INVITRO ANTI MALARIAL POTENTIAL OF *CHROZOPHORA SENEGALENSIS* EXTRACTS ON CYSTEINE PROTEASE OF *PLASMODIUM FALCIPARUM*

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ZARIA – NIGERIA

DECEMBER, 2017

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

DECEMBER, 2017

DECLARATION

I declare that this Dissertation entitled "In Vitro Anti-Malarial Potential of *Chrozophora Senegalensis* Extracts on the Cysteine Protease of *Plasmodium falciparum*" is a record of my own research work at Department of Biochemistry Ahmadu Bello University, Zaria under the supervision of Prof. I. A. Umar and Prof. H. C. Nzelibe.

The information derived from the literature has been duely acknowledged in the text and list of references provided. No part of this Dissertation has been previously presented and will not be presented elsewhere for another degree or diploma at any University.

_____ _____

Mrs. Umar Hauwa Date
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CERTIFICATION

This Dissertation entitled "In Vitro Anti-Malarial Potential of *Chrozophora senegalensis* Extract on the Cysteine Protease of *Plasmodium falciparum*" by Umar Hauwa meets the regulations governing the award of Masters of Science Degree (Biochemistry) of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This dissertation is dedicated to my family, my husband who by all means stood by me vividly to the completion of this work, and my kids, who endured my absence with patience and understanding.

ACKNOWLEDGEMENT

All thanks to Almighty Allah S.W.T, the oneness of whom is neither negotiable nor open to compromise, for His limitless mercies, favors and guidance over me. I beseech the peace and blessing of Allah unto the noble Prophet Muhammad (S.W.T), who is the mentor towards my steadfastness in the path of Islam.

My profound gratitude goes to my supervisor Prof. I. A. Umar, and Prof. H. C. Nzelibe of Biochemistry Department for all their support, commitments understanding and guidance towards the success of this work. I pray the Almighty god reward them abundantly. My endless thanks goes to the Post Graduate Co-ordinator Dr. A.Ibrahim for being there for me all the time and for his contributions, guidance and support. May Allah in his infinite mercy reward him and his family abundantly. Likewise the entire teaching and non-teaching staff of Biochemistry department, they have contributed enormously in one way or another. I am also very grateful to Prof. Sani Ibrahim who was always concernabout the progress of this work.

I am particularly grateful to my beloved and dear husband Dr. Idris Baba who stood by me, gave me all the support I needed and most of all, never got tired of helping out in times of needs, morally, spiritually and financially. My sincere appreciation goes to my parents for their prayers which made this work a successful one.

I will always be grateful to Dr. Baba Gabi for his uncommon support and assistance in the course of this work. May God reward you abundantly and help to succeed in all what you are doing.

ABSTRACT

Chrozophora senegalensis is traditionally used to treat malaria. The plant extracts were prepared by cold maceration with 4 different solvents n-hexane, ethyl ether, methanol, and aqueous. Phytochemical analysis shows the presence of tannins, alkaloids, saponins, flavonoids and phenolic in the methanol and aqueous extracts while the ethyl ether and n-hexane extracts contains terpenes, tannins and phenolics. Ethylether has flavonoids and n-hexane has traces of alkaloids.The extracts were tested in vitro against cultured *Plasmodium falciparum*. The highest inhibition of the *P. falciparum* with an IC_{50} of 2.37µg/ml` was demonstrated by the methanol extract followed by aqueous extract with IC_{50} of 13.36µg/ml, ethylether 32.47µg/ml and least by n hexane 37.68µg/ml. Further investigation against malarial cysteine protease with the four extracts shows highest inhibitory activity of the enzyme in the methanol extract with percentage inhibition of 80% and also 76%, 29%, and 15% for aqueous, n- hexane and ethyl ether respectively. Quantitative phytochemical screening of the methanol extract shows that tannins content was highest with 3.12mg/100g followed byalkaloids 3.10mg/100g, flavonoids 2.51mg/100g, phenolics 2.24mg/100g, saponins 1.69mg/100g and then terpenes 1.61mg/100g. Fractionation of the most active extract that is the methanol extract gave rise to 50 fractions which were pooled to ten different fractions according to their similarities in R_f values. The ten fractions were also further tested against the enzyme malarial cysteine protease. Fraction 3 showed highest inhibitory activity. Characterization of fraction three that shows the best inhibitory activity through gas chromatography/mass spectroscopy revealed the presence of nitro-benzoic acid and ellagic acid alone side other fatty-acids and their derivatives. The anti- plasmodial effect of the methanol extract of the plant could be due to its cysteine protease inhibitory activity. Further work on fraction 3 will be required to characterized and isolate the active compound.

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ABBREVIATIONS

ABU: Ahmadu Bello University

NARICT: National Research Institute of Chemical Technology

SEM: Standard Error of the Mean

%: Percentage

Kg: kilogram

ml: Milliliter

µl: Microliter

mg: Milligram

g: gram

WHO: World Health Organization

TLC: Thin Layer Chromatography

BANA N-Benzoyl DL-Arginine b-Naphthylamide.

RPMI: Roswell Park Memorial Institute

AOAC: Association of Official Analytical Chemists

RBC: Red Blood Cell

RNA: Ribonucleic Acid

PBS: Phosphate Buffer Saline

HCL: Hydrochloric Acid

SD: Standard Deviation

S/N: Serial Number

DNA: Deoxyribonucleic Acid

ATP: Adenosine Triphosphate

PFK: Phosphofructose -1- Kinase

pH: Hydrogen Ion Concentration

PIs: Plant Inhibitors

DPAP: Dipeptidyl Aminopeptidase

ABC: ATP-binding cassette

GS4H: Reduced Glutathione

ROI: Reaction Oxygen Intermediates

SOD: Superoxide Dismutase

CQ:Chloroquine

Hz: Hemozoin

DFMO: Difluoromethylornithine

GC/MS: Gas chromatography mass spectrometry

EI: Electron Ionisation

CI: Chemical Ionisation

PVM: Parallel Virtual Machine

MDR1:Multi-Drug Resistance 1

CRT: Chloroquine Resistance Transporter

CHAPTER ONE

1.0 INTRODUCTION

Malaria, a mosquito-borne disease is global. It is caused by a parasite in the blood called Plasmodia. Five species of this genus are implicated in human malaria, of which the most deadly is *Plasmodium falciparum*. They are usually transmitted via the bite of infected female Anopheles mosquitoes. The disease is mainly characterized by fever in uncomplicated cases but can develop into severe malaria as soon as 24 hours after it first appears (Trampuz *et al*., 2003). Malaria is an Infectious disease that continues to be associated with considerable morbidity and mortality. It was estimated that there were over 300 million cases of malaria every year in developing countries especially in Africa Sub-Sahara (90%) and other developing countries. Malaria kills over one million people a year-mainly children under five years and pregnant women (Christopher, 2002). Malaria is a major health problem in Nigeria. It constitutes 30% of all attendance to health facilities. It is the main cause of hospital death and the failure of malaria control is largely due to the increasing parasite resistance to chloroquine and vector resistance to insecticides used (Roll Back Malaria, 2002). In all malaria endemic countries, plants are used in traditional medicine for treatment of the disease. Examples are numerous with the urgent need to develop new, safe and effective drugs against malaria. Plants may provide such drugs directly as with quinine from Cinchona bark or artemisinine from the Chinese herb Artemisia annual and/or they may provide template molecules on which to base further novel structures by organic synthesis. In some rural areas of Nigeria, antimalarial traditional medicines are even preferred to pharmaceutical compound drugs, suggesting that the herbal preparations used by traditional healers contain useful active ingredients. Many gaps still remain to fully exploit the potential of herbal medicines as a solution to the malaria burden and this demands combined efforts from

public sector, research institutions, funding bodies and pharmaceutical groups, traditional healers, and the populations themselves.

The wide spread use of plant as medicaments is well documented. It has been reported that over 80% of the global population use plants their primary source of medications (Usman et al., 2014). This practice is common in northern Nigeria where a variety of plant species are used in the treatment and management of diseases often without proper scientific documentation. A common problem with the use of crude plant extracts in ethno medicine is the lack of toxicological evaluation of such plants. One of such plant that is used by many localities is *Chrozophora senegalensis.*

Chrozophora senegalensis is a medicinal plant that belongs to the family *Euphobiaceae*. The plant known variously among the Hausa tribes as "Damagi, Walkin-maciji or Bauren-Kiyashi" is a herb with deep red flowers, small green leaves and violet tingle capsules commonly found in dried up inundated flats or sandy river beds (Sofowora, 1993). In northern Nigeria, the plant is an astringent for diarrhea taken boiled with cereals food and also to treat boils. A decoction is used as a body wash by pregnant women. It is chiefly used as a remedy for syphilis, also as an ingredient in a mixture called "Rigakafi". The plant has been reported to serve as a remedy for intestinal pain, fever as well as conjunctivitis (Etkin, 1997 and Yusha'us, 2011). A leaf macerate is used in the savanna region for taenia and ascaris and it is said to be very efficacious (Etkin, 1997). A root decoction is a successful medicine in Senegal for suckling babies suffering from green diarrhea (Tignokpa *et al.,* 1986). In Ivory Coast an enema is made of the plant for administration to rickety children and the plant has a general application in stomach-aches and for venereal disease (Tignokpa *et al.,* 1986).

Proteases is an enzyme which appear to be required for a number of important functions in erythrocytic parasites, including haemoglobin hydrolysis, erythrocyte rupture, and erythrocyte invasion are important in the pathophysiology of malaria (Francis *et al.,*1994). The best characterized proteolytic function is the hydrolysis of haemoglobin, which provides amino acids for the parasite's protein synthesis and other necessary functions (Klemba, 2002). In *Plasmodium falciparum*, the most virulent human malaria, haemoglobinases have been identified from the aspartic (Francis *et al.,* 1994) cysteine (Shenai *et al.,* 2000) and metallo (Skiles, 2004) protease classes. Treatment with cysteine protease inhibitors blocks haemoglobin hydrolysis and parasite development in-vitro and in murine models (Rosenthal et al, 1998) suggesting that Plasmodia cysteine proteases are appropriate chemotherapeutic targets (WHO, 2010).

1.1 Statement of research problem

The emergence and spread of plasmodium resistance to antimalarial drugs is a great challenge facing the global effort to control malaria (WHO, 2010). Challenges such as the increased cost of anti-malaria, resistance of the parasite to drugs and lack of an effective vaccine, exacerbated and frustrated the global efforts in the control of malaria (WHO, 2010).

1.2 Justification

A previous review by WHO (2015) showed that over 200 different plant species have been identified in Nigeria for their use as antimalarial in folk medicine. But only a few species had been fully investigated. Therefore, there is a great need to pursue investigations on the other plants so far identified in order to foster and standardize their use either as herbal medicines or sources of new anti- malarial drug candidate molecules.

1.3 Aim and objectives

The research is aimed at fractionating, Characterizing and investigating the in-vitro anti-cysteine protease activity of the crude extracts of *Chrozophora senegalensis* using aqueous and organic solvents.

1.3.1 Specific objectives

- To determine qualitatively and quantitatively the phytochemicals in *Chrozophorasenegalensis* plant extracts.
- To investigate the in-vitro anti-plasmodial activity of aqueous, methanol, ethyl ether and n-hexane extracts of the plant.
- To determine the effect of the crude extracts on *P.falciparum* cysteine protease.
- To fractionate the most potent fraction using column chromatography and to determine the effects of the fractions on the cysteine protease.
- To characterize the most active sub-fraction using GCMS.

CHAPTER TWO

LITERATURE REVIEW

2.1 MEDICINAL PLANTS

Medicinal plants constitute an important natural wealth of a country. They play a significant role in providing primary health care services to rural people. They serve as therapeutic agents as well as important raw materials for the manufacture of traditional and modern medicine. Substantial amount of foreign exchange can be earned by exporting medicinal plants to other country (Sofowora,1993).The practice of traditional medicine is worldwide custom that has preoccupied mankind in most civilized countries. In Nigeria, both herbal and traditional orthodox medicines are practiced, though the conventionally trained medical doctors are contemplating the former. Despite the fact that traditional approach to health care delivery system is popularly practiced and in spite of the availability of medicinal herbs in our local market, there are very few if any, in government health centers. Thus it is believed that there are problems due to lack of adequate information of the subject, the crudity of the medicinal plant and technique involved in the traditional medicine. A number of plants have been used in traditional medicine for many years, some of which the therapeutic properties and constituents ave been established scientifically and other which have not yet been subjected to a thorough scientific investigation. A common problem with the use of crude plant extracts in ethno medicine is the lack of toxicological evaluation of such plants (Aliyu, 2006).

