite protease which are required for the rupture and subsequent reinvasion of erythrocytes by merozoite-stage parasite activities possibly to breach the erythrocyte cytoskeleton complex network of protein (Mckerrow *et al*,2001).They are also needed for the degradation of haemoglobin by intraerthrocyic tropozoite necessary for the growth of erythrocyte malaria parasite, apparently to provide free amino acid for parasite protein synthesis (Rosenthal *et al*,1994)

Proteases have been discussed as potential targets for antimalarial chemotherapy for a number of years. Among enzymes of interest are cysteine proteases that hydrolyze haemoglobin and possibly perform some additional necessary functions.As cysteine proteases appear to act cooperatively to degrade hemoglobin, and as inhibitors of such classes of proteases have antimalarial effects, it may be appropriate to use combinations of inhibitors to treat malaria. Such combination therapy might improve efficacy and also slow the development of resistance to new agents.

Hence malaria parasite cysteine protease are thus promising therapeutic target and characterization including the elucidation of properties that differ from those of host protease is an important goal (Barrett *et al*, 2000).

The key measures for control and subsequent elimination of malaria are prevention, vector control, and treatment.Even though many measures need to be used simultaneously in the fight against malaria, antimalarial drugs remain critical in the control and eventual elimination of malaria parasite (White, 2008).

This present study is focused at the antimalarial activity of leaf and stem bark methanolic extracts of *Diospyros mespiliformis,* and their inhibitory effect on cysteine protease in the development and treatment of diseases. Protease inhibitors have proven to be of great value in other diseases, including cardiovascular disease and AIDS, and they have generated interest as potential antimalarial therapies.

1. 2 STATEMENT OF RESEARCH PROBLEM

It has been estimated thatapproximately 250 million clinical cases of malaria occur every year (Wiesner *et al*,1999).Malaria is estimated to kill nearly one million people annually, with most of the deaths occurring in children under 5 years of age (Luzzatto,2010).Drugs such as quinine related drugs, antifolate combination drugs and artemisinin and its derivatives are antimalarial drugs used for effective treatment due to lack of ineffective vaccine and emergence of drug resistance (Croft,2007).Clinical resistance to these combinations has been reported (Noedl *et al*., 2008), suggesting that *P. falciparum* parasites have already developed the ability to grow in the presence of these antimalarials, which strongly suggests the need for further research into new antimalaria drugs..The discovery of new and effective antimalaria drugs based on new mechanisms of action or with new structures, is urgently needed to overcome the problem of rapid emergence of drug resistance and achieve long-term clinical efficacy.

Natural products (plants) have been a thriving source of discovery of new drugs due to their chemical diversity and ability to act on various biological targets. Also, the isolation of new bioactive compounds from medicinal plants based on ethnomedical and traditional data appears to be a very promising approach (Turschner *et al*, 2009)

The present study is looking at the antimalarial activity of *Diospyros mespiliformis* and effect of the plant extracts on cytsteine protease.

1.3 JUSTIFICATION

Ineffectiveness of chemotherapy constitutes the greatest threat to the control of malaria. Therefore, to overcome malaria, new knowledge, tools and products are urgently needed especially new drugs are of importance (Rasoanaivo *et al*., 2011). New drugs against malaria are urgently needed and the traditional methods of malaria treatment could be a promising source of new antimalarial compounds (Benoit-Vical,2005).The use of medicinal plants in modern medicine suffers from the fact that though hundreds of plants are used in the world to prevent or to cure diseases, scientific evidence in terms of modern medicine is lacking in some cases. Presently, it is necessary to provide scientific proof as to whether it is justified to use a plant or its active principles (Singh *et al.*, 2007).

Hence, the present study was prompted by the claim of some traditional health practitioners that *Diospyros mespiliformis* is an effective remedy for the management, control and treatment of malaria.

1.4 AIM AND OBJECTIVES OF THE STUDY

1.4.1 AIM

The aim of the present study was to investigate the *invivo* antimalarial potentials of leaf and stem bark methanolic extracts of *Diospyros mespiliformis* on *Plasmodium berghei* using animal models and also to determine the inhibitory effects of these extract on *Plasmodium* cysteine protease.

1.4.2 OBJECTIVES

The specific objectives of this study are:

I. Qualitative and quantitative phytochemical analysis of leaf and stem bark methanol extracts of *Diospyros mespiliformis.*

II. Determination of the antimalarial activities ofdifferent fractions of leaf and stem bark methanol extracts of *Diospyros mespiliformis* in *Plasmodium berghei* infected mice

III. Evaluation of the effect of different fractions of leaf and stem bark methanol extracts on some haematological and biochemical parameters in mice

IV. Investigate inhibitory effect of *Diospyros mespiliformis*the leaf and stem bark methanol extracts on cysteine protease.

CHAPTER TWO

LITERATURE REVIEW

2.1 GENERAL DISCRIPTION, HABITAT, AND CLASSIFICATION OF *Diospyros mespiliformis*

2.1.1 GENERAL DISCRIPTION AND HABITAT

Species of genus *Diospyros* belong to an extensive class of family *Ebenaceae* consisting of seven (7) genera. Yonemori *et al*. (2000) listed over 400 species forming the *Diospyros* genus and several of these species are of economic importance. The mild climatic region is suitable for growing and use in horticulture of three persimmon species – *Diospyros kaki* L. f. (japanese persimmon), *Diospyros virginiana* L. (persimmon virgin iana) and *Diospyros lotus* L. (date persimmon) (Harborne,1993).

Diospyros mespiliformis is a tall, evergreen tree 15-50m high, with dense, rounded and buttressed stem. Bark grey-black or black, smooth in young trees rough with small regular scales in older trees, pinkish when slashed. Young branchlets are green, tomentellous with pinkish-white hairs, glabrescent later. Crown is very branchy with dense foliage. Leaves alternate, shiny-green above, paler beneath, 4-7cm long, 1.5-5.5cm wide, oblong elliptic or oblolanceolate-elliptic, rarely lanceolate-elliptic, pubescent when young later becoming glabrescent or with few persistent, appressed hairs beneath, acute or subacuminate at the apex, cuneate or rounded at base with impressed midrib above, prominent beneath. Flowers pentamerous, white and fragrant. Male flowers sessile hairy and clustered on axillary peduncles. Female flowers solitary, shortly pedicellate and axillary with a 5-lobed calyx. Fruits usually globose, fleshy, up to 3cm in diameter, greenish and pubescent when young, yellowish to orange yellow and glabrous when ripe, bell shaped, with persistent style and enlarged calyx and contain 4-6 seeds. Seeds, dark brown, bean-shaped shiny and glabrous. The generic name Diospyros means ‘divine pear’, and the specific name mespiliformis is derived from two words,‘mesos’ meaning half, and ‘pilos’which is bullets.

The species occurs in woodlands, savannahs and along riverbanks. It prefers areas with permanent water that helps in natural regeneration, and it grows faster in frost-free areas. D. mespiliformis occurs naturally from Ethiopia in the north to Swaziland in the south. It favours heavy soils on riverbanks but also occurs in open woodland and is commonly found on termite mounds.

2.1.2 CLASSIFICATION OF THE PLANT

Kingdom: *Plantae*

Division: *Magnoliophyta*

Class: *Magnoliopsida*

Order: *Ericales*

Family: *Ebenaceae*

Genus: *Diospyros*

Species: *D. mespiliformis*

2.1.3 NUTRITIONAL AND MEDICINAL USE OF THE PLANT

The leaves, roots, bark and fruits contain antibiotic qualities and have many medicinal uses in West Africa. Roots and bark are used to stop purging and to enhance fertility, while the leaf decoction is used as remedy for fever, and wound dressing. Bark and roots for infections such as malaria, pneumonia, syphilis, leprosy, dermatomycoses, as an anthelmintic and to facilitate child birth. Different parts used against diarrhoea, headache, toothache and as a psycho pharmacological drug (Hong *et al*, 2000). The fruit is edible for humans; its flavor has been described as lemon-like, with a chalky consistency. They are sometimes preserved, can be dried and ground into a flour, and are often used for brewing beer and brandy.

Figure 2.1: *Diospyros mespiliformis* leaf (Zango village, Sabo-gari LGA.Kaduna state)

Figure 2.2: *Diospyros mespiliformis* Stem bark (Zango village, Sabo-gari LGA.Kaduna state)

2.2 MALARIA, THE DISEASE

Malaria is a vector borne infectious disease caused by protozoan, parasites from the phylum Apicomplexa (Heelan *et al*, 2002) and of genus *Plasmodium*. The genome of several *Plasmodium* species are as follows; *Plasmodium falciparum*, *Plasmodium knowlesi*, *Plasmodium vivax*, *Plasmodium berghei* and *Plasmodium yoelii*. Several species are found, of which only four cause malaria in humans namely *P. vivax, P. malariae, P. ovale* and *P. falciparum. P. falciparum* is the most serious form of the disease as it is responsible for the high malarial mortality and morbidity rates in Africa, and occurs predominantly in Nigeria. According to Rosenthal (2003) resistance to existing antimalarial drugs is mostly seen in *P. falciparum*. All these species have genomes of about 25 megabases organised into 14 chromosomes consistent with earlier estimates. The chromosomes vary in length from 500 kilobases to 3.5 megabases and it is presumed that this is the pattern throughout the genus. The Plasmodium contains a degenerated chloroplast called an apicoplast.

As a protist, the plasmodium is a eukaryote of the phylum *Apicomplexa*. Unusual characteristics of this organism in comparison to general eukaryotes include the rhoptry, micronemes, and polar rings near the apical end. The Plasmodium is known best for the infection it causes, malaria.

According to the latest estimates, there were about 219 million cases of malaria in 2010 and an estimated 660,000 deaths. Malaria mortality rates have fallen by more than 25% globally since the year 2000 and by 33% in the WHO African Region. Most deaths occur among children living in Africa where a child dies every minute from malaria. Country-level burden estimates available for 2010 show that an estimated 80% of malaria deaths occur in 14 countries and about

80% of cases occur in 17 countries (David *et al.,* 2004). Survey shows that 90% of the world's cases of malaria occur in sub-Saharan Africa. Nine out of ten cases of this disease occur in this region and record over one million deaths annually (World Malarial Report, 2005).

Malaria is a major public health problem in Nigeria. Every year, thousands of cases are reported from all over the country. It is the leading parasitic cause of morbidity and mortality in the tropics. One quarter of the world's population is at risk for malarial infection (Martens *et al*, 2002). The present global situation indicates a recent resurgence in the severity of the disease and that malaria could still be described as one of the most deadly diseases, with an annual incidence of 300-500 million clinically manifest cases and a death toll of 1-2 million people (Martins *et al.*, 2004). World Health Organization malaria report in 2008 puts the figure at 247 Million clinically manifest cases and a death toll of 881,000 people (Breiger et al, 2009). Mortality and morbidity due to malaria are a matter of great concern throughout the whole world, especially in the tropical and subtropical regions, even though casualty in children below the age of 5 years is very high, the disease affects all age groups (Bickii *et al.*, 2000).

2.2.1 TRANSMISSION

The main vector for the malaria parasites is the *Anopheles* mosquito. In Africa, the *Anopheles gambiae* complex is the most efficient in transmitting the disease (Nchinda, 1998). Malaria is also transmitted from mother to unborn baby (i.e. congenitally), by blood transfusions, as well as by sharing contaminated hypodermic needles (Heelan *et al*, 2002). The transmission of malaria corresponds with the rainy season which provides breeding sites for the *Anopheles* vector. The *Plasmodium* parasite is only carried by these mosquitoes in warm, humid and wet climates but disappears over winter. Larvae control is, therefore, imperative in the control of this epidemic disease. Other factors that contributes to the increase of malaria incidences include travelling to and from epidemic areas, the accidental imports of the malaria vectors (a phenomenon known as ‘’airport malaria‟) and the increase in agricultural activities (MoHSS, 2005).

2.2.2 LIFE CYCLE OF MALARIA

The agents of human malaria, i.e. *P. ovale, P. falciparum, P. malariae and P. vivax* all have a complex life cycle involving two hosts: the human and the *Anopheline* mosquito. Whenever an infected mosquito has a blood meal, it injects saliva containing the parasite (in the form of sporozoites) into the pierced skin serving as some kind of anesthesia. The sporozoites now travel through the bloodstream of the victim and infect the liver. In the liver, the sporozoites reside in the parenchyma cells (Mehlhorn, 2001) and asexual development or schizogony takes place. The parenchymal cells transform into a circular shape and start to divide, producing many merozoites, which are released into the bloodstream upon rupturing of the hepatocytes. Once in the bloodstream they infect the red blood cells (RBCs) and start to multiply. The erythrocytic life cycle, responsible for all clinical manifestations of malaria, begins when free merozoites invade erythrocytes. The intraerythrocytic parasites develop from small ring-stage organisms to larger, more metabolically active trophozoites and then to multinucleated schizonts. The erythrocytic cycle is completed when mature schizonts rupture erythrocytes, releasing numerous invasive merozoites**.** According to Mehlhorn (2001) the rest of the merozoites grow as trophozoites (i.e. the ring forms), increase in size, divides and mature into schizonts. Merozoites are formed from the schizonts as a result of erythrocytic schizogony. Within 48-72 hours the 40 infected erythrocytes burst/rupture and releases more merozoites, which infects more RBCs. Some of the merozoites undergo gametocytogenesis (Ingersoll *et al*, 2002) and develop into gametocytes (i.e.

macro- and microgametocytes) which can only mature outside the human body. When a mosquito takes a blood meal from an infected person, both the merozoites and gametocytes travels to the stomach/gut of the mosquito. The merozoites are digested, whereas the gametocytes mature or develop into gametes in the digestive gut. During fertilization, the gametes develop into a zygote and then into an ookinete (fertilized egg). The ookinete matures into an oocyst giving rise to sporozoites. These sporozoites, when released, travel to the salivary gland. When the female Anopheles bites the next victim, the cycle starts all over again.

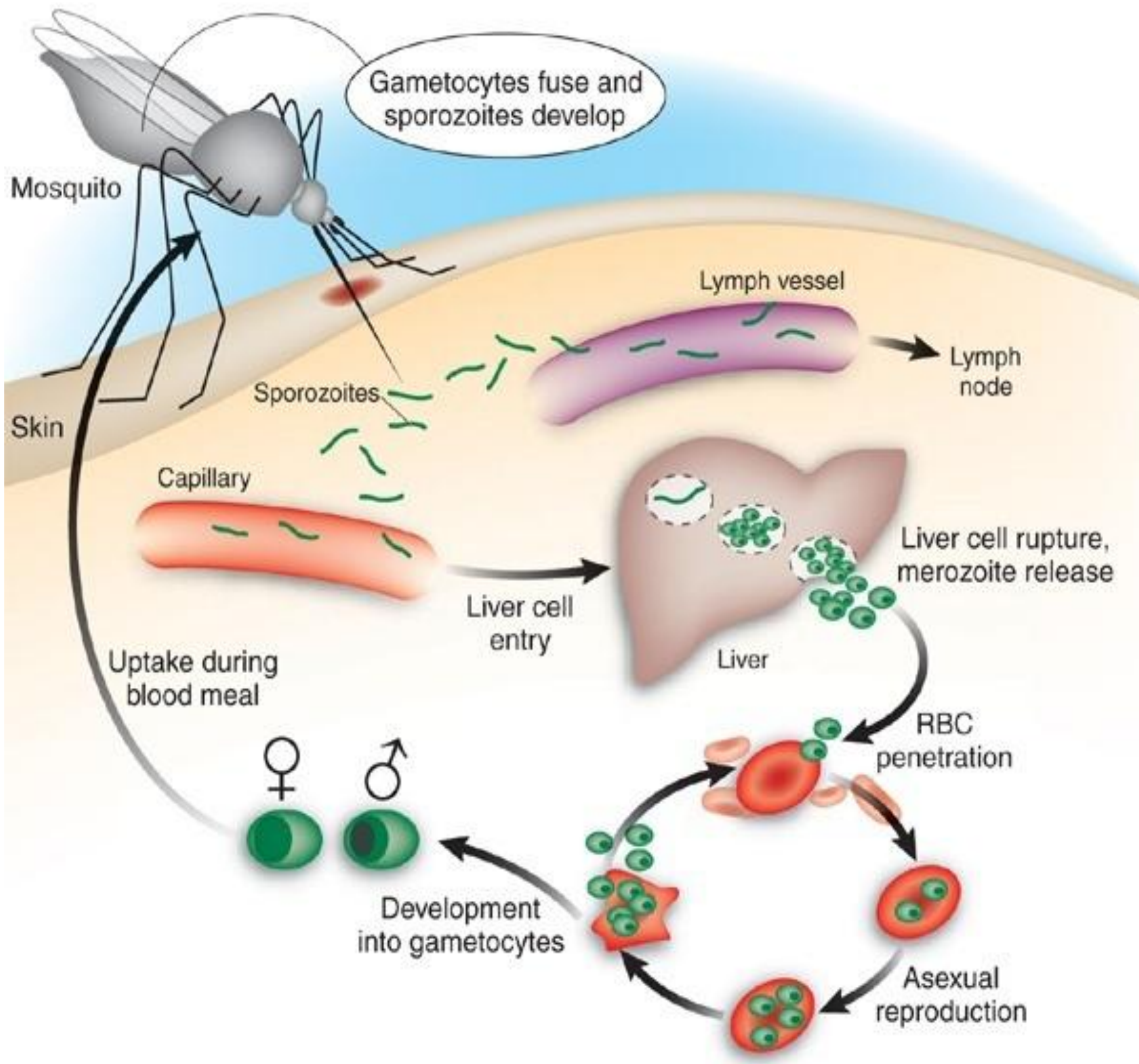


Figure 2.3: MALARIA LIFE CYCLE (Malcolm et al, 2006)**)**

2.2.3 DIAGNOSTICS AND CLINICAL SYMPTOMS

The severity of the disease varies from mild asymptotic infection to the critical complicated disease which causes death. Common symptoms include periodic flu-like symptoms such as fever, chills, sweating, muscle aches and headaches. Other symptoms include nausea, vomiting, coughing, diarrhoea, abdominal pain, myalgia (limbs and back), loss of appetite, orthostatic hypotension, jaundice (yellowing of skin and eye white), anemia, liver or kidney failure, enlarged liver and spleen and convulsions. Severe malaria is regarded as a multisystem disorder and includes cerebral malaria and severe malarial anemia (Weatherall, *et al.* 2002). Hans (2009) claims severe malaria delirium, metabolic acidosis and multi-organ dysfunction which may occur, if untreated could result in coma and eventually death.Cyclical symptoms such as fever, seizures, chills and anemia are said to correspond with the erythrocytic stage of the Plasmodium life cycle during which merozoites are released into the bloodstream (Rayner et al, 2005). Together with this release, there is also a deposit of parasitic waste products and debris, which is believed to give rise to the malarial paroxysm, i.e. the sequenced events of shaking chills, fever and sweating. Symptoms for complicated or severe malaria includes seizures, altered state of consciousness (or coma), excessive sleepiness, prostration (feelings of helplessness), respiratory distress, inability to ingest any fluids, bleeding problems, jaundice, the absence of urine and the dark coloration of urine if there is any on the other hand maintain that splenomegaly and anemia are the major/principal clinical symptoms of malaria. The latter is caused by the destruction of RBCs and the simultaneous loss of hemoglobin or by the removal of the infected erythrocytes as an immune response. Anemia may also be caused by the ceased production of RBCs due to the bone marrow suppression

2.2.4 PATHOGENESIS

Malaria is a disease caused by protozoan species (Plasmodium) and traditional medicines have been used to treat symptomatic malaria for hundreds of years (Willcox *et al,* 2004). These medicinal herbs are still used today by the majority of the rural populations in developing countries (Mohammed, 2009). A need for traditional medicine as a source of malaria treatment has been recognized in view of the difficulties faced in areas where populations are either unable to afford or access effective antimalarials or are unwilling to use allopathic medicines (Van, 2001). *Artemisia annua* plant has been used as a traditional remedy for chills and fevers for more than 2000 years by the Chinese. Artemether, a derivative from artemisinin, is found to be more active than its precursor (Alin *et al,* 1990) and is the most frequently used artemisinin derivative as a first line treatment for uncomplicated malaria (WHO, 2008). New antimalarial leads are, therefore directed towards plant sources. To assure the development of efficient and safe malaria phytomedicines, the uses of these medicinal plants needs to be scientifically studied (i.e. critiqued and standardized). The pharmacological efficacy, phytochemical composition, as well as the toxicity of these plants needs to be investigated as potential antimalarial medicines.

2.3 INTRACELLULAR SITES OF ACTION OF ANTIMALARIAL

Antimalarials have been investigated to be active during the stage when the parasite is located inside the RBC, in other words, the drug regimens target mainly the erythrocytic cycle of the *Plasmodium* life cycle (Ridley, 2002). During the blood stage of the *Plasmodium* parasites between 60-80 % of the haemoglobin is broken into haem (Egan, 2008). The latter is said to be harmful to the parasites. The parasites, however, neutralize the haem during the process of polymerization of haemazoin. This polymerization is inhibited by antimalarials such as chloroquine mefloquine, halofantrine, lumefantrine (Hempelmann, 2007) and artemisinins; resulting in the accumulation of haem and eventual death of the parasites. Antimalarial drugs, therefore, act by preventing globin lysis and haemazoin formation. Quinoline antimalarials such as chloroquine, for example, act by inhibiting the synthesis of the hemozoin pigment when they get trapped in the digestive vacuole of the parasite due to the extra proton they gain due to the acidic environment of the vacuole (Jiang *et al*., 2006). The malaria parasites use hemoglobin/ hemazoin to make amino acids which are vital for their existence. According to Gavigan and Machado et al, (2001), hemoglobin hydrolysis by the *Plasmodium* parasite (trophozoite) is mediated by a number of classes of enzymes (proteases) in the food vacuole including 49 aspartic and cysteine proteases and falcilysin. These proteases appear to mediate the invasion and rupture of infected erythrocytes as well, possibly to get through the erythrocyte cytoskeleton, a complex network of proteins (Rosenthal, 1998). In addition, a number of malarial proteins are made during the late schizont and merozoite life-cycle stages and likely to aid in the complex series of events involved in the RBCs (Hall et al, 2005). Although the exact roles of various classes of proteases are not entirely clear, inhibitors of cysteine and serine proteases have consistently repressed erythrocyte rupture and invasion.

