ANTI-PLASMODIAL, ANTI-INFLAMMATORY AND ANALGESIC ACTIVITIES OF ALKALOID FRACTION OF *Maytenus senegalensis* LEAF IN MICE

ABSTRACT

The treatment inadequacy and toxicity associated with conventional anti-malarial, anti-inflammatory and analgesic drugs calls for the search for alternatives from medicinal plants, particularly. In the present study, alkaloid fraction of *Maytenus senegalensis* leaf was evaluated for anti-malarial, anti-inflammatory and analgesic properties. Phytochemical screening and acute toxicity test were conducted using standard procedures. Antimalarial studies was conducted using *Plasmodium berghei*-infected mice and four days suppressive test was used at fraction concentrations of 75, 150 and 300 mg/kg bw. Analgesic and anti-inflammatory study were conducted using egg albumin induced paw-oedema and hot plate induced thermal stimuli respectively at fraction concentrations of 75 and 150 mg/kg bw. Sub-acute toxicity study was conducted by administering the extract to rats (5 each group) at dose of 0, 75 and 150 mg/kg bw for 28 days. Results obtained indicated that *Maytenus senegalensis* crude methanol extract contains alkaloids (198.46 ± 2.56 mg/g) as the most abundant phytochemical while tannins were the least (12.45 ± 0.95 mg/g). The alkaloid fraction had LD_{50} of> 5000 mg/kg bw and showed dose dependent anti-plasmodial activity with suppressive effects of $38.22 \pm 0.53\%$, $69.80 \pm 0.28\%$ and $79.43 \pm 0.42\%$ at 75, 150 and 300 mg/kg bw respectively. Alkaloid fraction also exhibited $53.16 \pm 4.09\%$ and $60.76 \pm 7.54\%$ anti-inflammatory effects, $43.35 \pm 4.98\%\%$ and $44.83 \pm 3.86\%$ analgesic effects at 75 and 150 mg/kg bw respectively. GC-MS analysis confirmed the presence of 3 alkaloids; 20α)-3-hydroxy-2-oxo-24-nor-friedela-1(10),3,5,7-tetraen-carboxylic acid-(29)-methylester, 2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro- and 3-hydroxy-20(29)-lupen-28-ol and a terpenes (phytol) as the major constituents of the alkaloid fraction. Alkaloid fraction increases the serum total proteins and transaminases concentrations but had no effect on sodium, potassium, chloride, alkaline phosphatase, triglyceride and glucose concentrations in the mice. Based on the result obtained, it can be concluded that the alkaloid fraction of *M. senegalensis* leaf exhibited promising antimalarial, analgesic and anti-inflammatory activities.

CHAPTER ONE

1.0   INTRODUCTION

1.1   Background to the Study

Malaria is a protozoan and parasitic infectious disease caused by five *Plasmodium* parasites: *vivax*, *falciparum*, *malariae*, *Knowlesi* and *ovale*. More than half of the world's population are at risk of malaria, which results in a global count of 212 million new infections and 429, 000 deaths per year (Odeghe *et al*., 2012; WHO, 2018). More than 90 percent of malaria infections and deaths in Sub-Saharan Africa mainly in children below five years and pregnant women (El Tahir *et al*., 2014). Poor rural dwellers in tropical and subtropical areas are highly vulnerable to this attack due to the favorable and ideal climate conditions for vector and parasite reproduction and growth. Additionally, drug resistance is one of the major challenges faced by the wide-ranging malarial eradication program (Da Silva *et al*., 2011).

Pain is an uncomfortable sensory and emotional experience associated with, or defined in terms of, real or possible tissue harm (IASP, 2015). Like Inflammation, pain may be acute or chronic depending on the lasting impact (Rajagopal, 2006). Acute pain has a sudden onset, occurs shortly after injury, is extreme in severity and generally short lived (under 30 days). This occurs as a result of stimulating nociceptors from tissue damage and usually disappears when the damage heals (WHO, 2012). Chronic pain is characterized as a pain that persists for one month in addition to the normal course of an acute illness or a fair duration of healing injury, or associated with a persistent pathological mechanism that causes constant pain or recurrent pain for months or years at intervals (Rajagopal, 2006).

Inflammation is part of a multifaceted biological process in vascular tissues attributable to bacteria, wounded cells or irritants causing injurious stimuli. It is an organism's defensive attempt to remove these injurious stimuli and begin the healing process (Medzhitov, 2008). However, prolonged response to injurious stimuli can result in progressive tissue damage resulting in various chronic conditions such as rheumatoid arthritis, asthma, Alzheimer's disease, cardiovascular disease, or inflammatory intestinal disease (Conforti *et al*., 2009; Beg *et al*., 2011). Symptoms such as swelling, tightness, irritation and pain are usually associated with inflammation and may cause discomfort to the patient (Beg *et al*., 2011).

Inflammation and pains are gaining research popularity owing to the etiologic role they play in various human diseases. Dexamethasone opioids, morphine and aspirin and other drugs have been established for the management of pain and inflammation, however, these drugs have recorded limited success due to unintended effects such as gastric lesions caused by non-steroidal anti-inflammatory drugs (Odeghe *et al*., 2012). Thus, the search for drugs alternative from natural product is recommended.

Natural products represent ample sources of natural bioactive metabolite with therapeutic values against several diseases (Mostafa *et al*., 2010). The therapeutic properties of these plants are however, attributed to the quality and quantity of the secondary metabolites particularly alkaloids, and flavonoids, which are known to play defensive role in plants but exert different pharmacological effects such as anti-inflammatory, analgesic, antidiabetics, antimicrobial, anti-parasitic and antioxidants effects in human and animals (Mohiuddin *et al*., 2018).

*Maytenus senegalensis (*family; *celastraccae)* is an African medicinal plant traditionally used in the folk medicine for the treatment of a number of ailments, including rheumatism, snake bites, diarrhoea, eye infection, and dyspepsia (Greenwood, 2008). *Maytenus senegalensis* (Lam.) is one of the most frequently used specie of the *Maytenus* Molina genus in the African traditional medicine (along with *M. Obsc) Maytenus senegalensis* is traditionally used as anti-microbial and anti-inflammatory agents, it is also used to treat respiratory ailments and inflammation. The use of this anti-inflammatory herbal drug is common in many African countries like Nigeria, Benin, Kenya, Zambia, Tanzania, Senegal and Zimbabwe. *Maytenus senegalensis* leaves are also used to treat toothaches in India (Gurib-Fakim, 2006). Previous study has demonstrated that the leaf, root and stem bark extracts of M. senegalensis possess *in vitro* anti-*plasmodial*, anti-leishmanial, and antibacterial activities (Odeghe *et al*., 2012). However, literature survey revealed dearth of scientific information on the pharmacological activities of alkaloids and flavonoid fraction.

1.2 Statement of the Research Problem

Anti-malarial drug resistance has emerged as one of the greatest challenges facing malaria control today and has also been implicated in the spread of malaria to new areas and re-emergence of malaria in areas where the disease had been eradicated. This resistance is common to numerous drugs used to treat malaria (Olasehinde *et al*., 2014). Thus, there is an urgent need for increased efforts in anti-malarial drug discovery especially in Africa. Fake medications are coming onto the scene as a result of deliberate criminal activity, while substandard drugs are becoming more common because of poor manufacturing practices. Not only are scores of patients being inadequately treated, but the presence of these undesirable and illegal medications significantly raises the risk of drug resistance by the malaria parasites.

Approximately 438,000 people are thought to have died from malaria in 2015 (World Health Organisation, 2015).

According to the 2006 National Center for Health Statistics Report, one in 10 Africans overall and three in five of those 60 years or older said that they experienced pain that lasted a year or more. More than one-quarter of adults said they had experienced low back pain, and 15 % of adults experienced migraine or severe headache in the past three months. Between the periods 1988-1994 and 1999-2002, the percentage of adults who took a narcotic drug to alleviate pain in the past month rose from 3.2 to 4.2 %. For the millions of people who experience persistent pain, the impact on function and quality of life can be profound (Kim *et al.*, 2004).

Due to extensive use of analgesic and anti-inflammatory agents, the toxicity and untoward effects do occur especially when pain and inflammation therapy involves the use of higher doses for longer periods. This result in a reduction of living quality and functional situation of the patients, increase in the fatigue levels and impairments in daily life activities in working capacity and social interactions (Allard *et al.*, 2001). Considering the probable adverse effects of these synthetic drugs, as well as their limited ability to provide long-term remission, there is a need of a new, effective and safe anti-inflammatory agent which can reduce pain and other associated symptoms. To overcome these problems the preparations from plant origin become a hope for mankind.

1.3   Justification for the Study

Medicinal plants, since time immemorial, have been used in virtually all cultures as a source of medicine (Hoareau and Dasilva, 1999). Traditional plants play an important role in medical system in Nigeria and plant materials remain an important resource to combat serious

diseases in the world. Pharmacognostic investigations of plants are carried out to find novel drugs or templates for the development of new therapeutic agents. Since many drugs, e.g. quinine and artemisinin were isolated from plants and because of the increased resistance of many pathogens, e.g. malaria parasites, towards established drugs, investigation of the chemical compounds within traditional plants is necessary (Phillipson and Wright, 1991). In recent times, natural products of plant sources have been the center of focus as the main source of new, safer and more effective bioactive compounds with medicinal properties (Ogbuehi *et al*., 2014).

1.4   Aim and Objectives of the study

Aim

To evaluate the anti-plasmodial, analgesic and anti-inflammatory activities of alkaloidal extract of *Maytenus senegalensis* leaf.

Objectives of the study

The objectives of the study are to:

i.   to determine the phytochemical compositions of *M. senegalensis* leaf extract

ii.   to determine the LD_{50} of the alkaloid extract of *M. Senegalensis*

iii.   to evaluate the antiplasmodial activity of the alkaloid extract of *M. Senegslensis*

iv.   to d*etermine* the anti-inflammatory and analgesic activities of the alkaloid extract of *M. Senegalensis*

v.   to deterrmine the effect of sub-acute administration of alkaloid extract of *M. senegalensis* on biochemical parameters in mice

vi.   to characterize the alkaloid extract of *M. Senegalensis* leaf using Gas Chromatography Mass Spectrometry (GC-MS).

CHAPTER TWO

2.0   LITERATURE REVIEW

2.1 Malaria

Malaria is a life-threatening disease caused by parasites that are transmitted to man and animal exclusively through the bites of infected *Anopheles* mosquitoes (WHO, 2014). Malaria causes great pain with associated morbidity and mortality. The cost of malaria in economic terms is quite enormous. Malaria maintains a vicious cycle of disease and poverty (WHO, 2015). There is also emerging drug resistance that is complicating the treatment and limiting the choice of antimalarial therapy thus creating a need for back up if the current therapies are phased out (Alemayehu *et al.,* 2015).

2.2   Transmission of Malaria

A person gets malaria when bitten by an infected female mosquito looking for blood meal. This occurs mainly between dusk and dawn. There are other rare mechanisms for the transmission of malaria which include congenitally acquired diseases, blood transfusion, sharing of contaminated needles and organ transplantation (Owusu-ofori *et al.,* 2013).

2.3   Symptoms of Malaria

The malaria destroys red blood cells causing anemia. It also adheres to cells in certain tissues and organs. They affect single or multiple organs with different levels of severity and which can be determined as neurologic, renal dysfunction, hematologic, cardiovascular and respiratory dysfunction as well as hepatic and metabolic dysfunctions. There is also a significant decrease in the level of hemoglobin and significant increase in the levels of SGOT,

SGPT, ALP, bilirubin, creatinine and urea (Mohapatra *et al.,* 2013). The common symptoms of malaria are sometimes similar to those of many other infectious diseases caused by bacteria, viruses or parasites. They include fever, chills, head ache, sweats, fatigue, nausea, vomiting, multi organ failure system which may progress to coma and death (Ronan *et al.,* 2009) .Other symptoms could be dry (nonproductive) cough, muscle or back pain and enlarged spleen (Web MD, 2014).

2.4   Malaria Parasites

Malaria parasites are microorganisms that belong to the genus *Plasmodium*. There are more than a hundred species of *Plasmodium* that infect many species of animals such as reptiles, birds and various mammals (CDC, 2015). Five species have long been recognized to infect humans; *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae*and *Plasmodium knowlesi.* The first two are the most important species because they pose the greatest risk to man. While the former is the most deadly form which is also responsible for the resistance and predominates in Africa (Talkmore *et al.,* 2015).

The first accurate clinical description of malarial fevers was given by Hippocrates in 400 B.C., who mentioned the classic triad of chills, fever and sweating, and analyzed the characteristic periodicity of various forms of malaria and associated splenomegaly with the endemicity of malaria and its topographic aspects (Wernsdorfer, 1980).

The first attempt to treat malaria came in the middle of the seventeenth century, with the introduction of the bark of a Peruvian tree with which the Countess of Chinchón was successfully treated for her febrile condition. The botanical description of the tree providing the Peruvian bark became known only 100 years after its introduction into Europe. Linné, in

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memory of the Countess of Chinchón's recovery, named the new genus of the bark-yielding tree *Cinchona*. Subsequently, the bark was generally called cinchona (Wernsdorfer, 1980).