2.2 GENERAL DESCRIPTION, HABITANT AND CLASSIFICATION OF *CHROZOPHORA SENEGALENSIS*

Plate 2.1: Image of *Chrozophora senegalensis* plant

*Chrozophora g*enus is a plant of the family *Euphorbiaceae* and the sole genus comprised in the subtribe *Chrozophorinae.* Plate 2.1, shows the image of *Chrozophora senegalensis* plant, it is a Monoecious, shrubby herb up to 60 cm tall; taproot stout and very long; stem ascending, knotty, much-branched from the base, densely short-hairy. Leaves alternate, simple; stipules small; petiole short; blade angular-ovate to triangular-ovate, 2.5–4 cm × 1.5–3 cm, base deeply cordate with 2 basal glands, apex rounded, margins undulate, upper surface sparsely hairy, lower surface densely short-hairy, 3-veined at base. Inflorescence a condensed axillary raceme, with male flowers at the top and female flowers at the base; bracts small. Flowers unisexual, regular, 5-merous; calyx short-hairy, petals deep red; male flowers with short pedicel, stamens up to 10, filaments fused into a column; female flowers with long pedicel, petals smaller than in male flowers, ovary superior, 3-celled, styles 3, fused at base, 2-fid at apex. Fruit a 3-lobed capsule c. 1 cm long, densely covered with white, shiny stalked scales, 3-seeded. Seeds ovoid, smooth, yellowish brown, covered by a thin, pale, shiny aril. *Chrozophora* comprises 7–8 species and is distributed in Africa, southern Europe and Asia.

Previous phytochemical investigation of the genus *Chrozophora* resulted in the isolation of several types of chemical constituents including essential oils, terpens, sterols, phenylpropanoid glycosides, xanthones, chromone and flavonoids. It was reported that the plant contained essential oils and flavonoid (Hombanje and Huang, 2010).

Species of *chrozophora* plant

1. *Chrozophora brocchiana* - Sahara and Sahel regions of Africa; Cape Verde
2. *Chrozophora gangetica* - India
3. *Chrozophora mujunkumi* - Uzbekistan
4. *Chrozophora oblongifolia*– E. Africa, Middle East, India, Pakistan
5. *Chrozophora plicata* - Sub-Saharan Africa, Arabian Peninsula, India, Pakistan, Thailand, Myanmar, Java
6. *Chrozophora rottleri* - Indian Subcontinent, Afghanistan, Indochina
7. *Chrozophora sabulosa* - Arabian Peninsula, Iran, Pakistan, Central Asia, Xinjiang
8. *Chrozophora sabulosa* - W Africa
9. *Chrozophora tinctoria* - Mediterranean, Middle East, India, Pakistan, Central Asia

The plant is not grazed by stock in Senegal except occasionally by goats and sheep. It causes vomiting and diarrhoea (Owhe-ureghe, et al 2001) but it is appreciated by camels (Caroline, 2002). A leaf-macerate is used in the savanna region for taenia and ascaris and it is said to be very efficacious (Bulus, et al 2003). This is not bitter, but a preparation of the whole plant however is astringent, and is taken in Nigeria boiled with cereal foods for diarrhoea (Owhe-ureghe *et al*., 2001). It is also used topically for rheumatism (Aliyu, 2006). A root-decoction is a successful medicine in Senegal for suckling babies suffering from green diarrhoea (Etkin, 1997 & Yushau, 2011). In Ivory Coast an enema is made of the plant for administration to rickety children and the plant has a general application in stomach-aches and for venereal diseases. In Nigeria it is used to treat syphilis and it is an ingredient of rigakafi (Hausa), a reputed preventive and cure. Some of the other ingredients are the roots and leaves of Crotalaria spp. (*Leguminosae:Papilionoideae*), *Portulaca oleracea(Portulacaceae), Feretia apodanthera*

(Rubiaceae), etc. This preparation is also regarded as a remedy for mental derangement. A decoction is used as a body-wash by pregnant women (Tignokpa et al., 1986).

2.3 MALARIA, THE DISEASE

Malaria is a vector borne infectious disease caused by protozoan, parasites from the phylum *Apicomplexa* (Heelanet al., 2002) and of genus *Plasmodium.* The genome of several plasmodium species Includes; *Plasmodium falciparum, Plasmodium knowlesi, Plasmodium vivax, Plasmodium berghei and Plasmodium yoelii.* Several species are found, of which only four cause malaria in human 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. Malaria is the 3rd leading cause of death for children under five years worldwide, after pneumonia and diarrheal disease (USAID, 2013). Thirty countries in Sub-Saharan Africa account for 90% of global malaria deaths (USAID, 2013). Nigeria, Democratic Republic of Congo (DRC), Ethiopia, and Uganda account for nearly 50% of the global malaria deaths (WHO, 2013). Malaria is the second leading cause of death from infectious diseases in Africa, after HIV/AIDS. Almost 1 out of 5 deaths of children under 5 in Africa is due to malaria (WHO, 2010).in Nigeria malaria is a major public health problem, where it accounts for more cases and deaths than any other country, about 97% of Nigeria's population are at risk. The remaining 3% of the population live in the malaria free highlands. There are an estimated 100 million malaria cases with over 300,000 deaths per year in the country. This compares with 215,000 deaths per year from AIDS. Malaria contributes to an estimated 11% of maternal mortality, it accounts for 60% of outpatient visits and 30% of hospitalizations among children under five years of age .Malaria has the greatest prevalence, close to 50% in children age 6 and below (WHO, 2010). According to Rosenthal (2003)

resistance to existing antimalarial drugs is mostly seen in *P.falciparum*. All these species have genomes of about 25 megabases organized 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 protest, 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.

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% cases occur in 17 countries (Davidet 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, 2000).

2.3.1 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 transfusion, 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).

2.3.2 LIFE CYCLE OF MALARIA PARASITE

Figure 2.1: Life Cycle of Malaria Parasite

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. In figure 2.1 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 trail through the bloodstream of the victim and infect the liver. In the liver, the sporozoites reside in the parenchyma cells (Mechado, 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 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 invastive merozoites. According to Mechado (2001) the rest of the merozoites grow as trophozoites (i.e. the ring forms), increase in size, divides mature into schizonts. Merozoites are formed from the schizonts as a result of erythrocytic schizogony. Within 48-72 hours the 40 infected merozoites undergo gametocytogenesis (Inga & Slems, 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 gametes develop into a zygote and then into an ookinete (fertilized egg). The ookinete matures into an ocyst 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.

2.3.3 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, diarrhea, abdominal pain, myalgia (limbs and back), loss of appetite, orthosatic hypotension, jaundice (yellowing of skin and eye white), aneamia, 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 malaria 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 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.

2.3.4 PATHOGENESIS

Malaria is a disease caused by protozoan species (*Plasmodium)* and traditional medicine 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 antimalarial or are unwilling to use allopathic medicines (Van, 2001). Artemisia annual 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). To assure the development of efficient and safe malaria phytomedicines, the use 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.

2.3.5 MECHANISM OF ACTION OF ANTIMALARIAL DRUG

The malaria parasite, like all organisms, must acquire nutrients from the environment and convert these nutrients to other molecules or energy (i.e. catabolism). These other molecules and the energy are then used to maintain the homeostasis of the parasite, and the growth and reproduction of parasite (i.e. anabolism). Both anabolic and catabolic processes are catalyzed by enzymes. Growing and reproducing organisms require high levels of macromolecules and other biochemical are for the maintenance of cellular structure and function. The malaria parasite needs to acquire these biochemical and precursors from the host.

The unique life cycle and resulting micro environments of the parasite has led to the evolution of metabolic pathways which differs from the human host. It may be possible to exploit these unique pathways and enzymes in the design of therapeutic strategies. For example, many anti-malarial are known to affect the food vacuole which is a special organelle for the digestion of host hemoglobin.

2.3.6 HEMOGLOBIN DEGRADATION AND THE FOOD VACUOLE

The malaria parasite requires amino acids for the synthesis of its proteins. The three sources of amino acids are: *de novo* synthesis, import from host plasma, and digestion of host hemoglobin. Hemoglobin is an extremely abundant protein in the erythrocyte cytoplasm and serves as the major source of amino acids for the parasite.

Figure 2.2 Ingestion of Host Cytoplasm by Plasmodium Parasite.

During the early ring stage, the parasite takes up to the host cell stoma by pinocytosis resulting in double membrane vesicles (Figure 2.2). The inner membrane, which corresponds to the PVM, rapidly disappears and digestion of hemoglobin takes place within these small vesicles during the early trophozoite stage. As the parasite matures, it develops a special organelle, called the cytostome, for the uptake of host cytoplasm and the small pigment-containing vesicles fuse to form a large food vacuole. (Gametocytes do not form the large food vacuole and are characterized by small pigment containing vesicles found throughout their cytoplasm. Double membrance vesicles pinch off from the base of the cytostome and fuse with the food vacuole. The inner membrane (originally the PVM) is lysed and the hemoglobin is released into the food vacuole.

Acid hydrolases (e.g., nucleases) have not been identified. Presumably other acid hydrolases are not needed since the microenvironment of the erythrocyte is almost exclusively protein, and in particular, hemoglobin.

Several distinct protease activities, representing three of the four major classes of proteases, have identified in the food vacuole. Multiple plasmepsins and falcipains have been identified. The

digestion of hemoglobin probably occurs by a semi-ordered process involving the sequential action of different protease (Goldberg, 2005). Several plasmespsin genes have been identified in the genome of *P. falciparum* and four of these appear to function in the food vacuole (Banerjee, 2002). Plasmepsin-1 and plasmepsin-2 are the best characterized and both are capable of cleaving undenatured hemoglobin between phenylalanine and leucine residues located at position 33 and 34 on the alpha-globin chains. These residues are located in a conserved domain known as the hinge region, which is believed to be crucial in stabilizing the overall structure of hemoglobin. Cleavage at this site presumable causes the globin subunits to dissociate and partially unfold. This unfolding will expose additional protease sites within the globin polypeptide chains. The other plasmepsins, as well plasmepsin-1 and plasmepsin-2, and the falcipains are then able to further degrade these large globin fragements. It has been suggested that falcipain-2 (Shenai, 2000), and possibly falcipain-3 (Sijwali, 2001), are capable of disgesting either native hemoglobin or therefore may also participate in the initial cleavage of hemoglobin.

Falcilysin cannot digest either native hemoglobin or denatured globin, but readily cleaves the small polypeptide fragments (up to 20 amino acids) generated by the action of falcipain and plasmepsin. The site specifity of falcilysin complements the plasmepsins and falcipains and leads to the formation of peptides 6-8 amino acids in length. Therefore, the digestion of hemoglobin is a semi-ordered process involving the initial degradation to large fragments followed by subsequent degradation to small peptides (Figure 2.3). The proposed pathway of hemoglobin digestion involves an initial cleavage by plasmepsin-1 (and possibly falcipain-2) followed by the combined actions of several plasmepsins and falcipains. The peptide fragments produced by these digestions are then digested into smaller peptides by falcilysin.

Figure 2.3: Food Vacoule Proteases of Plasmodium Parasite

Initially no food vacuole associated exopeptidase activity could be identified within the food vacuole (Kemba, 2004). However, recently two amino peptidase have been found in the food vacuole which can convert the peptides into amino acids. In addition, a dipeptidyl aminopeptidase (DPAP) activity has been identified within the food vacuole. It is postulated that the DPAP may remove dipeptides from the N-termini of the peptides generated through the digestion of the small peptides also takes place in the parasite cytoplasm, and therefore must be pumped out of the food vacuole. Pfmdr-1 has been localized to the food vacuole membrane and is a member of the ATP-binding cassette polypeptides (ABC) transporters super family. Some ABC transporters function to translocate polypeptides across membranes. For example, the *STE6* gene of yeast transports the a-type mating factor (a 12 amino acid peptide). *Pfmdr-1* can complement the *STE6* gene (Volkman, 1995) indicating that it could function to pump small peptides into the parasite cytoplasm.

In summary, a like scenario for the complete digestion of hemoglobin consists of the concerted actions of plasmepsins, falcipains and falcilysin leading to the production of small peptides. The small peptides are then converted into amino acids or dipeptides which are then converted in amino acids. Some of the small peptides may be pumped out of the food vacuole into the parasite cytoplasm, where an amino peptidase carries out the final conversion to amino acids.

2.3.7 DETOXIFICATION OF HEME AND REACTIVE OXYGEN INTERMEDIATES (ROI)

Digestion of hemoglobin also release heme. Free heme is toxic due to its ability to destabilize and lyse membranes, as well as inhibiting the activity of several enzymes. Three, and possibly four, mechanisms by which heme is detoxified has been identified as revealed in figure 3:

- Sequestration of the free heme into hemozoin, or the malaria pigment;
- A degradation facilitated by hydrogen peroxide within the food vacuole;
- A glutathione-dependent degradation which occurs in the parasite's cytoplasm;
- And possibly a heme oxygenase which has been found in *P. berghei* (rodent parasite) and *P.knowlesi* (simian parasite), but not *P.falciparum.*

Both the hemozoin formation pathway and the degradative pathways probably function simultaneously with 25-50% of the free heme being converted into hemozoin and the remainder being degraded (Figure 2.4). However, some studies suggest that up to 95% of the free iron released during hemoglobin digestion is found in hemozoin (Egan, 2008). X-ray crystallography and spectroscopic analysis indicates that hemozoin has the same structure as -hematin (Pagola, 2000). -hematin is a heme dimer formed via reciprocal covalent bonds between carboxylic acid groups on the protoporphyrin-IX ring and the iron atoms of two heme molecules. These dimmers interact through hydrogen bonds to form crystals of hemozoin. Therefore, pigment formation is best described as a biocrystallization, or biomineralization, *process (*Hempelmann,2007; Egan, 2008). The mechanism of hemozoin formation is not known, but recently a protein that may catalyze the formation of hemozoin has been described (Jani et al., 2008). Lipids may also participate in the process in that lipid bodies have been observed within the food vacuole and hemozoin is associated with lipids (Egan, 2008).