Studies have found that the biological activity caused by drugs results in the swelling of the parasite, as well as the inhibition of the proteolytic activity of these enzymes (Semenove *et al*, 1998).

Malaria parasites are said to be sensitive to the free radicals produced by some antimalarial drugs including artemisinin-related compounds. Artemisinin, in the presence of haem inhibits the polymerization of haemazoin by alkylation (Mishra *et al*., 2009). The haem or Fe^{2+} cleaves the peroxide bridge in the artemisinin compound leading to the formation of free radicals (Ridley, 2002). These radicals then alkylate one of the pyrazole rings in the haem nucleus, hence inhibiting the formation of haemazoin. Other modes of action shown by antimalarials include pyrimidine synthesis inhibition. Inhibiting this synthesis would mean inhibiting the growth of the parasites (Rosenthal, 2003)

An example of such an antimalarial drug is atovaquone which inhibits the respiratory chain of malaria mitochondria at complex III (Ridley, 2002). Some folate antimalarials such as pyrimethamine and sulfonamides inhibit the formation/ synthesis of dihydrofolate, which is necessary in the biosynthesis of pyrimidines. Iron chelators, on the other hand, are active by preventing iron from entering important metabolic pathways of parasites within the RBCs, as well as combining with iron, forming toxins to the parasites (Harbert *et al*, 1976).

Among potential targets for new modes of chemotherapy are malarial proteases. Cysteine and aspartic protease inhibitors are now under study as potential antimalarials. The increased understanding of the mode of action of a compound will contribute in the mitigation in the rapid spread of resistance against presently used antimalarials as some antimalarials given in combination treatments may have counter effects, causing a relapses of the parasite load (Gavigan *et al*., 2001).

2.4 ENZYMES

Enzymes are biologically active proteins that posses the ability to accelerate the rate of chemical reaction occurring in living cells without undergoing any permanent change. Enzymes are able to perform these catalytic functions at the physiological temperature and $_{P}$H because of their ability to lower the energies of activation of those reactions.

With the exception of a small group of catalytic RNA molecules, all enzymes are protein. Enzymes like other proteins have molecular weight ranging from 12000 to over 1million.some enzymes require no chemical groups for activity other than their amino-acid residues. Others require an additional chemical component called co-factor-either one or more inorganic ions such as Fe^{2+}, Mg^{2+}, Mn^{2+} or Zn^{2+} or a complex organic or metalloorganic molecule called a coenzyme. Some enzymes require both a coenzyme and one or more metal ion for activity. A coenzyme and/or metal ion that is very tightly or even covalently bound to the enzyme protein is called a prosthetic group. A complete, catalytically active enzyme together with its bound coenzyme and/or metal ions is called a holoenzyme or apoprotein.

The distinguishing feature of an enzyme-catalyzed reaction is that, it occurs within the confines of a pocket on the enzyme called the active site. The molecule that is bound in the active site and acted upon by the enzyme is called the substrate.

The entire enzymes discovered to date are grouped into six classes viz: oxido-reductase, transferases, hydrolases, lyases, isomerases, and ligases. These classes of enzyme have a unique identification number with which the various enzymes in each category can be located in the enzyme list. This code number consist of four digits or figures that is usually prefixed by EC (acronym for Enzyme commission) e.g. E.C.1.1.1.1 where the first digit shows the main classes listed above. The second and third are a little bit more difficult to define because they refer to different aspect of the reaction in different Enzyme classes. The fourth figure in the code simply gives the serial number of the Enzyme as assigned by the Enzyme nomenclature committee.

2.5 PROTEASES

Protease refers to a group of enzymes whose catalytic function is to hydrolyze (breakdown) peptide bonds of proteins. They are also called proteolytic enzymes or proteinases. Proteases differ in their ability to hydrolyze various peptide bonds. Each type of protease has a specific kind of peptide bonds it breaks. Examples of proteases include: fungal protease, pepsin, trypsin, chymotrypsin, papain, bromelain, and subtilisin. Proteolytic enzymes are present in bacteria and plants but are most abundant in animals.

Their catalytic actions had been divided into two different categories (Barrett *et al*, 2001) such as limited proteolysis which involves cleavage only one or a limited number of peptide of a target protein leading to activation or mutation of the formally inactive protein as can be seen in the conversion of prohormones to hormone and unlimited proteolysis which involves the degradation of the target protein conjugation to multiple molecule of ubiquitin, into its amino acids constituents.According to the Nomenclature Committee of the International Union of Biochemistry, proteases are classified in sub – group 4 of group 3 (hydrolases). Currently, proteases are classified on the basis of three major criteria:

i. Types of reaction catalyzed
ii. Chemical nature of the catalytic site and
iii. Evolutionary relationship with reference to structure (Barret, 1994).

Proteases are grossly subdivided into two major groups i.e. Exopeptidases and Endopeptidases depending on their site of action.

(I) **Exopeptidases:** act only near the end of the polypeptide chains. Based on their site of action at the N or C terminus, they are classified as amino and carboxy – peptidases respectively.

i. Amino – peptidases act at a free N terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide, or a tripeptide. They are known to remove the N terminal Met that may be found in heterologously expressed proteins but not in many naturally occurring mature proteins. They occur in a wide variety of microbial species including bacteria and fungi (kaou *et al*, 2008).

ii. Carboxy – peptidases act at C terminal of the polypeptide chain and liberate a single amino acid or a dipeptide. They can be divided into three groups, serine-, cysteine-, and metallocarboxy – peptidases based on the nature of the amino acid residues at the active site of the enzymes.

(2) **Endopeptidases:** are proteases that are characterized by their preferential action at the peptide bonds in the inner region of the polypeptide chains away from N and C termini.

Proteases play a critical role in many physiological and pathological processes such as protein catabolism, blood coagulation, cell growth and migration, tissue arrangement, morphogenesis in development, inflammation, tumour growth and metastasis, activation of zymogens, release of hormones and pharmacologically active peptides from precursor proteins and transport of secretary proteins across membranes (Jendrassik *et al.*, 2003). In general, extracellular proteases catalyze the hydrolysis of larger proteins to smaller molecules for subsequent absorption by the cell whereas intracellular proteases play a critical role in the regulation of metabolism. Some of the physiological functions performed by proteases are briefly discussed below:

i. **Regulation of Gene Expression:** Proteolysis of a repressor by an ATP – requiring protease resulted in a suppression or inactivation of a gene. A change in the transcriptional specificity of the B subunit of *Bacillus thuringensis* RNA polymerase was correlated with its proteolytic modification (Khafagi *et al,* 2001). Modification of ribosomal proteins by proteases has been suggested to be responsible for the regulation of translation.

Besides the general functions described so far, the proteases also mediate degradation of a variety of regulatory proteins that control the heat shock response, the SOS response to DNA damage, the life cycle of bacteriophages and programmed bacterial cell death (Lawrence *et al*,2001). Recently, a new physiological function has been attributed to the ATP – dependent proteases present in bacteria and eukaryotes. It is believed that they act as chaperons and mediate not only proteolysis but also the insertion of proteins into membranes and the disassembly or oligomerization of protein complexes (Suzuki *et al.,* 1997).

ii. **Protein Turnover:** All living cells maintain a particular rate of protein turnover by continuous, albeit balance degradation and synthesis of proteins. Catabolism of proteins provides a ready pool of amino acid as precursors of the synthesis of proteins. Proteases assist in the hydrolysis of large polypeptides into smaller peptides and amino acids, thus facilitating their absorption by the cell. The extracellular enzymes play a major role in nutrition due to their deploymerising activity. The microbial enzymes and the mammalian extracellular enzymes such as those secreted by pancreas are primarily involved in keeping the cells alive by providing them with the necessary amino acid pool as nutrition. In *Escherichia coli,* ATP – dependent protease La, the *lon* gene product is responsible for hydrolysis of abnormal proteins (Chung *et al*, 1981). The turnover of intracellular proteins in eukaryotes is also affected by a pathway involving ATP – dependent proteases (Hershko *et al.,* 1984). Evidence for the participation of proteolytic activities in controlling the protein turnover was demonstrated by the lack of proper turnover in protease deficient mutants.

iii. **Enzyme Modification:** Activation of the zymogenic precursor forms of enzymes and proteins by specific proteases represent an important step in the physiological regulation of many rate – controlling processes such as generation of protein hormones, assembly of fibrils and viruses, blood coagulation, and fertilization of ova by sperm. Activation of zymogen forms of chitin synthase by limited proteolysis has been observed in *Candida albicans, Mucor rouxii,* and *Aspergillus nidulans.* Pepsin, trypsin and chymotrypsin occur as their inactive zymogenic forms, which are activated by the actions of proteases. Proteolytic inactivation of enzymes leading to irreversible loss of *in vivo* catalytic activity is also a physiologically significant event. Several enzymes are known to be inactivated in response to physiological or developmental stages or after metabolic shift. Proteolytic modification of enzymes is known to result in a protein with altered physiological function e.g. leucyl – L – RNA synthetase from *E.coli* is converted into an enyme which catalyses leucine – dependent pyrophosphate exchange by removal of a small peptide from the native enzyme (Rao *et al.,* 1998).

Proteolytic enzymes are very important in digestion as they breakdown the protein foods to liberate the amino acids needed by the body. Additionally, proteolytic enzymes have been used for a long time in various forms of therapy. Their use in medicine is gaining more and more attention as several clinical studies are indicating their benefits in oncology, inflammatory conditions, blood rheology control, and immune regulation.

To understand why proteases are such important drug targets, and to be able to validate them as targets and identify or design efficient drugs against them, one has to understand the complexity of the biological processes they participate in, the mechanisms of protease activity and regulation and the biochemistry that relates their structure to function

Many proteolytic enzymes appear to play key roles in the life cycles of malaria parasites. In parallel with biochemistry advances, drug discovery projects designed to identify new antimalarial cysteine protease inhibitors are underway.

Studies with protease inhibitors have provided valuable information regarding the functions of cysteine proteases in malaria parasites. A number of older studies have supported this concept, with the demonstration that cysteine protease inhibitors have potent in vitro and in vivo antimalarial effects (Rosenthal, 2001). Plasmodium cysteine proteases are potential chemotherapeutic targets, and their characterisation should expedite drug discovery.

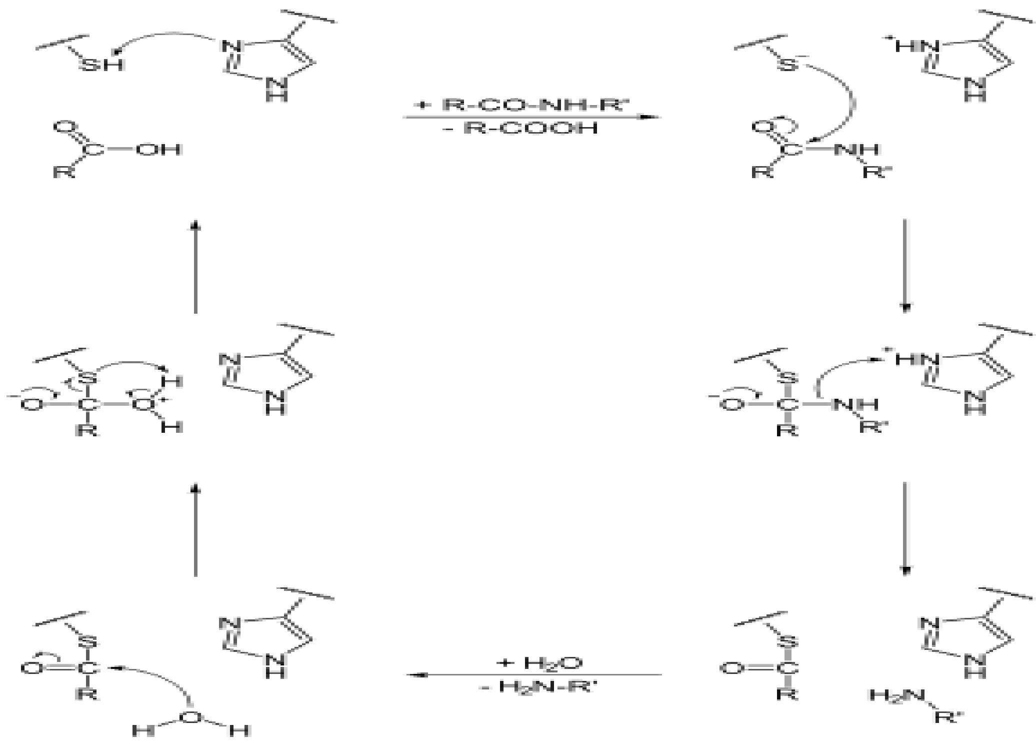


Figure 2.4; Reaction mechanism of the cysteine protease mediated cleavage of a peptide bond (Ekland *et al*, 2007)

Proteases are currently classified into six broad groups:

- Serine proteases
- Threonine proteases
- Cysteine proteases
- Aspartate proteases
- Glutamic proteases
- Metalloproteases

The threonine and glutamic-acid proteases were not described until 1995 and 2004, respectively. The mechanism used to cleave a peptide bond involves making an amino acid residue that has the cysteine and threonine (proteases) or a water molecule (aspartic acid, metallo- and glutamic acid proteases) nucleophilic so that it can attack the peptide carboxyl group. One way to make a nucleophile is by a catalytic triad, where a histidine residue is used to activate serine, cysteine, or threonine as a nucleophile.

Within each of the broad groups proteases have been classified, by Rawlings and Barrett, into families of related proteases. For example within the serine proteases families are labelled Sx where S denotes the serine catalytic type and the x denotes the number of the family, for example S1 (chymotrypsins). An up to date classification of proteases into families is found in the MEROPS database.

2.5.1 SERINE PROTEINASES

This class comprises two distinct families. The chymotrypsin family, which includes the mammalian enzymes such as chymotrypsin, trypsin, or elastase or kallikrein, and the substilisin family, which includes the bacterial enzymes like subtilisin. Though the general 3D structure is different in the two families, they have the same active site geometry, and catalysis proceeds via the same mechanism. The serine proteinases exhibit different substrate specificities, which are related to amino acid substitutions in the various enzyme subsites interacting with the substrate residues. Some enzymes have an extended interaction site with the substrate. Others have a specificity restricted to the P1 substrate residue (Schaller, 2004).

Three residues which form the catalytic triad are essential in the catalytic process, i.e. His 57, Asp 102, and Ser 195 (chymotrypsinogen numbering). The first step in the catalysis is the formation of an acyl enzyme intermediate between the substrate and the essential Serine at the active site. Formation of this covalent intermediate proceeds through a negatively charged tetrahedral transition state intermediate, and then the peptide bond is cleaved. During the second step, or deacylation, the acyl-enzyme intermediate is hydrolyzed by a water molecule to release the peptide and to restore the Ser-hydroxyl of the enzyme. The deacylation, which also involves the formation of a tetrahedral transition state intermediate, proceeds through the reverse reaction pathway of acylation. A water molecule is the attacking nucleophile instead of the Ser residue. The Histidine residue provides a general base and accepts the OH group of the reactive Ser (Haque *et al*., 2004).

2.5.2 CYSTEINE PROTEINASES

This family includes the plant proteases such as papain, actinidin or bromelain, several mammalian lysosomal cathepsins, and the cytosolic calpains (calcium-activated) as well asseveral parasitic proteases (e.g. Trypanosoma, Schistosoma). Papain is the archetype and the best studied member of the family. Recent elucidation of the X-ray structure of the Interleukin-1-beta Converting Enzyme has revealed a novel type of fold for cysteine proteinases. Like the serine proteinases, catalysis proceeds through the formation of a covalent intermediate and involves a cysteine and a histidine residue. The essential Cys 25 and His159 (papain numbering) play the same role as Ser 195 and His 57, respectively. The nucleophile is a thiolate ion rather than a hydroxyl group. The thiolate ion is stabilized through the formation of an ion pair with neighboring imidazolium group of His159. The attacking nucleophile is the thiolate-imidazolium ion pair in both steps, and then a water molecule is not required (Haque *et al*., 2004).

2.5.3 ASPARTIC PROTEINASES

Most of aspartic proteinases belong to the pepsin family. This family includes digestive enzymes like pepsin and chymosin, lysosomal cathepsins D, processing enzymes like renin, and certain fungal proteases (penicillopepsin, rhizopuspepsin, endothiapepsin). A second family comprises viral proteinases, such as the protease from the AIDS virus (HIV), also called retropepsin. Crystallographic studies have revealed these enzymes to be bilobed molecules with the active site located between two homologous lobes. Each lobe contributes one aspartate residue of the catalytically active diad of aspartates. These two aspartyl residues are in close geometric proximity in the active site, and one aspartate is ionized while the second one is unionized at the optimum pH range of 2-3. Retropepsins, are monomeric, i.e. carry only one catalytic aspartate, and then dimerization is required to form an active enzyme (Hamilton, 2004).

In contrast to serine and cysteine proteases, catalysis by aspartic proteinases does not involve a covalent intermediate though a tetrahedral intermediate exists. The nucleophilic attack is achieved by two simultaneous proton transfers: one from a water molecule to the diad of the two carboxyl groups and a second one from the diad to the carbonyl oxygen of the substrate with the concurrent CO-NH bond cleavage. This general acid-base catalysis, which may be called a "push-pull" mechanism, leads to the formation of a non-covalent neutral tetrahedral intermediate (Hamilton, 2004).

2.5.4 METALLO-PROTEINASES

The metallo-proteinases may be one of the older classes of proteinases and are found in bacteria and fungi as well as in higher organisms. They differ widely in their sequences and their structures, but the great majority contains a zinc atom which is catalytically active. In some cases, zinc may be replaced by another metal such as cobalt ornickel without loss of the activity. Bacterial thermolysin has been well characterized, and its crystallographic structure indicates that zinc is bound by two histidines and one glutamic acid. Many enzymes contain the sequence HEXXH, which provides two histidine ligands for the zinc while the third ligand is either a glutamic acid (thermolysin, neprilysin, alanyl aminopeptidase) or a histidine (astacin). Other families exhibit a distinct mode of binding the Zinc atom. The catalytic mechanism leads to the formation of a non-covalent tetrahedral intermediate after the attack of a zinc-bound water molecule on the carbonyl group of the scissile bond. This intermediate is further decomposed by transfer of the glutamic acid proton to the leaving group (Skiles *et al*., 2004).

Based on the specificity of their actions, metalloproteases can be divided into four groups

i. Neutral

ii. Alkaline

iii. Myxobacter I, and

iv. Myxobacter II.

The neutral proteases show specificity for hydrophobic amino acids while the alkaline proteases possess a very broad specificity. Myxobacter I protease is specific for small amino acids residues on either side of the cleavage bond, whereas myxobacter II protease is specific for lysine residue on the amino side of the peptide bond. All of them are inhibited by chelating agents such as EDTA but not by sulfhydryl agents of DFP.

2.6 CATALYTIC MECHANISM OF CYSTEINE PROTEASE

The first step in the reaction mechanism by which cysteine proteases catalyze the hydrolysis of peptide bonds is deprotonation of a thiol in the enzyme's active site by an adjacent amino acid with a basic side chain, usually a histidine residue. The next step is nucleophilic attack by the deprotonated cysteine's anionicsulfur on the substratecarbonylcarbon. In this step, a fragment of the substrate is released with an amine terminus, the histidine residue in the protease is restored to its deprotonated form, and a thioester intermediate linking the new carboxy-terminus of the substrate to the cysteine thiol is formed. Therefore they are also sometimes referred to as thiol proteases. The thioester bond is subsequently hydrolyzed to generate a carboxylic acid moiety on the remaining substrate fragment, while regenerating the free enzyme.

2.7 PROTEINASE INHIBITORS (Pls) IN PLANTS

Enzymes inhibitors are molecules that bind to enzymes and decrease their activities. They are substances which tend to decrease the rate of enzyme – catalyzed reaction. Since blocking an enzyme's activity can kill a pathogen or correct a metabolic imbalance, many drugs are enzyme inhibitors and they are also used as herbicides and pesticides (Berg *et al.,* 2002). The binding of an inhibitor can stop a substrate from entering the enzyme's active site and/or hinder the enzyme from catalyzing its reaction. This inhibitor binding can be either **irreversible** in which the inhibitor react with the enzyme and change it chemically, by modifying key amino acid residues needed for enzyme activity or **reversible** in which the inhibitor binds non – covalently to an enzyme in a reversible fashion and can be removed by dialysis. Reversible inhibitors usually form an equilibrium system with an enzyme which shows a definite degree of inhibition that remains constant over a period when initial velocity studies are normally carried out while the degree of inhibition by irreversible inhibitors may increase over this period of time (Berg *et al.*, 2002).

(I) Reversible Inhibition

Reversible inhibitors bind to enzymes with non – covalent interactions such as hydrogen bonds, hydrophobic interactions and ionic bonds. In contrast to substrates and irreversible inhibitors, reversible inhibitors generally do not undergo chemical reactions when bound to the enzyme and can be easily removed by dilution or dialysis. Examples of reversible inhibitors are seen in:

i. Peptide based protease inhibitor e.g. Ritonavir, a very successful class of antiretroviral drug used to treat HIV (Hsu *et al.,* 2006). This is a protein inhibitor with three peptides bonds which resembles the protein that is the substrate of the HIV protease and competes with these substrates in the enzyme's active site.

ii. Non – peptide protease inhibitor e.g. Tipranavir, another HIV protease inhibitor that is not based on a peptide and has no obvious structural similarity to a protein substrate. These non – peptide inhibitors can be more stable than peptide inhibitors because they will not be substrates for peptidases and are likely to be degraded (Fisher, 2003).