Since then, different therapeutic approaches have been established for the treatment of malaria, and the combination of approaches to fight malaria now includes long-lasting insecticidal nets, artemisinin-based combination therapy, supported by indoor residual spraying of insecticide, and intermittent preventive treatment in pregnancy. Nevertheless, malaria is estimated as an immense and persistent burden. According to the World Malaria Report 2016 (WHO, 2016), half of the world's population is at risk of malaria (Figure 2.1). About 1.2 billion of the people at high risk of malaria live in Africa and Southeast Asia. Eighty percent of the cases in Africa are concentrated in 13 countries and over half were in Nigeria, Democratic Republic of the Congo, Ethiopia, United Republic of Tanzania and Kenya (WHO, 2016). Figure 2.1 shows the world malaria distributions.
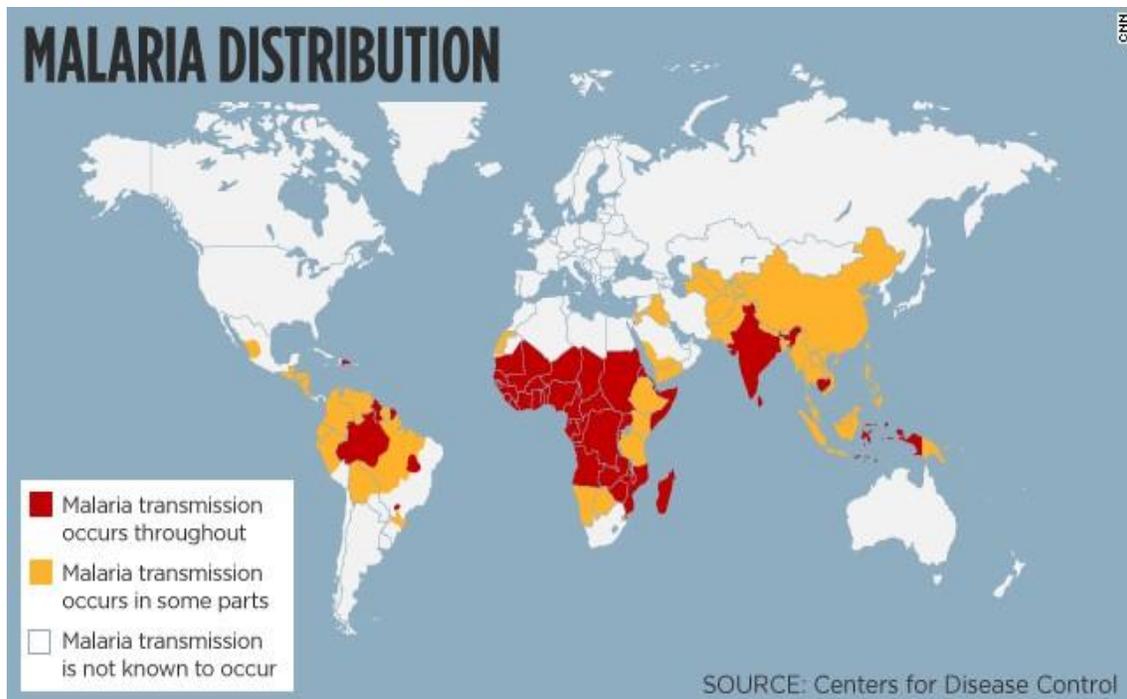
Figure 2.1: World Malaria Distribution (Centre for Disease Control, 2015).

There are many possible reasons for this situation, including the emergence of resistance to chloroquine and other known antimalarial drugs, resistance of parasite vectors to insecticides, environmental deterioration, and demographic growth with subsequent worsening of living and infrastructure standards in endemic areas, especially in Africa (Hyde, 2014). According to surveillance data reported by the World Health Organization, the main problems noticed in several African countries are associated with the gap observed between access and need in terms of prevention tools and artemisinin based treatment. A survey conducted in 18 African countries found that 34 % of households owned an insecticide treated net (ITN), 23 % of children and 27 % of pregnant women slept under an ITN. Thirty eight percent of children with fever were treated with antimalarial drugs, but only 3 % with artemisinin-based combination therapy and 18 % of women used preventive treatment during pregnancy (World Health Organization, 2012).

2.4.1 Life cycle of the *Plasmodium* Parasite

Malaria parasites undergo a complex life cycle alternating between vertebrate and arthropod hosts. As shown in Figure 2.2, the transmission to man starts after the inoculation of sporozoites into blood circulation by an infected mosquito. In the case of human malaria, only female mosquitoes of the genus *Anopheles* are potential vectors, since male anophelines do not feed on blood (Kreier, 2000). Possibly guided by chemotaxis and recognizing its targets, sporozoites leave the capillary lumen and enter hepatocytes (in the case of mammalian plasmodia). At this moment, parasites undergo a drastic morphological change. They appear round or oval and contain a chromatin nucleus surrounded by a cytoplasm. During the tissue (exo-erythrocytic) schyzogony, the nucleus divides and the cytoplasmatic mass grows. The number of nuclear divisions and their intervals vary among different species (Kreier, 2000).

By the end of nuclear division stage, the cytoplasm segregates and merozoites are formed, consisting of a single nucleus and cytoplasm (Figure 2.2). The number of merozoites produced by one hepatic schizont is estimated to be about 2000 in *P. malariae*, 10000 in *P. vivax*, 15000 in *P. ovale*and more than 30000 in *P. falciparum*. Hepatic merozoites are then delivered into the blood circulation to infect erythrocytes that will invaginate to form the parasitophorous vacuole. The erythrocyticmerozoites are ovoid or elongated structures and species-specific in size (Kreier, 2000). Once within the parasitophorous vacuole, the parasite rapidly transforms into an immature trophozoite (ring stage). Hemoglobin is ingested and digested to produce the typical malaria pigment (hemozoin). During this process, the parasite grows and the nuclear material of the mature trophozoite increases and undergoes several nuclear divisions to form a schizont. The mature schizont finally bursts to liberate individual

erythrocyticmerozoites. The latter differ from the hepatic form, mainly by the presence of malaria pigment. Erythrocyticmerozoites can now infect other erythrocytes. The duration of blood schyzogony is generally a multiple of 24 hours, usually 24, 48 or 72 hours and is related to the clinical manifestation of the disease (Kreier, 2000). Upon invading a new erythrocyte the merozoites can either initiate renewed blood schyzogony or develop into a gametocyte from the ring stage trophozoite (Kreier, 2000). Gametocytes can be ingested by the female *Anopheles* mosquito during a blood meal. The parasites' multiplication in the mosquito is known as the sporogonic cycle. Once in the mosquito's stomach, the microgametes (male gametes) penetrate the macrogametes (female gametes) generating zygotes (Figure 2.2). The zygotes in turn become motile and elongated (ookinetes) which invade the intestinal wall of the mosquito where they develop into oocysts. The oocysts grow, rupture, and release sporozoites, which migrate to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the life cycle (Kreier, 1980).
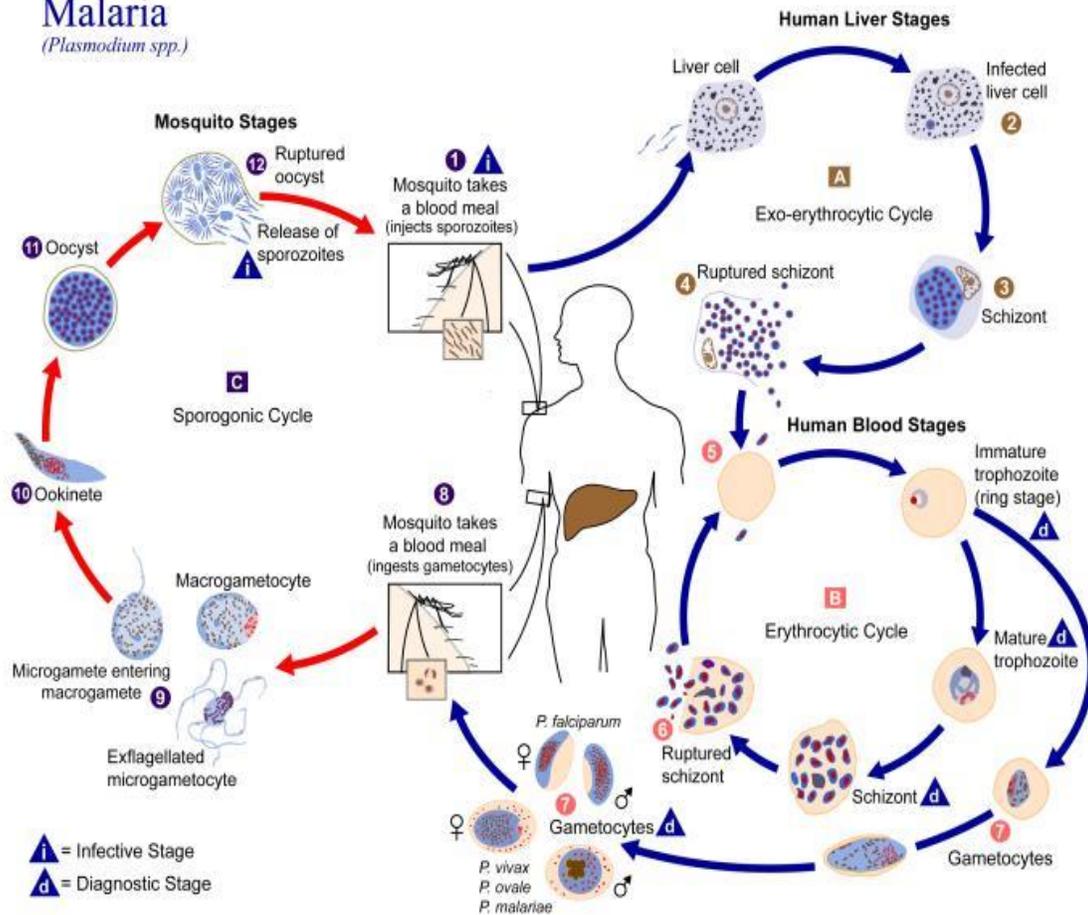
Figure 2.2: Life Cycle of *Plasmodium* (Centre for Disease Control, 2015)

2.5   General Clinical Manifestations of Malaria

More than 80 % of non-immune patients with malaria present with fever, rigor, malaise and

headache. Afebrile patients usually give a history of chills and sweat. Fever is usually

irregular initially, when symptoms may be non-specific and difficult to distinguish from those

caused by other infections, such as influenza, dengue and typhoid-fever. Vomiting occurs in

up to 34 % and diarrhea in about 16 % of patients. Classic periodic fever (every second day

in tertian parasites *P. falciparum*, *P. vivax* and *P. ovale*, and every third day in quartan parasite

P. malariae) is uncommon initially, although if present is highly suggestive of malaria.

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However, periodic fever is neither necessary nor sufficient for the diagnosis of malaria. Splenomegaly and splenic tenderness are the most common physical findings. Tachycardia, tachypnoea, icterus, hepatomegaly and hypotension also occur (Gillespie and Pearson, 2001). The incubation period is the time between the infective bite by the *Anopheles* mosquito and the first symptoms. Shorter periods are observed in general with *P.falciparum*, and longer with *P. malariae*.*P. vivax*and *P. ovale*can produce dormant liver stage parasites (hypnozoites), thus the liver stages may reactivate and cause disease several months after the infective mosquito bite. This is called a relapse (Kreier, 2000).

Malaria disease can be categorized as uncomplicated or severe (complicated). Severe malaria occurs when *P. falciparum* infections are complicated by serious organ failures or abnormalities in the patient's blood or metabolism. Severe malaria often develops very rapidly with specific complications, including cerebral malaria, severe anaemia, pulmonary oedema, acute black water fever, and renal failure. The clinical features of severe malaria depend on age and the immune status of the host. In endemic areas, most cases occur in young children (under five years old) and pregnant women (Gillespie and Pearson, 2001). The diagnosis of malaria may be obtained either by the observation of parasites on a blood smear microscopic analysis or by rapid diagnostic tests (RDTs). Light microscopy has the advantage of low cost and high sensitivity and specificity when used by well trained staff. RDTs are based on the detection of parasite antigens and are generally more expensive. In *P. falciparum* malaria, further laboratory results can be found, such as mild anaemia, mild thrombocytopenia, elevation of bilirubin and others (Gillespie and Pearson, 2001; WHO, 2012).

2.6   Current Chemotherapy in the Management of Malaria

Malaria control requires an integrated approach comprising prevention targeted at vector control and treatment with effective antimalarials. The successful management of malaria depends on prompt diagnosis, an accurate clinical assessment and instituting suitable chemotherapy as soon as possible. Treatment depends not only on the parasite species but also the susceptibility to antimalarial drugs, the severity of the illness, and the age and background immunity of the patient (Gillespie and Pearson, 2001). The affordable and widely available antimalarial drug chloroquine that was in the past the main treatment in malaria control is now ineffective in most *falciparum* malaria endemic areas and resistance to sulfadoxinepyrimethamine is increasing rapidly. The discovery and development of the artemisinin derivatives in China, and their evaluation in Southeast Asia and other regions, have provided a new class of highly effective antimalarials, and have already transformed the chemotherapy of malaria. Artemisinin based combination therapies (ACTs) are now generally considered as the best current treatment for uncomplicated *falciparum* malaria. The recommended ACTs are: artemether + lumefantrine, artesunate + amodiaquine, artesunate + mefloquine, artesunate + sulfadoxine–pyrimethamine. As second-line treatment for uncomplicated *falciparum* malaria, the following combinations are used: artesunate + tetracycline, or doxycycline or clindamycin; and quinine + tetracycline, or doxycycline or clindamycin. In the case of severe *falciparum* malaria, quinine and artemisinin derivatives (artesunate, artemether, artemotil, dihydroartemisinin) are basically used (WHO, 2012).

2.7   Targets in the Search for New Antimalarial Compounds

Many antimalarial targets can be related to the functions of distinct organellar structures within the parasite, mainly the lysossomal food vacuole (the site of extensive hemoglobin degradation), the apicoplast (a plastid organelle thought to originate from a cyanobacterium

symbiont) and the acrystate mitochondrion and its electron transport system (Sahu *et al.,* 2008).

2.7.1 Heme, hematin or hemozoin as targets

During the digestion of its host cell hemoglobin, large amounts of heme (Fe (II) PPIX) are released in the food vacuole of the intra-erythrocytic malaria parasite. This ferriprotoporphyrin is toxic for the parasite and its detoxification occurs via the formation of hematin (Fe (III) PPIX) and finally hemozoin, which is a dimer of Fe (III) PPIX, also known as malaria pigment (Egan, 2008).

Heme has been partially implicated in the mode of action of endoperoxideantimalarials, such as artemisinin, which specifically enters infected blood cells to form radical adducts with heme (Robert *et al.,* 2006). Hematin is believed to be the target of chloroquine and other quinolineantimalarials, and there is evidence suggesting that these drugs act by complexing with hematin, thus preventing its detoxification by blocking the formation of hemozoin (Ginsburg and Krugliak, 1999). The mechanism of hemozoin formation in the parasite is not completely elucidated, but histidine-rich protein-2 (HRP-2) has been implicated as an enzyme in the process and could be considered as a new target in the search for antimalarial drugs (Sahu*et al.,*2008).