Structures of Heme and Hemozoin

Heme	**□-Hematin**	**Hemozoin**

Legend. (Left) Chemical structure of heme. (Center) Ball and stick model of □-hematin. Reciprocal bonds between oxygen and iron shown in purple. (Right) Space

filling model of proposed hemozoin structure). A single □-hematin unit is outlined in yellow.

Figure 2.4: Structures of the Heme and Hemozoin

A portion of the free heme may be degraded into non-toxic metabolites. Three potential process have been described: in the food vacuole a hydrogen peroxide mediated oxidation of the porphyrin ring leads to its opening and subsequent breakdown; some of the heme translocates across the food vacuole membrane into the host cytoplasm where it is oxidized by reduced glutathione (GSH); and a heme oxygenase activity has been identified in some non-human malaria parasites. However, the role these processes play in the degradation of heme is not known.

Chloroquine and other 4-aminoquinolines inhibit pigment formation, as well as he heme degradative processes (Ginsburg, 1999), and thereby prevent the detoxification of heme. The free heme destabilizes the food vacuolar membrane and other membranes and leads to the death of the parasite. The fact that the biocrystallization of heme is a unique process to the parasite and not found in the host accounts for the high therapeutic index of such drugs in the absence of drug resistance. Many other anti-malarials target the food vacuole indicating the importance of this organelle and its various functions to the survival of the parasite.

The iron bound to hemoglobin is primarily in the ferrous state (Fe^{2+}). Release of the heme results in iron being oxidized to the ferric state (Fe^{2+}). Electrons liberated by this oxidation of iron promote the formation of reaction oxygen intermediates (ROI) such as superoxie anion radicals and hydrogen peroxide. ROI can cause cellular damage. Superoxide dismutase (SOD) and catalase are cellular enzymes that function to prevent oxidative stress by detoxifying the

superoxide ad hydrogen peroxide, respectively. Both of these activities are found in the food vacuole and may have been obtained from the host during ingestion of the erythrocyte cytoplasm. Hydrogen peroxide can also be exported into the parasite cytoplasm where it is detoxified by catalase and glutathione peroxidase. Some of the hydrogen peroxide produced as a result of the $Fe^{2+} \rightarrow Fe^{3+}$ conversion may also use for the peroxidative degradation of heme.

Figure 2.5 Functions of the Food Vacoule of Plasmodium Parasite

Chloroquine (CQ) accumulates in the food vacuole of the parasite. This accumulation may involve ion trapping following protonation, specific transport, and/or binding to a receptor (eg., heme) (Figure 2.5). The major action of chloroquine is to inhibit the formation of hemozoin (Hz) from the heme released by the digestion of hemoglobin (Hb). The free heme then lyses membranes and leads to parasite death. Chloroquine resistance is due to a decreased accumulation of chloroquine in the food vacuole. Two different transporters (CRT and MDRI) have been implicated in resistance. The functions of these transporter and their exact roles in chloroquine resistance are not known.

2.3.8 DRUG TREATMENT & RESISTANCE

One of the drugs that are used to treat malaria is chloroquine. Currently this is one the main drugs in the market alongside with artemisinin. The main use of the chloroquine drug is to inhibit hemoglobin degradation that is seen in the trophozoite stage of development. By inhibiting degradation, the trophozoite is not able to use hemoglobin as a food source, thus eventually causing cell death. However due to the parasites rapid resistant mechanism toward the drug, chloroquine is seen to accumulate in the digestive vacuole making it ineffective towards the

parasite. Resistance of the drug was initially attributed towards alterations in the copies of the gene called *pfMDR1* and was combined with isolated mutations. Other theories of resistant include a change in the pH gradient, or an alteration in membrane permeability or possibly both (Le Bras et al, 2003). There has also been a mutation of the *PfCRT* gene that codes for transmembrane protein on the digestive vacuole membarane conferring chloroquine resistance. A drug called Verapamil is being studied to reverse chloroquine resistance. Through this drug, a new membrane protein has been identified, Pgh1. Altered Pgh1 has shown to affect the transport of chloroquine (Le Bras et al, 2003) Chemo resistance is another hurdle that researchers are facing. Chemo resistance refers to the resistance caused by using multiple antimalarial drugs. By combining several drugs and using it in mass quantities, simultaneous resistance can occur. The mechanisms by which parasites become chemoresistance are by chromosomal mutations. Looking from a genetic standpoint, malaria is caused by three different groups – humans, *Anopheles* mosquitoes and *Plasmodium*. One can thus imagine the different mitotic mutations that can occur while the Plasmodium cells are replicating in the *Anopheles* and in the humans and the meiotic recombination that can occur just after the male and female gametocytes fuse together. Secondly, mosquitoes can carry different strains which is a problem because recombination of two different strains can occur causing a more resistance and possibly lethal strain. Thus highlighting the diversity of *P. falciparum* and the struggle to find an active drug to fight against the malaria parasite (Le Bras et al., 2003).

Currently, the world health organization recommends Artemisinin-based combination therapy (ACT) as a first-line drug to treat uncomplicated malaria. Artemisinin is a sesquiterpene lactone, derived from a plant that is grown in China and was used in traditional Chinese medicine. Although the exact mechanism of how the drug kills the parasite is still not fully understood. It is

thought to be an attack on a protein associated with the parasite. Nevertheless, studies have shown the drug acts very rapidly once inside the blood stream. Parasites are quickly eradicated from the body and have gametocytocidal properties. In turn, it reduces the number of gametocytes, reducing the toxicity of the bacteria and thus the transmission of the parasite to the host cell. Due to its short half-life, resistance is more likely. For that reason, a combination therapy is being used which is showing promising results. But recent in vivo studies have shown some resistance to the drug but more studies still need to be done (Neil, 2009).

Drug resistance is not a new issue that researchers are facing with. Combined use of chloroquine, amodiaquine, quinine, and other drugs have been used since the 1950s resulting in the development for resistance. Resistance outside of Africa is a lot higher as seen in the areas of Thailand, Myanmar and Cambodia. The overall prevalence in Africa to chloroquine resistance is 50% but this percentage has not increased in the last decade. But resistance to quinine, mefloquine and halofantrine is not high and low levels are still being used in remote areas in Africa. What studies do show that although levels of malaria are not as high compared to other Asian countries, the issue over multiple drug resistance is compelling and is ever more essential to find a solution before resistance spreads (Neil, 2009).

2.4 ENZYMES

Enzymes are biologically active proteins that poses 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 pH 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 1200 to over 1 million. Some enzymes require an additionals chemical

component called co-factor-either one or more inorganic ions such as Fe^{2+}, Mg^{2+} or Zn^{2+} or a complex organic or metallo organic 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 aproprotein.

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 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, typsin,

chymotrypsin, papain, bromelain, and subtilisin. Proteolyic enzymes are present in bacteria and plants but are most abundant in animals.

Their catalytic actions had been divided into two different categories (Barret *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 been 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 Nornenclature committee of the international union of Biochemistry, proteases are classified in sub – group 4 of group 3 (hydrolases). Currently, protease 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.

(1) **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 protein 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 – peptidaes based on the nature of the amino acid residues at the active site of the enzymes.

(2) Endopeptidases: are proteases that are character4ized 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 catabolilsm, 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, extracellur 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 wad correlated with its proteolytic modification (Khafagi & Dewedar 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 protease also mediate degradation of 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 & Cenedela 2001). Recently, a new physiological function has been attributed to ATP – dependent proteases present in bacteria and eukaryotes. It is belived 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 depolymerizing 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 lone gene product is responsible for hydrolysis of abnormal proteins (Chung & Goldberg 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 physiological significant event.

iv. Several enzymes are known to be inactivated in response to physiological or developmental stages or after metabolic shift. Proteolytic medication of enzymes is known to result in a protein with altered physiological function e.g. leucyl – L – RNA synthetase form E.coli is converted into an enzyme which catalyzes leucine – dependent pyrophosphate exchange by removal of a small peptide from the native enzymes (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 protease 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 characterization should expedite drug discovery.

Proteases are currently classified into six broad groups:

- Serine proteases
- Theronine 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 peptic carboxyl group. One way to make a

nucleophile is by a catalytic reaction, where a histidine residue is used to activate serina cysteine, or threonine as a nucleophile.

Within each of the broad groups proteases have been classified, by Rawlings and Barrett (2005), into families of realted proteases. For example within the seine proteases families are labeled SX where S denotes the serine catalytic type and the X denotes the number of the family, for example S1 (chymotrypsins).

2.5.1 SERINE PROTEASE

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 I 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 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. Amd 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 a general base and accepts the OH group of the reactive Ser (Haque *et al.,* 2004).

2.5.2 CYSTEINE PROTEASE

This family includes the plant proteases such as papain, actindin or bromelain, several mammalian lysosomal cathepasins, and the cytosolic calpains (calcium-activated) as well as several 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-l-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 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 (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 imidazolum group of His 159. The attacking nucleophile is the thiolate-imidazolium ion pair in both steps, and then a water molecule is not required (Haque *et a*l., 2004).

2.5.3 ASPARTIC PROTEASE

Most of aspartic proteases belong to the pepsin family. This family includes digestive enzymes like pepsin and Chymosin, lysosomal cathepsins D, processing enzymes like rennin, 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. Retropesins, 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 "push-pull" mechanism, leads to the formation of a non-covalent neutral tetrahedral intermediate (Hamilton, 2004).

2.5.4 METALLO-PROTEASE

The metallo-proteinases may be one of the older classes of proteinases 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 or nickel without loss of the activity. Bacterial thermolysin has been well characterized, and its crystallographic structure indicates that zinc is bound by two histidiness 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 glumatic 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, 2004).

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

i. Neutral

ii. Alkaline

iii. Myxobacter I, and

iv. Myxobater 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 peptide bond. All of them are inhibited by chelating agents such as EDTA.

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' 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 anionic sulfur on the substrate carbonyl carbon. In this step, a fragment of the substrate is released with an amino terminus, the histidine residue in the protease is restored to its deprotonated form, and a thioester intermediate linking the new carboxyl-terminus of the substrate to the cysteine thiol if 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 PROTEASE 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 inhibitor react with 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, theronine 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. Disopropyl 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, difluoromethylornithine or DFMO, which is an analogue of the amino acid ornithine and is used to treat African trypanosomiasis. Ornithine decarboxyase 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 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 the 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 plank 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 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. Chystatins 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, & Gaputo, 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.

2.8 PHYTOCHEMICALS

Phytochemicals are chemical compounds produced by plants, generally to help them thrive or thwart competitors, predators, or pathogens. The name comes from the Greek word *phyton*, meaning plant. Some phytochemicals have been used as poisons and others as traditional medicine.

2.8.1 CLASSIFICATION OF PHYTOCHEMICALS

Phytochemicals are classified as primary or secondary constituents depending on their role in plant metabolism.

2.8.1.1 PRIMARY PHYTOCHEMICALS

Primary constituents include the common sugar, amino acids, proteins, purines and pyramidines of nucleic acids, chlorophyll.

2.8.1.2 SECONDARY PHYTOCHEMICAL

Secondary constituents are the remaining plant chemicals such as alkaloids, terpenes, flavonoids, lignans, plants steroids, Saponins, flavonoids and glucosides (Hahn, 1998).

2.8.1.2.1 FLAVONOIDS

Flavonoids are water soluble polyphelonic molecules containing 15 carbon atoms. Flavonoids belong to the polyphenol family. Flaovonoids can be visualized as two benzene rings which are joined together with a short three-carbon chain. Many falvonoids are shown to have antioxidant

activity, free-radical scavenging capacity and anti-cancer activity, while some flavonoids exhibit potentials for anti-human immunodeficiency virus functions. Epidomological studies have illustrated that heart disease are inversely related to the flanovoid intake. Studies have shown that flavonoids prevent the oxidation of low-density lipoprotein thereby reducing the risk for the development of artherosclerosis (Batra *et al*., 2014). Fruits and vegetables are the main dietary sources of flavonoids for humans, along with tea and wine (Batra *et al*., 2004).

Figure 2.6: Structure of a Flavonoid

2.8.1.2.2 SAPONINS

Saponins are a group of secondary metabolites found widely distributed in the plant kingdom. They form stable foam characteristic. Saponins consist of a polycyclic aglycones attached to one or more sugar side chains. The aglycone part, which is also called sapogenin, is either steroid (C_{27}) or a triterpene (C_{30}). Saponins are phytochemicals which can be found in most vegetables, beans and herbs. The best known sources of saponins are peas and soyabeans (George *et al.*, 2002). The physiological role of saponins in plants is not yet fully understood, while there is a number of publications describing their identification in plants, and their multiple effects in animal cells and on fungi and bacteria, only a few have addressed their function in plant cells. Many saponins are known to be antimicrobial (Lacaille-Dubuis and Wagner, 2000).