Discussed below are the three kinds of reversible enzyme inhibitors which are classified according to the effect of varying the concentration of the enzyme's substrate on the inhibitor (Berg *et al.*, 2002).

(II) Irreversible Inhibition

An irreversible inhibitor binds to the active site of an enzyme by an irreversible reaction and hence cannot subsequently dissociate from it. A covalent bond is usually formed between the inhibitor and enzyme (Bickii,2002). The inhibitor may act by preventing substrate – binding or it may destroy some components of the catalytic site. Irreversible inhibitors often contain reactive groups such as aldehydes, haloalkanes, alkenes, phenyl sulphonates or fluorophosphates. These electrophilic groups react with amino acids side chains to form covalent adduct (Ryley et al, 1970). The residues modified are those with side chains containing nucleophiles such as hydroxyl or sulfhydryl groups; these include the amino acids serine, cysteine, threonine or tyrosine. Irreversible inhibitors display time dependent inhibition and their potency therefore, cannot be characterized by IC_{50} value. Examples of irreversible inhibition are seen in:

i. Diisopropyl fluorophosphates (DFP) which reacts with the active site of acetylcholine esterase in the synapses of neurons, and consequently is a potent neurotoxin, with a lethal dose of less than 100mg (Brenner, 2000).

ii. The inhibitor of polyamine biosynthesis, α - diifluoromethylornithine or DFMO, which is an analogue of the amino acid ornithine and is used to treat African trypanosomiasis. Ornithine decarboxylase can catalyze the decarboxylation reaction is followed by the elimination of a fluorine atom which converts the catalytic intermediate into a conjugated amine, a highly electrophilic species. This reactive form of DFMO then reacts with either a cysteine or lysine residue in the active site to irreversibly inactivate the enzyme (poulin *et al.,* 2007).

(III) Mixed Inhibition

In mixed inhibition, the inhibitor binds to the enzyme at the same time as the enzyme substrate. However, the binding of the inhibitor affects the binding of the substrate and vice versa. This type of inhibition can be reduced, but not overcome b increasing concentrations of substrate. Although, it is possible for mixed – type inhibitor to bind in the active site which generally results from an allosteric effect where the inhibitor binds to a different site on an enzyme. Inhibitor binding to this allosteric site changes the conformation of the enzyme so that the affinity of the substrate for the active site reduced (Berg *et al.*, 2002).

(IV) Competitive Inhibition

In this inhibition, the inhibitors often closely resemble in some respects to the substrates whose reaction is inhibited because of this structural similarity, they may compete for the same active site on the enzyme. The enzyme – bound inhibitor then either locks the appropriate reactive group or it is held in an unsuitable position with respect to the catalytic – site of the enzyme or to other potential substrates for a reaction to take place. This type of inhibition can be overcome by sufficiently high concentrations of substrate i.e. by out – competing the inhibitor (Berg *et al.*, 2002).

(V) Non – Competitive Inhibition

This is a form of mixed inhibition where the binding of the inhibitor to the enzyme reduces its activity but does not affect the binding of substrate. As a result, the extent of inhibition depends only on the concentration of the inhibitors ((Berg *et al.,* 2002).

Serine proteinase inhibitors are universal throughout the plant kingdom and have been described in many plant species. Therefore, the number of known and partially characterized inhibitors of serine proteinases is enormous (Haque *et al*., 2004). Serine proteinase inhibitors have been reported from a variety of plant sources and are the most-studied class of proteinase inhibitors. Chiche *et al.* (2004) first introduced the squash inhibitor, a well-established family of highly potent canonical serine proteinase inhibitors isolated from Cucurbitaceae. The squash inhibitors were among the first discovered proteins with the typical knottin fold shared by numerous peptides extracted from plants, animals, and fungi (Melo *et al*., 2002).

Plant cystatins or phytocystatins are the second most-studied class of inhibitors and have been identified and characterized from several plants, viz., cowpea, potato, cabbage, ragweed, carrot, papaya, apple fruit, avocado, chestnut, and Job's tears. Cystatins have also been isolated from seeds of a wide range of crop plants. These crop plants include those of sunflower, rice, wheat, maize, soybean, and sugarcane (Kuroda *et al.*, 2003).

Aspartic proteinase inhibitors are a relatively less-studied class partly due to their rarity, and the metallo-proteinase inhibitors in plants are represented by the metallo-carboxypeptidase inhibitor family in tomato and potato plants.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 CHEMICALS/REAGENTS

Reagent kits from Randox Laboratory Ltd, Ardmore Co,Antrm UK for the determination of the following biochemical parameters were used: Aspartate aminotransferase (AST), Alanine aminootransferase (ALT), alkaline phosphatase (ALP), Total protein, Albumin, Bilirubin (Indirect and Direct), serum creatinine and urea. Chemicals were purchased from (Sigma chemical company St Louis U.S.A).These include: Sodium dihydrogen phosphate,disodium hydrogen orthophosphate, Tris, Coomassie Brilliant Blue R-250,Benyl arginine para-nitroanilide, (BAPNA), Trichloroacetic acid, ethylenediaminetetraacetic acid (EDTA) was employed as standard reference for the antimalarial screening in this study.

3.1.2 EQUIPMENT

Micropipettes (Labsystems E25631) (Bishop Meadow Road, Loughborough Leicestershire LE 11 SRG United Kingdom).Spectrophotometer (Jenway 2028) (820 Linden Avenue, Rochster, NY 14625, USA), Microscope (Olympus) (141 Palmgroove Avenue, London, United Kingdom),Glass Slides/Cover slips (Uniscope) (Surgifriend Medicals, Middlessex England).Water Bath (Bird and Tatlak 72358/33) (Shephrat Cambridgeshire, SGB 6GB, England).Microheamatocrit BX32RS2 (Olympic Cooperation Tokyo Japan) , Haematocrit Reader BX45RC4 (Olympic Cooperation Tokyo, Japan) Pasteur pipette 63A54 (Emma Optical Works Ltd Tokyo,Japan), Weighing Scale BS200S (AND GF-2000 Nettles Instrument, Switzerland) Colorimeter (Jenway Laboratory UK) Jenway 6405 UW/VIS Spectrophotometer

((Jenway Laboratory UK) Eppendorf 5430R Centrifuge (Eppendorf Company), Water Bath (Grant Instrument (Cambridge) Ltd) 760 pH Meter (Metrohm Herisan Switzerland) Refrigerator (Thermocool Ltd, Lagos), Cuvette (AU480 Beckman coultier-Inc,(congo-Brazzaville), syringe and needles (E.O Modesty venture,Idumota Island,Lagos),

3.1.3 PLANT MATERIAL

Fresh leaf and stem bark of *Diopsyros mespiliformis* were collected from Zango village, Sabo Gari LGA of Kaduna State, in the month of June 2013 and authenticated in the herbarium at the Department of Biological sciences, Ahmadu Bello University Zaria where a voucher specimen with voucher number 901431 was deposited.

3.1 4 EXPERIMENTAL ANIMALS

A total of hundred and sixty (160) mice of both sexes(4 weeks) weighing between 18-28g were purchased from the animal house, Department of Pharmacology, Ahmadu Bello University, Zaria. The animals were housed in well-ventilated cages and allowed to acclimatize under standard laboratory condition for a period of two weeks before commencement of the experiment.They were fed daily with grower mash from vital feed (Vital Feeds, Jos, Nigeria) and water ad libitum.

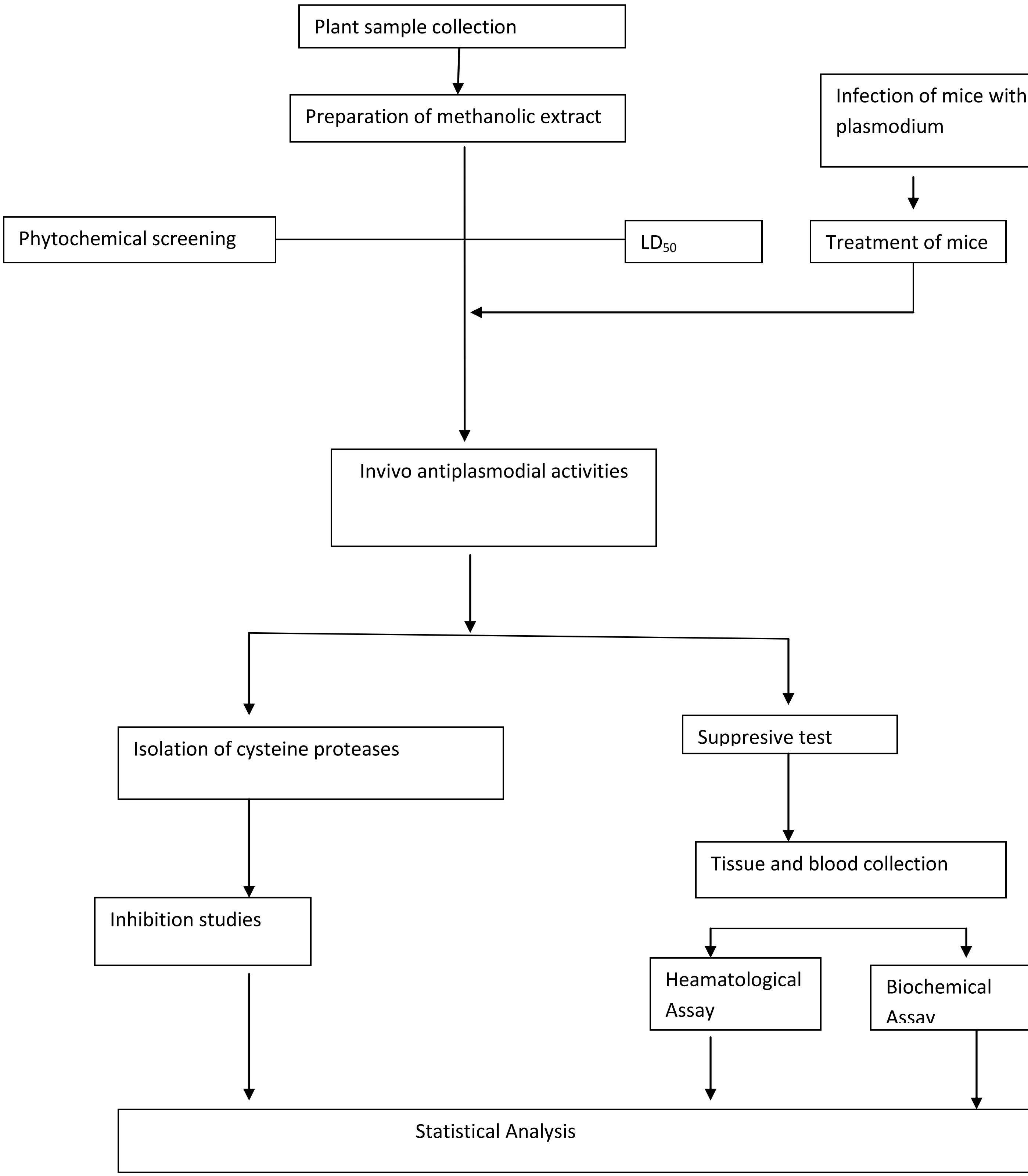
3.1.5 PARASITES

The choloquine sensitive *Plasmodium berghei* (NK-65) used in this study was obtained from Biochemistry Department, Nigerian Institute of Medical Research (NIMR), Yaba, Lagos, Nigeria.

The parasite was maintained by sub-passaging into healthy mice throughout the duration of the study. Although *P. berghei* is generally used in rodent model for malaria, mice model was used in this study because of the high susceptibility of mice to *P. berghei* infection compared to laboratory rats and hamsters which are less susceptible (Kellick-Kendrick, 1978). The susceptibility of mice to *P. berghei* infection is equally supported by the study conducted by Pavia (1983).

The infection of the recipient mice was initiated by needle passage of the parasite preparation from the donor to healthy test animals via an intraperitoneal route as described by Peter and Anatoli (1998) and David *et al*. (2004). Briefly, *P. berghei* infected red blood cells obtained from the tail vein of infected mice was diluted with Phosphate Buffered Saline (PBS) so that each 0.2 mL that was subsequently injected contained approximately 10^6-10^7 infected red cells (parasite) .

EXPERIMENTAL DESIGN



3.2.1 PLANT EXTRACT PREPARATION AND PERCENTAGE YIELD CALCULATION

Fresh leaf and stem bark of *Diospyros mespiliformis* were rinsed in clean water and air dried at room temperature for two weeks. The dried leaf and stem bark were pulverized into powder using Thomas-Wiley laboratory mill (Model 4) manufactured by Arthur H. Thomas Company, Philadelphia, PA., U.S.A. before being extracted.

A portion of five hundred grams (500g) of the pulverized plant leaves was suspended in 2.5 L of methanol and the solution was left standing for 48 hours in large amber bottles with intermittent shaking. At the end of the extraction, the crude methanol extract was filtered using Whatman No. 1 filter paper (1mm mesh size) and then concentrated in a water bath maintained at 45 C until greenish black residues were obtained. A portion of the crude extract was then subjected to phytochemical analysis using standard procedures (Sofowora, 1998) and the portion was reconstituted for further fractionation and the fractions were kept in sealed containers and refrigerated at 2-4^{o}C for further use. The crude extraction and fractionation process was repeated thrice. The percentages yield of both the crude methanol, leaves extract and fractions were determined as percentage of the weight (g) of the extract to the original weight (g) of the dried sample used, using the formular below;

$$\text{Percentage yield of crude extract} = \frac{\text{Weight of extract}}{\text{Weight of sample}} \times 100$$

$$\text{Percentage yield of fractions} = \frac{\text{Weight of fraction}}{\text{Weight of crude extract}} \times 100$$

3.2.2 Fractionation of Crude Extract

The methanol crude extract of *Diospyros mespiliformis* was subjected to liquid-liquid partition to separate the extract into different fractions. 250ml of the reconstituted extract was placed in a separator funnel and 250ml of ethylacetate and *n*-butanol solvents were added sequentially as a 1:1 (v/v) solution and rocked vigorously (Abbot and Andrews, 1970). The sample was left standing for 30 minutes for each solvent on the separator funnel until a fine separation line appear clearly indicating the supernatant from the sediment before it was eluted. The process was repeated thrice in order to get adequate quantity for each fraction. The ethylacettate, *n*-butanol as well as the aqueous residue fractions were concentrated over a water bath maintained at 45^oC. The concentrated fractions were kept in sealed containers and refrigerated at 2-4^oC for further use.

3.2.3 ACUTE TOXICITY EVALUATION/DETERMINATION OF MEDIAN LETHAL DOSE (LD_{50})

The median lethal dose (LD_{50}) of the plant extract was conducted in order to select a suitable dose for the evaluation of antimalarial activity.This was done using the method described by Lorke (1983) using 13 mice for each extact. In the initial phase, mice were divided into 3 groups of 3 mice each and were treated with 10mg, 100mg and 1000mg of the extract per kg body weight intraperitoneally. They were observed for 24hours for signs of toxicity, including death. If no death is observed, the second phase follows. In the second phase, 3 mice were divided into 3 groups of one mouse each, and were treated with the extract based on the findings in the first phase. The LD_{50} was calculated from the results of the final phase as the square root of the product of the lowest lethal dose and the highest non-lethal dose.

The results of the second phase experiment were used to calculate the LD_{50} of both plant extracts.The LD_{50} was calculated according to the method outlined by Lorke, (1983) as:

$$Geometric mean of the doses for which there was no mortality \times$$

$$the next doses for which mortality occurred \ = \times mg/kg.$$ i.e.

$$\sqrt{Dose with mortality \times Next dose without mortality}$$

3.3 PRELIMINARY PHYTOCHEMICAL SCREENING

Phytochemical screening of the methanolic stem bark and leaf extracts of *Diospyros mespiliformis* were carried out according to standard methods of Sofowora (1982); Trease and Evans (1983) and Brain and Turner (1975).

3.3.1 Test for Alkaloids

Dragendroff's, Wagner's and Mayer's Reagent for Alkaloids

Three milliter of the methanolic extracts solution was stirred with 5ml of 10% aqueous hydrochloric acid on water bath and filtered. The filtrate was divided into three portions of 1ml each. To the first portion was added few drops of Dragendroff's reagent to give an orange-red precipitate. To the second portion was added few drops of Wagner's reagent to give reddish-brown colour. To third portion was added few drops of Mayer's reagent to give a buff precipitate. Colour change as stated above indicated the presence of alkaloids while no colour change indicates the absence of alkaloids (Trease and Evans, 1983).

3.3.2 Test for Cardiac Glycosides

Salkowskii's Test for Cardiac Glycosides

Small amount of the extracts was dissolved in 2ml chloroform followed by the addition of concentrated H_2SO_4 to form a lower layer. A reddish-brown colour at the interphase indicated the presebce of steroidal nucleus (Sofowora, 1982).

Keller-Killiani's Test for Digitalis Glycoside

Small quantity of the extracst was dissolved in 2ml of 3.5% ferric chloride in glacial acetic acid. This was then transferred to the surface of the 2ml concentrated H_2SO_4. Reddish-brown ring obtained at the interphase of the liquid indicated the presence of digitoxose sugar component (deoxy sugar) characteristics of cardenolide (Trease and Evans, 1983).

3.3.3 Test for Flavonoids

Test for Flavonoids

The following constituents; flavones, flavonoids and phenolic group were tested with small portion of the extract. To test for flavones and phenolic group, a small quantity of the sample was put in a test-tube and a few drops of ethanol added to make it alcoholic before being subjected to different tests.

Ferric Chloride Test for Flavonoids

About 5ml distilled water was added to extract and boiled on water bath for about 2 min and then filtered. To 2ml of the filtrate, few drops of 10% alcoholic ferric chloride solution were added. Effervescence occurred and the dark brown solution change to green, blue to violent colouratioon indicated the presence of phenolic group (OH group) (Trease and Evans, 1983).

3.3.4 Test for Anthraquinones

Test for Anthraquinone Derivatives:

Borntrager's Test for Combined and Free Anthraquinone Derivatives

Small quantity of the extracts was boiled with 2ml of 10% HCL for 30 min and the mixture filtered while still hot and the filtrate allowed to cool. The cooled filtrate was then shaken with equal volume of chloroform layer which was then transferred into a clean test-tube and treated with equal volume of 10% ammonia solution. The mixture was then shaken and the colour of the upper layer noted. Colourless layer indicated that anthracene derivatives i.e. free or combined anthraquinone was absent (Trease and Evans, 1983).

3.3.5 Test for Carbohydrates

Molisch's Test (General Test for Carbohydrates)

A few drops of Molisch's reagent were added to the extract dissolved in distilled water. This was followed by the addition of 1ml of concentrated H_2SO_4 down the side of the test-tube, so that the acid formed a layer beneath the aqeuous layer. Mixture was allowed to stand for 20 min and then diluted with 5ml of distilled water. Formation of a red to dull violet colour at the interface of the two layers was taken as positive test (Trease and Evans, 1983).

3.3.6 Test for Glycosides

Five millimeter of concentrated sulphuric acid was added to the extract and boiled for 15 minutes. This was then cooled and neutralized with 20% KOH and was divided into two portions. Another part of extract was dissolved in distilled water, this was used as a control; no acid hydrolysis.

Fehling's solution Test: Fehling's solution A and B was added to one portion and boiled for few minute, red precipitate indicates the aglycone portion as a result of hydrolysis of glycosides.

Ferric Chloride Test: Ferric chloride solution (3 drops) was added to the other portion. Green to Black precipitate indicates phenolic aglycone as a result of hydrolysis of glycoside (Trease and Evans, 1983).

3.3.7Test for Saponins

Fronthing Test: Small quantity of the extract was dissolved in 10ml of distilled water. This was then shaken vigorously for 30seconds and was allowed to stand for 30minutes.A honey comb formed for more than 30minutes indicates saponin (Trease and Evans, 1983).

3.3.8 Test for Tanins

Lead Sub-Acetate Test: Lead Sub-Acetate solution (3drops) was added to a solution of the extract. A red precipitate indicates tannins.

Ferris Chloride Test: About 0.5g of extract was dissolved in 10ml of distilled water and then filtered. Few drops of ferric chloride solution were added to the filtrate. Formation of blue black precipitate indicates hydrolysable tannins and green precipitate indicate the presence of condensed tannin (Trease and Evans, 1983)

3.4 QUANTITATIVE ANALYSIS OF PHYTOCHEMICALS

3.4.1 Determination of Saponin

A gravimetric method of Association of Official Analytical Chemist (AOAC, 1984) employing the use of a soxhlet extractor and two different organic solvents was used. The first solvent (Acetone) extracts lipids and interfering pigments while the second solvent (methanol) extracts saponin.

Procedure: Two grams of *Diospyros mespiliformis* sample was weighed into a thimble and put in a soxhlet extractor with a condenser fitted on top. Extraction was done with acetone in a 250ml round bottom flask for 3hrs, after which the other weighed 250ml round bottom flask containing methanol was fitted to the same extractor continued for another 3hrs. At the end of second extraction, the methanol was recovered by distillation and the flask oven-dried to remove the remaining solvent in the flask. The flask was allowed to cool in a dessicator and weighted. This test was carried out in triplicates.

Calculation:

$$\text{Saponin} = A\text{-}B/W$$

Where A = Weight of flask and extractor (saponin), B = Weight of empty flask, W=Weight of sample

3.4.2 Determination of Tannin

Concentration of tannin was determined using the standard method described by AOAC (1980).

Procedure: Two grams of the dried sample was boiled with 300ml of distilled water. This was diluted in a standard volumetric flask and filtered through a non-absorbent cotton wool. A volume of 25ml of the infusion was measured into a 2 litre porcelain dish and titrated with 0.1N potassium permanganate (0.1N potassium permanganate was standardized against 0.1N Oxalic acid) until the blue changed green, Then few drops of 0.1N potassium permanganate was added at a time until the solution turns golden yellow. This test was carried out in triplicates.