2.7.2 Apicoplast targets

The apicoplast has been discovered as a relict chloroplast organelle in malarial parasites and other apicomplexan parasites. The apicoplast apparently resulted from endosymbiosis with cyanobacteria and – although no longer photosynthetic – is essential to parasites. The organelle maintains certain specific functions and more than 500 proteins were identified.

Apicoplast function includes several biochemical pathways such as fatty acid, isoprenoid, and heme synthesis, which are present in bacteria, plants, and apicomplexan parasites, but are fundamentally different to the analogous pathways in the human host. Hence, these parasite-specific metabolic pathways are potentially good targets for drug development (Ralph *et al.,* 2004). One of the new approaches involving these metabolic pathways is fatty acid synthesis in the intra-erythrocytic parasite, which is crucial for the synthesis of cell and organellar membranes. Higher eukaryotes normally use a type I fatty acid synthase (FASI) system, while in the apicoplast a type II fatty acid synthase (FASII) system takes place. In the FASII system, each fatty acid biosynthetic pathway is carried out by an enzyme encoded by a different gene.

In *P. falciparum*, this biosynthetic pathway incorporates several enzymes susceptible to inhibition by drugs. Some examples of FASII inhibitors are isoniazide and triclosan (FabI inhibitors), as well as thiolactomycin and derivatives (FabB and FabG inhibitors). Among these enzymes, the *P. falciparum* enoyl-ACP reductase (*Pf*FabI) has been particularly investigated for inhibition, since its active site is known and a crystal structure of the enzyme is available incorporating the inhibitor triclosan and the cofactor NADH in the active site. Triclosan binding traps the *Pf*FabI enzyme in the nonproductive NAD+ cofactor state and prevents the binding of NADH (Carballeira, 2008; Sahu *et al.,* 2008). Some natural products, such as alkaloids have been demonstrated to inhibit FabG, FabZ and FabI (Tasdemir *et al.,* 2006).

2.8 The Role of Plants in Antimalarial Drug Discovery

The estimated 300,000 species of higher plants contain a pharmacopeia of complex and unique chemical compounds that are employed *in planta* toward numerous ends from

primary metabolism and growth, to anti-pathogenic activity and reproduction (Fabricant and Fansworth, 2001). Humans have taken advantage of this fact by utilizing plants medicinally for millennia. In our current medicinal repertoire, the plant derived compounds quinine, artemisinin, and their derivatives account for more than half of the WHO's accepted antimalarial remedies (WHO, 2014). Quinine, being derived from *Cinchona* spp. and artemisinin, derived from *Artemisia annua*, represent inspiring and triumphant stories of ethnobotanical knowledge influencing the discovery and development of potently bioactive compounds that have been used to save countless lives. However, more than 1,200 plant species are known to be traditionally used for malaria treatment (Wilcox *et al.,* 2005). This ethnobotanical knowledge may similarly serve as a guide in elucidating novel antimalarial compounds of comparable or superior activity to quinine and artemisinin. In fact, hundreds of antimalarial compounds have already been described from plants and other natural sources, some of which hold clinical appeal (Kaur *et al.,* 2009).

While a select number of these compounds have garnered attention as drug candidates, few of this number have been viewed as integrated botanical therapies. On the one hand, this neglect originates in the highly complex nature of bioactive plant extracts, with many compounds of varied activity. For example, quinine, long thought to be far superior to crude cinchona bark, has been shown to be less effective than a mixture of cinchona alkaloids (Honigsbaum and Wilcox, 2004). On the other hand, plant extracts containing important medicinal compounds are often scientifically dismissed because their activities cannot be traced to a single active chemical. The biological activity of extracts *in situ* may contrast sharply with that measured in bioassays of isolated components (Tyler, 1999). For this

reason, drug discovery programs have failed to capture the value of multi-functional, multi-faceted agents such as phyto-medicines and botanical therapeutics.

Luckily, with a little effort, previously complex plant extracts can now be subject to isolation and fractionation methods that preserve phytochemical integrity and deliberately seek to quantify incidences of chemical interactions. In this manner, we can begin to understand and exploit the entire range of efficacies of phytochemical mixtures and their key components. This is truly important considering recent calls from the World Health Organization for the discontinuation of all artemisinin monotherapies, while favoring the use of Artemisinin Combination Therapies (ACTs; mixtures of slow acting artemisinins with longer acting quinolone antimalarials in an effort to delay resistance) (WHO, 2014). Chemical complexity found in synthetically, carefully constructed drug therapy programs and naturally in plants is believed to help delay or avoid the emergence of resistant *Plasmodium* strains.

2.9   The Current Status of Antimalarial Drugs

There are a number of approaches for the prevention and control of malaria. These include vector avoidance (i.e. insecticide treated bed nets), vector control (residual indoor and outdoor mosquito spraying), vaccine development and chemotherapy (Taubs, 2000). However, with a successful vaccine still in the works, our current best defense against malaria is chemotherapy, and in populations where prophylactic treatment is simply too expensive, quick acting drugs that clear *Plasmodium* infections are most often used. Currently used antimalarial drugs fall into several categories: aminoquinolines, arylaminoalcohols, 8 aminoquinolines, artemisinins, antifolates, inhibitors of the respiratory chain and antibiotics (Schlitzer, 2014). Quinine, which is derived directly from *Cinchona* spp., was isolated in 1820 and was the only drug in pure form used to treat malaria for more than one hundred

years (Rocco, 2003). In the 1940's, chloroquine was introduced as a less expensive and more effective alternative and quickly became the mainstay of antimalarial drug therapy and pioneer of the malaria eradication era in the 1950's. The natural endoperoxide, artemisinin, was discovered from the traditional Chinese medicinal plant *Artemisia annua*in 1972 and influenced the semi-synthesis of several analogues, which have become the current first choice in front line antimalarial treatment (Graziose *et al.,* 2010). Unfortunately, the successes of modern drug development were not long lived. When chloroquine resistant *P. falciparum* strains first began to appear in Africa during the late 1970s (Campbell *et al.,* 1979), medicinal advantage over malaria began to fade. Since then, antimalarial chemotherapy has been dominated by the cyclical development of new drugs (often as modifications of existing drugs) and the subsequent appearance of drug resistance. This fact is nowhere more disturbing then in reports of resistance to artemisinin in Southeast Asia (Noedl *et al.,* 2009). The therapeutic 'arms race' between new drug entities and resistance is a constant concern as the synthetic drug pipeline dries up (Schlitzer, 2014). Antimalarial drugs are also becoming progressively more costly in a time when increasing world population, climate change, and political distress are exacerbating an overall inability to afford antimalarial treatment (Stratton *et al.,* 2014). These two problems; resistance and cost, must be addressed for malaria to be successfully controlled.

2.10   Anti Malaria Compounds and their Mechanism of Action

Antimalarials are used in three different ways: prophylaxis treatment of *falciparum* malaria, and treatment of non-*falciparum* malaria. Prophylactic antimalarials are used almost exclusively by travelers from developed countries who are visiting malaria endemic countries. Treatment protocols for *falciparum* malaria vary, depending on the severity of the

disease; fast-acting, parenteral drugs are best for severe, life threatening disease. In addition, treatment protocols for *falciparum* malaria vary geographically and depend on the resistance profiles for strains in particular regions. Non-*falciparum* malarias, in contrast, rarely are drug resistant. In addition, *P. vivax* and *P.ovale* have dormant liver stages that can cause relapses months to years after an infection is cleared, so they need to be treated with an additional agent that can clear this stage. The antimalarials in common use come from following classes of compounds: the quinolines (chloroquine, quinine, mefloquine, amodiaquine, primaquine), the antifolates (pyrimethamine, proguanil and sulfadoxine),the artmisinin derivatives (artemisinin, artesunate, artemether, arteether) and hydroxyl-naphthaquinones (atovaquine).

2.10.1 Chloroquine

Chloroquine was first synthesized in Germany, but it was not recognized as a potent antimalarial drug until the 1940s during the US World War II military effort. By 1946, it was found to be far superior to other contemporary synthetic antimalarials (Coggeshall and Craige, 1949). The structure of chloroquine is presented in Figure 2.3. Chloroquine became the cornerstone of antimalarial chemotherapy for the next 40 years. It quickly became the drug of choice globally to treat uncomplicated *P. falciparum* infections, and it was used as part of the Global Malaria Eradication campaign launched by the World Health Organisation in 1955. Chloroquine is one of the least expensive antimalarials available and is still in widespread use. This drug can be taken both as a prophylactic and as a treatment.

Despite much research during the last 40 years, the exact mechanism by which chloroquine kills the malaria parasite remains controversial (Foley and Tilly, 2007). Once in the food vacuole, chloroquine is thought to inhibit the detoxification of heme. Chloroquine becomes protonated (to CQ^{2+}) because the digestive vacuole is acidic (pH 4.7) and subsequently

cannot leave the vacuole by diffusion. Chloroquine caps hemozoin molecules and prevents the further bio-crystallization of heme, thus leading to heme buildup. Chloroquine binds to heme (or FP) to form what is known as the FP-chloroquine complex; this complex is highly toxic to the cell and disrupts membrane function. The actions of the toxic FP-chloroquine complex and FP result in cell lysis and ultimately the auto-digestion of the parasite cell. In essence, the parasite cell drowns in its own metabolic products.
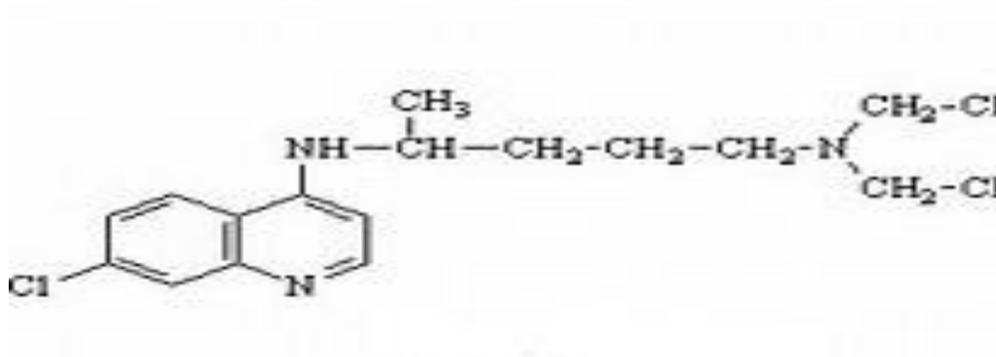

Figure 2.3: Structure of Chloroquine (Harrison, 2004)

2.10.1.1 Mechanism of Resistance

Resistance to chloroquine was slow to develop, taking almost 20 years, despite extensive use of the drug, suggesting that mutations in several genes were required to produce the resistance phenotype. The mechanism of chloroquine resistance also is uncertain. Chloroquine resistant parasites accumulate less chloroquine in the food vacuole than do sensitive parasites (Fitch, 1970) and one assumption is that chloroquine resistance is not based on the mode of action

of the drug but on the access of the drug to the parasite food vacuole. Early studies indicated that chloroquine resistance was associated with an elevated level of drug efflux. Drug resistant parasites were reported to release pre-accumulated chloroquine almost 50 times faster than chloroquine-sensitive isolates (Krogstad *et al.,* 1987). Another proposal is that chloroquine is transported actively through the parasite by the Na^+/H^+ exchanger (NHE) and that resistance to chloroquine is mediated by mutations in the NHE (Wunsch *et al.,* 2010) but this suggestion has been disputed. Wellems *et al*. (2010) analyzed a cross between a chloroquine resistant and a chloroquine-sensitive strain of *P.falciparum*, and identified a chloroquine-resistance locus within a 400 kb segment of chromosome. Su *et al*. (2014) mapped the putative chloroquine-resistance locus to a 36 kb region and identified the open reading frames of 8 potential genes within this region. Initially, chloroquine resistance was thought to be caused by cg2, a gene coding for a polymorphic protein located at the parasite periphery. However, recent transformation studies have ruled out cg2 and suggest another gene, PFTCR within this region (Fidock *et al.,* 1999).

2.10.2 Quinine

Quinine is derived from the bark of the cinchona tree and was used for treating fevers as early as the 17th century, although not until 1820, it was the active ingredient of the bark, isolated and used in its purified form. The structure of Quinine is shown in Figure 2.4. Quinine is used as a treatment for uncomplicated and severe malaria in many different therapeutic regimens. Quinimax, which is a combination of quinine, quinidine, and cinchonine (all

derived from cinchona bark), is also used (Deloron *et al.,* 1985). Quinine must be administered for at least 7 days to non-immune populations (Krishna and White, 1996) but it is effective in immune populations (such as in sub-Saharan Africa) when given for 3 to 5 days because it appears to be potentiated by the host immune system (Miller *et al.,* 1989). Quinine is also used in combination with antibiotics (tetracycline or doxycycline).