Figure2.7: Structure of a Saponin

2.8.1.2.3 TANNINS

Tannins are heterogeneous group of high molecular weight polyphenolic compounds with the capacity to form reversible and irreversible complexes with proteins (mainly), polysacharides (cellulose, hemicellulose, pectin, etc), alkanoids nucleic acids and minerals, etc. (Scholfeild *et al*., 2001). Tannins are common in fruits (grapes, persimmon, blueberry, etc), in chocolate, in legume forages (trefoil, etc.), in legume trees, in grasses (sorghum, corn, etc.).

In medicine, specifically in Asian (Japanese and Chinese), natural healing, the tannins containing plants extracts are used as astringents against diarrhea, as diuretics against stomach and duodenal tumours (Dolara *et al*., 2005).

Figure2.8: Structure of a Tannin

2.8.1.2.4 PHENOLIC ACID

Phenolic acid are found in a variety of plant-based foods; the seeds, skins of fruits and leaves of vegetables contain the highest concentrations. Examples of foods rich in phenolic acid include mango, berries, apples, citrus fruits, plums, cherries, kinois, onions, tea, coffee, red wine, and flour made from whole wheat, rice corn or oats (Shereen *et al.*, 2006). Their role as dietary antioxidants has received the most attention in recent literatures, it is also involved in immune regulation diseases, asthma and allergic reactions. (Andreasen *et al*; 2001).

Figure2.9: Structure of a Phenolic Acid

2.8.1.2.5 TERPENES

Terpenes are a large and diverse class of organic compounds, ZSQA produced by a variety of plants, particularly conifers (Mc Naught & Wikinson, 1997) and by some insects such as termites or swallowtail butterflies, which emit terpenes from their osmeteria, they are derived biosynthetically from units of isoprene, which has the molecular formula C_5H_8. The basic molecular formula of terpenes are multiples of that, $(C_5H_8)_n$ where n is the number of linked isoprene units. Terpenes and terpenoids are the primary constituents of the essential oils of many types of plants and flowers. Essential oils are used widely as fragrances in perfumery, and in medicine it is known to have anti-inflammatory, antibacterial and pain-relieving (analgesic) properties. Myrcene has a sedative, calming and relaxing effect. It is known to increase the psychoactive effects of THC and alternative medicines such as aromatherapy. Synthetic variations and derivatives of natural terpenes and terpenoids also greatly expand the variety of aromas used in perfumery and flavors used in food additives. Vitamin A is a terpenoid.

Figure 2.10: Structure of a Terpene

2.8.1.2.6 ALKALOID

Alkaloids are a group of naturally occurringchemical compounds that mostly contain basicnitrogen atoms. This group also includes some related compounds with neutral and even weakly acidic properties (Robert, 1998). Some synthetic compounds of similar structure are also termed alkaloids. In addition to carbon, hydrogen and nitrogen, alkaloids may also contain oxygen, sulfur and, more rarely, other elements such as chlorine, bromine, and phosphorus (Kittakoop et al.,2014). Alkaloids are produced by a large variety of organisms including bacteria, fungi, plants, and animals. They can be purified from crude extracts of these organisms by acid-base extraction. Alkaloids have a wide range of pharmacological activities including antimalarial (*e.g.*quinine), antiasthma (*e.g.*ephedrine), anticancer (*e.g.*homoharringtonine) (Russu et al.,2013), cholinomimetic (*e.g.*galantamine), vasodilatory (*e.g.*vincamine), antiarrhythmic (*e.g.*quinidine), analgesic (*e.g.*morphine) (Raymond, 2010) antibacterial (*e.g.*chelerythrine), and antihyperglycemic activities (*e.g.*piperine) (Cushnie, 2014). Many have found use in traditional or modern medicine, or as starting points for drug discovery. Other alkaloids possess psychotropic (*e.g.*psilocin) and stimulant activities (*e.g.*cocaine, caffeine, nicotine, theobromine) (Robbers,1996), and have been used in entheogenic rituals or as recreational drugs. Alkaloids can be toxic too (*e.g.*atropine, tubocurarine) (Qiu, 2014).Although alkaloids act on a diversity

of metabolic systems in humans and other animals, they almost uniformly evoke a bitter taste (Russo et al.,2013).

Figure 2.11: Structure of an Alkaloid

2.9 PRINCIPLES OF GAS CHROMATOGRAPHY- MASS SPECTROMETRY

Gas chromatography mass spectrometry (GC/MS) is an instrumental technique, comprising a gas chromatograph (GC) coupled to a mass spectrometer (MS), by which complex mixtures of chemicals may be separated, identified and quantified. This makes it ideal for the analysis of the hundreds of relatively low molecular weight compounds found in environmental materials. In order for a compound to be analysed by GC/MS it must be sufficiently volatile and thermally stable. In addition, functionalised compounds may require chemical modification (derivatization), prior to analysis, to eliminate undesirable adsorption effects that would otherwise affect the quality of the data obtained. Samples are usually analyzed as organic solutions consequently materials of interest (e.g. soils, sediments, tissues etc.) need to be solvent extracted and the extract subjected to various 'wet chemical' techniques before GC/MS analysis is possible.

The sample solution is injected into the GC inlet where it is vaporized and swept onto a chromatographic column by the carrier gas (usually helium). The sample flows through the column and the compounds comprising the mixture of interest are separated by virtue of their relative interaction with the coating of the column (stationary phase) and the carrier gas (mobile phase). The latter part of the column passes through a heated transfer line and ends at the entrance

to ion source (Fig 2.12) where compounds eluting from the column are converted to ions.Two potential methods exist for ion production. The most frequently used method is electron ionisation (EI) and the occasionally used alternative is chemical ionisation (CI). For EI a beam of electrons ionise the sample molecules resulting in the loss of one electron. A molecule with one electron missing is called the molecular ion and is represented by $M^{+\cdot}$ (a radical cation). When the resulting peak from this ion is seen in a mass spectrum, it gives the molecular weight of the compound. Due to the large amount of energy imparted to the molecular ion it usually fragments producing further smaller ions with characteristic relative abundances that provide a 'fingerprint' for that molecular structure. This information may be then used to identify compounds of interest and help elucidate the structure of unknown components of mixtures. CI begins with the ionisation of methane (or another suitable gas), creating a radical which in turn will ionise the sample molecule to produce $[M+H]^+$ molecular ions. CI is a less energetic way of ionising a molecule hence less fragmentation occurs with CI than with EI, hence CI yields less information about the detailed structure of the molecule, but does yield the molecular ion; sometimes the molecular ion cannot be detected using EI, hence the two methods complement one another. Once ionised a small positive is used to repel the ions out of the ionisation chamber.

The next component is a mass analyser (filter), which separates the positively charged ions according to various mass related properties depending upon the analyser used. Several types of analyser exist: quadrupoles (Figure 2.13), ion traps, magnetic sector, time-of-flight, radio frequency, cyclotron resonance and focusing to name a few. The most common are quadrupoles and ion traps. After the ions are separated they enter a detector the output from which is amplified to boost the signal. The detector sends information to a computer that records all of the data

produced, converts the electrical impulses into visual displays and hard copy displays. In addition, the computer also controls the operation of the mass spectrometer.

Figure 2.12: A schematic of an ion source

Figure 2.13: A schematic of a quadrupole

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant sample collection

The plant material was collected fresh from Hussainiyya area, Zaria Local Government, Kaduna State in the month of July 2014 and identified at the herbarium unit Biological science department Ahmadu Bello University Zaria where a voucher specimen with a voucher number 689 was deposited

3.1.2 Chemical/Reagents

N-benzoyl- DL-arginine b-naphthylamide hydrochloride (BANA), RPMI 1640, were purchased from sigma chemical company, UK. And all other chemicals and reagents used were of analytical grade.

3.2 METHODS

3.2.1 Preparation of Extracts

The whole plant of the *C. senegalensis* was collected fresh and was dried under the shade for two weeks which was finally grounded to powder. The extraction was performed by maceration process. This involved soaking 100g of the crude powder of *C. senegalensis* in each of 1000mls of aqueous, methanol, ethyl ether and n-hexane, covered and allowed to stand for 72 hours with intermittent shaking. The extract was sieved, and filtered. The solvents were evaporated leaving the extract to dry off. The dried extracts were kept in tight bottle and kept for further use. The percentage yield of all the crude extracts were

determined as percentage of weight (g) of the extract to the original weight (g) of the dried sample used, using the formula below;

Percentage yield of crude extract =

3.2.2 Phytochemical Screening of Plant Extracts

3.2.2.1 Qualitative screening

Tannins: **To 2 ml extract of *C. senegalensis*2 ml of 5% $FeCl_3$ was added. Formation of yellow brown precipitate indicated the presence of tannins (Jigna et al., 2007).**

Alkaloids: **To 2 ml extract *C. senegalensis*, 1.5 ml of 1% HCl was added. Then the solution was heated in water bath, 6 drops of Mayors reagents/Wagner's reagent/Dragendroff reagent was added. Formation of Orange precipitate indicated the presence of alkaloids (Oguyemi, 2008).**

Saponins**: Exactly 2 g of powdered extract *C. senegalensis* was subjected to frothing test. Frothing persistence indicated presence of saponins. Latter the froth was mixed with few drops of olive oil. Formation of emulsion indicated presence of saponins (Sofowora, 1993).**

Terpenes; **To 2 ml of the extract of *C. senegalensis*, 5 ml chloroform, 2ml acetic anhydride and concentrated H_2SO_4 were added carefully to form a layer. Reddish brown coloration of interface indicated the presence of terpenes (Harbone 1999).**

Flavonoids: **Exactly2 g powdered extract of *C. senegalensis* was extracted in 10 ml of water. To 2 ml of the filtrate few drops of concentrated HCl followed by 0.5 g of magnesium turnings was added. After 3 minutes magenta red or pink color indicated the presence of flavonoids (Jigna, 2007).**

Phenolic: **To 2 ml of aqueous extract, 1 ml of 1% ferric chloride solution was added. Blue green color indicated the presence of phenolics (Martin, 2004).**

3.2.2.2 Quantitative screening

Alkaloid determination: **Exactly 2.5 g of the powdered extract of *C. senegalensis* was extracted using 100 ml of 20% acetic acidin ethanol. The solution was covered for 4 hours, then Filtered and the filtrate was concentrated to 25ml.Concentrated ammonium hydroxide was added stepwise to attain precipitation. The whole solution was kept as such that precipitate will settle. The collected precipitate was washed with dilute ammonium hydroxide and finally filtered. The filtrate was discarded and pellet obtained was dried and weighed using method described by (Edeoga 2005 and Okwu & Josiah, 2006).**

% Alkaloid = x 100

Flavonoids determination: **Ten grams of the plant samples were extracted with 100ml of 80% aqueous methanol at room temperature. The whole solution was filtered through whatman filter paper no.42 (125mm), the filtrate was later transferred into a crucible and was evaporated to dryness in a water bath and weighed as flavonoid (Edeoga 2005 and Okwu & Josiah, 2006).**

Calculation:

% Flavonoid = weight of flavonoids/ weight of sample x 100.

Tannins determinations**: concentration of tannin was determined using the standard method described by AOAC, (1984). Two grams of the dried extract was boiled with 300ml of distilled water, this was diluted (3.33times) 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 permanganent until the blue solution changed to green, then 10 drops of 0.1N potassium permanganate was added at a time until the solution turns golden yellow (Edeoga 2005 and Okwu & Josiah, 2006).**

Calculation:

The tannin content in the sample was calculated by multiplying the volume of 0.1N potassium permanganate used (titre value) by 0.006623g

Saponin determination**: Ten g of the powdered extract of *C.senegalensis* sample was mixed with 100 ml of 20% aqueous ethanol. The mixture was kept for 4 hours on water bath shaker at 55° C.It was then filtrated and the filtrate was again be extracted in same manner. The combined extract was concentrated to 40 ml over water bath at 90°C. The Concentrate obtained was transferred into a separating funnel and 10 ml of diethyl ether was added and was vigorously shaken to obtain aqueous and ether layers. Aqueous layer was recovered while ether layer was discarded. The process was repeated. To the aqueous layer n-butanol was added and the whole mixture was washed in separating funnel twice with 10 ml 5% of aqueous NaCl. Upper part was retained in a flask and heated in water bath until evaporated and then dried in oven to a constant weight (Obadoni and Ochuko, 2001; Edeoga, 2005).**

Calculation:

Percentage Saponins=

Where A= weight of flask and extract (saponin), B=weight of empty flask

W= weight of samples.

Phenolics determination**: Exactly 1 g of sample powder was extracted with 10% ethanol and centrifuge. Supernatant was evaporated to dryness and was further redissolved in 5ml of water. Different aliquots 0.5ml was pipetted into test tubes and final volume was made to 3ml by water. 0.5 ml of folin's reagent followed by 20% Na_2CO_3 equal to 2ml was added respectively. Tubes were vortexed and kept outside at room temperature for minute. Absorbance was read at 650 nm against blank. Standard curve was then plotted using various different concentrations of 0.001M Gallic acid). The data obtained was calculated by comparism between the standard curve (mg gallic acid/ml) and the absorbance of each sample. The results obtained were expressed as mg gallic equivalents per gram of dry matter(Obadoni and Ochuko, 2001; Edeoga, 2005).**

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3.2.3 Antimalarial Assay

3.2.3.1 Selection of Patients

First of all ethical consideration was approved on 17/5/2014 by Ministry of Health Kaduna State. The inclusion criteria for the patient selection according to WHO 2001 were patients having mono infection with *P.falciparum,* parasitaemia density not less than 1000 and not more than 80 000 asexual parasites/1ml of blood following microscopic examination were adapted for the screening. Patients with severe malaria, pregnant women and patients who took antimalarial drugs during the previous 14 days were excluded. Three fresh blood specimens were collected from patients suffering from fever and other malaria symptoms that are confirmed infected by *P. falciparum* using the RDT method of confirmation.