The tannin content in the sample was calculated by multiplying the volume of 0.1N potassium permanganate used (titre value) by 0.0066235g. Using the equation below;

1ml of 0.1N potassium permanganate (titre value) = 0.0066235g Tannins

Therefore Tannins content in the sample = titre value x 0.0066235g

3.4.3 Determination of Glycosides

The procedure of AOAC (1984) was used in determination of cyanogenic glycosides.

Procedure: Five grams of sample was placed in a 200ml conical flask; 50ml of distilled water was added. The content of the flask was stirred and allowed to stand for 4hrs. The filtrate was steam distilled into 20ml of 2.5% NaOH. About 75ml of the distillate was collected. This was titrated with 0.02N $AgNO_3$ (Silver nitrate) after the addition of 8ml of 6N Ammonium hydroxide and 2ml of 2% potassium iodide. Permanent turbidity indicated end-point. This test was carried out in triplicates. The cyanogenic glycosides was calculated by multiplying the volume of 0.0N $AgNO_3$ used (titre value) by 1.08mg, using the equation below;

1ml 0.02N $AgNO_3$ = 1.08mg HCN

Therefore cyanogenic glycosides content in the sample = titre value x 1.08mg.

3.4.4 Determination of Alkaloids

This was carried out using the procedure described by Harbone (1973) with slight modification by Edeoga *et al.* (2005).

Procedure: Five grams of the sample was weighed into 250ml beaker, 100ml of 10% acetic acid in ethanol was then added. The mixture was covered and allowed to stand for 4 hours. It was then filtered and the extract concentrated on a water bath to ¼ of the original volume. Thereafter, concentrated ammonium hydroxide was added drop wise until precipitation was completed. The solution was then allowed to settle and precipitate collected, washed with diluted ammonium hydroxide and filtered. The filtrate was concentrated in a water bath maintained at $45^{o}{}_{C}$. The dry weight of the filtrate is equivalent to the weight of alkaloids in mg/g of the dry sample weight. This was carried out in triplicates.

3.5 ANIMAL GROUPING

A total number of 160 mice were used for this determination. The mice were randomly divided into sixteen (16) groups each of leaf and stem bark respectively, with five animals per group.

Group 1: Non infected and not treated (Normal control)

Group 2a: Non infected and treated with medium dose (ethylacetate fraction) leaf methanol extract (200mg/kg)

Group 2b: Non infected and treated with medium dose (ethylacetate fraction) stem bark methanol extract (100mg/kg)

Group 3a: Non Infected and treated with medium dose (n-butanol fraction) leaf methanol extract (200mg/kg)

Group 3b: Non Infected and treated with medium dose (n-butanol fraction) stem bark methanol extract (100mg/kg)

Group 4a: Non Infected and will be treated with medium dose (Aqeous fraction) leaf methanol extract (200mg/kg)

Group 4b: Non Infected and will be treated with medium dose (Aqeous fraction) stem bark methanol extract (100mg/kg)

Group 5: Infected and administered 0.2ml normal saline **(**Negative control)

Group 6: Infected and treated with standard chloroquine (5mg/kg)

Group 7: Infected with parasite and treated with (ACT) Artimisinin Combination Therapy (10mg/kg)

Group 8a: Infected and treated with low dose ((ethylacetate fraction) leaf methanol extract (100mg/kg)

Group 8b: Infected and treated with low dose ((ethylacetate fraction) stem bark methanol extract (50mg/kg)

Group 9a: Infected and treated with medium dose (ethylacetate fraction) leaf methanol extract (200mg/kg)

Group 9b: Infected and treated with medium dose (ethylacetate fraction) stem bark methanol extract (100mg/kg)

Group 10a:Infected and treated with high dose ((ethylacetate fraction) leaf methanol extract (400mg/kg)

Group 10b:Infected and treated with high dose ((ethylacetate fraction) stem bark methanol extract (200mg/kg)

Group 11a: Infected and treated with low dose ((n-butanol fraction) leaf methanol extract (100 mg/kg)

Group 11b: Infected and treated with low dose ((n-butanol fraction) stem bark methanol extract (50mg/kg)

Group 12a:Infected and treated with medium dose ((n-butanol fraction) leaf methanol extract (400mg/kg)

Group 12b:Infected and treated with medium dose ((n-butanol fraction) stem bark methanol extract (200mg/kg)

Group 13a: Infected and treated with high dose ((n-butanol fraction) leaf methanol extract (400 mg/kg)

Group 13b: Infected and treated with high dose ((n-butanol fraction) stem bark methanol extract (200mg/kg)

Group 14a: Infected and treated with low dose (Aqeous fraction) leaf methanol extracts (100 mg/kg)

Group 14b: Infected and treated with low dose (Aqeous fraction) stem bark methanol extracts (50mg/kg)

Group 15a: Infected and treated with medium dose (Aqeous fraction) leaf methanol extract (200 mg/kg)

Group 15b: Infected and treated with medium dose (Aqeous fraction) stem bark methanol extracts (100mg/kg)

Group 16a: Infected and treated with high dose (Aqeous fraction) leaf methanol extracts (400 mg/kg)

Group 16b: Infected and treated with high dose (Aqeous fraction) stem bark methanol extracts (200mg/kg)

3.6 *IN- VIVO* ANTIPLASMODIAL ACTIVITY

In-vivo study of antimalarial property of the plant was evaluated by determining its suppressive antiplasmodial properties by using the method of Bulus *et al.,* (2003).

3.6.1 SUPPRESSIVE TEST

Malaria parasite innoculum was prepared and collection of blood samples from the mice (donor mice) infected with *Plasmodium berghei* (NK-65) was diluted with normal saline such that 0.2ml will contain 10^6-10^7 of the parasite.

% Suppression = APC – APT / APC X 100

APC = Average Parasitaemia in the Negative Control,

APT = Average Parasitaemia in the Test group

3.7 TISSUE COLLECTION AND PREPARATION

At the end of the experiments, the animals was sacrificed by anesthetic ether six hours after the last treatment and blood samples were drawn from the heart of each sacrificed animal from all groups with 5ml sterile syringe (individually for each animal) and transferred into plane sample bottles immediately (non- heparin or non- EDTA anticoagulant bottles). The blood was left to clot and the serum separated by centrifugation using Labofuge 300 heraeus centrifuge at 1500 x g for 10 minutes and the supernatant (serum) was then be collected with Pasteur pipette into another set of tubes. The liver and kidney were collected, weighed, minced and homogenized using 50 mM potassium phosphate buffer (pH 7.4). The homogenate was centrifuged at 3,000 x g for 15 minutes. The supernatant was decanted and used for further analysis.

The percentage change in organ weight of each of the animals was calculated as follows:

$$\text{\% Change in weight} = \frac{\text{Organweight}}{\text{Animalweight}} \times 100$$

3.8 HAEMATOLOGICAL ASSAYS

Packed cell volume (PCV) and haemoglobin concentration (Hb) was determined. The estimation of Hb concentration was carried out using the cyanomethaemoglobin and PCV by microhaematocrit methods of Alexander and Griffins (1993).

3.8.1 Determination of Packed Cell Volume (PCV)

The method described by Esievo *et al*, (1982) was used in the determination of packed cell volume (PCV). Recommended anticoagulant used is ethylene-diamino-tetra-acetic acid

(EDTA). Capillary haematocriit tubes approximately 7cm in length, with a bore of about 1cm were used.

Procedure: The tubes were filled with blood by capillary action (to about 7.5% of its length).The outside was dried carefully with a piece of gauze.The blood/reagent mixture was allowed to stand for about 10 min for maximum conversion of haemoglobin to cyanmethaemoglobin.The cuvette was wiped with tissue paper and placed in a spectrophotometer. Reading was taken and the absorbance or the percentage transmission recorded at 540nm.Values obtained were compared with the readings obtained using standard cyanmethaemoglobin solution.Values are expressed in gm of haemoglobin per deciliter of blood (g/dL)

3.8.2 Determination of Haemoglobin Concentration (Hb)

Haemoglobin concentration (Hb) was determined using the cyanomethaemoglobin method of Alexander and Griffins (1993).

Principle: Blood is mixed with dilute hydrochloric acid. This process haemolyses the red cells, disrupting the integrity of the red cell membrane and causing the release of haemoglobin, which in turn, is converted to a brownish-colored solution of acid haemastin when compared with a color standard.

Procedure: Blood from the tail of the mice was allowed by capillary action to flow through the heparinized capillary tube. Sample solutions and standard solutions were prepared as follows.

Random Sample: 5000µl of Drabkin reagent was mixed with 20µl of distilled water.

Standard: 5000µl of Drabkin reagent was mixed with 20µl of sample solution of haemoglobin.

Target Sample: 5000µl of Drabkin reagent + 20µl of blood

The concentration of haemoglobinn determined using Drabkin's method (spectrophotometric method).Drabkin reagent was mixed with the blood, the solution was incubated at room temperature (28^{o}$_{C}$) for the duration of 5 minutes and absorbance was measured at 540nm against distilled water.

The concentration of haemoglobin was calculated according to the following formula:

Hb concentration (g/dl) = $\frac{\text{Absorbance of tested sample}}{\text{Absorbance of standard}} \times \text{Concentration of standard in umol/L}$

3.9 BIOCHEMICAL ASSAYS

3.9.1 Estimation of Serum Alkaline Phosphatase (ALP) using the method of Rosalki et al, 1993

Principle: Alkaline phosphatese (ALP) catalyses the transfer of the phosphate group from 4-nitrophenylhosphate to diethanolamine (DEA), liberating 4-nitrophenol in alkaline medium. The catalytic concentration is determined from the rate of 4-nitrophenol formation, measured at 405nm.

4-nitrophenyphosphate + DEA $\longrightarrow$ DEA-phosphate + 4-nitrophenol

Procedure: Substrate solution (3 ml) was incubated at 37^{0}C for 15 min and then 0.5ml of the sample was added. This was mixed well and immediately 0.05 ml of the mixture was removed and mixed with 9.5 ml of NaOH. This corresponded to zero time assay (blank). The remaining solution (substrate+enzyme) was incubated for 15min at 37^{0}C and then 0.5 ml was drawn and mixed with 9.5ml of 0.085N NaOH. Absorbance was measured at 405 nm against the reference blank. Specific activities were expressed as moles of p-nitrophenol produced per min per g tissue.

3.9.2 Estimation of Serum Alanine Aminotransferase (ALT or GPT) using the method of Reitman and Frankel 1957

Principles: Alanine aminotransferase (ALT or GPT) catalyses the transfer of the amino group from alanine to 2-oxoglutarate, forming pyruvate and glutamate. The catalytic concentration is determined from the rate of decrease of NADH, measured at 340nm by means of the lactate dehydrogenase (LDH) coupled reaction. For this assay, the method of Gella *et al.,* (1997) was used.

$$\text{Alanine} + \text{2-Oxoglutarate} \longrightarrow \text{Pyruvate} + \text{Glutamate}$$

$$\text{Pyruvate} + \text{NADH} + H^{+} \longrightarrow \text{Lactat} + NAD^{+}$$

Procedure: Exactly 0.5ml of reagent 1 which is made up of phosphate buffer, L-alanine and α-oxoglutarate was added into two clean test tubes, one containing 0.1ml of serum and the other containing 0.1ml distilled water (blank). The content in each test tube was mixed, incubated for exactly 30minutes at 37^{0}C. 0.5ml of reagent 2 which is made up of 2,4-dinitrophenylhydrazine was added to each of the test tubes, mixed and allowed to stand for exactly 20minutes at 20-25^{0}C. Then 0.5ml of sodium hydroxide solution was added to each of the test tubes, the content in each of test tubes was mixed and absorbance was read against the blank at 540nm after 5minutes. The ALT concentration (U/I) was determined from the standard calibration table provided in the manual of Randox Lab. Ltd, UK Reagent Kit.

3.9.3 Estimation of Serum Aspartate Aminotransferase (AST or GOT) using the method of Reitman and Frankel 1957

Principle: Aspartate aminotransferase (AST or GOT) catalyses the transfer of the amino group from aspartate to 2-Oxoglutarate, forming Oxaloacetate and glutamate. The catalytic concentration is determined from the rate of decrease of NADH, measured at 340nm by means of the malate dehydrogenase (MDH) coupled reaction. For this assay, the method of Gella *et al.*, (1997) was used.

$$\text{Aspartate + 2-oxoglutarate} \longrightarrow \text{Oxaloacetate + Glutamate}$$

$$\text{Oxaloacetate + NADH + H}^{+} \longrightarrow \text{Malate + NAD}^{-}$$

Procedure: Exactly 0.5ml of reagent 1 which is made up of phosphate buffer, L-aspartate and α-oxoglutarate was added into two clean test tubes labelled as test sample and reagent blank, containing 0.1ml of serum and 0.1ml of distilled water, the content in each test tubes was mixed and incubated for exactly 30minutes at 37^0C. 0.5ml of reagent 2 which is made up of 2,4-dinitrophenylhydrazine was added to each of the test tubes, the content of each of the test tubes was mixed and allowed to stand for exactly 20minutes at 20-25^0C. To each of the test tubes, 0.5ml of sodium hydroxide solution was added, mixed and absorbance was read against the blank at 540nm after 5minutes. The AST concentration (U/l) was determined from the standard calibration table provided in the manual of Randox Lab. Ltd, UK Reagent Kit.

3.9.4 Determination of Serum Bilirubin Concentration

The serum total and direct bilirubin concentrations were determined by the method of Jendrassik and Grof (1938).

Principle: Bilirubin is estimated by reacting with diazotized sulfanilic acid obtained from sodium nitrite and sulfanilic acid solutions. Bilirubin when reacted with diazotized sulfanilic acid forms a pink colored azocompound that is measured at 546nm. The unconjugated or free bilirubin takes longer time to react and requires caffeine as accelerator. The indirect bilirubin is calculated from the difference between the total and direct bilirubin.

Procedure for Total Bilirubin

Sample **(200µl)** was pipetted into both the standard and test tubes. Then to the standard tube, 0.1ml of 2-bilirubin solution was added followed by the addition of 1.0ml of 3-bilirubin solution. Also, to the test tube, 0.1ml of working reagent was added followed by 1.0ml of 3-bilirubin solution. The reaction was incubated at $30^0{}_C$ for 5 minutes and absorbance was read at 546nm against sample blank.

Procedure for Direct Bilirubin

Sample (0,05mml) was pipette into both the standard and test tubes. Then to the standards tubes, 1.0ml of normal saline was added and followed by the addition of 0.1ml of 2-bilirubiin solution. Also, 1.0ml of normal saline was added to the test tubes and followed by the addition of 0.1ml of working reagent. The reaction was incubated at $30^0{}_C$ for 3 minutes and absorbance was read at 546nm against sample blank.

Calculation: Serum Bilirubin (mg/dl) = (Absorbance of sample-Absorbance of blank) x 26.312. Total Bilirubin - Direct Bilirubin = Indirect Bilirubin.

3.9.5 Estimation of Serum Total Protein using Biuret method of Tietz (1975)

Principle: Proteins contain a large number of peptide bonds. When treated with Cu^{2+} ions in a moderately alkaline medium, a blue colour chelated complex of unknown composition is formed between the Cu^{2+} ion, carbonyl group and amide group of the peptide bonds. An analogous reaction takes place between the cupric ion and the organic compound Biuret (NH_2-C-NH_2), hence, the reaction is called Biuret reaction. The intensity of the colour produced is proportional to the number of peptide bonds undergoing the reactions.

Procedure: Exactly 1ml of biuret reagent was added to three clean test tubes labelled as sample, standard and blank containing 0.02ml of serum, 0.02ml of standard and 0.02ml of distilled water respectively, the content in each of the test tube was mixed, incubated for 10minutes under room temperature. The absorbance was read at 540nm. The total protein concentration was calculated using the formula below

Total protein concentration= Change in absorbance for sample/change in absorbance for standard × Concentration of the standard

3.9.6 Estimation of Serum Total Albumin using method of Doumas *et al.*, (1971)

Principle: Albumin in the sample reacts with bromoocresol green in acid forming a coloured complex that can be measured by spectrophotometrically at 630nm. The deeper the colour complex, the greater the concentration of albumin and vice versa.

Procedure: Exactly 1ml of Bromocresol green reagent (R1) was added to three clean test tubes labelled test sample, standard and reagent blank containing 0.02ml of serum sample, 0.02ml of standard and 0.02ml distilled water respectively. The mixtures in each of the test tubes were incubated for 10minutes under room temperature. The absorbance of the serum sample (A sample) and of the standard (A standard) was read at 630nm against the reagent blank.

Calculation

The albumin concentration in the sample was calculated from the following formula in g/l:

Albumin concentration (g/l) = Change in absorbance of sample/change in absorbance of standard × Concentration of the standard

3.9.7 Determination of Serum Urea Concentration by the method of Fawcett and Scout (1960)

Principle: Urease breaks down urea into ammonia and carbon dioxide. In alkaline medium, ammonia reacts with hypochlorite and salicylate to form dicarboxyindophenol, a blue coloured compound. The reaction is catalyzed by sodium nitroprusside. The intensity of colour produced is measured spectrophotometrically at 578nm.

$$\text{Urea} + H_2O \longrightarrow 2NH_3 + CO_2$$

$$NH_3 + \text{Hypochlorite} + \text{Salicylate} \longrightarrow \text{Dicarboxyindophenols (Blue compound)}$$

Procedure: Reagent (1ml) containing sodium nitropusside and urease was added into three clean test tubes labeled as test sample, standard and reagent blank containing 0.01ml sample, 0.01ml standard reagent and 0.01ml distilled water respectively. The content in each of the test tubes was mixed and incubated at room temperature (25-30^{o}C) for 10 minutes. The absorbance of the test sample and standard were read against reagent blank at 578nm.

Calculation:

The serum urea concentration was calculated using the formula below;

$$\text{Urea Concentration (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \text{Standard Concentration}$$

BUN Concentration (mg/dl) = 0.467 x Urea Concentration (mg/dl)

3.9.8 Determination of Serum Creatinine Concentration by using Bertels and Bohmer (1973) colorimetric method

Principle: Creatinine present in the serum reacts with alkaline picrate to form a colored complex. The rate of formation of colored complex is directly proportional to creatinine concentration. This rate of reaction (intensity of orange color produced) is measured colorimetrically at 510nm and is compared with that of the standard.

$$\underset{\text{(Yellow)}}{\text{Creatinine + Picric acid}} \xrightarrow[\text{(Alkaline Medium)}]{NaOH} \underset{\text{(Orange)}}{\text{Creatinine picrate}}$$

Procedure: Working reagent (1ml) containing picric acid and sodium hydroxide was added into two clean test tubes labeled sample test and standard, containing 0.1ml of test sample and 0.1ml of standard solution. The content in each test tube was mixed and after 20 seconds, the absorbance of the Standard (ST1) and Test Sample (TS1) was read. Exactly 80 seconds later, absorbance for (ST2) and (TS2) of the standard and sample was read at 510nm against distilled water (blank).

Calculation: The Conc. of creatinine in serum (mg/dl) was calculated using the formular below;

$$\frac{\text{TS2} - \text{TS1}}{\text{ST2} - \text{ST1}} \times \text{StandardConc. (mg/dl)}$$

(ST = Standard, TS = Test Sample)

3.10Fractionation of Leaf and Stem-bark Extracts of *Diospyros mespiliformis* by Preparative Thin Layer Chromatographic (TLC)

Preparative thin layer chromatography was carried out on the methanol leaf and stem-bark extracts of *Diospyros mespiliformis* using pre-coated TLC plates. Capillary tube was filled by dipping one end into each extracts, and lightly applied to the thin layer plate. Ascending thin layer chromatography (TLC) was conducted in a wide chromatographic tank containing butanol, ethylacetate, and water as the solvent system (6:2:1) for the leaf and Stem-bark extract of *Diospyros mespiliformis*. The solvent system in the chamber was allowed to mix before loading the TLC plates. After that, the plates were allowed to dry under room temperature; the TLC plates were sprayed with *para*-anisaldehyde for detection of the bands. The spots were scraped off from the TLC plate and suspended in suitable solvent and centrifuged at 3000 ×g for 10 min. The supernatants were concentrated to dryness at room temperature and reconstituted with distilled water. Each fraction obtained was tested for inhibitory activity against the partially purified cysteine protease.

3.11 Isolation of Cysteine Protease

The malaria parasite was separated by Triton X-100 temperature-induced phase separation procedures, using the protocol of Smythe *et al.* (2004) with slight modifications. Briefly, 0.5 % Triton X-100 in Tris-buffered saline was added to the parasites and incubated at 4^{o}C for 90 min. The supernatant was collected after centrifuging at 10,000 x g for 30 min at 4^{o}C and was layered on 6 % sucrose containing 0.06 % Triton X-100 followed by incubation at 37°C for 5 min. The cytosolic phase was collected after initial centrifugation at 900xg for 5 min at 37^{o}C and precipitated with cold acetone. The pellets from each preparation was suspended in 6 ml at 50 mM phosphate buffered saline, pH 7.2 (Emmanuel et al, 2011).

3.11.1 Determination of Total Protein

Principle: The protein concentration was quantified according to the method described by Bradford (1976). The assay is based on the principle is based on an absorbance shift of the dye Coomassie Brilliant Blue G-250 in which under acidic conditions the red form of the dye is converted into its bluer form to bind to the protein being assayed. Both the hydrophobic and ionic interactions stabilize the ionic form of the dye, causing a visible color change.