2.10.2.1   Mode of action

Quinine acts in a manner similar to that of chloroquine but with some differences; chloroquine causes clumping of the malaria pigment, whereas quinine antagonizes this process (Peters, 1993). In addition, quinine is a weaker base than chloroquine and has less affinity for heme, implying that mechanisms other than ion transport into the food vacuole and heme-drug interactions are required for the action of these drugs (Foley and Tilly, 2007). Quinine also interacts rather weakly with heme (Kd = $2.63 \times 10\text{-}6$ M) (Chou *et al.,* 1980), but has been shown to inhibit heme polymerization (Chou and Fitch, 1993) and heme catalase activity (Ribeiro *et al.,* 2009). In the absence of a specific transporter, quinine is likely to be accumulated less efficiently in the food vacuole than chloroquine. Further work is required to determine whether the mechanism of action of quinine is more closely aligned to that of chloroquine.
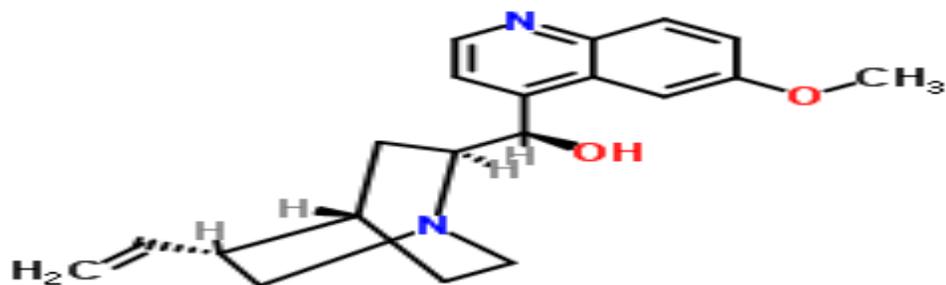
Figure 2.4: Structure of Quinine Sulfate (Harrison, 2004)

2.10.3 Mefloquine and halofantrine

Mefloquine was developed in the 1970s by the United States Army in response to the increasingly poor cure rates of chloroquine, with clinical trials beginning in 1972 (Davidson *et al.,* 1975). Mefloquine has a very long half-life both in patients with malaria (10.3 to 20.5 days) (Na-Bangchang *et al.,* 2010) and in healthy volunteers (13.8 to 27.5 days). Mefloquine is recommended for prophylaxis and therapy in chloroquine-resistant areas. Despite considerable publicity about possible neuropsychiatric side-effects of mefloquine, the same evidence is not conclusive (Choo, 1996). Halofantrine is a tricyclic compound that was developed at approximately the same time as was mefloquine and has been used as a second-line agent; its use may be limited by its cardio-toxic side-effects and variable pharmacokinetics.

2.10.3.1 Mode of action

Mefloquine interacts relatively weakly with free heme, with reported Kd values ranging from $3.3 \times 10\text{-}7$ to $1.63 \times 10\text{-}5$ M (Chou *et al.,* 1980). Mefloquine has been shown to inhibit heme polymerization in vitro with a similar (Slater, 1993) or lower efficiency than chloroquine (that is, close to millimolar levels). Given the lower basicity of mefloquine, it seems unlikely that it would reach the intravacuolar concentration required to inhibit heme polymerization. Furthermore, while chloroquine treatment of *P*.berghei infected mice was found to cause a decrease in hemozoin production, mefloquine and quinine had no effect (Chou and Fitch, 1993). Mefloquine is also a much less potent enhancer of the peroxidase activity of heme than chloroquine and has been shown to interfere with the ability of chloroquine to enhance heme-induced cell lysis (Dutta and Fitch, 2013). The available data suggest therefore that, mefloquine interferes with a different step in the parasite-feeding process than chloroquine (Geary *et al.,* 1986). Desneves *et al*. (1996) used the technique of photo affinity labeling to identify two high-affinity, mefloquine-binding proteins with apparent molecular masses of 22 to 23 kDa and 36 kDa in *P.falciparum* infected erythrocytes. The identities of these polypeptides have not been established yet, but they may be involved in mefloquine uptake or action. There is also increasing evidence to suggest a role for the *plasmodial* P-glycoprotein (P-glycoprotein homolog-1, Pgh-1) in mefloquine resistance. This raises the possibility that Pgh-1 may also be the target of action of mefloquine.

2.10.3.2 Mechanism of resistance

Mefloquine resistance in field isolates of *P.falciparum* is associated with amplification of the pfmdr1 gene (Cowman *et al.,* 1994) and over-expression of its protein product Pgh-1 (Cowman *et al.,* 1994). Moreover, selection for mefloquine resistance in vitro leads to

amplification and over expression of the pfmdr1 gene (Cowman *et al.,* 1994). This has led to the idea that Pgh-1 is responsible for at least some forms of mefloquine resistance.

2.10.4 Antifolates

Some of the most widely used antimalarial drugs belong to the folate antagonist class, albeit their role in malaria control is hampered by rapid emergence of resistance under drug pressure (Plowe *et al.,* 1998). Inhibition of enzymes of the folate pathway results in decreased pyrimidine synthesis, hence, reduced DNA, serine, and methionine formation. Activity is exerted at all growing stages of the asexual erythrocytic cycle and on young gametocytes. Traditionally, antifolates are classified into two:

1. Type-1 antifolates (sulfonamides and sulfones) mimic p-aminobenzoic acid (PABA). They prevent the formation of dihydropteroate from hydroxyl-methyl dihydropterin catalyzed by dihydropteroate synthase (DHPS) by competing for the active site of DHPS (a bifunctional enzyme in plasmodia coupled with 2-amino-4-hydroxy-6hydroxymethyl-dihydropteridine pyrophosphokinase [PPPK]).

2. Type-2 antifolates (pyrimethamine, biguanides and triazine metabolites, quinazolines) inhibit dihydrofolate reductase (DHFR, also a bifunctional enzyme in plasmodia coupled with thymidylate synthase [TS]), thus preventing the NADPH-dependent reduction of H2folate (DHF) to H4folate (THF) by DHFR.
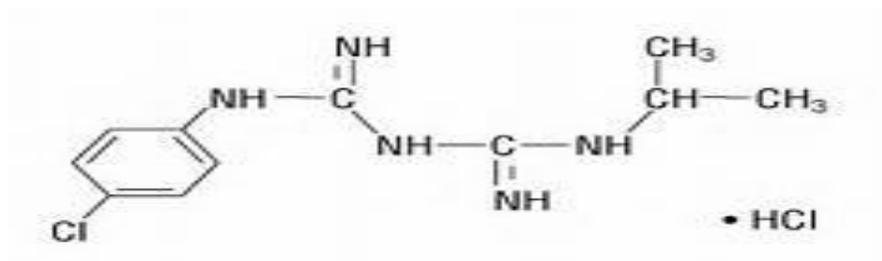
Figure 2.5: **Structure of Proguanil** (Sweeney, 2003)

2.10.4.1 Mode of action

The antifolate drugs inhibit either dihydrofolate reductase (DHFR) (pyrimethamine, cycloguanil) or dihydropteroate synthase (DHPS) (sulfadoxine). These are two key enzymes in de novo folate biosynthesis; inhibition of this metabolic pathway leads to the inhibition of the biosynthesis of pyrimidines, purines, and some amino acids. Antifolate antimalarial drugs interfere with folate metabolism, a pathway essential to malaria parasite survival.

2.10.4.2 Mechanism of resistance

This class of drugs includes effective causal prophylactic and therapeutic agents, some of which act synergistically when used in combination. Unfortunately, the antifolates have proven susceptible to resistance in the malaria parasites. Resistance is caused by point mutations in DHFR and DHPS, the two key enzymes in the folate biosynthetic pathway which are targeted by antifolates. Resistance to these drugs arises relatively rapidly in response to drug pressure and is now common worldwide.

2.10.5 Artemisinin derivatives

For nearly 2,000 years, a cold-water extract of sweet wormwood (*Artemisia annua,* ''qinghao'') has been used in China for the treatment of fevers. The structure of artemisinin is shown in Figure 2.6.The active ingredient of this plant was isolated in 1970 by Chinese

scientists. Artemisinin (or Qinghaosu) and its derivatives (artesunate, artemether, and arteether) have been used extensively in China and Southeast Asia, where there are high levels of resistance to the majority of the quinolone containing drugs and to all the antifolate drugs (Meshnick, 2002). They achieve higher reduction rates of parasitaemia per cycle than any other drug known to date (White, 1997).
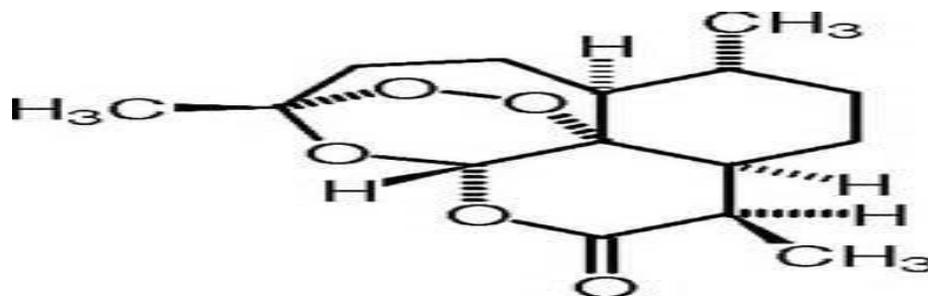

Figure 2.6: Structure of Artemisinin (Krishna and White, 1996)

2.10.5.1 Mode of action

Artemisinin and its derivatives are sequiterpene lactones. Once administered, the artemisinin derivatives are hydrolyzed rapidly to the biologically active metabolite dihydroartemisinin. The mode of action of the artemisinin drugs has not been completely elucidated. The structure of artemisinin is unusual, and its activity is thought to depend on the presence of the endoperoxide bond, as molecules without it have no antimalarial activity (Brossi *et al.,* 2000). The endoperoxide bond may interact with iron or heme, decomposing into free radicals (Meshnick, 2002). Unlike many redox reactions, this process is not reversible, so a single drug molecule will produce only one free radical. The effect of free radicals on the malaria parasite is still not fully understood. Because the concentration of free radicals is insufficient to cause general membrane damage, one theory is that a ''specific free radical target'' exists

(Meshnick, 2002). The artemisinin free radical can form a covalent bond with either heme or other parasite proteins and an initial hypothesis was that a heme-artemisinin compound might inhibit the production of hemozoin. No evidence, however, of reduced quantities of hemozoin in artemisinin-treated *P.falciparum* cultures has been found.

2.10.5.2 Mechanism and status of resistance

Artemisinin-resistant strains have been developed both in *P.falciparum* cultures and in *P.yoelii* mouse models (Chawira *et al.,* 1986). There also have been some indications of increasing in vitro resistance in field isolates (Gay *et al.,* 1994). *P.falciparum* resistance to artemisinins, which was confirmed on the Cambodia Thailand border in 2009, is now suspected in parts of Myanmar and Viet Nam. However, Artemisinin-based combination therapies (ACTs) remain highly effective in almost all settings, so long as the partner drug in the combination is locally effective. Artemisinin derivatives have a gametocytocidal activity (Peters, 1993), a feature that, in combination with their pharmacokinetic and pharmacodynamic properties, may well delay the development of drug resistance in the field. Resistance is difficult to induce experimentally, and is labile (low levels of resistance are achieved after sustained drug pressure) but not retained once drug pressure is removed (Peters, 1993). Available data suggest that resistance to this class of compounds would be multigenic and share similarities with the quinoline family, as demonstrated in vitro on a series of parasite isolates (Meshnick, 2002).

2.10.6 Atovaquone

The antimalarial activities of hydroxynaphthoquinones were discovered during World War II. Atovaquone is the first effective compound in this class. Currently, it is being marketed as Malarone, which contains a fixed combination of atovaquone and proguanil (Figure 2.5).
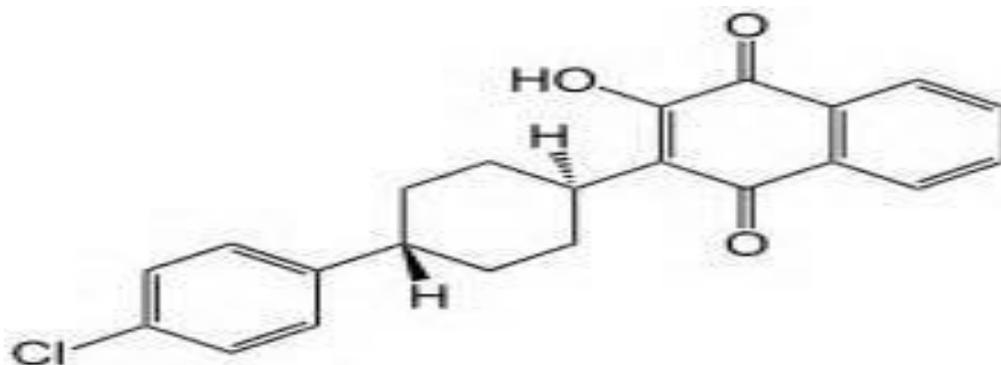

Figure 2.7: Structure of Atavaquone (Harrison, 2004)

2.10.6.1   Mode of action

Atovaquone {2-[Trans-4-(40-chlorophenyl) cyclohexyl]-3hydroxy-1, 4-naphthoquinone}, hydroxyl naphthoquinone is used for both the treatment and prevention of malaria in a fixed combination with proguanil. Whilst known to act primarily on mitochondrial functions, its mode of action and synergy with proguanil are not completely understood. This matter is further complicated by the diverse functions of mitochondria in various organisms and by technical difficulties with experiments. It is generally agreed that atovaquone acts on the mitochondrial electron transfer chain, although more recently, its activity and synergy with proguanil has been ascribed to its interference with mitochondrial membrane potential. Atovaquone inhibits cytochrome c reductase activity in *P. falciparum* (Fry and Pudney, 1992). Atovaquone is a ubiquinone analogue that binds to the cytochrome bc1 complex of the parasite mitochondrial electron transport chain. The malaria mitochondria electron

transport chain disposes of electrons generated by dihydroororate dehydrogenase during the synthesis of pyrimidines (Gutteridge *et al.,* 1979) and the inhibition of this process by atovaquone may kill the parasite. More recently, it has been shown in *P. yoelii* that atovaquone also dissipates the mitochondrial membrane potential of the parasite which may kill the parasite by initiating a process similar to apoptosis.

2.10.6.2 Mechanism and status of resistance

When atovaquone was first used in clinical trials in Thailand, the treatment failure rate was 33 %, regardless of duration of therapy. This high level of treatment failure suggests that either a natural background of resistant mutants exists or resistance arises rapidly by the acquisition of point mutations in the cytochrome b gene. Mutations in cytochrome b have been found in atovaquone-resistant Pneumocystis carini and *P. yoelii* strains, indicating that they may be the cause of atovaquone resistance (Walker *et al.,* 1998). Because of the high rate of treatment failure, atovaquone has been combined with other drugs, including proguanil, doxycycline, and tetracycline. All of these combinations yielded high cure rates (Looareesuwan *et al.,* 2014; Radloff *et al.,* 2014), and the atovaquone-proguanil combination (Malarone) also is effective as a prophylactic.