3.2.3.2 Preparation of Culture Medium for Cultivation of *Plasmodium falciparum*

The cultivation of the plasmodia parasite was carried out using the technic described by Trager & Jensen (1976) in which one packet of RPMI 1640 (containing 25 mM of HEPES buffer, glucose) dissolved in 960 ml of double distilled water. 40 µg/ml of gentamycin sulfate (1.2 ml of Gentamycin/L) was added. This solution was passed through a Millipore filter of 0.22 µm porosity and store at 4°C as 96 ml aliquots in glass media bottle.

3.2.3.3 Preparation of Washing Medium (Incomplete medium)

Exactly 4.2 ml of 5 % sodium bicarbonate (5 gms of sodium bicarbonate was dissolved in 100 ml double distilled water and filtered through a Millipore filter of 0.22 µm porosity and store at 4°C) was added to 96 ml of stock RPMI 1640 media.

3.2.3.4 Serum Preparation

O+ blood was collected in centrifuge tube without anticoagulant and kept at 4°C. Next day it was centrifuged at 10000 x g for 20 min at 4°C. Serum was separated aseptically and kept in aliquots. The serum was inactivated by keeping it at 56°C water bath for half an hour.

3.2.3.5 Complete Medium

Normal inactivated O+ human serum (10 ml) was added to 90 ml of incomplete media.

3.2.3.6 Preparation of Erythrocytes (RBCs) for Culture

O+ blood was collected in anticoagulant into centrifuge tubes. Then this was centrifuged at 1500 x g for 10 min at room temperature. Plasma and buffy coat was removed with sterile Pasteur pipette. After this washing media was added, centrifuged at 1500 x g for 10 min and supernatant was removed. This washing process was repeated thrice. Equal amount of complete media was added to the pellet and stored at 4°C. In vitro cultivation of *Plasmodium falciparum*.

3.2.3.7 Invitro Cultivation of *P.falciparum*.

Suspension of (50 %) of infected cells with complete media (with 15 % serum) was prepared. Appropriate amount of uninfected cells was added to get an initial parasitaemia of 0.5 % and diluted with complete media to get5 % cell suspension (5 % hematocrit). Culture was kept in a candle jar to generate CO_2 at 37°C.

Monitoring Culture Growth

After every 24 hrs. The media was removed using a sterile Pasteur pipette without disturbing the cells that settled down. Then the cells were mixed without frothing and a drop of blood was placed on the slide and a thin film was made. Two ml of Fresh complete media (with 10 % serum) was added, mixed properly, and kept back in the incubator. Thin film was stained and examined for parasitaemia. It was then compared with initial parasitaemia.

Smear Preparation

Culture (3.0 µl) was placed on a slide toward left side. The cells ware smeared across the slide with the help of another slide, to get a thin film and dried the slide at room temperature. Slide was fixed by immersing in methanol for 30 seconds followed by drying. The slides was then be stained with Field A stain for 15 seconds and washed with water to remove excess stain. The slide was then dried and consequently stained with Field B stain for 20 seconds and washed with water to remove the excess stain. It was then dried and observed under the microscope. An area of stained thin blood film where the erythrocytes were evenly distributed was observe using 100 X objective (under oil immersion). Approximately 100 erythrocytes in this area was counted. Without moving the slide, the number of infected erythrocytes amongst the 100 erythrocytes were also counted. The slide was moved randomly to adjacent fields and counting was continued as mentioned above. An equivalent of 1,000 erythocytes was counted. The counting was repeated twice for a total examination of three different parts of the slide, i.e., 3 areas 1000 cells. The mean number of infected RBCs per 1,000 RBCs was taken by dividing the infected RBCs by 3.

Sub culturing (Passaging**)**

Old media was removed and freshly washed RBCs and complete media (with 10 % serum) was added. For example to 20 µl of infected RBCs with around 5 % parasitaemia, 160 µl freshly washed RBCs (i.e. 80 µl RBCs) suspension (concentration of prasitaemia = 1 %) and 2000 µl of complete media was added. Culture was dispensed to more vials.

Synchronization of *Plasmodium falciparum*

Sorbitol (5 %) was prepared in distilled water and passed through a Millipore filter of 0.22 µm porosity. To 1 ml of the culture pellet, 9 ml of sorbitol was added and kept at room temperature for 5 minutes. Supernatant was removed by centrifugation at 10000 x g and pellet was washed thrice in complete media (Washing procedure same as mentioned for preparation of erythrocytes (RBCs) for culture). Freshly washed erythrocytes was added to the culture.

3.2.3.8 Invitro Anti-plamodial Activity of the Plant Extracts

The assay was performed in triplicate in a 96-well microtiter plate, according to WHO method [in vitro micro test (Mark III)] that is based on assessing the inhibition of schizont maturation (WHO 2001). RPMI 1640 (Sigma Company, USA) was the culture medium used for cultivation of *P. falciparum* (Flores et al., 1997). Dilution was prepared from the crude plant extract with concentrations 200, 100, 50, 25, 12.5, 6.25 µg/ml. Negative controls treated by solvent and positive controls (Chloroquine phosphate) was added to each set of experiments. Twenty five microliters from blood mixture media was added to each well in plate and incubated in CO_2 condition at 37^0C for 24–30 h. After incubation, contents of the wells were harvested and stained for 30 min in a 2% Giemsa solution pH 7.2, after which the developed schizonts were counted against the total asexual parasite count of 200. The counting process was done in triplicate. Parasites count for each blood film was done. Each film was observed at three different visual fields. The number of schizonts with two or more nuclei per 200 parasites was counted. The control and the test wells were compared for the determination of the percentage inhibition. The inhibition of parasite growth in the drug treated groups were calculated as follows:-

Percentage inhibition

3.2.4 Preparation of Crude Enzyme Lysate.

The released parasite was isolated using the protocol described by Amlabu et al. 2011. As described briefly, 0.5 % Triton X-100 in Tris-buffer saline was added to the parasites and incubated at 4°C for 90 min. An initial centrifugation of the blood at 10,000 x g for 30 min at 4°C was carried out and the supernatant was collected. It was then layered on 6 % sucrose containing 0.06 % Triton X-100 followed by incubation at 37°C for 5 min. the separation was further enhanced by further centrifugation at 1000xg for 5 min at 37°C in aqueous and detergent phases which was precipitated with cold acetone. The resulting precipitates was referred to as the aqueous and detergent phase proteins, respectively. The pellets from each preparation was suspended to 6 ml in 50 mM phosphate buffered saline, pH 7.2.

3.2.5 Cysteine Protease activity Assay

The detergent phases of the malaria parasite was used for the enzymatic activity assays as described by (Amlabu. 2011). 50 µl of the enzyme lysate with 500 µl of 100 mM sodium acetate buffer, pH 4.5, and 100 µl of 1% (w/v) N-benzoyl- DL-arginine b-naphthylamide hydrochloride (BANA), The reaction volume was adjusted to 1 ml using distilled water and the Assays was carried out at 37 ºC for an hour. The assay activity was stopped by the adding 200 µl of 20 % (v/v) trichloroacetic acid. Centrifugation process was carried (10,000 x g for 5 min at room temperature) to remove the protein precipitate and absorbance of the supernatant was read at 405 nm.

3.2.6 Cysteine Protease Inhibitor Assay:

The extract inhibitory activity against cysteine protease was assay usingN-benzoyl- DL-arginine b-naphthylamide hydrochloride (BANA), as substrate. Aliquots of 500µl from fractions was pre-incubated for 10 min at 37^{0}C with 500 µl crude enzyme lysate. The assayswas initiated by the addition of 1ml substrate solution (1% (w/v) BaNA), incubated for 20 min at 37^{0}C, and 2 ml of trichloroacetic acid (TCA, 20% w/v) was added to terminate the reaction. After 20 min at room temperature, the mixture was centrifuged at 10,000g for 10 min and the absorbance of the supernatant was measured at 405nm.

3.3.1 Thin Layer Chromatography (TLC) for Solvent Development and Pooling of Fractions.

A strip of the pre-coated silica gel was cut out and a spot of the sample was applied on the plate with a dropper about 1.0 cm from the edge. It was then dried using hot air dryer. The strip was lowered into a small chromatographic jar containing the solvent system. The jar was covered with a glass lid and the solvent was allowed to ascend until the solvent front was about ¾ of the length of the strip. The strip was removed and dried by a hot air dryer and viewed under UV light to identify the fluorescing spot. The fluorescent spot was marked and then sprayed with spray reagent: vanillin in concentrated tetraoxosulphate (IV) acid. The strip was placed in hot oven at 110^{0}C for 5 seconds for visibility of fluorescent bands. The dark blue colour reaction was recorded and the relative Retention factor (R_f) value was calculated based on the formula described by Patra et al. (2012).

3.3.2 Column Chromatography

Silica gel was used as the stationary phase while varying solvent combinations of increasing polarity was used as the mobile phase (Patra et al., 2012). The slurry was prepared by Mixing 150 g of silica gel and 350 ml of hexane was poured down carefully into the column. The tap of the glass column was left open to allow free flow of solvent into a conical flask below. At the end of the packing process, the column was allowed 24 h to stabilize. The sample was prepared by adsorbing 8.0 g of the extract to 20 g of silica gel 60G in methanol and dried on a hot plate. Sample was continuously stirred to dryness by using Spatula, while guarding against getting it burnt. Dry powder was allowed to cool and gently layered on top of the column. The column tap was opened to allow the eluent to flow at the rate of 40 drops per minute. Elution of the extract was done with solvent systems of 8:2 methanol to chloroform, the eluted fractions were then collected in aliquots of 1 ml in test tubes. . Similar fractions were pooled together according to the TLC profile, which were then used for further in-vitro anti-plasmodia activity. The lead fraction obtained was characterized using GC/MS methods.

3.4 Methodology for GC/MS

The sample solution was injected into the GC inlet where it is vaporized and swept onto a chromatographic column by the carrier gas (usually helium). The sample flowed through the column and the compounds comprising the mixture of were separated by virtue of their relative interaction with the coating of the column (stationary phase) and the carrier gas (mobile phase).

The latter part of the column passes through a heated transfer line and ends at the entrance to ion source where compounds eluting from the column were converted to ions. A beam of electrons ionised the sample molecules and a peak from this ion was seen in a mass spectrum. After the ions were separated they enter a detector the output from which was amplified to boost the signal. The detector sent information to a computer that records all of the data produced, converts the electrical impulses into visual displays and hard copy displayed.

3.5 Statistical Analysis

The data were analysed by the analysis of variance (ANOVA) using SPSS program (version 20.0 SPSS Inc. Chicago,IL, USA). The results were expressed as mean standard deviation (SD). P value less than 0.05 was considered as significant (P<0.05) (Mahajan, 1997)

CHAPTER FOUR

4.0 RESULTS

4.1 THE PERCENTAGE YIELD OF *C.SENEGALENSIS* EXTRACTS

The percentage yield of the *C. senegalensis* extracts are shown in Table 4.1. the percentage yield (w/w) of the various extract have methanol fraction 29.95 ± 0.60% as the highestyield, followed by aqueous fraction with 26.87 ± 0.53%, ethylether fraction with 15.48 ± 0.57% and n-hexane has the lowest with 9.93 ± 0.29%.

4.2 PHYTOCHEMICAL ANALYSIS OF *C. SENEGALENSIS* EXTRACTS

The preliminary qualitative phytochemical screening of the aqueous, methanol, ethyl ether and n–hexane extracts of the whole plant *C. seregalensis* revealed the presence of tannins, alkaloid, saponins , flavonoid and phenolic in aqueous and methanol extract while terpenes are absent in both the extracts as shown in table 4.2. In the ethylether and n-hexane extracts the preliminary qualitative phytochemical screening shows the presence of the tannins, terpenes, and phenolics. Alkaloids are only present in ethylether extract and flavonoids only in n-hexane but saponins are absent in both the extracts.

In the quantitative phytochemicals of the extracts Table 4.3, the aqueous extracts of *C. senegalensis*, phenolics content was high with 3.24mg/100g followed by saponins 2.69mg/100g, alkaloids 2.88mg/100g, flavonoids 2.21mg/100g then terpenoids with 1.09mg/100g then the least was Tannins 0.21mg/100g. In the methanol extract, tannins content is highest with 3.12mg/100g followed by alkaloids with 3.10, flavonoids 2.51mg/100g, then phenolics, 2.24mg/100g, saponins 1.69mg/100g, and then least was terpenoids 1.61mg/100g. Ethyl ether extract has higher concentration of terpenoids 6.21mg/100g and lower concentration of tannins 4.09mg/100g and the alkaloid content also is not as high as it was in both the aqueous and methanol extract only 0.11mg/100g was estimated. Lastly, the n-hexane extract that contain only 4 phytochemicals has highest concentration of terpenes 4.25mg/100g followed by tannins 2.68mg/100g, flavonoid 1.53mg/100g and then phenolics with 0.98mg/100g.