Procedure: Coomassie Brilliant Blue G-250 (25 mg) was dissolved in 12.5 ml 95% ethanol. To this solution, 25 ml 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 250 ml. Protein solution (0.1 ml) was pipetted in test tubes. Five milliliters of protein reagent was added to the test tube and the contents mixed by vortexing. The absorbance at 595 nm was measured after 2 min and before 1hr against a reagent blank prepared from 0.1 ml of the appropriate buffer and 5 ml of protein reagent. The concentration of protein was plotted against the corresponding absorbance resulting in a standard curve.The latter is used to determine the concentration of protein in unknown samples

3.12 PARTIAL PURIFICATION OF CYSTEINE PROTEASE

3.12.1 Purification with Ammonium Sulphate

Ammonium sulphate was added slowly to the crude enzyme with constant stirring starting from 40% until 70% saturation was achieved. The sample was allowed to stand overnight at 4^{o}C, thereafter, it was centrifuged at 14,000×g for 30 minutes, the supernatant was then discarded and the pellet was suspended in the assay buffer (10mM sodium phosphate, pH 8.0, 1 mM Dithiothreitol, 1 mM EDTA, and 10% glycerol) and tested for the enzyme activity as described by Zhang *et al*., (2010).

3.12.2 Gel filtration on Sephadex G-50 Column

Gel filtration was carried out using Sephadex G-50 column. The pooled fractions from the ammonium sulphate precipitation were loaded onto Sephadex G-50 column (1.6 cm ×90cm) previously equilibrated with 10mM sodium phosphate buffer, pH 8.0. The column was eluted with the same buffer, and 20 fractions of 2ml each were collected at a flow rate of 14 minutes. Cysteine protease activity was then determined in each of the fractions. Fractions containing the enzyme activity were pooled together and the protein concentration was estimated by the method of Bradford (1976).

3.13 Studies of Initial Velocity

The Michaelis-Menten constant (K_M) and maximum velocity (V_{max}) of the enzyme cysteine protease was determined for the activity using N-benzylarginine paraaminoanilide (BApNA) as substrate. Different concentrations of the substrate, 0.1mM, 0.2mM, 0.3mM, 0.4mM and 0.5mM were prepared from a stock solution of 2.5mM and incubated with the enzyme to determine its activity. The absorbance was read at 405nm. Initial velocity data obtained were used to plot Lineweaver-Burk's plot to determine the $K_M$and V_{max} (Lineweaver and Burk, 1934).

3.13.1 Inhibition Studies

The inhibition studies were carried out by modifying the method described by Emmanuel *et al.*, (2011). The activity of the purified enzyme was determined in the presences of varying concentrations (0mg/ml, 0.2mg/ml, 0.4mg/ml, 0.6mg/ml, 0.8mg/ml, and 1mg/ml) of the leaf and Stem bark methanol extracts. The incubation was carried out at 37^0C and each reaction mixture was allowed to stand for 30 min prior to substrate addition. The reaction mixture was incubated further for10 min before taking the reading at 405 nm. Values obtained were used in Lineweaver

Burk's plot to determine the pattern of inhibition. A secondary plot was used plotted to determine the inhibition binding constant (K_i).

Table 3.1: Incubation Pattern using *Diospyros mespiliformis* leaf extract

Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
0mg/ml [I]	0.2mg/ml [I]	0.4mg/ml [I]	0.6mg/ml [I]	0.8mg/ml [I]	1.0mg/ml [I]
0mg [I]+E+ 0.1mM [S]	0.2mg [I]+E+ 0.1m M[S]	0.4mg [I]+E+ 0.1mM [S]	0.6mg [I]+E+ 0.1mM [S]	0.8mg [I]+E+ 0.1mM [S]	1.0mg [I]+E+ 0.1mM [S]
0mg [I]+E+ 0.2mM [S]	0.2mg [I]+E+ 0.2m M[S]	0.4mg [I]+E+ 0.2mM [S]	0.6mg [I]+E+ 0.2mM [S]	0.8mg [I]+E+ 0.2mM [S]	1.0mg [I]+E+ 0.2mM [S]
0mg [I]+E+ 0.3mM [S]	0.2mg [I]+E+ 0.3mM [S]	0.4mg [I]+E+ 0.3mM [S]	0.6mg [I]+E+ 0.3mM[S]	0.8mg [I]+E+ 0.3m M[S]	1.0mg [I]+E+ 0.3mM [S]
0mg [I]+E+ 0.4mM [S]	0.2mg [I]+E+ 0.4mM [S]	0.4mg [I]+E+ 0.4mM [S]	0.6mg [I]+E+ 0.4mM [S]	0.8mg [I]+E+ 0.4mM [S]	1.0mg [I]+E+ 0.4mM [S]
0mg [I]+E+ 0.5mM [S]	0.2mg [I]+E+ 0.5m M[S]	0.4mg [I]+E+ 0.5mM [S]	0.6mg [I]+E+ 0.5mM [S]	0.8mg [I]+E+ 0.5mM [S]	1.0mg [I]+E+ 0.5mM [S]

Table 3.2: Incubation Pattern using*Diospyros mespiliformis* stem bark extract

Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
0mg/ml [I]	0.2mg/ml [I]	0.4mg/ml [I]	0.6mg/ml [I]	0.8mg/ml [I]	1.0mg/ml [I]
0mg [I]+E+ 0.1mM [S]	0.2mg [I]+E+ 0.1m M[S]	0.4mg [I]+E+ 0.1mM [S]	0.6mg [I]+E+ 0.1mM [S]	0.8mg [I]+E+ 0.1mM [S]	1.0mg [I]+E+ 0.1mM [S]
0mg [I]+E+ 0.2mM [S]	0.2mg [I]+E+ 0.2m M[S]	0.4mg [I]+E+ 0.2mM [S]	0.6mg [I]+E+ 0.2mM [S]	0.8mg [I]+E+ 0.2mM [S]	1.0mg [I]+E+ 0.2mM [S]
0mg [I]+E+ 0.3mM [S]	0.2mg [I]+E+ 0.3mM [S]	0.4mg [I]+E+ 0.3mM [S]	0.6mg [I]+E+ 0.3mM[S]	0.8mg [I]+E+ 0.3m M[S]	1.0mg [I]+E+ 0.3mM [S]
0mg [I]+E+ 0.4mM [S]	0.2mg [I]+E+ 0.4mM [S]	0.4mg [I]+E+ 0.4mM [S]	0.6mg [I]+E+ 0.4mM [S]	0.8mg [I]+E+ 0.4mM [S]	1.0mg [I]+E+ 0.4mM [S]
0mg [I]+E+	0.2mg [I]+E+	0.4mg [I]+E+	0.6mg [I]+E+	0.8mg [I]+E+	1.0mg [I]+E+

0.5mM [S] 0.5m M[S] 0.5mM [S] 0.5mM [S] 0.5mM [S] 0.5mM [S]

3.14 CALCULATION OF PERCENTAGE PARASITAEMIA

Percentage parasitemia was calculated using Giemsa-stained thin blood films prepared from blood collected from the tail vein of each mouse in all the groups. The mean % parasitemia was recorded for each mouse and for each group.

3.14.1 Estimation of Parasitemia

Parasitemia was monitored in all the groups for 14 days starting from day 1 using thick and thin smears of blood films made from tail vein of mice (David *et al.,* 2004). The percentage parasitemia was also calculated according to the method outline by Iwalewa *et al.,* (1997).

Parasitaemia was monitored in all the groups for 14 days using thick and thin smears of blood films made from tail vein of mice (WHO, 1980; David *et al.,* 2004). The smears were stained with 10% Giemsa stain at pH 7.2 for 15 min and examined under the microscope. The percentage parasitaemia was calculated according to the method outlined by Iwalewa *et al.,* (1997) as:

$$\text{Percentageparaesitemia} = \frac{\text{Noofparasitesintreated}}{\text{Noofparasitesincontrol}} \times 100$$

3.15 DATA ANALYSIS

The data was analyzed by the analysis of variance (ANOVA). The results were expressed as Mean ± Standard Deviation (SD) except where otherwise stated. P value less than 0.05 ($P< 0.05$) were considered to be statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1 The percentage yield of leaf and stem bark methanol fractions of *Diospyros mespiliformis*.

The percentage yield of the leaf and stem bark methanol fractions of ***Diospyros mespiliformis*** are shown in table 4.1. The percentage yield (w/w) of the various extracts have ethyl acetate fraction (29.95±6.60 and 22.75±1.12) as the highest yield, followed by aqueous fraction (26.87±2.53 and 14.65±3.32), and n-butanol fraction (15.48±4.57 and 9.93±0.29) has the lowest yield. In both leaf and stem bark extracts, the % yield of ethyl acetate fraction is higher than that of aqueous fraction; though the difference is far greater with the stem bark extract.

4.2 Phytochemical analysis of *Diospyros mespiliformis*.

Qualitative phytochemical constituents of leaf and stem bark methanol extracts of *Diospyros mespiliformis* is presented in Table 4.2. The results reveal the presence of saponin, alkaloids, tannin, cardiac glycosides, free anthraquinone, glycosides, flavonoids and carbohydrate while cardiac glycosides, free anthraquinone and flavonoids were absent in stem bark extract

The quantitative analysis of some phytochemical is summarised in Tables 4.3 and 4.4. The result shows significant ($P<0.05$) higher glycosides content in the leaf extract and significant ($P<0.05$) higher saponin and tannins contents in the stem bark extract.

Table 4.1: Percentage yield of fractions of *Diospyros mespiliformis* leaf and stem bark methanol extract

	Percentage yield (%)	
Fractions	**Leaf**	**Stem bark**
Ethyl acetate fraction	29.95 ± 6.60^{b}	22.75 ± 1.12^{c}
n-Butanol fraction	15.48 ± 4.57^{a}	9.93 ± 0.29^{a}
Aqueous fraction	26.87 ± 2.53^{b}	14.65 ± 3.32^{b}

Values are Mean ± SD for triplicate determinations. Values with different superscripts down the column are significantly different (P<0.05)

Table 4.2: Phytochemical composition of leaf and stem bark extract of *Diospyros mespiliformis*

Phytochemical	Leaf	Stem bark
Carbohydrate	+	+
Glycoside	+	+
Saponin	+	+
Cardiac glycoside	+	-
Flavonoids	+	-
Tannin	+	+
Free Anthraquinone	+	-
Alkaloids	+	+

(+ve)=Present: (-ve) = absent

Table 4.3: Quantitative Composition of some phytochemicals in Leaf Methanol Extract of *Diospyros mespiliformis*

PHYTOCHEMICALS (mg/g)	ETHYLACETATE FRACTION	N-BUTANOL FRACTION	AQUEOUS FRACTION
Saponins	0.24 ± 0.04^{b}	0.17 ± 0.03^{a}	0.22 ± 0.03^{ab}
Glycosides	0.35 ± 0.03^{a}	0.34 ± 0.03^{a}	0.45 ± 0.03^{b}
Alkaloids	0.06 ± 0.02^{a}	0.06 ± 0.01^{a}	0.04 ± 0.01^{a}
Tanins	0.25 ± 0.04^{b}	0.37 ± 0.05^{c}	0.14 ± 0.04^{a}

Values are mean ± Standard Deviation (n=3) .Values with different superscripts in the row differ significantly ($p<0.05$)

Table 4.4: Quantitative Composition of some phytochemicals in the Stem Bark Methanol Extracts of *Diospyros mespiliformis*

PHYTOCHEMICALS (mg/g)	ETHYLACETATE FRACTION	N-BUTANOL FRACTION	AQUEOUS FRACTION
Saponins	0.06±0.02[a]	0.08±0.04[a]	0.24±0.03[b]
Glycosides	0.23±0.04[a]	0.38±0.03[b]	0.46±0.03[c]
Alkaloids	0.05±0.03[a]	0.04±0.02[a]	0.03±0.02[a]
Tanins	0.25±0.04[b]	0.14±0.03[a]	0.16±0.03[a]

Values are mean ± Standard Deviation (n=3) .Values with different superscripts in the row differ significantly ($p<0.05$)

4.3 Median Lethal Dose of Leaf and Stem Bark Methanol Extracts of *Diospyros mespiliformis*

The median lethal dose (LD_{50}) determination for leaf and stem bark extract of *Diospyros mespiliformis* are represented in table 4.5 and 4.6. No death was recorded after the oral administration up to a dose of 5000mg/kg body weight for the leaf and stem bark extracts.

Table 4.5: Median Lethal Dose (LD_{50}) Determination for Leaf Methanol Extract of *Diospyros mespiliformis*

Phase	No of mice	Dosage of leaf extract (mg/kg)	Mortality
I	3	10	NO DEATH
	3	100	NO DEATH
	3	1000	NO DEATH
II	1	1600	NO DEATH
	1	2900	NO DEATH
	1	5000	NO DEATH

Table 4.6: Median Lethal Dose (LD50) Determination for Stem Bark Methanol Extract of *Diospyros mespiliformis*

Phase	No of mice	Dosage of stem bark extract (mg/kg)	Mortality
I	3	10	NO DEATH
	3	100	NO DEATH
	3	1000	NO DEATH
II	1	1600	NO DEATH
	1	2900	NO DEATH
	1	5000	NO DEATH

4.4 Effect of different fractions of leaf and stem bark methanol extracts of *Diospyros mespiliformis* on Liver and Kidney organ weight

Effects of different fractions of the leaf and stem bark Methanol Extracts of *Diospyros mespiliformis* on mean change in Organ Weight of *Plasmodium berghei* (NK-65) infected Mice are shown in Table 4.7 and 4.8. Table 4.7 showed no significant ($p<0.05$) difference between the percentage change in liver and kidney weight of the infected treated group compared with normal control group. Ethyl acetate fraction showed large difference in liver and kidney weight. N-butanol and aqueous fraction showed appreciable increase except the infected groups treated with 200 and 400mg/kg body weight of the liver. Table 4.8 showed significant increase in all cases.

Table 4.7: Effect of Different Fractions of the leaf Methanol Extract of *Diospyros mespiliformis* on mean change in Organ (Liver and Kidney) Weight of *Plasmodium berghei* (NK-65) Infected Mice

TREATMENT/DOSE	ETHYL ACETATE FRACTION		N-BUTANOL FRACTION		AQUOEUS FRACTION	
(n=5)	LIVER(g)	KIDNEY(g)	LIVER(g)	KIDNEY(g)	LIVER(g)	KIDNEY(g)
Normal Control (NC)	1.33 ± 0.22^{a}	0.96 ± 0.06^{a}	1.33 ± 0.22^{a}	0.96 ± 0.06^{a}	1.33 ± 0.22^{a}	0.96 ± 0.06^{a}
NC + 200mg/kg	1.42 ± 0.39^{a}	2.57 ± 0.70^{b}	4.46 ± 0.85^{c}	1.50 ± 1.21^{ab}	6.72 ± 2.92^{e}	1.40 ± 0.59^{ab}
IM+ normal saline	4.65 ± 1.17^{d}	2.12 ± 0.97^{b}	4.65 ± 1.17^{c}	2.12 ± 0.97^{b}	4.65 ± 1.17^{d}	2.12 ± 0.97^{b}
IM+ CQ (5mg/kg)	2.29 ± 0.53^{b}	1.82 ± 0.58^{ab}	2.29 ± 0.53^{b}	1.82 ± 0.58^{ab}	2.29 ± 0.53^{bc}	1.82 ± 0.58^{ab}
IM+ ACT (10mg/kg)	1.55 ± 0.54^{ab}	1.54 ± 0.76^{ab}	1.55 ± 0.54^{ab}	1.54 ± 0.76^{ab}	1.55 ± 0.54^{ab}	1.54 ± 0.76^{ab}
IM + 100mg/kg	3.74 ± 1.03^{bcd}	2.00 ± 0.40^{b}	1.86 ± 0.30^{ab}	1.39 ± 0.21^{a}	1.84 ± 0.65^{ab}	1.77 ± 0.85^{ab}
IM+ 200mg/kg	3.80 ± 1.45^{bcd}	1.97 ± 0.95^{b}	1.01 ± 0.27^{a}	1.20 ± 0.30^{a}	1.72 ± 0.31^{ab}	1.31 ± 0.26^{ab}
IM+ 400mg/kg	4.00 ± 0.39^{cd}	2.43 ± 0.74^{b}	1.85 ± 0.78^{ab}	1.53 ± 0.37^{ab}	0.92 ± 0.16^{a}	1.74 ± 0.18^{ab}

Values are Means ± SD. (n=5)..Values with different superscript down the columns are significantly different ($p<0.05$)

NC=Normal Control, CQ=Chloroquine, IM=Infected Mice, ACT=Arthemisinin Combination Therapy.

Table 4.8: Effect of Different Fractions of the Stem Bark Methanol Extract of *Diospyros mespiliformis* on mean change in Organ (Liver and Kidney) Weight of *Plasmodium berghei* (NK-65) Infected Mice

TREATMENT/DOSE	ETHYL ACETATE FRACTION		N-BUTANOL FRACTION		AQUOEUS FRACTION	
(n=5)	LIVER(g)	KIDNEY(g)	LIVER(g)	KIDNEY (g)	LIVER(g)	KIDNEY(g)
Normal Control (NC)	1.33 ± 0.22^{a}	0.96 ± 0.06^{a}	1.33 ± 0.22^{a}	0.96 ± 0.06^{a}	1.33 ± 0.22^{a}	0.96 ± 0.06^{a}
NC + 100mg/kg	4.28 ± 2.28^{bc}	1.44 ± 0.68^{ab}	5.09 ± 1.88^{cd}	1.63 ± 0.10^{ab}	5.08 ± 1.64^{c}	1.95 ± 1.16^{a}
IM+ normal saline	4.65 ± 1.17^{c}	2.12 ± 0.97^{abc}	4.65 ± 1.17^{cd}	2.12 ± 0.97^{b}	4.65 ± 1.17^{bc}	2.12 ± 0.97^{a}
IM+ CQ (5mg/kg)	2.29 ± 0.53^{ab}	1.82 ± 0.58^{ab}	2.29 ± 0.53^{ab}	1.82 ± 0.58^{ab}	2.29 ± 0.53^{ab}	1.82 ± 0.58^{a}
IM+ ACT (10mg/kg)	1.55 ± 0.54^{a}	1.54 ± 0.76^{ab}	1.55 ± 0.54^{a}	1.54 ± 0.76^{ab}	1.55 ± 0.54^{a}	1.54 ± 0.76^{a}
IM + 50mg/kg	2.26 ± 0.45^{ab}	2.11 ± 0.61^{abc}	6.21 ± 2.10^{d}	1.04 ± 0.29^{a}	1.93 ± 0.34^{a}	2.15 ± 1.05^{a}
IM+ 100mg/kg	4.20 ± 1.42^{bc}	2.00 ± 0.44^{abc}	3.94 ± 1.00^{cd}	1.45 ± 0.46^{ab}	8.43 ± 4.10^{d}	2.13 ± 0.96^{a}
IM+ 200mg/kg	5.42 ± 2.91^{d}	2.68 ± 0.88^{bc}	6.30 ± 2.23^{d}	1.71 ± 0.40^{ab}	6.69 ± 3.00^{cd}	1.48 ± 0.52^{a}

Values are Means ± SD. (n=5).Values with different superscript down the columns are significantly different ($p<0.05$).

NC=Normal Control, CQ=Chloroquine, IM=Infected Mice, ACT=Arthemisinin Combination Therapy.

4.5 CHANGES IN PACKED CELL VOLUME AND HAEMOGLOBIN LEVEL IN MICE

4.5.1 Effect of leaf and stem bark fractions of *Diospyros mespiliformis* on packed cell volume and haemoglobin levels

The effect of oral administration of leaf and stem bark fractions of *Diospyros mespiliformis* on packed cell volume are shown in Figure 4.1 and 4.2 and haemoglobin level are shown in Figure 4.3 and 4.4 respectively. The result showed that, the packed cell volume (PCV) and haemoglobin (Hb) level in the infected normal saline group was significantly ($P<0.05$) lower than the PCV and Hb level of the normal control group and infected treated group.

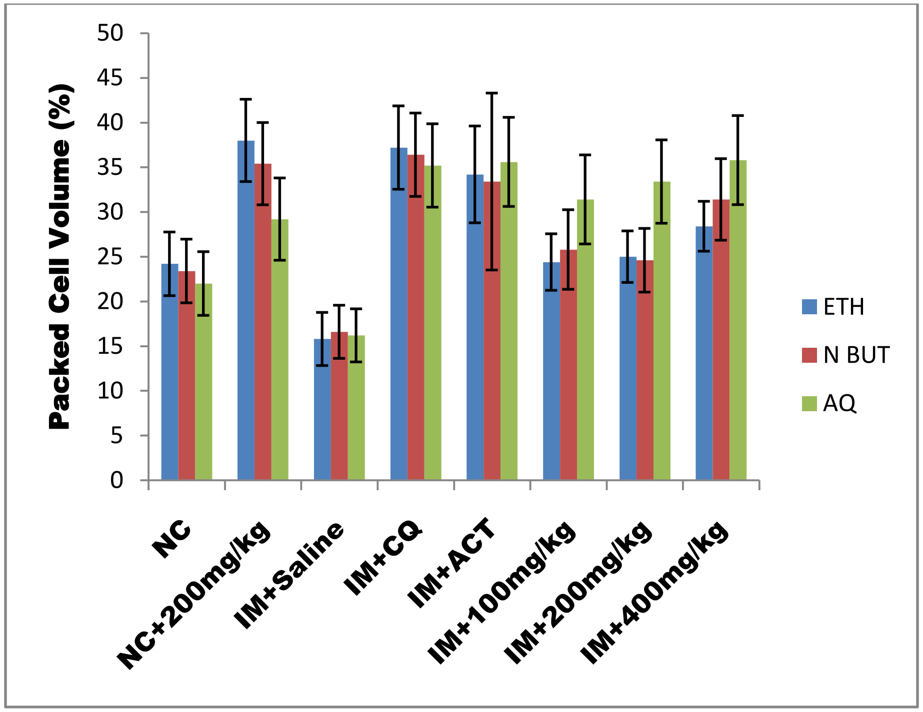


Figure 4.1: Effect of Different Fractions of Leaf Methanol Extract of *Diospyrosmespiliformis* on Packed Cell Volume (PCV) of *Plasmodiumberghei* Infected Mice

Values are Means ± SD. (n=5). significantly different ($p<0.05$)

NC=Normal Control, CQ=Chloroquine,

IM=Infected Mice, ACT=Arthemisinin Combination Therapy.