2.11 Anti Malaria Potentials of Some Alkaloidal Compounds

Alkaloids are one of the major classes of natural products that exhibit antimalarial activity. Indeed, quinine, the first antimalarial drug, belongs to this class. Over 100 alkaloids from higher plants were reported to demonstrate significant antimalarial activity in studies published from 1990; some of these were more potent than chloroquine (Saxena *et al.,* 2003). Alkaloids with antimalarial activity grouped according to their structural classes.

2.11.1 Bis-benzylisoquinolines

They are a large and diverse group of alkaloids that occur in many plant species, particularly in members of the *Menispermaceae, Berberidaceae, Ranunculaceae, Annonaceae* and *Monimiaceae.* Many of the plants that contain these compounds have reputations as medicinals in the folklore of various cultures. In an effort to discover new antimalarial agents from natural sources, Angerhofer and co-workers tested bisbenzylisoquinoline alkaloids that were isolated via phytochemical studies and bioassay-directed fractionation. The cytotoxicity of the isolates was assayed against KB cells (human oral epidermoid carcinoma), and their selectivity for inhibiting the growth of intra-erythrocytic malaria parasites was evaluated.

2.11.2 Morphinan alkaloid

These were biogenetically derived from benzylisoquinolines via aporphines, was recently isolated from *Strychnopsisthouarssi* (Menispermaceae plant species that is endemic to Madagascar) and it was named tazopsine (tazo = malaria) (Carraz *et al.,* 2006). This plant is the only ingredient in a widely used remedy that is reputed to provide specific protection against malaria. Stem bark decoction has shown weak activity against the FcB1 strain of *P.falciparum*erythrocytic stages in vitro (IC$_{50}$ 34.0 ± 9.4 µg/mL). The traditional use of this plant to prevent malaria led to the in vitro evaluation of its effect on liver stages of *P.yoelii* and *P.falciparum*.

2.11.3 Naphtylisoquinolines

Comprising more than 70 natural alkaloids and over 150 derivatives are a new class of structurally unique acetate biogenetically-derived alkaloids that have been isolated from tropical lianas belonging to the families Dioncophyllaceae and Ancistrodaceae (Bringmann and Feineis, 2001). Plant species of these families are widely used in the traditional medicine

of West African countries, and Southern and Southeast Asia to treat malaria and other diseases, such as dysentery, leprosy, fever, and measles. Good correlations between in vitro (CQR *P.falciparum* NF54 strain) and in *vivo* (*P.*berghei, Anka strain) antimalarial activities were observed for representatives of this group of alkaloids. Dioncophylline C, dioncophylline B and dioncopeltine A caused complete clearance of parasites after oral administration to *P.*berghei-infected mice, without noticeable toxic effects (Bringmann *et al.,* 2003).

2.11.4 Cryptolepine, an indoloquinoline alkaloid

These are the major constituent (this alkaloid constitutes over 1 % of its weight) and the most potent anti-*plasmodial* compound derived from *Cryptolepissanguinolenta*. A decoction of the roots of this climbing shrub is used in West Africa for the treatment of malaria. Furthermore, its major constituent, cryptolepine, has potent effects against both CQS (D6 IC_{50} 27.0 ± 0.3 ng/mL) and CQR *P.falciparum* strains in vitro (K1 IC_{50} 33.0 ± 0.1 ng/mL, W2 IC_{50} 41.0 ± 0.5 ng/mL); however, cytotoxic effects have been observed. It has been demonstrated that cryptolepine intercalates with DNA and stabilizes the topoisomerase II-DNA covalent complex; thus, the scission of DNA by topoisomerase is stimulated (Wright *et al.,* 2001).

2.11.5 Mono- and bis-indole alkaloids

They have been isolated from several plants that are traditionally used to treat malaria in different continents. The most active compounds are those that originate from plants that belong to the genera *Strychnos* (*Loganiacae*) and *Alstonia* (*Apocynaceae*). A review covering the indole alkaloids that have high antiplasmodial activities in *vitro* and in *vivo*, and favourable selectivity indices (SI=CC_{50}/IC_{50}) was published recently (Frederich *et al.,* 2011).

The antiplasmodial activity of 69 indolo mono terpenoid alkaloids from various *Strychnos* species (Loganiaceae) have been evaluated against CQR and CQS lines of *P.falciparum* in vitro (Frederich *et al*., 2011). The most active alkaloids were also tested for cytotoxicity against HCT-1116 (colon cancer cells) and their antiplasmodial SIs was calculated (Philippe *et al.,* 2013).

2.11.6 Benzofenantridine alkaloids

These were isolated by bioassay-guided fractionation of the trunk bark of Zanthoxylumrhoifolium; this species was traditionally used in French Guiana to treat and prevent malaria. The antiplasmodial activity was concentrated in the alkaloid fraction, which comprised seven benzophenantridine alkaloids, of which nitidine was the most potent against *P. falciparum* (IC$_{50}$< 0.27µM). The investigation of a trunk bark decoction that was employed as a traditional remedy revealed the presence of alkaloids, including nitidine; therefore the traditional use of Z. rhoifolium for the treatment of malaria was justified (Julian *et al.,* 2006).

Seven alkaloids were isolated from Tecleatrichocarpa (synon.Toddaliatrichocarpa) from Kenya. Of these alkaloids, two (normelicopicine and arborinine) displayed limited in *vitro* activity against *P.falciparum* strains (HB3 and K1). Normelicopicine was also shown to be active against *P.*berghei-infected mice (32 % suppression of parasitaemia at a dose of 25 mg/kg/day) in addition to having low in *vitro* KB cell cytotoxicity (IC$_{50}$> 328µM) (Mauriithi *et al.,* 2002).

2.11.7 Acridone alkaloids

They are derived from species that belong to the genera Citrus (*Glycosmis* and *Severimia*) and are members of the family Rutaceae were tested for antimalarial activity in vitro against *P.yoelii* and in *vivo* against *P.berghei-* and *P.vinckei*-infected mice. At a concentration of 10 μg/mL in vitro, seven out of the 30 tested alkaloids inhibited 90 % or more of the parasite growth. Against *P.yoelii,* they were shown to be either equally or more effective than chloroquine in vitro (94 ± 4 % inhibition).

2.11.8 Furoquinoline and acridine alkaloids

They have been isolated from plants that belong to the Rutaceae family. The in vitro and in *vivo* activities of acridones against rodent malaria (Fujioka *et al.,* 1989) and the in *vitro* effect of 23 furoquinoline and acridone alkaloids against CQR (W2) and CQS (HB3) clones of *P. falciparum* have been reported (Basco *et al.,* 1994). The assayed alkaloids included isolates from three New Caledonian plants (Geijerabalansae, Sarcomelicopeglauca and Sarcomelicopedogniensis) and derivatives that were obtained by chemical modifications and the dimerization of acronycine. Fourteen alkaloids had $IC_{50}< 10$ μg/mL against the W2 strain. Most of the active alkaloids were more than twice as active against the resistant clone as they were against the susceptible one. The most active alkaloid was an O-pyranoglycoside derivative of acronycine, which had an IC_{50} of 0.60 μg/mL (Basco *et al.,* 1994).

2.12   Malaria Vaccine and its Challenges

2.12.1 Vaccines

Our relationship with parasites has been a long one on the evolutionary scale. The methods adopted by parasites to thrive and colonize living organisms are truly fascinating. Along with basic features such as fecundity and resistant cyst structures, the parasites exhibit a fine-tuning of modifications in response to the attack by the host immune system. While the host fights the parasites through its armory of immune as well as certain behavioral responses, the parasites appear to use the host immune responses towards quorum sensing, limiting their own number, but surviving. The human malarial parasite, *Plasmodium falciparum*, which appears to have an ancient origin and has evolved in parallel with humans (Hughess and Verra, 2002), is known to possess a complex arsenal of defenses against man, and therefore the efforts to generate an effective malaria vaccine have been fraught with obstacles.

A good estimation of the current burden of malaria has been difficult, but nevertheless it is apparent that over one million persons succumb to malaria every year in Africa (Cox *et al.,* 2015). Devising an effective malaria vaccine would certainly help in limiting such morbidity. Over the years, numerous attempts have been made to develop a vaccine against malaria. The possibility of using inactivated sporozoites was first demonstrated in 1910 in avian malaria (Sergent *et al.*, 2007). It was followed by studies in 1941 that showed immunization with irradiated sporozoites could prevent infection. Besides irradiated sporozoites, the other observation that holds promise for a vaccine comes from the documented 'clinical immunity' observed in adult residents of malaria endemic areas (Butcher, 2007; Girard *et al.,* 2007).

2.12.2 Pre-erythrocytic stage (PE) vaccine

Sporozoites constitute the infective stage of the malarial parasite and they are ideally the target for a malaria vaccine. The pre-erythrocytic phase of infection, which lasts for a few

days, is particularly an attractive target of protective immunity, since this phase is clinically silent. In the 1960s, a series of experiments was stematically established that irradiated sporozoites do confer protection to the respective vertebrate host (Valenzuela *et al.,* 2002). However, a large number of mosquito bites or sporozoites are required to produce such a state of immunity. This led to an immediate hunt for protective antigens of the sporozoite, and the circumsporozoite protein (CSP) was identified. At that time recombinant DNA technology had just taken off and the hepatitis B antigen, expressed in yeast, was already showing characteristics of a promising subunit vaccine (Valenzuela *et al.,* 2002). It was anticipated that one had to simply follow a similar path for the CSP antigen to have a successful malaria vaccine.

2.12.3 Asexual stage vaccines

For a person naïve to malaria, clinical disease is concomitant with the occurrence of erythrocytic stages of *Plasmodium* in the blood. Vaccination against the asexual stages of *Plasmodium* is therefore not an option for the prevention of malaria but more towards diminution of the severity of the disease. Such a 'clinically immune' state is observed in adults resident in malaria hyper- or hollo-endemic regions and is termed 'pre-munition'. It has been demonstrated that administration of gamma-globulins from such malaria immune adults results in the clearance of parasites in the susceptible patients, establishing the therapeutic potential of such antibodies (Edozien *et al.,* 1962). Immunoglobulin G subtypes (Bouharoun-Tayoun and Druihle, 2012) and monocytes (Bouharoun-tayoun *et al.,* 2013) have been proposed to play important roles in such a protection.

For an asexual stage malaria vaccine, the impetus came from the establishment of parasite culture by Trager and Jensen 1976 and peptide biology. Using peptide fractions from cultured

parasites, four synthetic peptides were identified which resulted in a strong immune response in murine models and a formulation of these synthetic peptides gave birth to the first asexual stage vaccine SPf6645. It moved rapidly from primate studies to clinical trials (Valero *et al.,* 2013). However, the results were variable in Africa (efficacy of 2 to 30 %). A systematic trial in Thailand failed to show any efficacy in 1996, and further vaccine trial has been abandoned by the world health organization (WHO). The vaccine is now being resurrected with new adjuvants and is in the Phase I trial.

2.12.4 Malaria vaccine for pregnant women

Although individuals living in malaria endemic areas achieve 'clinical immunity' by the time they reach sexual maturity, the one exception to this rule is pregnancy. PAM is an important cause of maternal and perinatal morbidity and mortality in endemic areas. Pregnant women are more susceptible to malaria than non- pregnant women, and this susceptibility is greatest in first and second pregnancy. Central to the pathogenesis of *P.falciparum* infection in pregnancy is the ability of infected erythrocytes to accumulate in the maternal vascular area of the placenta. Trophozoite and schizont stages of the parasite display this ability to sequester in the placenta. Chondroitin sulphate A (CSA) has been consistently identified as the dominant placental adhesion receptor, although the possibility of the existence of additional receptors cannot be ignored. It has been shown that serum IgG from multi-gravidae exposed to *P. falciparum* could substantially inhibit the adhesion of infected erythrocytes from pregnant women to CSA.

2.12.5 Transmission blocking vaccine

Mosquito stage transmission blocking (MSTB) or transmission blocking vaccines (TBV) is anti-mosquito stage vaccine that targets antigens on gametes, zygotes or ookinetes. This

strategy can be used in malaria control due to the particular biology of *plasmodia*, where transmission-inducing and pathology- inducing parasite forms occur in two different hosts. The idea for TBVs emerged from the observations of Carter and Chen (1976), who showed that antibodies elicited by gametocytes from the avian malarial parasite, *P. gallinaceum* were capable of killing the emerging gametocytes not in the avian host but in the mosquito vector.

The ultimate goal of TBVs is the interruption of malaria transmission from human to mosquito populations through prevention of parasite development in the mosquito mid-gut. Antibodies generated in the host as a result of vaccination may kill the gametocyte within the host or get ingested with the gametocytes and kill the gametes when they emerge from the human host red cell in the mosquito mid-gut. Unlike vaccines against the other stages of the parasite, antibodies elicited by TBV kill the parasite outside the person immunized. Since the hepatic or blood-stage cycle is not targeted, these vaccines are not expected to protect the individual who is vaccinated but instead protect people who live in the immediate area.

2.12.6 Whole organism vaccine

Sporozoites: The 'subunit' vaccines that target the PE stages were developed in the hope of reproducing the immunity generated by irradiated sporozoites. But most of these vaccines, which deliver one or a few parasite antigens, induce only partial protection with rapidly fading immune responses (Moorthy *et al.,* 2004). The mechanisms by which irradiated sporozoites induce protective response is yet unclear. Over the years, studies using irradiated sporozoites have given us some valuable insights into the immune responses to the PE stages of the parasite. Murine models have been the most studied and in these systems, both the T-cells (mainly CD8+) that target intra-hepatocytic stages, and antibodies that recognize antigens on the sporozoite surface and block sporozoite invasion, appear to be important for

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protection. In addition, the proteins interferon-g, interleukin-12 and nitric oxide also seem to be critical (Doolan and Martinez, 2007).