Table 4.1: Percentage yield of *C. senegalensis* Extracts

Fractions	**Percentage yield (%) of *C. senegalensis***
Aqueous	26.87 ± 0.60^{a}

Methanol	29.95 ± 0.53[a]
Ethyl ether	15.48 ± 0.57[b]
N-hexane	9.93 ± 0.29[c]

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

Table 4.2: Qualitative Phytochemical Composition of Aqueous, Methanol, Ethylether and N-hexane Extracts of *C.senegalensis*.

Solvents	Phytochemicals					
	Tannins	Alkaloids	Saponins	Terpenes	Flavonoid	Phenols
Aqueous	+	+	+	+	+	+
Methanol	+	+	+	+	+	+
Ethyl-ether	+	+	–	+	–	+
N-hexane	+	–	–	+	+	+

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

Table 4.3: Quantitative Phytochemical Composition of Aqueous, Methanol, Ethylether and N-hexane Extracts of *C.senegalensis*.

	Concentration (mg/100g)			
Phytochemical	Aqueous	Methanol	Ethylether	N-hexane
	a	a	b	
Alkaloids	2.88 ± 1.24	3.10 ± 0.01	0.11 ± 0.15	ND
	a	a		b
Flavonoids	2.24 ± 0.09	2.51 ± 0.03	ND	1.53 ± 0.02
	a	b		
Saponins	2.69 ± 0.02	1.69 ± 0.02	ND	ND
	a	b	b	c
Phenols	3.24 ± 0.02	2.24 ± 0.02	2.84 ± 0.01	0.98 ± 0.05
	a	a	b	c
Terpenes	1.09 ± 0.06	1.61 ± 0.03	6.21 ± 0.17	4.25 ± 0.03
	a	b	c	d
Tannins	0.21 ± 1.09	3.12 ± 0.01	4.09 ± 0.65	2.68 ± 0.03

Value are mean ± standard deviation n=3 values with different superscripts in the row differ significantly ($P<0.05$)

4.3 PERCENTAGE PARASITAEMIA IN THE CULTURE FOR SEVEN DAYS DURING CULTIVATION

The three slides (Plate I-III) have shown the microscopic visualization of the parasite for 3 days in the cause of cultivation. The 1st day, the culture was initiated with 0.5 % parasitaemia(plate I). On the 3rd day (plate II) there was significant increase in the level of parasitaemia with 0.61% and it raised up to 1.87% parasitaemia on the 7th day (plate III). Figure 4.1 shows the increase in the parasites level for 7 days. The result shows constant increase in the level of parasitaemia throughout the cultivation which was even more prominent at day 4 to day 7. The Percentage parasitaemia of *Plasmodium falciparum* in the culture for 7 days was represented graphically from the first day of cultivation with 0.5% parasitaemia up to the last day that was day seven (1.87 %).

4.4 ANTI–PLASMODIAL ACTIVITY OF AQUEOUS, METHANOL, ETHYLETHER AND N–HEXANE EXTRACTS OF *C.SENEGALENSIS*.

The result for the anti-plasmodial activity of the extracts at different concentrations of the plants was represented in Figure 4.2 which shows higher activity in methanol extract at all the 4 different concentrations. . Ethyl ether and n-hexane extracts showed no significant difference at ($P<0.05$) in the anti-plasmodial activity when compared to negative control. Methanol extract showed large significant difference in the anti-plasmodial activity when also compared to negative control, but they all have significant difference with the positive control.

In Figure 4.3, $1C_{50}$ value which is the concentration require to inhibit 50% of the parasite show that the methanol extract has the lowest $1C_{50}$ of 2.37 µg/ml which made it the most potent among the 4 extracts and n hexane that has the highest $1C_{50}$ of 32.47 µg/ml is the least potent

PLATES a-c: Microscopic Visualization of *P. falciparum* on the 1st, 2nd and 3rd Days of Cultivation Respectively

Figure4.1: Percentage Parasitaemia in the Culture for Seven Days during Cultivation

Figure 4. 2: Anti-Plasmodial Activity of Aqueous, Methanol, Ethylether and N-hexane Extracts of*C.senegalensis*.

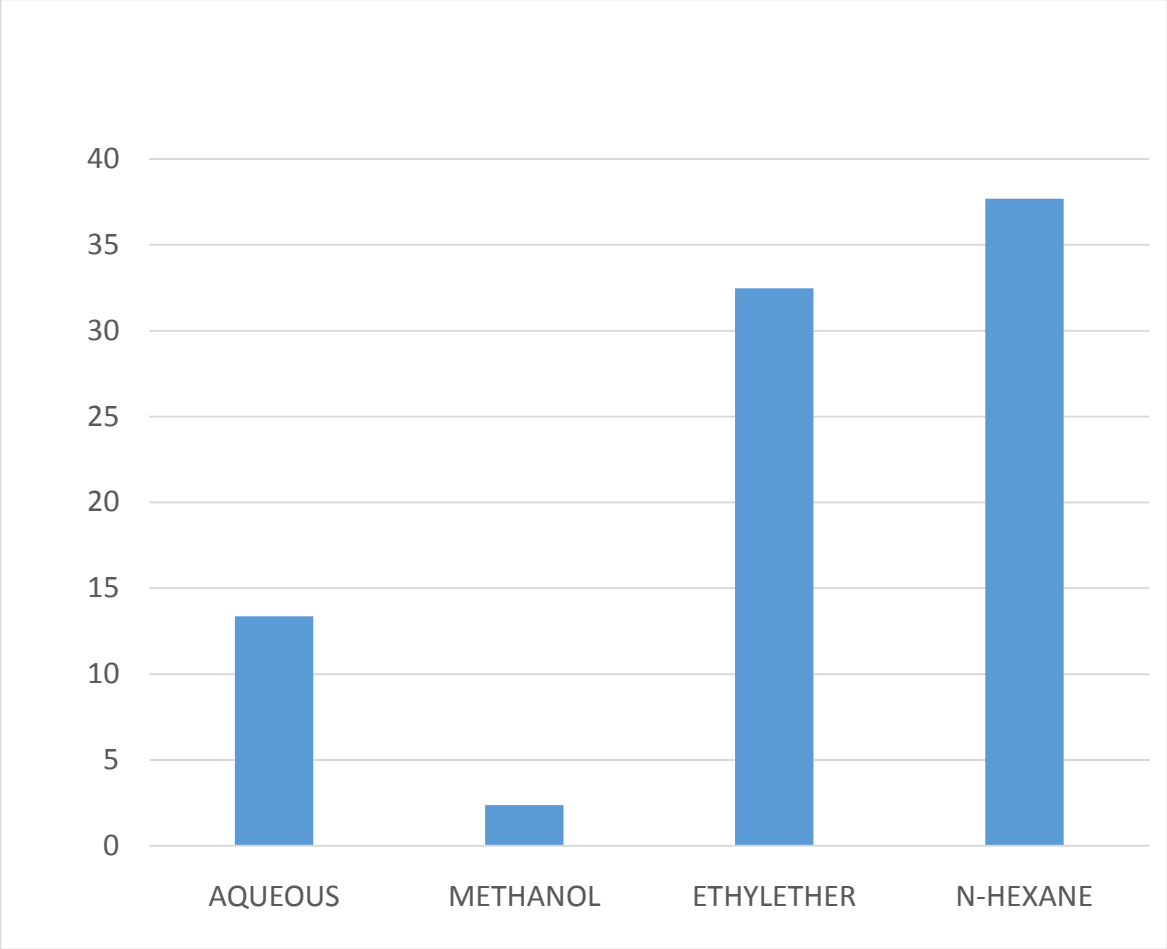


Figure 4.3: Median Inhibitory Concentration (IC_{50}) of Aqueous, Methanol, Ethylether andN-hexane Extract of *C. senegalensis*.

4.5 CYSTEINE PROTEASE INHIBITON OF THE AQUEOUS, METHANOL, ETHYL ETHER AND N–HEXANE EXTRACTSOF *C. SENEGALENSIS*

Figure 4.4 shows the inhibition of the enzyme cysteine protease by the whole plant crude extracts of the 4 solvents, aqueous, methanol ethyl ether and n–hexane the result shows that for the enzyme activity also methanol extract has the highest tendency to inhibit the activity of the enzyme and n-hexane was the least. When compared together statistically at $P<0.005$ there was no significant difference between the aqueous and methanol extracts, but they differ significantly with ethyl ether and the n-hexane extracts

4.6 R_f VALUES FOR THE 50 FRACTION OF THE *C. SENEGALENSIS* METHANOL EXTRACTS AND THE POOLING TO 10 FRACTIONS.

In Table 4.4: It shows the R_f values of the 50 fraction base on the result of the thin layer and column chromatography collected from the most potent fraction that was the methanol. The 50 fractions collected were pooled together to 10 fractions according to their similarities in their R_f values. Fractions 1-6 have 4 different R_f values with R_f 1 ranging from o.71-0.86, R_F 2 ranging from 0.68-0.8, R_f3 from 0.59-0.67 and R_f 4, 0.44 - 0.68. Fractions 7-10 are also pooled as a single fraction 2, which also have 4 different R_f values ranging from 0.46 - 0.74, fraction 11-15 have values from 0.27 - 0.58 and are pooled as fraction 3, fractions 16-18 were pooled as fraction 4 with R_f values of 0.26-0.61, fractions 19-24 have R_f values of 0.18-0.44 and are pooled as fraction 5, fraction 25-28 were pooled as fraction 6 with R_f values of 0.19-0.27, fraction 29-33 as fraction 7 with R_f values of 0.11-0.19, fractions 34-37 as fraction 8 with R_f values of 0.05-0.14, fractions 38-43 as fraction 9 with R_f values of 0.03-0.09 lastly fractions 44-50 were pooled as fraction 10 with a single spots and values ranging from 0.03-0.05.

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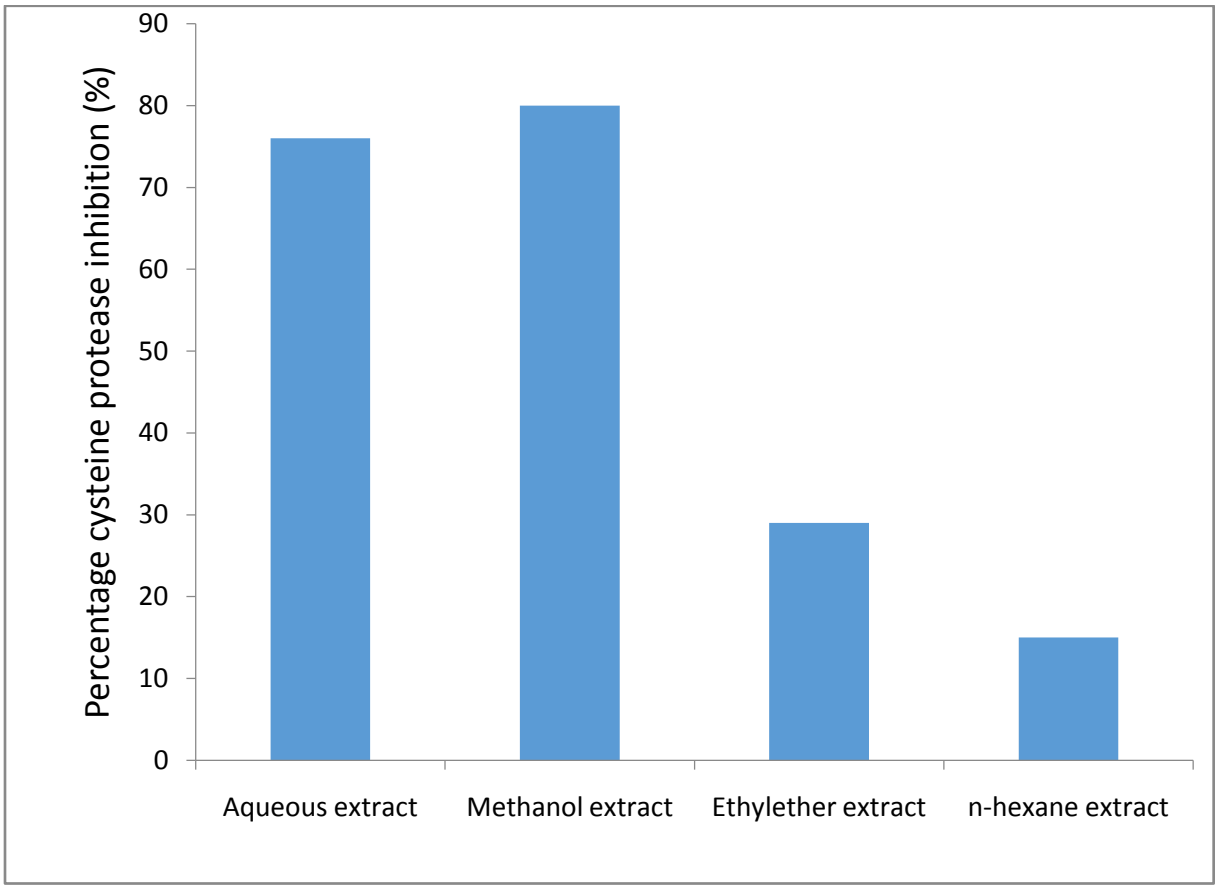


Figure 4.4: Percentage Cysteine Protease Inhibition of Aqueous, Methanol, Ethyl ether and N-hexane Extracts of *C. senegalensis.*

Table 4.4: R_fValues for the 50 Fractions of the *C. senegalensis* Methanol Extracts and the Pooling to 10 Sub-fraction.