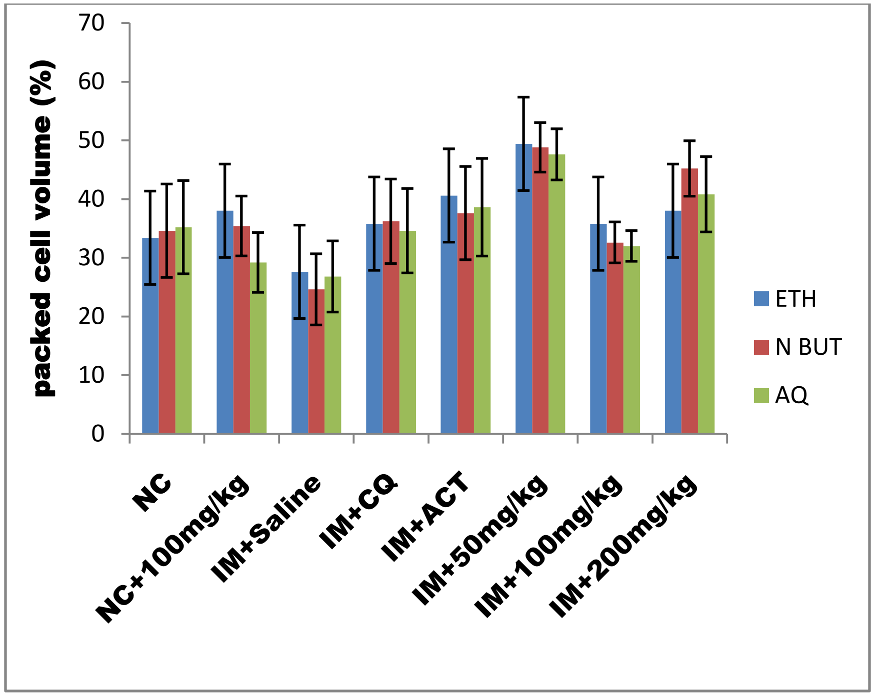


Figure 4.2: Effect of Different Fractions of Stem Bark Methanol Extract of *Diospyrosmespiliformis* on Packed Cell Volume (PCV) of *Plasmodiumberghei* Infected Mice

Values are Means ± SD.(n=5). significantly different (p<0.05)

NC=Normal Control, CQ=Chloroquine,

IM=Infected Mice, ACT=Arthemisinin Combination Therapy.

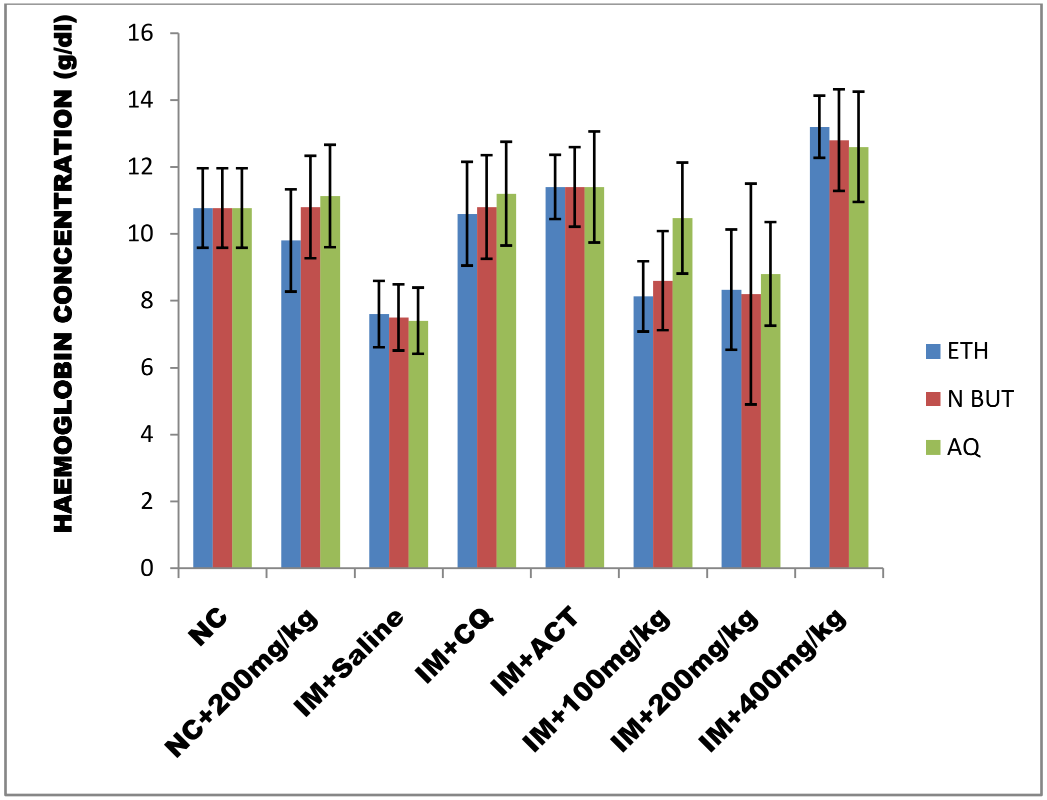


Figure 4.3: Effect of Different Fractions of Leaf Methanol Extract of *Diospyrosmespiliformis* on Haemoglobin level (Hb) of *Plasmodiumberghei* Infected Mice

Values are Means ± SD. (n=5). significantly different (p<0.05)

NC=Normal Control, CQ=Chloroquine,

IM=Infected Mice, ACT=Arthemisinin Combination Therapy.

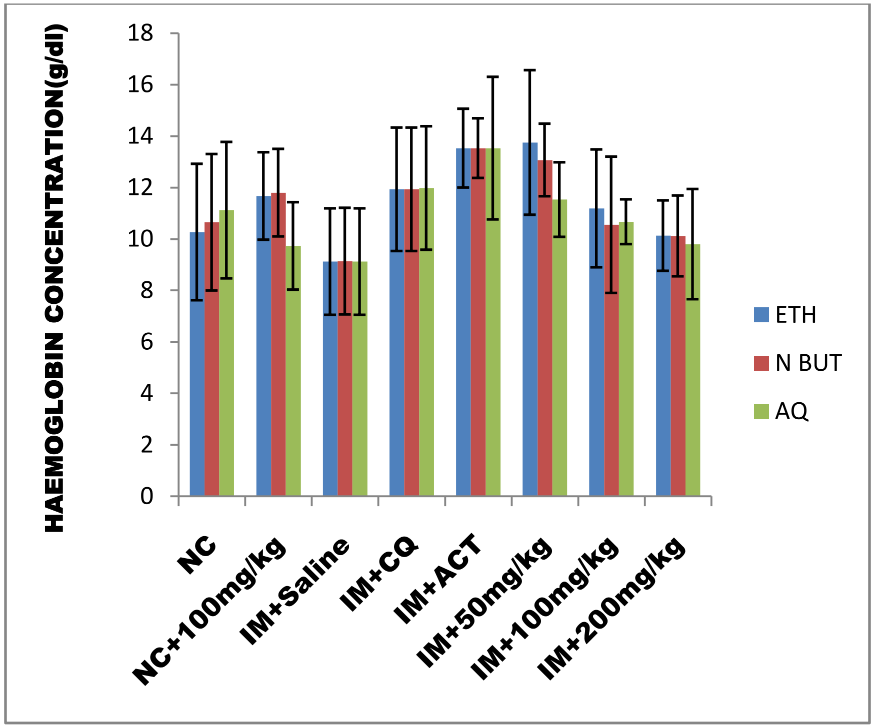


Figure 4.4 Effect of Different Fractions of Stem Bark Methanol Extract of *Diospyrosmespiliformis* on Haemoglobin level (Hb) of *Plasmodiumberghei* Infected Mice

Values are Means ± SD. (n=5). significantly different ($p<0.05$).

NC=Normal Control, CQ=Chloroquine,

IM=Infected Mice, ACT=Arthemisinin Combination Therapy.

4.6 In-vivo Parasiteamia Level and Percentage Suppression of *Plasmodium berghei* (NK-65) by Different Fraction of the Leaf and Stem Bark Methanol Extracts of *Diospyros mespiliformis*

At the end of the four (4) days suppressive test, table 4.9 and 4.10 showed no significant ($P<0.05$) change in parasiteamia level in both the normal control group and the normal control group treated with 200mg/kg body weight. Significant increases in parasiteamia level were recorded in the infected group and treated group (dose dependent). However, significant increase ($P<0.05$) in % suppression of the infected treated group compare to the infected group (Negative control group).

Table 4.9: In-vivo Parasiteamia Level and Percentage Suppression of *Plasmodium berghei* (NK-65) by Different Fractions of the Leaf Methanol Extract of *Diospyros mespiliformis*

TREATMENT/DOSE (n=5)	ETHYL ACETATE FRACTION		N-BUTANOL FRACTION		AQUEOUS FRACTION	
	PARASITEAMIA LEVEL	% SUPPRESSION	PARASITEAMIA LEVEL	% SUPPRESSION	PARASITEAMIA LEVEL	% SUPPRESSION
Normal Control (NC)	0.00±0.00[a]	0	0.00±0.00[a]	0	0.00±0.00[a]	0
NC + 200mg/kg	0.00±0.00[a]	0	0.00±0.00[a]	0	0.00±0.00[a]	0
IM+ normal saline	1.94±0.08[e]	0	1.94±0.08[e]	0	1.94±0.08[d]	0
IM+ CQ(5mg/kg)	0.22±0.21[b]	88.66	0.22±0.21[ab]	88.66	0.22±0.21[a]	88.66
IM+ ACT(10mg/kg)	0.37±0.22[bc]	80.93	0.37±0.22[bc]	80.93	0.37±0.22[ab]	80.93
IM + 100mg/kg	0.42±0.81[c]	56.19	0.48±0.26[bc]	58.24	0.73±0.52[bc]	56.70
IM+ 200mg/kg	0.54±0.92[c]	72.16	0.62±0.31[cd]	68.04	0.77±0.27[c]	60.30
IM+ 400mg/kg	0.85±0.21[d]	78.35	0.81±0.21[d]	75.25	0.84±0.47[c]	62.37

Values are Means ± SD. (n=5).Values with different superscript down the columns are significantly different ($p<0.05$).

NC=Normal Control, CQ=Chloroquine,

IM=Infected Mice, ACT=Arthemisinin Combination Therapy.

Table 4.10: In-vivo Parasiteamia Level and Percentage Suppression of *Plasmodium berghei* (NK-65) by Different Fractions of the Stem bark Methanol Extract of *Diospyros mespiliformis*

TREATMENT/DOSE	ETHYL ACETATE FRACTION		N-BUTANOL FRACTION		AQUEOUS FRACTION	
(n=5)	PARASITEAMIA LEVEL	% SUPPRESSION	PARASITEAMIA LEVEL	% SUPPRESSION	PARASITEAMIA LEVEL	% SUPPRESSION
Normal Control (NC)	0.00 ± 0.00^{a}	0	0.00 ± 0.00^{a}	0	0.00 ± 0.00^{a}	0
NC+ 100mg/kg	0.00 ± 0.00^{a}	0	0.00 ± 0.00^{a}	0	0.00 ± 0.00^{a}	0
IM+ normal saline	1.94 ± 0.08^{e}	0	1.94 ± 0.08^{e}	0	1.94 ± 0.08^{d}	0
IM+ CQ(5mg/kg)	0.22 ± 0.21^{b}	88.66	0.22 ± 0.21^{ab}	88.66	0.22 ± 0.21^{a}	88.66
IM+ ACT (10mg/kg)	0.37 ± 0.22^{bc}	80.93	0.37 ± 0.22^{bc}	80.93	0.37 ± 0.22^{ab}	80.93
IM + 50mg/kg	0.22 ± 0.07^{b}	72.68	0.56 ± 0.19^{cd}	64.43	0.11 ± 0.09^{a}	76.29
IM+ 100mg/kg	0.29 ± 0.28^{b}	85.05	0.62 ± 0.13^{d}	68.04	0.46 ± 0.22^{b}	87.5
IM+ 200mg/kg	0.53 ± 0.30^{c}	88.66	0.69 ± 0.22^{d}	71.13	0.50 ± 0.20^{c}	94.33

Values are Means ± SD. (n=5).Values with different superscript down the columns are significantly different ($p<0.05$).

NC=Normal Control, CQ=Chloroquine,

IM=Infected Mice, ACT=Arthemisinin Combination Therapy.

Table 4.11: Effect of Different Fractions of the Leaf Methanol Extract of *Diospyros mespiliformis* on Liver Function Parameters of *Plasmodium berghei* (NK-65) Infected Mice

TREATMENT/DOSE	**AST (IU/L)**			**ALT (IU/L)**			**ALP (IU/L)**		
(n=5)	ETHYL ACETATE FRACTION	N-BUTANOL FRACTION	AQUOEUS FRACTION	ETHYL ACETATE FRACTION	N-BUTANOL FRACTION	AQUOEUS FRACTION	ETHYL ACETATE FRACTION	N-BUTANOL FRACTION	AQUOEUS FRACTION
Normal Control (NC)	42.66±6.71^{c}	42.66±6.71^{bc}	42.66±6.71^{d}	43.61±3.21^{b}	43.61±3.21^{b}	43.61±3.21^{b}	33.07±7.31^{ab}	33.07±7.31^{ab}	33.07±7.31^{ab}
NC + 200mg/kg	30.55±1.57^{a}	36.03±3.94^{ab}	41.74±3.86^{d}	31.03±5.35^{a}	36.87±5.21^{ab}	40.08±5.99^{ab}	26.33±4.66^{a}	29.51±6.02^{a}	31.51±3.62^{ab}
IM+ normal saline	50.78±5.42^{d}	50.78±5.42^{c}	50.78±5.42^{e}	56.42±5.61^{c}	56.42±5.61^{c}	56.42±5.61^{c}	55.91±14.43^{c}	55.91±14.43^{c}	55.91±14.43^{c}
IM+ CQ (5mg/kg)	34.20±3.51^{ab}	34.20±3.51^{ab}	34.20±3.51^{c}	40.41±2.57^{b}	40.41±2.57^{ab}	40.41±2.57^{ab}	30.84±1.02^{a}	30.84±1.02^{ab}	30.84±1.02^{ab}
IM+ACT(10mg/kg)	34.48±3.89^{ab}	34.48±3.89^{ab}	34.48±3.89^{c}	42.54±2.27^{b}	42.54±6.27^{b}	42.54±10.27^{b}	35.08±8.34^{ab}	35.08±8.34^{ab}	35.08±8.34^{ab}
IM + 100mg/kg	38.28±2.29^{bc}	32.68±9.46^{a}	28.72±4.95^{bc}	27.62±4.18^{a}	32.43±8.66^{a}	30.57±5.27^{a}	36.87±3.84^{ab}	34.14±3.82^{ab}	29.01±3.71^{a}
IM+ 200mg/kg	41.00±7.65^{bc}	30.44±6.53^{a}	22.60±2.33^{a}	36.11±2.00^{ab}	40.39±5.28^{ab}	32.29±9.08^{ab}	41.85±9.33^{b}	36.28±2.70^{ab}	41.81±11.53^{b}
IM+ 400mg/kg	49.80±5.99^{d}	42.60±9.83^{bc}	25.88±3.61^{ab}	58.44±5.20^{c}	45.41±8.97^{b}	36.66±10.79^{ab}	42.30±4.07^{b}	40.20±2.10^{b}	42.09±7.32^{b}

Values are Means ± SD. (n=5).Values with different superscript down the columns are significantly different ($p<0.05$).

NC=Normal Control, CQ=Chloroquine,

IM=Infected Mice, ACT=Arthemisinin Combination Therapy.

4.12: Effect of Different Fractions of the Stem Bark Methanol Extract of *Diospyros mespiliformis* on Liver Function Parameters of *Plasmodium berghei* (NK-65) Infected Mice

TREATMENT/DOSE	AST (IU/L)			ALT (IU/L)			ALP (IU/L)		
(n=5)	ETHYL ACETATE FRACTION	N-BUTANOL FRACTION	AQUOEUS FRACTION	ETHYL ACETATE FRACTION	N-BUTANOL FRACTION	AQUOEUS FRACTION	ETHYL ACETATE FRACTION	N-BUTANOL FRACTION	AQUOEUS FRACTION
Normal Control (NC)	42.66±6.71^{c}	42.66±6.71^{d}	42.66±6.71^{b}	43.61±3.21^{e}	43.61±3.21^{c}	43.61±3.21^{b}	33.07±7.31^{ab}	33.07±7.31^{a}	33.07±7.31^{a}
NC + 100mg/kg	40.07±2.12^{bc}	32.57±2.30^{bc}	42.89±7.09^{b}	33.31±5.51^{bc}	36.49±4.75^{bc}	38.93±4.84^{b}	32.67±2.44^{ab}	33.25±5.75^{a}	38.25±6.24^{ab}
IM+ normal saline	50.78±5.42^{d}	50.78±5.42^{e}	50.78±5.42^{c}	56.42±5.61^{f}	56.42±5.61^{d}	56.42±5.61^{c}	55.91±14.43^{c}	55.91±14.43^{c}	55.91±14.43^{d}
IM+ CQ (5mg/kg)	34.20±3.51^{b}	34.20±3.51^{c}	34.20±3.51^{a}	40.41±2.57^{cde}	40.41±2.57^{bc}	40.41±2.57^{b}	30.84±1.02^{a}	30.84±1.02^{a}	30.84±1.02^{a}
IM+ ACT(10mg/kg)	34.48±3.89^{b}	34.48±3.89^{c}	34.48±3.89^{a}	42.54±10.27^{de}	42.54±10.27^{c}	42.54±10.27^{b}	35.08±8.34^{ab}	35.08±8.34^{a}	35.08±8.34^{ab}
IM + 50mg/kg	33.93±3.99^{b}	25.35±4.25^{ab}	31.97±5.83^{a}	35.29±6.50^{bcd}	38.49±6.97^{bc}	30.27±2.26^{a}	42.09±3.97^{b}	45.71±3.51^{b}	39.65±2.95^{ab}
IM+ 100mg/kg	34.51±5.52^{b}	21.89±5.90^{a}	38.47±2.63^{ab}	31.85±2.77^{b}	34.70±2.50^{b}	38.37±2.04^{b}	39.51±5.67^{ab}	40.89±2.84^{ab}	44.65±2.41^{bc}
IM+ 200mg/kg	25.69±5.92^{a}	30.73±5.40^{bc}	42.97±4.23^{b}	23.65±1.78^{a}	26.03±0.71^{a}	42.05±7.37^{b}	31.81±3.25^{a}	35.23±2.96^{a}	49.97±2.63^{cd}

Values are Means ± SD. (n=5).Values with different superscript down the columns are significantly different ($p<0.05$).

NC=Normal Control, CQ=Chloroquine,

IM=Infected Mice, ACT=Arthemisinin Combination Therapy.

Table 4.13: Effect of Different Fractions of the Leaf Methanol Extract of *Diospyros mespiliformis* on Serum Total Protein and Albumin Parameters of *Plasmodium berghei* (NK-65) Infected Mice

TREATMENT/DOSE	TOTAL PROTEIN (g/L)			ALBUMIN (g/L)		
(n=5)	ETHYL ACETATE FRACTION	N-BUTANOL FRACTION	AQUOEUS FRACTION	ETHYL ACETATE FRACTION	N-BUTANOL FRACTION	AQUOEUS FRACTION
Normal Control (NC)	44.72±9.36^{ab}	44.72±9.36^{bc}	44.72±9.36^{b}	16.60±9.70^{a}	16.60±9.70^{a}	16.60±9.70^{a}
NC + 200mg/kg	38.67±12.88^{a}	47.96±4.44^{cd}	43.65±7.21^{b}	32.00±5.10^{d}	36.20±6.06^{b}	18.60±3.78^{a}
IM+ normal saline	54.99±4.17^{b}	54.99±4.17^{e}	54.99±4.17^{c}	29.00±5.70^{cd}	29.00±5.70^{b}	29.00±5.70^{b}
IM+ CQ(5mg/kg)	36.06±6.74^{a}	36.06±6.74^{ab}	36.06±6.74^{ab}	17.80±1.92^{a}	17.80±1.92^{a}	17.80±1.92^{a}
IM+ ACT (10mg/kg)	36.16±10.94^{a}	36.16±10.94^{ab}	36.16±10.94^{ab}	23.60±8.50^{abc}	23.60±8.50^{ab}	23.60±8.50^{ab}
IM + 100mg/kg	37.05±9.49^{a}	31.10±5.35^{a}	30.92±10.87^{a}	19.20±3.90^{ab}	16.80±2.68^{a}	19.00±6.69^{a}
IM+ 200mg/kg	39.56±10.42^{a}	36.44±3.28^{ab}	33.40±7.93^{ab}	23.40±3.05^{abc}	17.80±4.44^{a}	21.40±4.22^{ab}
IM+ 400mg/kg	46.40±15.14^{ab}	40.56±5.19^{abc}	39.44±5.08^{ab}	25.60±3.05^{bcd}	21.20±3.83^{ab}	22.40±7.30^{ab}

Values are Means ± SD. (n=5).Values with different superscript down the columns are significantly different ($p<0.05$).

NC=Normal Control, CQ=Chloroquine,

IM=Infected Mice, ACT=Arthemisinin Combination Therapy.