It is now known that irradiated sporozoites do penetrate hepatocytes and begin intracellular development in the parasitophorous vacuole, but subsequently stop growing. Irradiated parasites persist in the hepatocytes for up to six months in rats and mice. Eradicating these parasites by chemotherapy abrogates protection in these rodents (Scheller and Azad, 2007), suggesting that continued synthesis of parasite antigens is required for maintaining protection. Thus, one is perhaps back to square one in the sense that the irradiated sporozoites might constitute the best PE vaccine. Indeed, an assay of liver burden of parasites in the murine model demonstrates that the best reduction is obtained with irradiated sporozoites, and the inhibition is several- fold higher than any of the other formulations. The generation of sporozoites has been refined and recently it has been shown that irradiated sporozoites of *P.falciparum* can generate strong, strain independent protection for at least 10 months in more than 90 % of human recipients. Large scale production of sporozoites may lead to the development of an effective sporozoite vaccine. However, the radiation has to be just right; too much will render the vaccine in- effective, too little may result in some parasites remaining virulent and causing the disease rather than protecting against it.

2.12.7 Challenges for an effective malaria vaccine

Lack of good animal models for testing of human malaria vaccines, the difficulties in evaluation of efficacy of the vaccine in endemic areas, and the lack of understanding of the immunosuppressive mechanisms of the parasite are the foremost reasons for the failure of an effective vaccine. The use of murine models to demonstrate robust protection has failed to stand true in most human trials. One of the possible reasons for the same is because we use

unnatural rodent hosts, and not the natural host (tree shrews) of the murine malaria (Druihle *et al.,* 2002).

Vaccine development and field trials are lengthy and expensive. In most of the trials, including those in naïve volunteers, it is important to note that sterile immunity was not observed in a large proportion of the subjects for a significant length of time. This has important implications. Are children from endemic areas in a position to take frequent vaccination doses? The RTS, as well as irradiated sporozoite vaccine might prove useful for transient visitors to endemic areas, such as tourists or military personnel. However, to manage the current burden of malaria in endemic areas, alternative methods such as insecticide spraying, insecticide-treated bed nets, long-lasting insecticidal nets and combination drug therapies should be used to their maximum.

2.13   Pain and its Management

The International association for the study of pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with an actual or potential tissue damage, or described in terms of such damage" (IASP, 2015). Pain is always subjective; each individual learns the application of the word through experiences related to injury in early life. Accordingly, pain is the experience that is associated with actual or potential tissue damage (IASP, 2015). It varies from person to person and in the same person from time to time. There may be a strong emotional component contributing to the pain experiences; but that does not mean that the suffering is less important (Rajagopal, 2006). It is also a specific enteroceptive sensation; as it can be perceived as arising from a particular portion of the body, its temporal properties can be detailed, it can be differentiated qualitatively and it involves dedicated subsets of peripheral and central neurons (Craig and Sorkin, 2001).

Pain is the most common reason patients need a physician. For most patients, it is of short duration and quickly forgotten. Unfortunately, for some, the pain does not pass but becomes a continuous burden and an unrelenting suffering (Debon *et al.*, 2013). Common chronic pain conditions affect approximately 100 million adults in the United States at a cost of $560-635 billion annually in direct medical treatment costs and lost productivity (Committee on Advancing Pain Research, Care, and Education, 2011). Pain, especially when chronic, markedly decreases individuals' health status and quality of life and can detrimentally affect the families of patients. It often interferes with every day work activities (Breivik *et al.*, 2013). In addition, the presence of a long lasting pain syndrome is a leading risk factor for suicide (Jamison and Edwards, 2012). It is thus, a serious and costly public health problem (Holtman *et al.*, 2010).

2.13.1 Nociceptive transmission

Nociception is the encoding and processing of noxious stimuli in the nervous system that can be measured with electrophysiological techniques (Schaible, 2006). Primary afferent sensory nerve fibers with cell bodies in the dorsal root ganglia (DRG) innervate all tissues of the body. Among the small myelinated (Aδ) and the unmyelinated (C) sensory fibers are those that respond selectively to noxious or potentially damaging stimuli, called nociceptors (Craig and Sorkin, 2001). Mechanical, chemical, or thermal nociceptive stimulation will recruit peripheral nociceptors that conduct the nociceptive signal in the primary somatosensory neuron to the dorsal horn of the spinal cord (Marchand, 2008). The diagrammatic reperesentation mechanism of nociceptive transmission is shown in Figure 2.8.

In the dorsal horn, the primary neuron will make a synaptic contact with the secondary or projection neuron. Secondary neurons from the spino-thalamic (lateral) and spino-reticular

(medial) tracts will immediately cross in the spinal cord and send afferent projections to higher centers. A large proportion of afferents will make a second synapse in the lateral and medial nuclei of the thalamus, which subsequently make synaptic contact with tertiary neurons. Tertiary neurons from the thalamus send impulses to the primary and secondary somatosensory cortices and project to limbic structures (Marchand, 2008). Nociceptive primary afferent fibers communicate with second-order dorsal horn cells by release of neurotransmitters, neuromodulators and trophic agents (Craig and Sorkin, 2001). Descending tracts reduce or facilitate the spinal nociceptive processing. The descending tracts are formed by pathways that originate from brain stem nuclei (particularly the periaqueductal grey (PAG) and the rostral ventromedial medulla (RVM)) and descend in the dorsolateral funiculus of the spinal cord. Descending inhibition is part of an intrinsic anti nociceptive system (Fields and Basbaum, 1999).
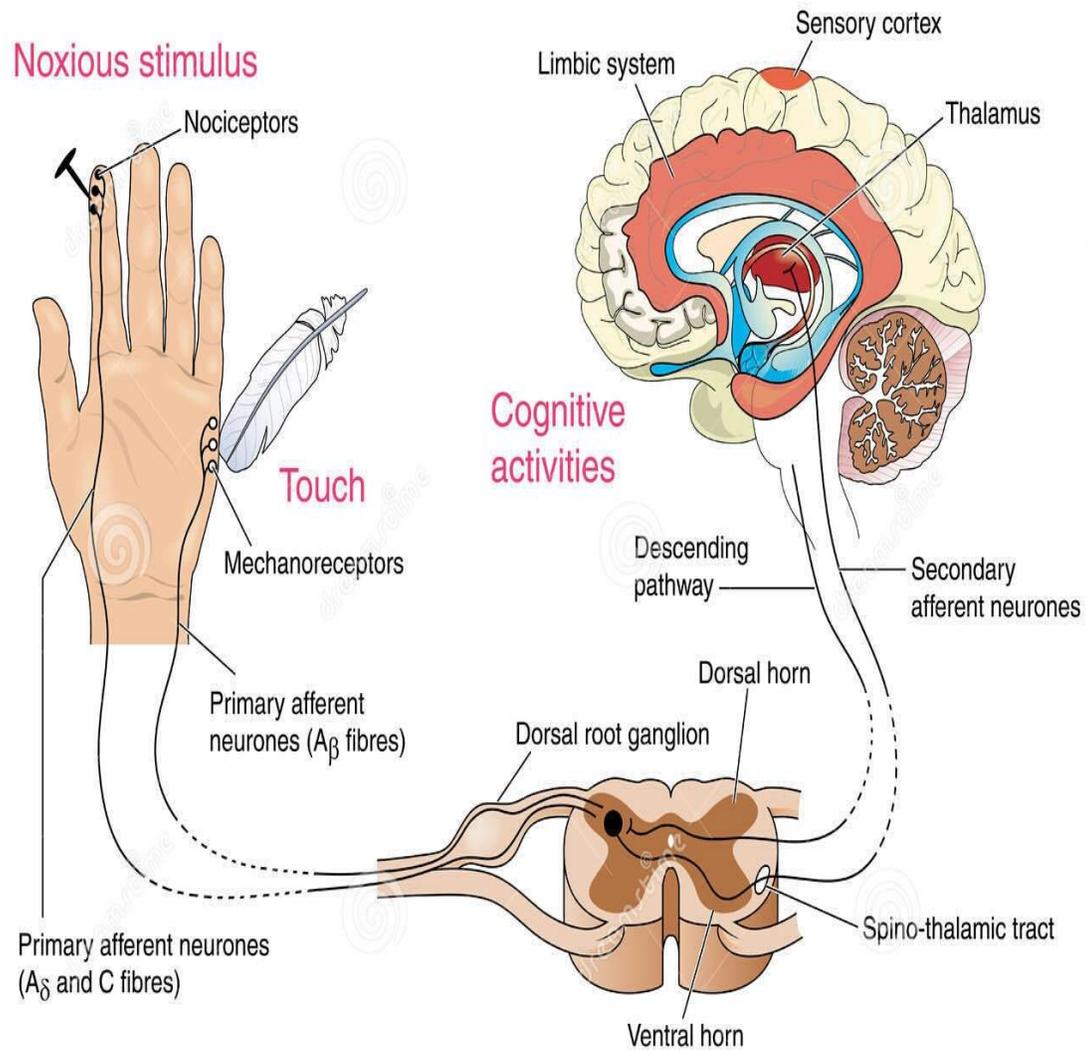
Figure 2.8: Mechanism of Nociceptive Transmission (Marchand, 2008)

2.13.2 Classification of pain

Pain can be categorized according to several variables with the general classification being based on its location, duration, frequency, underlying cause and intensity. Classification of pain is thus complicated and can be a source of confusion for many clinicians. Duration of the pain process is the most obvious distinction (Cole, 2002) for classifying pain as acute and chronic (Oertel and Lötsch, 2013).

2.13.2.1   Acute pain

Acute pain has a sudden onset, felt immediately following injury, is severe in intensity, and is usually short-lasting (less than 30 days). It arises as a result of tissue injury stimulating nociceptors and generally disappears when the injury heals (WHO, 2012).

Acute pain develops when a stimulus, such as pressure, heat or inflammation, is presented to the body (Lee *et al.*, 2011). It has the primary biological function of warning an organism of impending or immediate threat of tissue damage (Helms and Barone, 2008; Segall *et al.*, 2012).

2.13.2.2   Chronic pain

Chronic pain is defined as a pain which persists a month beyond the usual course of an acute disease or a reasonable time for an injury to heal, or is associated with a chronic pathological process which causes continuous pain, or pain which recurs at intervals for months or years (Rajagopal, 2006).

Due to the apparent changes in neurophysiology, it can be considered as a pathological condition itself (Lamont *et al.*, 2000; Australian Physiotherapy Association, 2012). Chronic pain may result from a chronic disease and may then actually result from persistent nociceptive processes (Schaible, 2006).

2.13.3 Management of pain

Unrelieved acute pain can cause chronic pain, and long-standing pain can cause anatomical and even genetic changes in the nervous system (Rajagopal, 2006). Management of pain should be tailored to the individual patient on the basis of pain type(s), the causative disease(s), and psychosocial aspects. Evidence-based symptomatic pharmacotherapy is the

mainstay of the treatment of pain, and it should be treated individually according to the efficacy and possible contraindications or side effects (Attal *et al.*, 2006). Non-pharmacological measures of pain management may be helpful in pain management including the application of heat or cold, massage, therapeutic touch, guided imagery, and relaxation techniques (Helms and Barone, 2008).

Analgesic drugs are mainly divided into two classes: opiate receptor agonists and non-steroidal anti-inflammatory drugs (NSAIDs) (Ye *et al.*, 2012) or opioids or non-opioids (Vittalrao *et al*., 2011). Other currently available analgesic drugs include antidepressants, anticonvulsants (Holtman *et al*., 2010), sodium (Na+) channel blockers, Glutamate receptors antagonists (Gao and Ji, 2010).

2.13.3.1 Non-steroidal anti-inflammatory drugs (NSAIDs)

This group of drugs remains the mainstay as a potent analgesic and anti-inflammatory agents (Viljoen *et al.*, 2012). They are often referred to as peripherally acting non-opioid analgesics. All of NSAIDs appear to share at least one common mechanism, namely inhibition of cyclo-oxygenase (COX) enzyme(s) which leads to a decrease in the synthesis of various prostaglandins and thromboxanes (Modi *et al.*, 2012). Depending on their mechanism of action, NSAIDs are broadly divided into two major classes as nonselective COX inhibitors and selective COX-2 inhibitors (Vane *et al.,* 1998).

There are many potentially significant adverse events that may occur with prolonged use of NSAIDs. Patients may develop gastrointestinal (dyspepsia, bleeding, and peptic ulcer formation through inhibition of protective prostaglandin formation), hematologic (platelet inhibition due to inhibition of thromboxane synthesis (Niemi *et al.*, 1997) and renal dysfunction (Munir *et al.*, 2007).

2.13.3.2 Acetaminophen

Acetaminophen or paracetamol, is a very popular and among commonly used analgesics (Rezende *et al.*, 2008) for the management of acute and chronic pain. It is generally well-tolerated with demonstrated analgesia (Anderson and Holford, 2008). It is generally accepted that the two systemic effects of paracetamol of therapeutic significance are analgesia and anti-pyresis, while its anti-inflammatory and anti-rheumatic activities are negligible (Bruton *et al.*, 2006).

Multiple targets have been the mechanisms of action proposed for its activity, including COX-1, COX-2, a putative COX-3 isozyme (Rezende *et al.*, 2008; Raffa *et al.*, 2010), reinforcement of descending inhibitory serotonergic pain pathways (Pickering *et al.*, 2008), inhibition of the L-arginine-nitric oxide (NO) pathway (Bujalska, 2004), mediated through substance P or N-methyl-D-aspartate (NMDA) (Hunskaar *et al.*, 1985) and active paracetamol metabolites have effect on cannabinoid (CB) receptors (Ottani *et al.*, 2006). Its use is closely being monitored by Food and Drug Administration (FDA) in terms of inducing adverse skin reactions (J'èwiak-benista and Nowak, 2014) and also severe liver impairment after paracetamol overdose is documented (Toms *et al.*, 2008).