	Initial fraction	R_f1	R_f2	R_f3	R_f4
Fraction 1	1	0.78	0.75	0.64	0.6
	2	0.79	0.72	0.67	0.58
	3	0.81	0.71	0.6	0.56
	4	0.86	0.8	0.59	0.54
	5	0.81	0.76	0.59	0.55
	6	0.84	0.73	0.64	0.58
Fraction 2	7	0.71	0.68	0.59	0.44
	8	0.68	0.55	0.49	
	9	0.72	0.67	0.61	0.48
	10	0.72	0.61	0.57	0.46
Fraction 3	11	0.58	0.45	0.33	0.29
	12	0.58	0.42	0.35	0.3
	13	0.6	0.47	0.29	0.27
	14	0.56	0.49	0.32	
	15	0.55	0.51	0.36	
Fraction 4	16	0.57	0.29		
	17	0.61	0.32	0.26	
	18	0.57	0.27		
Fraction 5	19	0.36	0.31	0.19	
	20	0.36	0.32	0.21	
	21	0.41	0.35	0.26	
	22	0.44	0.33	0.18	
	23	0.39	0.29		
	24	0.38	0.31		
Fraction 6	25	0.22	0.17		
	26	0.27	0.19		
	27	0.3	0.21	0.19	
Fraction 7	29	0.19	0.15	0.12	
	30	0.17	0.15	0.11	
	31	0.19	0.17		
	32	0.18	0.14		
	33	0.2	0.16	0.12	
Fraction 8	34	0.13	0.09	0.05	
	35	0.14	0.08		
	36	0.14	0.08	0.05	
	37	0.12	0.06		
Fraction 9	38	0.07	0.05		
	39	0.06	0.04		
	40	0.06	0.03		
	41	0.06	0.04		
	42	0.08	0.05		
	43	0.09	0.05		
Fraction 10	44	0.04			

45	0.03
46	0.04
47	0.05
48	0.03
49	0.05
50	0.04

4.7 CYSTEINE PROTEASE INHIBITION OF THE METHANOL FRACTIONS

The fractions collected and pooled after column chromatography of the most active fraction of the methanol extract were also tested for cysteine protease inhibition and the result was shown in figure 4.5. Fraction 3 has the highest inhibition with 81% inhibition. Fractions 1,2,3,4 and 5 shows no significant difference at ($p<0.05$) in their anti- cysteine protease activity but they differ significantly with the rest of the fractions.

4.8 RESULTS FOR GC/MS OF METHANOL MOST ACTIVE FRACTION

Figure 4.6 shows the spectra for the result of the most active fraction that is fraction 3 of the methanol extract of *C senegalensis.* Table 4.5 shows the acids in the GC/MS result.

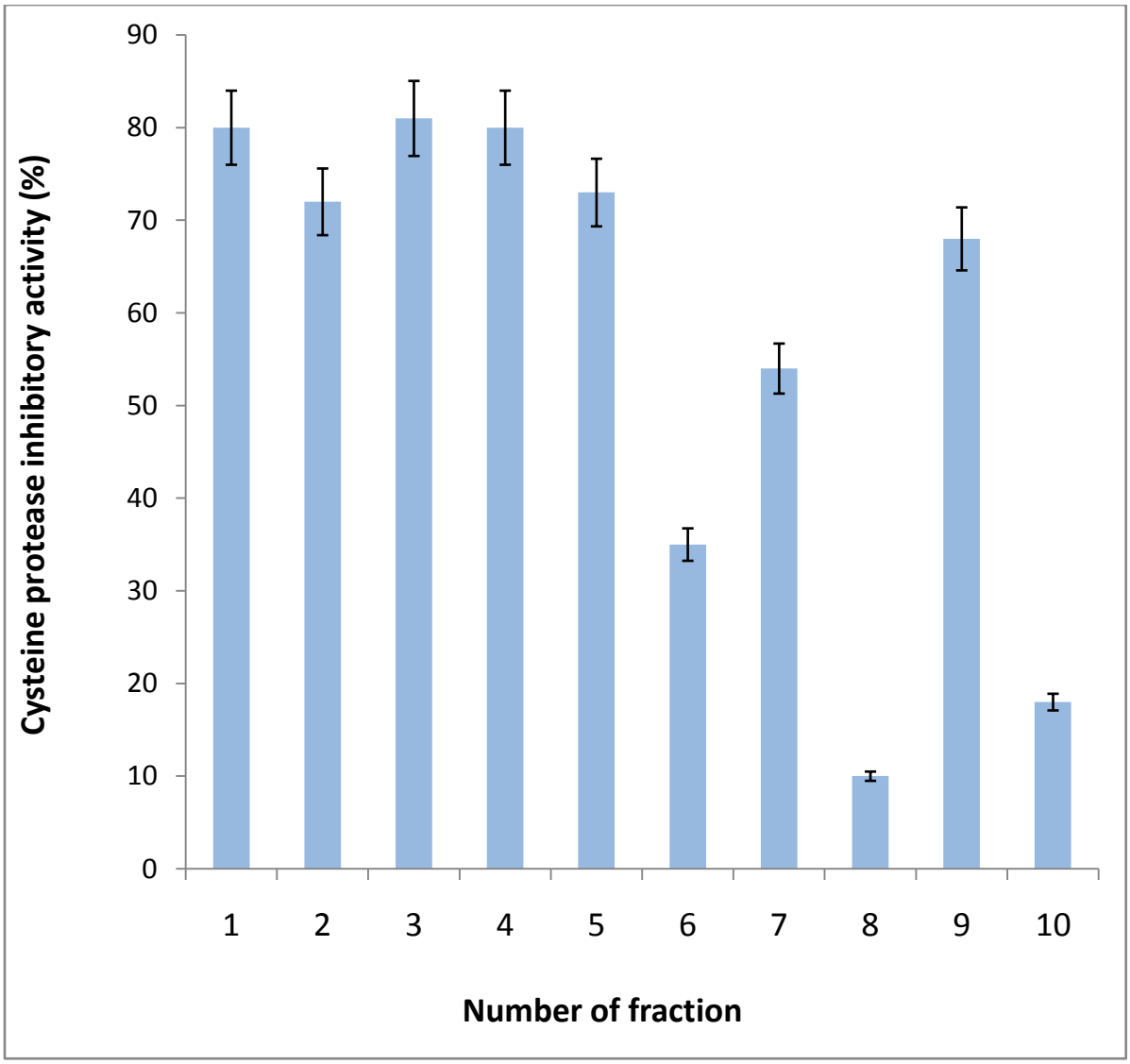


Figure 4.5; Percentage Cysteine Protease Inhibitory Activity of the 10 Fractions of Methanol Extracts of*C.senegalensis.*

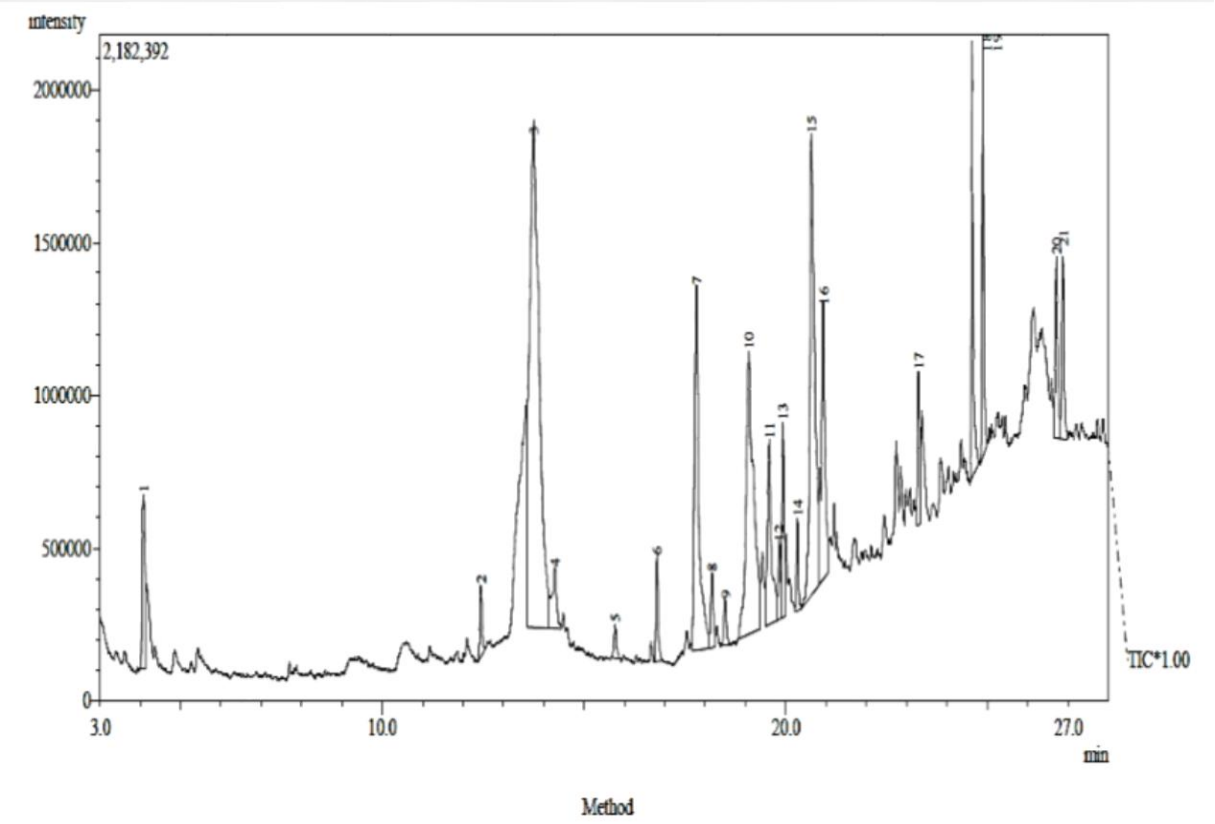


Figure 4.6: Spectra for the GC/MS Result for the Most Potent Fraction of Methanol Extract of *C.senegalensis.*

Table 4.5: The Acids in the GC/MS result of the Methanol Most Active Extract

S/No	Retention time	Common name	Similarity Index
1	12.45	Palmitic acid	89
2	14.26	Petroselinic acid	80
3	16.81	Isopalmitic acid	92
4	18.51	Stearic acid	89
5	19.08	Oleic acid	94
7	19.83	Linoleic acid	88
9	20.29	Hexyamine	80
10	23.29	Phthalic acid	83
11	24.88	Nitrobenzoic acid	84
12	26.88	Ellagic acid	97

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 (Venkatalakshion, 2012). This plant based traditional medicinal system continues to play an essential role in health care, with about 80% of the world` inhabitants relying mainly on traditional medicines for their primary health care (Omonkhelin et al, 2007). Plant materials remain an important source of medicine in the fight against malaria (Aderounmu, 2007) since widely used antimalarial drugs such as quinine and artemisinin are isolated from plants (Rosenthal, 2001). The *Chrozophora* s*enegalensis* plant material used for this research was dried, ground and stored at low temperatures. This was done to preserve the presence and the quality of the compounds found within the plant. According to Makkar (2000) the moisture content of fresh plant material changes the chemical composition and properties of the plant over time. Grinding of the plant material into fine particles was to create a larger surface area to allow higher extraction of bioactive compounds.

The successful extraction of bioactive compounds from plants, according to Parekh et al., (2005) is largely dependent on the type of solvent used in the extraction procedure. Effective extraction from the dried plant material was achieved using different solvent. The dried plant material was

extracted using water (a similar extraction method employed by traditional healers in preparing most of their herbal remedies) and some organic solvents system (methanol, ethylether and n-hexane) to obtain the bioactive compounds present in the plant under pharmalogical investigation.

Both aqueous and organic extractions were carried out in order to isolate both polar and non-polar compounds. Whilst the pattern for finding the compounds was similar, the abundance varied with methanol showing higher levels of the compounds isolated. This is consistent with other studies which illustrate that plant phytochemicals are more soluble in organic solvents (Willcox et al., 2004). Willcox *et al.*, (2004) further maintains that organic solvent extractions are used as a good alternative in evaluating the antimalarial activities of plants, as organic solvents are able to extract a broad spectrum of chemical constituents. Even though, it is interesting to note that most medicinal plant preparations are conducted in aqueous solvents (water) traditionally, it is noteworthy to observe the effect of organic solvents on the plant material and to describe them.