Table 4.14: Effect of Different Fractions of the Stem Bark Methanol Extract of *Diospyros mespiliformis* on Serum Total Protein and Albumin Parameters of *Plasmodium berghei* (NK-65) Infected Mice

TREATMENT/DOSE	TOTAL PROTEIN (g/L)			ALBUMIN (g/L)		
(n=5)	ETHYL ACETATE FRACTION	N-BUTANOL FRACTION	AQUOEUS FRACTION	ETHYL ACETATE FRACTION	N-BUTANOL FRACTION	AQUOEUS FRACTION
Normal Control (NC)	44.72±9.36^{ab}	44.72±9.36^{abc}	44.72±9.36^{a}	16.60±9.70^{a}	16.60±9.70^{a}	16.60±9.70^{a}
NC + 100mg/kg	43.88±12.77^{ab}	45.00±6.68^{abc}	61.72±8.16^{b}	33.60±4.45^{b}	27.80±6.38^{bc}	23.20±5.93^{ab}
IM+ normal saline	54.99±4.17^{c}	54.99±4.17^{c}	54.99±4.17^{b}	29.00±5.70^{b}	29.00±5.70^{c}	29.00±5.70^{b}
IM+ CQ (5mg/kg)	36.06±6.74^{a}	36.06±6.74^{a}	36.06±6.74^{a}	17.80±1.92^{a}	17.80±1.92^{ab}	17.80±1.92^{a}
IM+ ACT (10mg/kg)	36.16±10.94^{a}	36.16±10.94^{a}	36.16±10.94^{a}	23.60±8.50^{ab}	23.60±8.50^{abc}	23.60±8.50^{ab}
IM + 50mg/kg	42.52±7.59^{a}	48.40±2.73^{bc}	61.12±5.22^{b}	23.40±9.81^{ab}	24.80±9.01^{abc}	18.80±3.35^{a}
IM+ 100mg/kg	40.80±4.01^{a}	38.80±4.23^{ab}	57.48±8.26^{b}	16.80±8.14^{a}	20.80±6.61^{abc}	15.20±1.92^{a}
IM+ 200mg/kg	45.32±3.75^{ab}	54.04±10.30^{c}	57.92±6.21^{b}	29.00±10.12^{b}	26.00±8.06^{abc}	17.20±3.90^{a}

Values are Means ± SD.(n=5).Values with different superscript down the columns are significantly different ($p<0.05$).

NC=Normal Control, CQ=Chloroquine,

IM=Infected Mice, ACT=Arthemisinin Combination Therapy.

Table 4.15: Effect of Different Fractions of the Leaf Methanol Extract of *Diospyros mespiliformis* on Serum Bilirubin Parameters of *Plasmodium berghei* (NK-65) Infected Mice

TREATMENT/DOSE	ETHYL ACETATE FRACTION		N-BUTANOL FRACTION		AQUOEUS FRACTION	
(n=5)	DIR BIL(g/L)	IND BIL(g/L)	DIR BIL(g/L)	IND BIL(g/L)	DIR BIL(g/L)	IND BIL(g/L)
Normal Control (NC)	$4.57±0.65^{bc}$	$1.29±0.65^{a}$	$4.57±0.65^{bc}$	$1.29±0.65^{a}$	$4.57±0.65^{a}$	$1.29±0.65^{ab}$
NC + 200mg/kg	$4.80±1.15^{b}$	$1.21±0.24^{a}$	$4.42±1.07^{bc}$	$1.70±1.67^{a}$	$4.23±3.26^{ab}$	$2.00±1.54^{ab}$
IM+ normal saline	$5.75±1.25^{c}$	$1.89±1.57^{a}$	$5.75±1.25^{c}$	$1.89±1.57^{a}$	$5.75±1.25^{b}$	$1.89±1.57^{ab}$
IM+ CQ (5mg/kg)	$3.45±0.87^{ab}$	$1.15±1.05^{a}$	$3.45±0.87^{ab}$	$1.15±0.15^{a}$	$3.45±0.87^{a}$	$1.15±0.15^{b}$
IM+ ACT (10mg/kg)	$3.76±1.71^{b}$	$2.91±2.07^{a}$	$3.76±1.71^{ab}$	$2.91±0.07^{a}$	$3.76±1.71^{a}$	$2.91±1.07^{bc}$
IM + 100mg/kg	$4.12±1.24^{b}$	$1.28±1.16^{a}$	$2.80±1.41^{ab}$	$3.04±1.34^{a}$	$4.35±3.73^{ab}$	$0.56±0.12^{a}$
IM+ 200mg/kg	$2.10±0.69^{a}$	$1.58±1.34^{a}$	$3.08±1.78^{ab}$	$2.09±1.09^{a}$	$3.77±1.80^{a}$	$2.15±0.99^{abc}$
IM+ 400mg/kg	$2.20±1.14^{a}$	$1.58±0.98^{a}$	$2.07±0.79^{a}$	$1.91±0.62^{a}$	$2.97±0.12^{a}$	$3.87±1.34^{c}$

Values are Means ± SD. (n=5).Values with different superscript down the columns are significantly different ($p<0.05$).

NC=Normal Control, CQ=Chloroquine,

IM=Infected Mice, ACT=Arthemisinin Combination Therapy.

Table 4.16: Effect of Different Fractions of the Stem Bark Methanol Extract of *Diospyros mespiliformis* on Serum Bilirubin Parameters of *Plasmodium berghei* (NK-65) Infected Mice

TREATMENT/DOSE	**ETHYL ACETATE FRCTION**		**N-BUTANOL FRACTION**		**AQUOEUS FRACTION**	
(n=5)	DIR BIL(g/L)	IND BIL(g/L)	DIR BIL(g/L)	IND BIL(g/L)	DIR BIL(g/L)	IND BIL(g/L)
Normal Control (NC)	4.57±0.65^{b}	1.29±0.65^{a}	4.57±0.65^{b}	1.29±0.65^{a}	4.57±0.65^{b}	1.29±0.65^{ab}
NC + 100mg/kg	0.54±0.23^{a}	2.26±0.67^{ab}	0.42±0.24^{a}	1.94±0.65^{ab}	0.40±0.16^{a}	0.56±0.43^{a}
IM+ normal saline	5.75±1.25^{c}	1.89±1.57^{ab}	5.75±1.25^{c}	1.89±1.57^{ab}	5.75±1.25^{c}	1.89±1.57^{ab}
IM+ CQ (5mg/kg)	3.45±0.87^{b}	1.15±0.15^{a}	3.45±0.87^{b}	1.15±0.15^{a}	3.45±0.87^{b}	1.15±0.15^{a}
IM+ ACT (10mg/kg)	3.76±1.71^{b}	2.91±1.07^{b}	3.76±1.71^{b}	2.91±1.07^{b}	3.76±1.71^{b}	2.91±2.07^{b}
IM + 50mg/kg	0.74±0.34^{a}	0.98±0.68^{a}	1.20±0.52^{a}	0.92±0.33^{a}	0.36±0.11^{a}	1.86±0.48^{ab}
IM+ 100mg/kg	0.48±0.30^{a}	0.82±0.16^{a}	0.38±0.08^{a}	1.10±0.21^{a}	0.34±0.17^{a}	1.16±1.15^{a}
IM+ 200mg/kg	0.56±0.24^{a}	0.66±0.53^{a}	0.48±0.15^{a}	0.94±0.42^{a}	0.38±0.08^{a}	1.86±0.91^{ab}

Values are Means ± SD. (n=5).Values with different superscript down the columns are significantly different ($p<0.05$).

NC=Normal Control, CQ=Chloroquine,

IM=Infected Mice, ACT=Arthemisinin Combination Therapy.

4.7.2 EFFECT OF DIFFERENT FRACTIONS OF THE LEAF AND STEM BARK METHANOL EXTRACTS OF *DIOSPYROS MESPILIFORMIS* ON KIDNEY FUNCTION PARAMETERS

Creatinine and urea concentration in the serum of normal and infected mice of different fractions of leaf and stem bark methanol extracts of *Diospyros mespiliformis* are represented in Table 4.17 and 4.18 respectively. The result showed that the concentration of serum creatinine of the leaf extract significance ($P<0.05$) increase in the ethyl acetate and n-butanol fractions of the infected saline group, infected low and medium dose group compared to the normal control. Kidney function studies of stem bark extract showed that there were significance ($P<0.05$) increase in creatinine serum level of the non-infected medium dose group, and significance increase in aqueous fraction of infected saline group compare to the normal control. No significant difference in urea level down the group.

Table 4.17: Effect of Different Fractions of the Leaf Methanol Extract of *Diospyros mespiliformis* on Serum Creatinine and Urea Parameters of *Plasmodium berghei* (NK-65) Infected Mice

TREATMENT/DOSE	**CREATININE(Mg/dl)**			**UREA(Mg/dl)**		
(n=5)	Ethyl acetate fraction	N-Butanol fraction	Aqueous fraction	Ethyl acetate fraction	N-Butanol fraction	Aqueous fraction
Normal Control (NC)	37.00 ± 1.54^{ab}	37.00 ± 1.54^{ab}	37.00 ± 1.54^{ab}	2.30 ± 0.84^{a}	2.30 ± 0.84^{a}	2.30 ± 0.84^{a}
NC + 200mg/kg	40.10 ± 4.60^{b}	42.20 ± 5.14^{cd}	39.10 ± 2.53^{b}	2.20 ± 0.45^{a}	4.20 ± 0.76^{ab}	3.84 ± 1.45^{ab}
IM+ normal saline	44.90 ± 5.52^{c}	44.90 ± 5.52^{d}	44.90 ± 5.52^{c}	3.60 ± 2.19^{ab}	3.60 ± 2.19^{ab}	3.60 ± 2.19^{ab}
IM+ CQ (5mg/kg)	36.90 ± 1.64^{ab}	36.90 ± 1.64^{ab}	36.90 ± 1.64^{ab}	2.32 ± 0.81^{a}	2.32 ± 0.81^{a}	2.32 ± 0.81^{a}
IM+ ACT(10mg/kg)	40.30 ± 1.04^{b}	40.30 ± 1.04^{bc}	40.30 ± 1.04^{b}	2.56 ± 1.17^{a}	2.56 ± 1.17^{ab}	2.56 ± 1.17^{ab}
IM + 100mg/kg	35.00 ± 3.22^{a}	35.00 ± 1.27^{a}	44.20 ± 1.64^{c}	3.38 ± 1.80^{ab}	3.70 ± 2.14^{ab}	4.74 ± 2.48^{b}
IM+ 200mg/kg	46.40 ± 1.47^{c}	44.50 ± 1.84^{d}	34.00 ± 2.43^{a}	3.62 ± 1.30^{ab}	4.64 ± 1.40^{b}	3.62 ± 1.60^{ab}
IM+ 400mg/kg	35.10 ± 2.70^{a}	34.30 ± 1.92^{a}	34.10 ± 1.14^{a}	4.90 ± 2.25^{b}	4.48 ± 1.73^{ab}	4.26 ± 1.64^{ab}

Values are Means ± SD. (n=5).Values with different superscript down the columns are significantly different ($p<0.05$).

NC=Normal Control, CQ=Chloroquine,

IM=Infected Mice, ACT=Arthemisinin Combination Therapy.

Table 4.18: Effect of Different Fractions of the Leaf Methanol Extract of *Diospyros mespiliformis* on Serum Creatinine and Urea Parameters of *Plasmodium berghei* (NK-65) Infected Mice

TREATMENT/DOSE	CREATINIE (Mg/DL)			UREA (Mg/DL)		
(n=5)	Ethyl acetate fraction	N-Butanol fraction	Aqueous fraction	Ethyl acetate fraction	N-Butanol fraction	Aqeous fraction
Normal Control (NC)	37.00±1.54^{ab}	37.00±1.54^{ab}	37.00±1.54^{bc}	2.30±0.84^{a}	2.30±0.84^{a}	2.30±0.84^{a}
NC + 100mg/kg	50.60±7.19^{d}	53.60±3.30^{e}	46.60±6.77^{e}	4.34±1.23^{b}	4.60±2.88^{b}	3.50±1.17^{ab}
IM+ normal saline	44.90±5.52^{c}	44.90±5.52^{c}	44.90±5.52^{d}	3.60±2.19^{ab}	3.60±2.19^{ab}	3.60±2.19^{ab}
IM+ CQ (5mg/kg)	36.90±1.64^{a}	36.90±1.64^{ab}	36.90±1.64^{bc}	2.32±0.81^{a}	2.32±0.81^{a}	2.32±0.81^{a}
IM+ ACT(10mg/kg)	40.30±1.04^{b}	40.30±1.04^{b}	40.30±1.04^{bc}	2.56±1.17^{a}	2.56±1.17^{a}	2.56±1.17^{a}
IM + 50mg/kg	42.20±5.14^{bc}	35.90±3.01^{a}	32.20±1.44^{b}	3.92±1.31^{ab}	3.50±1.03^{ab}	2.92±0.73^{a}
IM+ 100mg/kg	45.20±2.64^{c}	46.40±3.23^{cd}	28.50±2.82^{a}	4.60±2.88^{b}	4.20±0.97^{b}	4.60±2.46^{b}
IM+ 200mg/kg	41.10±5.09^{b}	44.60±2.10^{c}	40.20±4.70^{bc}	4.06±1.55^{b}	3.28±0.61^{ab}	4.10±2.88^{b}

Values are Means ± SD. (n=5).Values with different superscript down the columns are significantly different ($p<0.05$).

NC=Normal Control, CQ=Chloroquine,

IM=Infected Mice, ACT=Arthemisinin Combination Therapy.

4.8 Purification Profile of *Plasmodium berghei* cysteine protease

Results of the partially purified *Plasmodium berghei* cysteine protease are summarized in Table 4.19. The crude protein contained approximately 2.79mg/ml total protein with a specific activity of 3.86µmol/min/mg. Precipitation of the crude protein with ammonium sulphate resulted to a purification fold of 2.55 and a 46.00% yield. The result obtained following gel filtration on Sephadex G-50 column revealed a total yield of 35.69% with a specific activity of 47.30µmol/min/mg.

Figure 4.1 is a plot showing the elution profile of protein cysteine protease after gel filtration on Sephadex G-50 column. The plot resulted to a two-peak fraction of the enzyme. From the plot, the first peak has about 0.05 mg/ml of protein concentration with a corresponding enzyme activity of 0.41 µmol/min. The second peak with a reduced height has about 0.001 mg/ml of protein concentration and an activity of about 0.145 µmol/min.

Table 4.19: Purification Profile for *Plasmodium berghei* cysteine protease

Purification Step	Total Protein (mg)	Total Activity (µmol/min)	Specific Activity (µmol/min/mg)	Purification Fold	Percentage Yield
Crude Protein	2.79	10.76	3.86	1.00	100
Ammonium Sulphate Fractionation	0.50	4.95	9.85	2.55	46.00
Gel Filtration on Sephadex G-50 Column	0.08	3.84	47.30	12.25	35.69

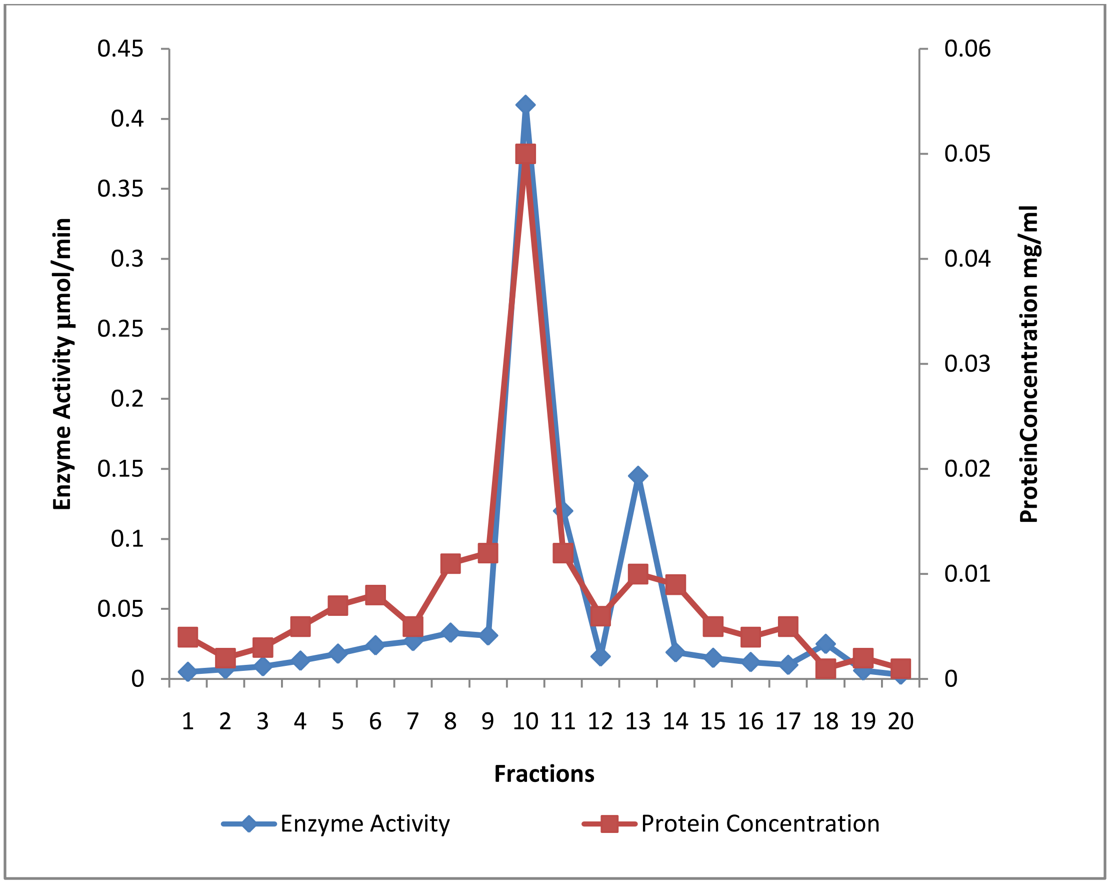


Fig 4.5: Elution Profile of Plasmodium berghei cysteine protease on Sephadex G-50 Column Chromatography.

4.8.1 Inhibition Studies and Determination of Inhibition Pattern Using the Plant Extracts

The double reciprocal plot (in Figure 4.8) for the leaf bioactive fraction of *Diospyros mespiliformis* shows a mixed non-competitive pattern of inhibition which was dose dependent. Figure 4.9 presents a double reciprocal plot describing the pattern of inhibition of the stem-bark bioactive fraction of *Diospyros mespiliformis* on the *Plasmodium berghei* protein cysteine protease. It clearly depicts a competitive inhibition pattern which was as well a dose dependent. Figure 4.10 and 4.11 present secondary plots showing how the Ki (Inhibition binding constant) for the extracts were estimated for the *Diospyros mespiliformis* leaf and stem bark extracts respectively, against the *Plasmodium berghei* cysteine protease. The points of intersection of the plots on the x-axis (representing the concentration of the extracts) was used to deduce the Ki, they were found to be 0.83mg/ml for *Diospyros mespiliformis* leaf extract against the purified enzyme, and 2.68mg/ml for *Diospyros mespiliformis* stem bark extract against the purified enzyme.

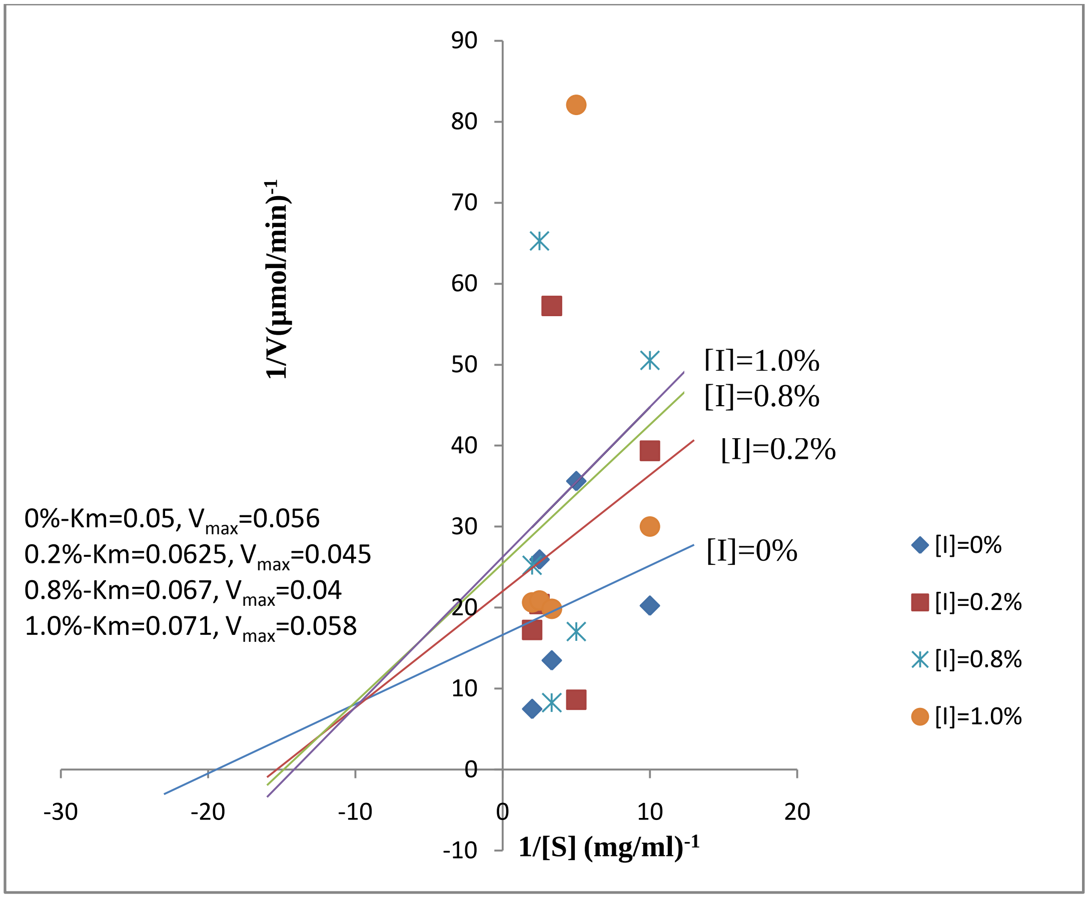


Figure 4.6: Lineweaver-Burk Plot Showing the Inhibitory Effect of Leaf Bioactive fraction of *Diospyros mespiliformis* on *Plasmodium berghei* cysteine protease (Mixed non-competitive pattern of inhibition).