2.13.3.3 Opioids

Opioid analgesics are widely accepted for first-line treatment of severe acute pain and chronic pain (Fine, 2012). Opioid receptors (μ, δ, κ) are G protein-coupled receptors distributed throughout the central nervous system. Activation of opioid receptors by endogenous or synthetic opioids results in closing of the voltage sensitive calcium channels, K+ (potassium) efflux leading to hyperpolarization; and inhibition of adenylyl cyclase to produce cyclic adenosine monophosphate (cAMP). This results in reduced neuronal excitability and a

reduction in transmission of nerve impulses and release of excitatory neurotransmitters (McDonald and Lambert, 2008).

When used over a protracted period of time, prescription opioid abuse may become a concern, especially in patients with a prior history of a substance use disorder (Ives *et al.*, 2006). Major side effects of opiates include physical dependence, tolerance, respiratory depression and constipation (Köksal *et al.*, 2007).

2.14   Inflammation and its Management

2.14.1 Overview of inflammation

It is a pervasive form of defense that is broadly defined as a nonspecific response to tissue malfunction and is employed by both innate and adaptive immune systems to combat pathogenic intruders (Noah *et al.*, 2012). Based on visual observation, it is characterized by the cardinal signs of: sensation of heat, redness, swelling, pain and loss of function (Punchard *et al.*, 2004).

The primary functions of inflammation are to rapidly destroy or isolate the underlying source of the disturbance, remove damaged tissue, and then restore tissue homeostasis (Medzhitov, 2008). Although the inflammatory response is essential for host defense, it is very much a double-edged sword (Wu *et al.*, 2013) because, inflammation itself can damage otherwise healthy cells which could then further stimulate inflammation. This runaway inflammation can lead to an organ failure and/or death (Kumar *et al.*, 2004).

Inflammation is a major feature of many diseases (Shu *et al.*, 2013). Inflammatory reactions are not only the response of living tissues to injury and infection, but also are relevant to

disease developments, such as asthma, multiple sclerosis, colitis, inflammatory bowel disease and atherosclerosis (Das *et al.*, 2012).

2.14.2 The inflammatory response

The inflammatory response is a complex process that includes activation of white blood cells, the release of immune system chemicals such as complements and cytokines, and the production and release of inflammatory mediators and prostaglandins (Cotran *et al.*, 2001). At its basic level, it is a tissue-destroying process that involves the recruitment of blood-derived products, such as plasma proteins, fluid, and leukocytes, into perturbed tissue (Noah *et al.*, 2012).

Alarmins or damage-associated molecular patterns (DAMPs) are endogenous molecules that signal damage or necrosis and are also recognized by the innate immune system. Toll like receptors (TLRs), which are membrane-spanning pattern-recognition receptors, recognize molecular patterns; represent a germ line encoded non self-recognition system that is hardwired to trigger inflammation (Lawrence, 2009; Shelton and Miller, 2010).

Once recognition of ligands occurs, TLRs activate common signaling pathways; NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and interferon regulatory factors. Activation of TLRs also activate mitogen-activated protein kinase (MAPK) cascade (Shelton and Miller, 2010; Slavich and Irwin, 2014). The two principal transcription factors in turn, drive the expression of pro-inflammatory immune response genes such as tumor necrosis factor alpha (TNF-α) and interleukin (IL-1) that produce cytokines (Slavich and Irwin, 2014). They also result in an increased expression of chemokines and other inflammatory mediators (Shelton and Miller, 2010). Cytokines are classified either pro-inflammatory or anti-inflammatory, depending on the way they influence inflammation

(Viljoen *et al*., 2012). Many of these cytokines are pro-inflammatory (e.g., IL-1α/β, IL-6, IL-18, TNF-α) (Shelton and Miller, 2010; Viljoen *et al.*, 2012). IL-1 activation of IL-1 receptors induces the transcription of other pro-inflammatory (IL-6 and TNF-α, which can also increase IL-1 expression) and anti-inflammatory (IL-10) cytokines. TNF-α also induce secretion of other inflammatory cytokines (Shelton and Miller, 2010; Ma *et al.*, 2013). The inflammatory cytokines also increase vascular permeability. Due to these reasons, they allow immune cells to leave the blood vessels and migrate to tissues. This process of redistributing cells of the innate immune is aided by chemokines (Slavich and Irwin, 2014).

2.14.3 Types of inflammation

Acute inflammation is an immediate and early response to an injurious agent and it is relatively of short duration, lasting for minutes, several hours or few days. It is characterized by exudation of fluids and plasma proteins and the emigration of predominantly neutrophilic leukocytes to the site of injury (Bezabeh *et al.*, 2004). Acute inflammation has a limited beneficial response, particularly during infectious challenge (Gabay, 2006). Chronic inflammation can be defined as a prolonged inflammatory process (weeks or months) where an active inflammation, tissue destruction and attempts at repair are proceeding simultaneously (Bezabeh *et al.*, 2004).

One hallmark of acute inflammation is that initially the leukocyte infiltrate is mostly neutrophilic, but after 24 to 48 hours monocytic cells predominate. In contrast, chronic inflammation is histologically associated with the presence of mononuclear cells, such as macrophages and lymphocytes (Gabay, 2006).

2.14.4 Management of inflammation

2.14.4.1 Corticosteroids

Corticosteroids (CS) are among the most widely used drugs and are effective in many inflammatory and immune diseases (Barnes, 2006). Corticosteroids therapy affects endogenous corticosteroid production and has a suppressive effect on Hypothalamo-Pituitary Adrenal (HPA) axis. The actions of all CS are mediated by interaction of hormone with CS receptor, which regulates gene transcription. They continue to act inside the cell even after their disappearance from the circulation, as the events initiate and the products of these events (such as specific proteins) may be present even after disappearance of CS from the circulation (Gupta and Bhatia, 2008).

They are potent inhibitors of T cell activation and cytokine secretion (Farrell and Kelleher, 2003). The glucocorticoid-receptor complex can either induce key anti-inflammatory genes that encode anti-inflammatory molecules or selectively repress specific inflammatory genes that encode cytokines, chemokine, adhesion molecules, inflammation associated enzymes, lipid mediators of inflammation and receptors (Derendorf and Meltzer, 2008). Their use, however, requires close monitoring because their side effects are widespread to every organ of the body (Viljoen *et al.*, 2012).

2.15   Natural Products in Drug Discovery

Medicinal plants use is widespread. Life saving and essential drugs from medicinal plants such as morphine, digoxin, aspirin, and emetine were introduced into modern therapeutics several centuries ago. However, plants have been used as drugs for over millennia by human beings (Sittie, 2005). Plants historically have served as models in drug development for some major reasons: the first being that plants are unique chemical factories capable of

synthesizing large numbers of highly complex and unusual chemical substances. It has also been estimated by the World Health Organization (WHO) that about 80 % of the population of the developing countries rely exclusively on plants to meet their health care needs (WHO, 2012). The second reason is that biologically active substances derived from plants have served as templates for synthesis of pharmaceuticals; while the third reason concerns the fact that highly active secondary plant constituents have been instrumental as pharmacological tools to evaluate physiological processes (Paddon and Keasling, 2014).

2.15.1 Herbal analgesics and anti-inflammatory agents used in Nigeria

There are 6500 species of higher plants in Nigeria making the country one of the most diverse floristic regions in the world (Bekele, 2007). Many plants are used as an analgesic and/or anti-inflammatory agents in traditional medicine practice in Nigeria. Some of these plants include: *Anogeissusleio carpus, Azanza garckeana, Alliums ativum* (Giday, 2001), *Zingiberofficinale* (Yirga, 2010), *Moringastenopetala* (Teklehaymanot and Giday, 2010)*, Tavernieraabyssinica and Nigella sativum* (Yirga, 2010; Wabe *et al.*, 2011), *Albucaabyssinica* (Mesfin *et al*., 2013), *Rutachalepensis* (Mesfin *et al*., 2009; Bekele and Reddy, 2015), *Zehneriascabra* (Akele, 2012) *and Lepidiumsativum* (Bekele and Reddy, 2015)

2.16   *Maytenus senegalensis* (LAM.)

Maytenus senegalensis (Lam.) is an African shrubs or tree that goes under the common name of Red spike-thorn, which belong to the *celastraccae* family. *Maytenus senegalensis* is one of the most frequently used specie of the *Maytenus* Molina genus in the African traditional medicine (along with *M. Obscura, M. peduncularis, M. putterlichwides, M. serrata and M. endata*) (Neuwinger, 2011). *Maytenus senegalensis* is traditionally used as anti-microbial and

anti-inflammatory agents; it is also used to treat respiratory ailments and inflammation. The use of this anti-inflammatory herbal drug is common in many African countries like Nigeria, Benin, Kenya, Zambia, Tanzania, Senegal and Zimbabwe. *Maytenus senegalensis* leaves are also used to treat toothaches in India (Gurib-Fakim, 2006). In Zulu territories and Mozambique the leaves of *Maytenus senegalensis* are macerated in water to be consumed twice a day for the treatment of tuberculosis. Recently, the anti-inflammatory activities of *Maytenus senegalensis* ethanol extract (70 %) were determined in Wistar albino rats by the carrageenan-induced paw oedema method. These extracts exhibited significant anti-inflammatory activity (120 mg/kg, per os), reducing oedema by 51 % and 35 % respectively. (Fansworth and Soejarto, 2009)

Figure 2.9: *Maytenus senegalensis* **(Lam.)** (Muregi *et al*., 2012)

2.16.1 Cultivation of *Maytenus senegalensis*

The plant does not show a specific preference for a particular soil type. It is more easily found where it grows in groups. It also prefers a well-drained humus rich fertile soil (Vuorella *et al.,* 2004). It tolerates floods, heavy wind or stagnant water. It is reported to have tolerated an animal precipitation. *Maytenus senegalensis* is mainly cultivated in East coast of Africa, Afghanistan, Indian and Arabia it is best suited to tropical climates with a well distributed rainfall of 1500 – 2000 mm yearly from sea level to about 600mm altitude (Simmons, 2015). *Maytenus senegalensis* tolerates warmer and more insipid climate than *Annona senegalensis.*

2.16.2 Climatic condition of *Maytenus senegalensis*

Celastraceae is a large family comprising trees, shrubs and woody lianas with a Gondwanan distribution (Simmons *et al.,* 2011). Geographically, *Maytenus senegalensis* occupies a wide variety of habitats, from deciduous woodland, Hucket, sarab and woody grassland and also on river band and swamp margins.

2.16.3 Botanical description

Maytenus senegalensis is an evergreen shrub tree or more rarely a shrublet, often straggling which grows up to 1m high, charmed or with green to brown spines up to 24 cm long glabrous or very rarely with young branches lined or angular (Figure 2.9). The lamina of *Maytenus senegalensis* is a pale to deep green with a pale mudrib petiole up to 10mm long. Spathilated or blanceolated to ovated or elliptic margins up to 9.5 and 5 cm (Figure 2.9). The flowers are dioecious, unscented with peduncle 1-30 mm long, pectical up to 7 mm, circular to triangular lanceolate sepals (Hutchings *et al.,* 2013). It has an untidy sparse, multi-stemmed evergreen shrub; it branches into many long, thin whitish-grey branchlets and twigs that curve down,

with grey-green leaves towards the tips. The flowers bloom in dense, short racemes between the leaves (Figure 2.8). The capsule is reddish, glubsoid or pyriform, 2-6 mm long, ovary 2-3 locular, smooth. Reddish-brown 1-2 seeds, glossy with a fleshy smooth rose-pink in yellowish arid obliquely covering the lower (Fabricant *et al.,* 2001).

2.16.4 Uses of *Maytenus senegalensis* as a medicinal plant

Maytenus senegalensis (Lam.) grows in the semi desert regions of Asia and tropical regions Africa. Its roots and bark are traditionally used in the folk medicine of some African regions for the treatment of a number of ailments, including chest pains, rheumatism, snakebites, diarrhoea, eye infection, and dyspepsia (indigestion). It is also used as an antibacterial, antimicrobial and antibiotic. An extract of the roots and barks is used for severe headaches, an analgesic, for skin rashes, muscle spasms, excessive sweating, fevers, parasitic intestinal infections, as an anti-inflammatory, for arthritis and muscle pain, for nausea, vomiting and diarrhea The leaves are used for malaria, yellow fever, and tryanosomiasis (El Tahir *et al.,* 2014). It is also used for fertility problems, venereal diseases, pneumonia, epilepsy, and as a tonic (Jorge, 2004). Following the traditional use of the plant in Sudan, Kenya or Tanzania, it was demonstrated that leaf, root and stem bark extracts of *M. senegalensis* possess in vitro anti-*plasmodial*, anti-leishmanial, and antibacterial activities (El Tahir *et al.,* 2014).

Roots of the related *Maytenus senegalensis* chipped into beer have been used in Zambia as an aphrodisiac (Muregi *et al.,* 2012). The roots, which are slightly bitter, are also mildly laxative and are used in various parts of tropical Africa for gastro-intestinal troubles, especially dysentery and a poultice of the green leaves has been put on sores in Nigeria (Matu and Steven, 2003). *Maytenus senegalensis* is a potential alternative chemotherapeutic agent of *Schistosomamansoni.* The activity of the plant has been shown to be directly proportional

to the concentration of plant extracts (Fansworth and Soejarto, 2009). Screening studies on antitumor properties of the root and stem extracts revealed *in vitro* cytotoxic activity against carcinoma cells and in *vivo* anti-leukemia effects. Interestingly, plants of the genus *Maytenus* are used in South America to prepare infusions or decoctions as anti-inflammatory and analgesic remedies for oral and/or topical administration. Evidence for the in *vivo* anti-inflammatory activity of *M. senegalensis* root extracts has been scientifically established. Such extracts contain maytenoic acid, which was found to be an anti-inflammatory triterpene twice as active as the NSAID indomethacin and only three times less active than hydrocortisone. *M. senegalensis* roots could be employed in therapeutic preparations for the treatment of inflammatory-based diseases. The plant material can be also regarded as a source of the potent anti-inflammatory principle, maytenoic acid (Fabricant *et al.,* 2001).