The percentage yield (w/w) of the various extracts of the plant has methanol fraction 29.95 ± 0.60% as the highest yield, followed by aqueous fraction with 26.87 ± 0.53%, ethylether fraction with 15.48 ± 0.57% and n-hexane has the lowest with 9.93 ± 0.29%. The extract yield of the plants was higher in the aqueous and methanol extracts than for the ethylether and the n-hexane organic extracts. Parekh et al (2005) maintains that these observations can be explained due to the polarity of the compounds extracted. The high polarity of water and methanol probably accounted for the high yield in the dry extracts because both polar and some less polar compounds were extracted. Hence, the extract yield increased when solvents of polarity were used. In a similar extraction conducted by Usman et al., (2014) using ethanol, ethyl acetate, n-

butanol, and aqueous the percentage yield (w/w) of the extracts were 28.6% from ethanol, 16.6% in the ethyl acetate, 6.43% in n-butanol and 40.1% in aqueous, in this research also the percentage yield is higher in the polar solvents than in the non-polar solvents even though the higher yield of the plant in the extraction conducted by Usman et al., (2014) might be due to the soxhlet extraction method, polarity of solvents used, climate and environmental condition.

The preliminary qualitative phytochemical screening of the aqueous, methanol, ethyl ether and n–hexane extracts of the whole plant *C. senegalensis* revealed the presence of tannins, alkaloid, saponins , flavonoid and phenolic in aqueous and methanol extract while terpenes are absent in both the extracts. In the ethylether and n-hexane extracts the preliminary qualitative phytochemical screening shows the presence of the tannins, terpenes, and phenolics. Alkaloids are only present in ethylether extract and flavonoids only in n-hexane, but both extracts have absence of saponins. In a research conducted by Isyaku, (2016) on the phytochemical of the ethanol extract of the root of *C. senegalensis*, it revealed the presence of flavonoids, tannins, alkaloids and saponins. These classes of compounds are known to show curative activity against several pathogens (Hassan et al., 2004) and therefore could explain its use traditionally for the treatment of wide array of illnesses.

In the quantitative phytochemicals of the 4 extracts, in the aqueous extracts of *C. senegalensis* phenolics content is highiest with 3.24mg/100g concentration and the least is Tannins 0.12mg/100g. In the methanol extract, Tannins content is highest with 3.10mg/100g the least is terpenoids 1.61mg/100g. Ethyl ether extract has highiest concentration of terpenoids 6.21mg/100g and lowest concentration of tannins 4.09mg/100g. Lastly, the n-hexane extract that contain only 3 phytochemical has highest concentration of terpenes 4.25mg/100g and lowest concentration of phenolics 0.98mg/100g. Generally majority of the secondary metabolites are

higher in the aqueous and methanol extracts than in the ethyl ether and n-hexane extracts, it was explained that polarity level and species nature are playing major role in extracting secondary metabolites. Past studies have revealed that compounds with antimalarial activity are commonly polar (willcox et al 2004). In a research conducted by Hamilton (2004), terpenoids have an important role in producing antimalarial activity, proved by the high inhibition percentage of *Diaspyros jarica* stem bark extract which only contained terpernoids however, result in inhibition percentage greater than 50%.Terpenoids as an antimalarial acts by inhibiting the growth phase of the plasmodium parasite from ring form to tropozoites and inhibited nutrient intake by the parasites by inhibiting the permeation pathway. Flavonoids was thought playing an important role in generating antimalarial activity. The proof was the inhibition percentage of extracts containing high flavonoids in *Alectryon senratus* leaves extract. The plant contains flavonoids and has inhibition percentage of 81.1%. Flavonoids work by inhibiting the influx of L-glutamine and myoinosol into the infected red blood cell (Hamilton, 2004). Anthraquinone could kill the parasites through various mechanisms, resulting from aldehyde at C-2. Alkaloids are one of the major classes of natural products that exhibit antimalarial activity, indeed, quinines, the first antimalarial activity drug belongs to the class. Over100 alkaloids from higher plants were reported to demonstrate significant antimalarial activity in studies published from 1990 to 2010 some of these were more potent than chloroquine (Hombanje and huang, 2010). These research results from literature review are in line with the findings in this research work giving an idea on why the activity is higher in methanol and aqueous extracts than in the ethylether and n-hexane extract with a simple reason that all the phytochemicals having anti-plasmodial activity are present in a higher concentration. than in the ethylether and n-hexane extracts.

Cultivation of the*P.falciparum* parasite involved growing the parasite in human red blood cells outside the body in cell culture flasks at 37^{o} C. The results revealed that at the initial stage of cultivation of synchronized parasite, percentage parasitaemia was 0.5%, the second day it decreases a little bit to 0.45% which may be due to instability of the parasite, the 3^{rd} day it increases to 0.61%, then to 0.73% on the 4^{th} day from 0.73% it raises of to 1.02 the 5^{th} day and this might be due to acclimatization of the environment by the parasite. The 6^{th} day it increases to 1.1% and then to 1.87% on the 7^{th} day. The images in plate I-III shows the microscopic visualization of the parasite with their percentage parasitaemia on each day. The plant extracts were screened for anti-plasmodial activity when the parasitaemia level was at its peak using the *in vitro* model at different concentrations for each of the extracts, 6.25 µg/mL, 12.5 µg/mL, 25.5 µg/mL, 50.0 µg/mL, 100 µg/mL and 200 µg/mL and it was found that in all the 4 different extracts, the least activities is at concentration of 6.25 µg/mL with percentage inhibition values of 22.65% for aqueous, 36.58% for methanol, 12.37% ethylether and 11.08% for n-hexane. They all have highest activity at highest concentration with percentage inhibition values of 58.81%, 87.10%, 14.43% and 13.45% respectively which implies that the activity is concentration dependent. Among the 4 extract methanol has highest activity in all concentration probably due to the higher concentrations of all the phytochemical (alkaloids, terpenoids, flavonoids) that have the inhibition capacity of the malarial parasite activity in different ways. The activity is followed by aqueous extracts, ethyl ether and n-hexane has the least. These explains way the IC_{50} value of methanol extracts is lower than that of all the 4 extract. The IC_{50} value is the concentration require to inhibit 50% of the parasite and that as the concentration increases the activity also increases. Since methanol extract can have up to 87.10% inhibition at concentration of 200mg/mL, it means a little quantity is needed for its $IC_{50.}$ N-hexane that has the lowest

percentage inhibition capacity among the 4 extracts a therefore has the highest IC_{50} value. Ettebong, (2012) conducted a research to evaluate the *in vivo* anti-plasmodial activities of the extract and fractions (*n*-hexane, chloroform, ethylacetate, butanol, aqueous) of the whole plant of *C. senegalensis* in *Plasmodium berghei* infected mice. The extract showed significant ($P< 0.05–0.001$) dose-dependent anti-plasmodial activity in the 4-day repository and curative tests and increased the survival times of the infected mice. All the fractions exhibited significant anti-plasmodial activity with the highest being ethylacetate fraction). The activity is highest in the ethylacetate extract in his research work probably due to highest concentration of alkaloids in the extracts and alkaloids were found to have up to 76.91 % inhibition capacity of*Plasmodium falciparum* in a research conducted by (Hombanje & Huang, 2010).

After the screening of the 4 crude extracts of the plant on anti-plasmodial activity, cysteine protease inhibitory activity of both the crude extracts and methanol fractions were also conducted. Extensive evidence suggests that the degradation of hemoglobin is necessary for the growth of erythrocytic malaria parasites, apparently to provide free amino acids for parasite protein synthesis (Mckerrow et al., 1993 and Rosenthal & Meshrick 1996). In *P. falciparum,* hemoglobin degradation occurs predominantly in trophozoites and early schizonts, the stages at which the parasites are most metabolically active. Incubation of cultured *P. falciparum* parasites with the protease inhibitor leupeptin caused trophozoite food vacuoles to fill with apparently undegraded erythrocyte cytoplasm (Dluzewski et al., 1986 & Vander et al., 1989). Analysis of the leupeptin-treated parasites showed that they contained large quantities of undegraded globin, while minimal globin was detectable in control parasites (Rosenthal, 1995). Leupeptin inhibits both cysteine and some serine proteases, but the highly specific cysteine protease inhibitor E-64 also caused undegraded globin to accumulate. After parasites were incubated with inhibitors of

other classes of proteases including the aspartic protease inhibitor pepstatin (Bailly et al., 1992), globin did not accumulate. More recent studies that used non-denaturing electrophoretic methods demonstrated that cysteine protease inhibitors not only blocked malarial globin hydrolysis, but also inhibited earlier steps in hemoglobin degradation, including denaturation of the hemoglobin tetramer and the release of heme from globin (Gambia et al 1996). Another study showed that E-64, but not pepstatin, inhibited the production of hemozoin (the malarial end product of heme) by cultured parasites (Asawamahasakda et al., 2014). These results suggest that a cysteine protease is required for initial steps in hemoglobin degradation by *P. falciparum* .In this research result the plant methanol extract which was found to have highest anti-plasmodial activity also had the highest activity in cysteine-protease inhibition test. This shows the tendency that the extract anti-plasmodial activity is due to cysteine protease enzyme inhibition so also the hierarchy of activity is exactly the same with that of anti-plasmodial activity that is from methanol to aqueous, ethylether and then n-hexane had the least activity.

Bio guided assay fractionation of the methanol extract increases the specific activity of the enzyme which could be attributed to the removal of interacting protein components attached to the enzyme. This observed inhibition implies that the group of compounds in the extract through individual or synergistic action inhibited the enzyme activity. The fractions collected and pooled after column chromatography of the most active fraction of the methanol extract were also tested for cysteine protease inhibition and the result is shown in figure 4.8. Fraction 3 has the highest inhibition with 81% inhibition. Fraction 1,2,3,4 and 5 shows no significant difference at ($p<0.05$) in their anti- cysteine protease activity but they differ significantly with the rest of the fractions. The dose dependent inhibition of the activities of cysteine protease 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 virulence posed by the parasite. Characterization of fraction three that shows the best inhibitory activity through gas chromatography/mass spectroscopy revealed the presence of nitro-benzoic acid and ellagic acid alone side other fatty-acids and their derivatives which shows a high binding strength towards the enzyme cysteine protease. Ellagic acid was tested for antiprotozoal activity against *Entamoeba histolytica* and *Giardia lambia* and was found to be very effective (Alanis et al.,2003) These compounds could serve as a good source of antidote for malaria and could as well guide the design of a novel drug of malaria.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 SUMMARY

From the present study the qualitative phytochemical screening of the plant extracts reveals, the presence of tannins, alkaloid, saponins, flavonoids and phenolics, in methanol and aqueous extracts while ethyl ether and n-hexane extracts contain terpenes, flavonoids and phenolics. Ethyl ether also contain traces of tannins and alkaloid.

i. Quantitative phytochemical analysis of the methanol extracts of C *senegalensis* shows alkaloid content is highest with (3.10 ± 0.01 mg/100g) followed by tannins (3.12 ± 0.01mg/100g), flavonoids (2.51 ± 0.03mg/100g), phenolics with (2.24 ± 0.02 mg/100g). Saponins (1.69 ± 0.02mg/100g) and then the least terpenes with (1.61 ± 0.03 mg/100g).

ii. The percentage parasite level for 7 days during cultivation showed constant increase throughout the 7 days which is more prominent at day 4 to day 7.

iii. The C. *Senegalensis* extracts possessed anti-plasmodial effect and the methanol extract has the highest activity with percentage inhibition at dose of 200µg/mL. The dose dependent inhibition of the extract implies that the higher the concentration of the compound in the extracts the higher the inhibition. The IC_{50} value for the methanol extract of the *C. senegalensis* also shows a greater activity with IC_{50} of 2.37µg/mL.

iv. Cysteine protease inhibition activity for the 4 extracts of *C. senegalensis*. Aqueous, methanol, ethyl ether and n-hexane also shows highest activity in methanol extract with percentage inhibition of 80.1%.

v. Fractions of the methanol extract were also tested against the enzyme and this enabled the isolation of some fatty acids. Ellagic acid and Nitrobenzoic acid were documented to have anti-protozoa activity from the literature review.

6.2 CONCLUSION

The antimalarial activity shown by these plant also confirms the importance of using an ethnopharmacological approach in screening plants as a source for potential antimalarials. The result shows that methanol extract fractions were found to have some level of inhibition in the activity of the cysteine protease enzyme with 80.1% inhibition capacity, this study has scientifically validated probable principle and site of action in the ethno-medicinal practice in the use of *Chrozophora senegalensis* in the treatment of malaria and having explored the antimalarial properties in the plant extracts with the active compounds identified which may be effective therapeutic agents for the treatment and management of malaria. In conclusion this study shows the value of traditional herbs in the fight against malaria. The validation of plants used by the indigenous populations as described will facilitate in the ongoing efforts to eliminate malaria by providing treatment to people more especially, rural populations that do not have access or do not believe in conventional medicine and rely mostly on ethnomedicine.

6.3 RECOMMENDATION

1. Chronic toxicity testing of the plant should be carried out to determine the safety of the plant.
2. Isolation and identification of the constituents of the plant extracts responsible for the observed anti-malarial activities should also be study.
3. Antioxidant activity of the plant extract should be done.
4. Invivo an Ex-vivo activity of the plant extracts should also be carried out

5. The enzyme kinetic studies should also be studied to know the mode of the inhibition of the enzyme

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LIST OF APPENDICES

Percentage Inhibition

Percentage Parasitaemia in the blood

R_f

Percentage inhibition

Percentage yield of crude extract

APPENDICE II