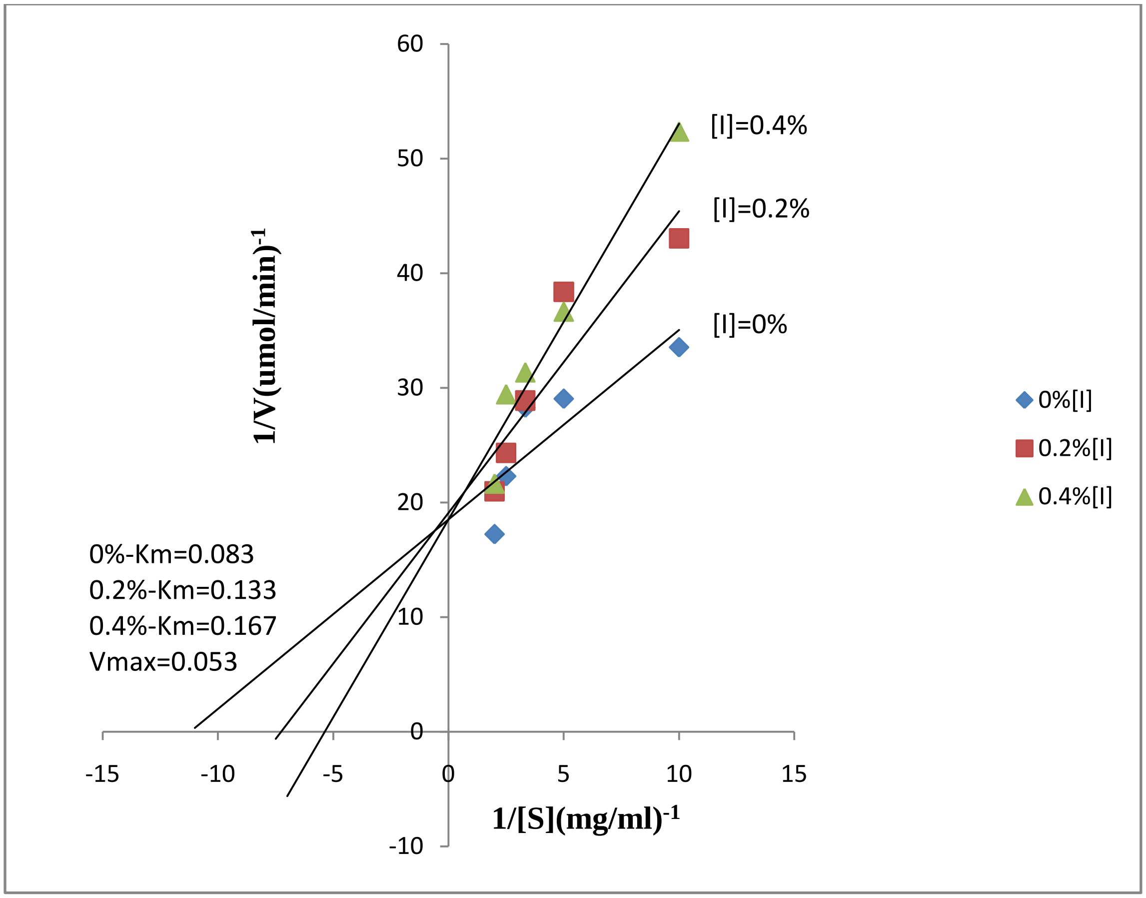


Figure 4.7: Lineweaver-Burk Plot Showing the Inhibitory Effect of Stem-bark Bioactive fraction of *Diospyros mespiliformis* on *Plasmodium berghei* cysteine protease (competitive pattern of inhibition).

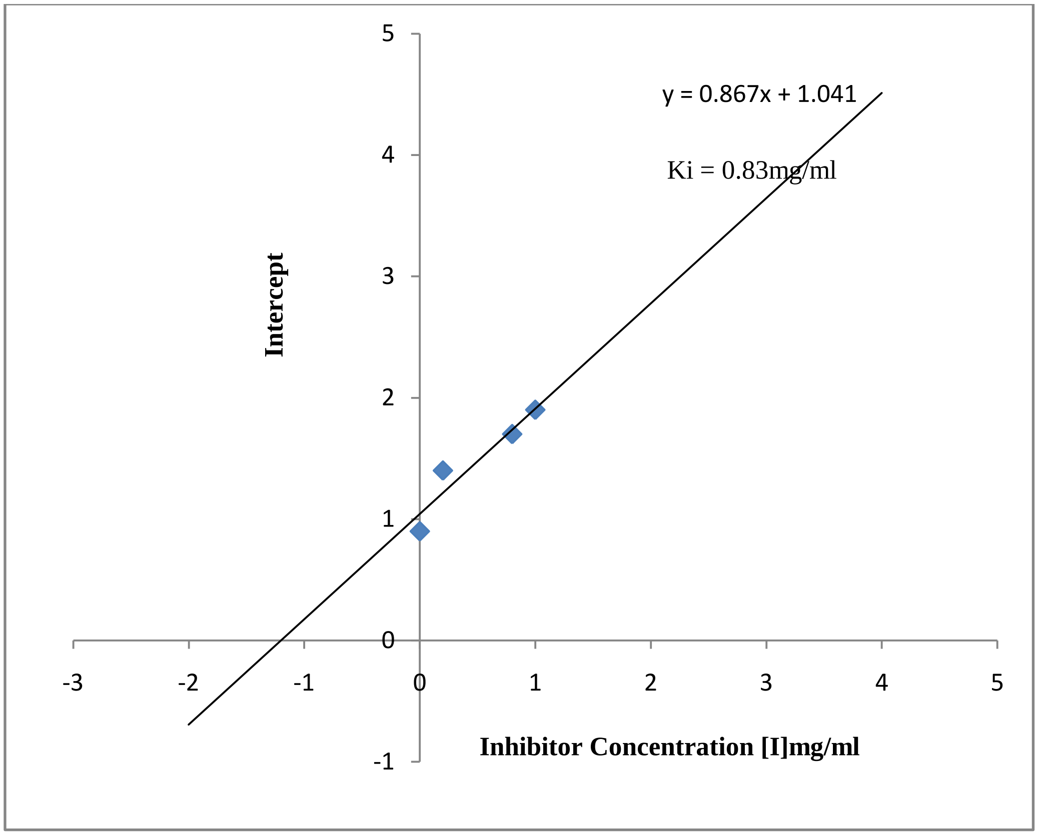


Figure 4.8: Secondary Plot Showing the Inhibition binding constant (Ki) of the bioactive fraction of *Diospyros mespiliformis* leaf extract against the Partially Purified *Plasmodium berghei* cysteine protease.

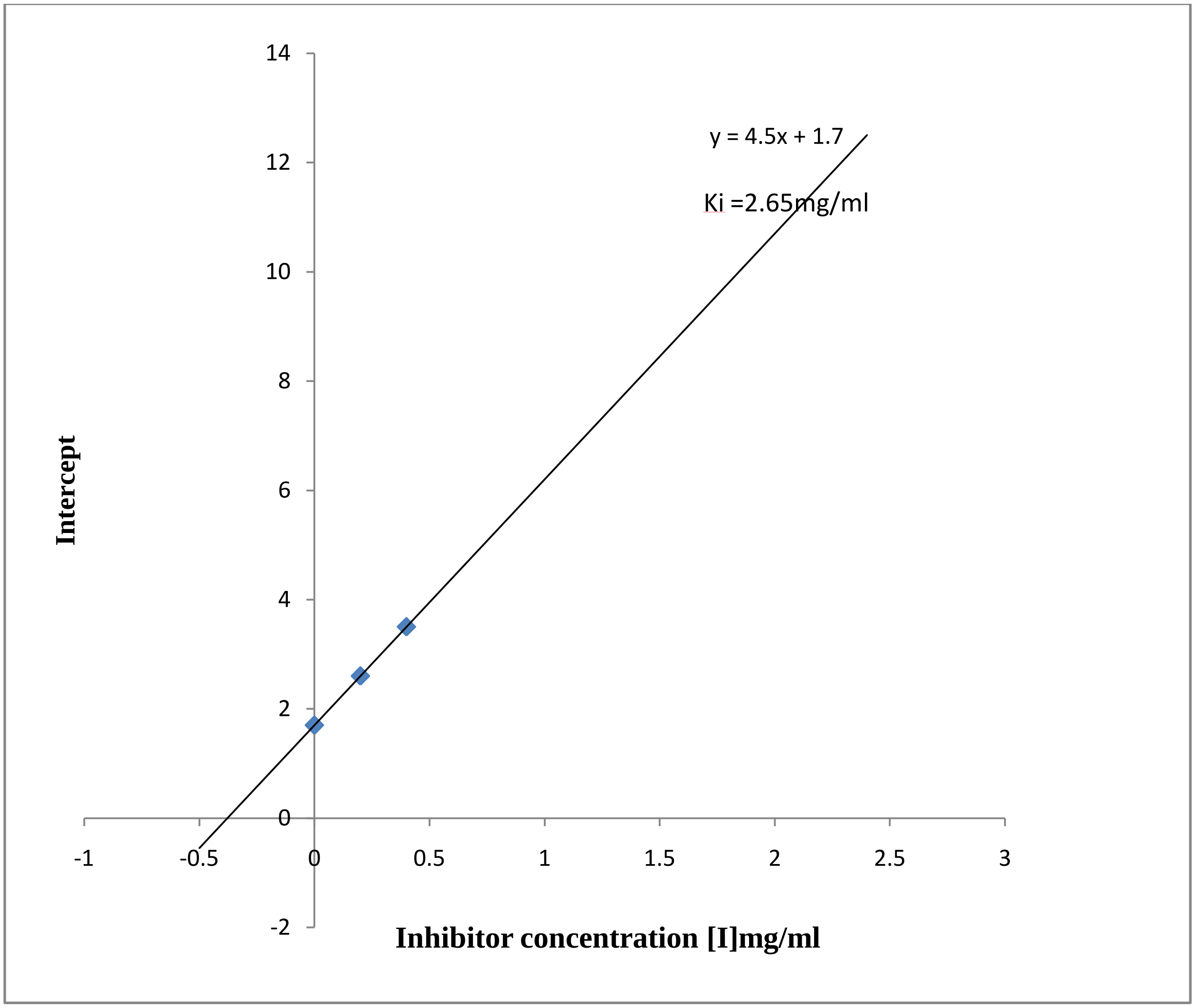


Figure 4.9: Secondary Plot Showing the Inhibition binding constant (Ki) of the bioactive fraction of *Diospyros mespiliformis* stem bark extract against the Partially Purified *Plasmodium berghei* cysteine protease.

CHAPTER FIVE

5.0 DISCUSSION

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources (Venkatalakshimi *et al.* 2012). This plant based traditional medicinal system continues to play an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care (Omonkhelin *et al.,* 2007). It is well known that these plants generally contain secondary metabolites, which have been reported to be responsible for protecting them (Smith and Eyzaguine, 2007; Kumar *et al.,* 2009) and some of these secondary metabolites have been associated with numerous physiological activities in mammalian cells in various studies (Mishra *et al.,* 2009). The preliminary phytochemical studies revealed the presence of carbohydrates, free anthraquinone, cardiac glycosides, glycosides, saponins, tannins, alkaloids and flavonoids in the crude leaf methanol extract and absence of carbohydrates, glycosides, saponins, and tannins in the stem bark methanol extract of *Diospyros mespiliformis*. The presence of these phytochemicals in the plant, accounts for its usefulness as medicinal plant (Jayathilakan *et al.*, 2007). The quantitative phytochemical analysis showed that glycosides had the highest concentration in the leaf and stem bark extracts (Table 4.4).

In-vivo parasiteamia level and percentage suppression (table 4.10) revealed significant increase in the infected saline group and infected treated with chloroquine had the highest percentage suppression which almost cleared the parasite.

Haematological investigation provides information on the general reticuloendothelial system and pathophysiology of the blood .Baker and Silverton, (1995) showed that xenobiotics causes low packed cell volume and haemoglobin (Hb) levels which may be associated with the oxidation of sulphyldryl groups of the erythrocyte membrane thus, inflicting injury to the erythrocytes membrane and causing the release of haemoglobin in the blood plasma. This is in agreement with the present study as PCV and Hb values in mice infected and treated with chloroquine, gave significantly low levels. The infected normal saline group of the stem bark extract appeared to boost blood cells as the values of PCV and Hb approached the normal level (figure 4.1 to 4.4). This may be as a result of reduction in oxidative damage to red blood cells that may eventually lead to reduction in haemoglobin breakdown by the liver. This finding suggests that the administration of stem bark methanol extract of *Diospyros mespiliformis* to patient with remarkable low PCV level may increase their PCV and Hb level. It implies that the stem bark extract, may possess constituents that would trigger the production of more blood cells and thus, boost PCV (Patrick-iwuanyanwu *et al.*, 2007; Emeka *et al.*, 2009).

Assessment of damage to the liver can be made by estimating the activities of serum ALT, AST and ALP which are enzymes originally present at higher concentration in cytoplasm (Reham *et al,* 2009). When there is hepatopathy, these enzymes leak into the blood stream in conformity with the extent of liver damage (Dominic *et al.*, 2012).

In the intestine, conjugated bilirubin may be metabolized by colonic bacteria, eliminated or reabsorbed. Metabolism of bilirubin into urobilinogen followed by reabsorption of urobilinogen accounts for the yellow color of urine as we urinate which is a downstream product of urobilinogen. Furthermore, metabolism of urobilinogen iinto stercobilin while in the bowels accounts for the brown color of stool. Elevated bilirubin level may also be associated with reduced hepatocyte secretion of bilirubin (Nkosi *et al.,* 2005; Dominic *et al.,* 2012). Also, elevated bilirubin may also be due to possible obstruction in the flow of the bile within the liver (hepatic jaundice) or in the bile duct (post jaundice) (Emeka *et al.,* 2009).However, the elevation of bilirubin levels in the infected normal saline group of stem bark extract, may be as a result of haemolytic anemia that may be associated with oxidative damage to red blood cells or internal haemorrage thus, leading to elevated bilirubin level (prehepatic jaundice) since bilirubin is an intermediate product in heam (either from haemoglobin or myoglobin) break down in the liver (Reham *et al.,* 2009). This is in agreement with the commonly accepted view that serum levels of transaminases, bilirubin and serum proteins returned back to normalcy with the healing of hepatic parenchyma cells which lead to the regeneration of hepatocytes (Chavan *et al.,* 2012).

The kidney helps in maintaining homeostasis of the body by reabsorbing important material and excreting waste products. Creatinine is a break down waste product formed in the muscle by creatine phosphate metabolism. Creatine is synthesized in the liver, passes into the circulation and is taken up almost entirely skeletal muscle for energy production. Creatinine retention in the blood is evidence of kidney impairment. Urea is the main end product of protein catabolism. Amino acid deamination takes place in the liver, which is also the site of urea cycle, where ammonia is converted into urea and excreted through urine. It represents 90% of the total urinary nitrogen excretion. Urea varies directly with protein intake and inversely with the rate of excretion. From the present study it is evident that significant elevation in serum creatinine levels can be attributed to the damaged nephron structural integrity (Khan and Siddique, 2012). However, the significant reduction in urea and creatinine levels in the normal control group can be attributed to the curative properties of the leaf and stem bark extracts, of *Diosp*yros *mespiliformis* on the glomerular and tubular cells which may improve renal function in kidney disease.

This work reveals the partial purification of cysteine protease isolated from *Plasmodium berghei*, and the inhibitory effects of fractionated leaf and stem-bark extracts of *Diospyros mespiliformis* on the enzyme. The purification profile revealed increase in specific activity of the enzyme at each purification step. Increase in the specific activity could be attributed to the removal of interacting protein components attached to the enzyme. It could also be explained to be as a result of the purification matrices in which the enzyme was subjected during the purification process. The mechanism of inhibition exhibited by the bioactive fractions of the *Plasmodium berghei* cysteine protease reveals a mixed non-competitive pattern of inhibition. This implies that other sites on the enzyme apart from the active site could as well be involved in the inhibition, although dialysis of the pre-incubated bioactive fractions and the enzyme was not carried out to determine whether the inhibition could be reversed. Other findings revealed that steroids, saponins and tannins constitute the component that interacts with enzyme-substrate complex resulting in the reduction of enzyme activity (Abubakar *et al*; 2000). Reduction in the enzyme activities could be attributed to the phytochemical constituents present in the extracts, revealing their capacity to interact with the enzyme. This observed inhibition implies that the group of compounds in the extract through individual or synergistic action inhibited the enzyme activity. The dose dependent inhibition of the activities of cysteine protease (shown in figure 4.6 and 47) implies that the higher the concentration of the compounds in the fractions the higher the inhibition of the enzyme activities and as such the lower the transmissibility and virulence posed by the parasite. The low $K_i$values obtained from the plots for both compounds towards the enzyme reveals a high binding strength of the inhibiting compounds against cysteine protease. Much lower K_i value obtained for the compound in the leaf extract is a clear indication of higher binding strength of the compound towards the enzyme. These compounds could serve as a good source of antidote for malaria and could as well guide the design of a novel drug without side effects.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 SUMMARY

I From the present study, the qualitative phytochemical screening of the crude methanol leaf extract of *Diospyros mespiliformi*s showed the presence of carbohydrates, cardiac glycosides, free anthraquinone , glycosides, saponins, tannins, alkaloids and flavonoids and absence of flavonoids, free anthraquinone, cardiac glycosides, and glycosides in the stem bark methanol extract.

II The quantitative phytochemical analysis of the fractions of leaf and stem bark methanol extract of *Diospyros mespiliformis* showed that, saponins (0.57±0.06 mg/gl), ,alkaloids (0.12±0.04 mg/gl), tannins (0.73±0.36 mg/gl) and .saponins (0.36±0.21 mg/gl),alkaloids (0.67±0.01 mg/gl), tannins (0.51±0.22 mg/gl) and glycoside had the highest concentration of (1.15±0.10 mg/gl) and (0.97±0.33 mg/gl).

III *Invivo* parasiteamia level and percentage suppression revealed significant ($P<0.05$) increase in the infected saline group and infected treated with chloroquine had the highest percentage suppression which almost cleared the parasite.

IV The leaf methanol extract significantly ($P<0.05$) increase packed cell volume and heamoglobin level in the infected saline group and infected treated with arthemisinin combination therapy. Stem bark methanol extract significantly ($P<0.05$) reduce packed cell volume and heamoglobin level in the infected treated with standard drug and different doses of the extract.

V The leaf and stem bark methanol extracts significantly ($P<0.05$) increased AST, ALT, ALP and total protein in the infected saline group as well as ALT of the stem bark methanol extract showed significant ($P<0.05$) reduction in the infected but treated but with low and high doses of the extracts. Stem bark methanol extract significantly ($P<0.05$) increased direct bilirubin level in the infected saline group.

VI The leaf and stem bark methanol extracts possessed anti-nephrotoxic effect as evident by the significant ($P<0.05$) reduction in the level of creatinine in the infected treated group and significant ($P<0.05$) increase in the infected saline group.

VII Protein cysteine protease isolated from *Plasmodium berghei* was purified to 12.25-fold and a yield of 35.69% on Sephadex G-50 column with a final specific activity of 47.30µmol/min.

6.2 CONCLUSION

The result of this study has scientifically validated the traditional use of *Diospyros mespiliformis* in the management and treatment of malaria. The result showed that, *Diospyros mespiliformis* leaf and stem bark methanol extracts possess antimalarial activity. Plant components like carbohydrates, glycosides, saponin, cardiac glycosides, flavonoids, tannins, free anthraquinone and alkaloids are contained in the leaf and absence of cardiac glycosides, flavonoids, free anthraquinone and alkaloids in the stem bark extract. The results indicate that Stem bark and Leaf methanol extracts of *Diospyros mespiliformis* is relatively safe at doses ≤ 200mg/kg and ≤ 400mg/kg body weight respectively which possesses antiplasmodial effects. The Stem bark and leaf methanol extracts of *Diospyros mespiliformis* were found to produce some level of suppression at 100 and 200mg/kg body weight which were the effective therapeutic doses. Chloroquine cleared almost all the parasite at 5mg.kg body weight. This study has scientifically validated the most probable principle and site of action in the ethno-medicinal practice on the use of *Diospyros mespiliformis* in the treatment of malaria. This may be due to the inhibitory effect of the methanol extracts of on *Diospyros mespiliformis* cysteine protease, and having explored the antimalarial properties in the plant extracts with the bioactive compounds identified which may be effective therapeutic agents for the treatment and management of malaria.

6.2 RECOMMENDATION

The results presented herein validate that the leaf and stem bark methanol extracts of *Diospyros mespiliformis* are safe and possess potent anti-malarial activity which justifies its continuous use in traditional medicine as an antimalarial remedy. By extension, the following recommendations are suggested to further these research findings:

I. Chronic toxicity testing of the plant should be carried out to determine the safety of the plant.

II. Characterization of cysteine protease inhibiting activity of *Diospyros mespiliformis* could be further studied.

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APPENDICE

Appendix 1.0: Flowering leaf of *Diospyros mespiliformis*

APPENDIX 2.0

CALCULATION FORMULA FOR THE PURIFICATION PROFILE

$$\text{Total Protein} = \frac{\text{Total Activity}}{\text{Specific Activity}}$$

$$\text{Total Activity} = \text{Specific Activity} * \text{Total Protein}$$

$$\text{Specific Activity} = \frac{\text{Enzyme Activity (Total Activity)}}{\text{Total Protein}}$$

$$\text{Purification Fold} = \frac{\text{Specific Activity of Protein at a Purification Step}}{\text{Original Specific Activity (Crude)}}$$

$$\text{Percentage Yield} = \frac{\text{Total Activity of protein at a step}}{\text{Total Activity of Original Purification (Crude)}} * 100$$

APPENDIX 3.0

STANDARD CURVE FOR PROTEIN ESTIMATION USING BSA