2.16.5 Chemical constituents of *Maytenus senegalensis*

The *Celastraceae* family is a source of important bioactive secondary metabolites. Alkaloid amines such as cathine often occur in this botanical family as also, rarely, benzylisoquinolide alkaloids. Celastraceae members are commonly tanniferous, containing anthocyanins, sometimes saponiferous, only rarely cyanogenic and without iridoid compounds. Among the compounds isolated from their species, triterpenes and triterpenoidquinonemethides are of great interest due to their wide range of biological activities. Constituents as β-amyrin, lupane derivatives and quinoid pigments are considered typical of the *Celastraceae* family members. Compounds

isolated from the *Maytenus* genus include the ansa macrolide, maytansine, and related macrolides such as nor-maytansine, maytanprine and maytanbutine (Da-Silva *et al.,* 2009). Two of the more well-known chemicals are mayteine and maytansine - alkaloids long

documented with antitumor activitity and which occur in other *Maytenus* plants as well. Other isolated compounds include spermidine alkaloids (celacinnine and celallocinine) and nicotinoylsesquiterpene alkaloids (maytoline and maytolidine) as well as catechin, procyanidins and phenoldienonetriterpenoids (Pistelli *et al.,* 2008).

CHAPTER THREE

3.0   MATERIALS AND METHODS

3.1   Materials

3.1.1   Collection and identification of plant material

The plant *Maytenus senegalensis* was collected from Bida, Niger State. The plant was authenticated by a botanist, from Department of Biological Sciences, Federal university of Technology Minna, Nigeria. The plant was further identified and authenticated at the department of Botany Ahmadu Bello University, Zaria, Kaduna State, Nigeria, where a voucher number 900199 was deposited at the herbarium unit of the same institute.

3.1.2   Reagents and chemicals

Organic solvents (methanol, ethanol, chloroform and ethylacetate) used in this study were products of Sigma Chemical Co St. Louis M.O (USA) All other chemicals used were also of analytical grade and were freshly prepared with distilled water.

3.1.3   Apparatus and equipment

The apparatus and glass wares used include beakers, volumetric flasks, measuring cylinders, test tubes, syringes, spatula, pestle and mortar, micropipettes. Equipment used in this study include: rotary evaporator (Batch no: 5111, made in Germany), heating mantle (Batch no: MSM9204-03, made in Leighton) and water bath (Batch no: 85104, made in Britain). All analyses were performed with commercial kits from Randox Laboratories Limited, United Kingdom, Aggape Diagnostics (Switzerland) and Spectrum.

3.1.4   Experimental animals

A total of ninety (90) adult swiss albino mice weighing 25.34±0.98 g were obtained from Animal Holding Unit, Department of Biochemistry, Federal University of Technology Minna, Nigeria. They were housed in clean cages with wood shavings as beddings under standard environmental conditions of temperature and relative humidity, 12 hours day light/night cycle) with access to commercial feed pellets (growers) and water *ad libitum*. The cages were cleaned regularly throughout the experimental periods (their beddings were changed every two days). The rules governing the use of laboratory animals as laid out by the Federal University of Technology Minna Committee on Ethics for Medical and Scientific Research and also existing internationally accepted principles for laboratory animal use and care as contained in the Canadian council on Animal Care Guidelines and Protocol Review (CCAC, 1997) were duly observed.

3.1.5   Parasite

Plasmodium berghei NK65 chloroquine-sensitive strain was obtained from National Institute of Pharmaceutical Research and Development (NIPRD) Abuja, Nigeria and maintained in the   laboratory by serial passage in mice.

3.2   Methods

3.2.1   Sample extraction

One hundred grams (100 g) of the plant powder was extracted with absolute methanol using reflux method at a temperature of 45 °C for 2 hours and the extract was filtered using muslin cloth followed by further filtration using whattman No 1 filter paper with pore size of 0.7 μm

to obtain a fine filtrate. The filtrate was then concentrated using water bath at 45 °C into fine paste and kept in the refrigerator for further analysis.

3.2.2 Qualitative phytochemical analysis

3.2.2.1 Flavonoids

Half gram (0.5 g) of the extract was heated with 10 mL of ethylacetate in a test tube over a steam bath for 3 minutes. The mixture was filtered and 4 mL of the filtrate was shaken with 1 mL of dilute ammonia solution. The yellow coloration observed indicated the presence of flavonoids (Harborne, 1973; Sofowora, 1993).

3.2.2.2 Tannins

Exactly 0.5 g of the crude extract was boiled for 10 mins in 20 mL of distilled water in a test tube and filtered. Few drops of 0.1 % ferric chloride ($FeCl_3$) solution was added to the filtrate and observed for brownish green or a blue-black coloration (Harborne, 1973).

3.2.2.3 Saponins

The froth test method was used to test for the presence of saponins. 0.2 g of the extract was boiled in 20 mL of distilled water in a test tube in boiling water bath and filtered. Ten milliliters (10 mL) of the filtrate was mixed with 5 mL of distilled water and shaken vigorously to form a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously for the formation of emulsion characteristic of saponins (Obadoni and Ochuko, 2002).

3.2.2.4 Phenols

Equal volumes of extract and Iron (iii) chloride were mixed; a deep bluish green solution indicates the presence of phenol (Trease and Evans, 1989).

3.2.2.5   Alkaloids

The extract (0.5 g) was stirred with 5 mL of 1 % aqueous hydrochloric acid on a steam bath. Few drops of picric acid solution were added to 2 mL of mixture. The formation of a reddish brown precipitate was taken as a preliminary evidence for the presence of alkaloids (Harborne, 1976; Trease and Evans, 1989).

3.2.3   Quantitative phytochemical analysis

3.2.3.1   Determination of total flavonoids

This method is based on the formation of flavanoid-aluminium complex with its maximum spectrophotometric absorption at 415 nm. Fifty micolitre (50 µL) of extract was mixed with 50 µL of 10 % aluminiumtrichloride in methanol and drop of acetic acid was added. This was then diluted with distilled water to 5 mL. Absorbance was read at 412 nm after 30 minutes. Blank was prepared from 50 µL of distilled water in place of extract and same procedures for the sample was repeated. Quercetin was used as standard (Ejikeme *et al*., 2014).

3.2.3.2   Determination of total phenol

The 100 mg extract was dissolved in 100 mL of distilled water. One milliliter (1 mL) of the mixture was pipetted into sample test tube, 0.5 mL of 2 M Folin-Ciocalteu reagent and 1.5 mL 20 % of Na_2CO_3 solution were added to the sample test tube. Distilled water was added to make up the volume to 8 mL. This was then mixed thoroughly and incubated for two hours at room temperature. Thereafter, tube content was mixed again and absorbance was read at

765 nm. The total phenol content was then determined using the standard calibration curve gotten from various dilution concentration of garlic acid as standard (Hagerman *et al.,* 2000).

3.2.3.3   Determination of total alkaloids

The extract (0.5 g) was dissolved in 1 mL of 96 % ethanol: 20 % H_2SO_4 (1:1) and filtered. One millitre (1 mL) of filtrate was added to 5 mL of 60 % H_2SO_4 and allowed to stand for 5 minutes. Thereafter, 5 mL of 0.5 % formalaldehyde was added and allowed to stand again for further 3 hours. Absorbance was read at 565 nm and vincristine was used as standard at 0.1 M concentration (Harborne *et al.,* 1973).

3.2.3.4   Determination of total tannins

The extract (100 mg) was weighed into a beaker. Fifty millilitres (50 mL) of distilled water was added and shaken in a mechanical shaker for one hour. One millilitre (1 mL) of the filtrate was pipetted into sample test tube. Two millilitres (2 mL) of 0.1 M $FeCl_3$ in 0.1 M HCl and 0.008 M potassium ferrocyanide were added to the filtrate and mixed thoroughly. Absorbance was read at 120 nm within 10 minutes. Tannic acid (1 M) was used as standard (Van *et al.,* 1981).

3.2.3.5   Determination of total saponin

The extract (0.5 g) was added to 20 mL of 1 M hydrochloric acid and boiled for 4 hours. This was filtered after cooling and 50 mL of petroleum ether was added to the filtrate and evaporated to dryness. Five millilitres (5 mL) of acetone/ethanol was added to the residue. 0.4 mL was taken into 3 different test tubes. 6 mL of ferrous sulphate reagent was added and 2 mL of concentrated H_2SO_4 was added after. It was thoroughly mixed after 10 mins and the absorbance was read at 490 nm (Obadoni and Ochuko, 2002).

3.2.4　Extraction of alkaloids

Total alkaloids were extracted from *Maytenus senegalensis* leaf according to Harborne, (1984). Briefly, 10 g of plant dry powder was extracted with 80 % ethanol for 24 hours in a continuous extraction by soxhlet apparatus 1000 mL volume. The extract was filtered by Whatman No1 filter paper and then, the filtrate was concentrated by a rotary evaporator under vacuum 45 ^{o}C until the solution reached 10 mL. The concentrated extract was transferred to a beaker and 2 N HCL was added gradually to adjust the pH value up to 2. Then, the pH value of the extract was adjusted to 10 using NH OH, and washed with 10 mL chloroform 3 times. The chloroform portion was dried to obtain the total alkaloid fraction. The dried alkaloid fraction was weighed and preserved in a clean container at 4 ^{0}C for use as described by Jigam *et al.* (2017).

3.2.5 Acute toxicity

Acute toxicity of the alkaloid was determined in 2 phases according to Lorke's (1987). In Phase 1, a total of 9 mice were grouped into 3 of three (3) mice each and were given a single dose of 10, 100 and 1000 mg/kg bw of the alkaloid fraction respectively. A control group was also set up comprising of 3 mice and was given 2 mL/kg bw normal saline. The absence of death after 24 hours of extract administration led to the initiation of Phase II which was set up with another 3 groups of 3 mice each and were given a single dose of 1600, 2900 and 5000 mg/kg bw of the alkaloid fraction respectively. The fraction was administered orally using esophageal cannula. The mice were observed for any adverse effect and mortality within 24 hours of treatment and after a week.

3.2.6　Anti-plasmodial screening

Four days (4) suppressive test were used to evaluate the antimalarial properties of the alkaloid fraction of *Maytenus senegalensis* as described by Jigam *et al.* (2011). A total of 25 *P. berghei* infected mice were randomly grouped into five (I- V) of 5 mice each. Groups I – III animals were treated with 75, 150 and 300 mg/kg bodyweight of alkaloids respectively. Groups IV and V received normal saline (2 mL/100g) and chloroquine (5 mg/kg bodyweight) to serve as negative and positive drug controls respectively. All the treatments were done orally for 4 consecutive days. Daily parasitaemia count were carried out by preparing a Giemsa stained-thin film and viewed under microscope as described by Jigam *et al*. (2011).

$$\% \text{ inhibition} = \frac{\text{Mean parasitemia in negative control} - \text{Mean parasitemia in treated}}{\text{Mean parasitemia in negative control}} \times 100$$

3.2.7 Anti- inflammatory study

Anti-inflammatory activity of the extract was tested using egg albumin induced paw oedema in mice according to the methods of Jigam *et al.* (2012). A total of 20 mice were randomly grouped into four (A- D) of 5 mice each. Group A and B were administered a single dose of 75 and 150 mg/kg bw alkaloid fraction respectively, Group C were administered a dose of 150 mg/kg bw acetyl salicylic acid and Group D were administered a dose of 2 mL/kg bw normal saline 30 minutes before the injection of the albumin into the right hind limb. The increase in paw was recoreded at 20, 40, 60, 80, 100 and 120 minutes after injecting egg albumin. The percentage inhibition of oedema was calculated for each dose using the formula:

$$\% \text{ inhibition}$$

$$= \frac{\text{Mean increase in paw in negative control} - \text{Mean increase in paw in treated}}{\text{Mean increase in paw in negative control}} \times 100$$

3.2.8 Analgesic study

The hot plate test (Thermal stimuli) was used to measure analgesic activity of the extract according to the method described by Mohiuddin *et al.* (2018). A total of 20 mice were randomly grouped into four (A- D) of 5 mice each. Group A and B were administered a single dose of 75 and 150 mg/kg bw alkaloid fraction respectively, Group C and D were administered 150 mg/kg bw sodium diclofenac and 2 mL/kg bw normal saline respectively 30 mins before the hot plate test. The hot plate was set at 45 ^{0}C and the time (seconds) taken by the mice to react to the thermal pain by licking their paw or jumping were recorded at 0, 15, 30, 45 and 60 minutes of treatments.

% inhibition

$$= \frac{\text{Mean increase in thermal stimuli in negative control} - \text{Mean increase in thermal stimuli in treated}}{\text{Mean increase in thermal stimuli in negative control}}$$

$\times 100$

3.2.9 Toxicological study

Animals (5 each) were dosed with 0 (control), 75 mg/kg and 150 mg/kg bwt of alkaloid fraction of *Maytenus senegalensis* orally for 4 wk. The procedure described by Yusuf *et al.* (2018) was adopted for the preparation of serum. Briefly, the animals were anaesthetized with diethyl ether and the blood was collected through cardiac puncture into sample bottles and left for fifteen minutes to clot, and then centrifuged at 3000 rpm for 15 minutes in order to get the serum. The sera were stored in the refrigerator at -20 ^{o}C for subsequent analysis.

3.2.9.1 Quantitative determination of triacylglycerols

Principle: Enzymatic determination of triacylglycerol (TGL) is based on the reactions involving hydrolysis of triacylglycerol to glycerol and free fatty acids by lipoprotein lipase

(LPL). The glycerol is phosphorylated by adenosine triphosphate (ATP) in the presence of glycerol kinase (GK) to form glycerol-3-phosphate (G-3-P) and adenosine diphosphate (ADP). Glycerol-3-phosphate is oxidized by glycerophosphate oxidase (GPO) to form dihydroxyacetone phosphate (DHAP) and hydrogen peroxide (H_2O_2). A violet coloured complex is formed by peroxidase (POD) catalysed coupling of 4-aminoantipyrine and phenol with H_2O_2 which is proportional to concentration of triacylglycerol in the sample (Fossati and Prencipe, 1982).

$$TGL + H_2O \xrightarrow{\text{LPL}} Glycerol + fatty\ acids)$$

$$Glycerol + ATP \xrightarrow[\text{Mg}^{2+}]{\text{GK}} Glycerol\text{-}3\text{-}phosphate + ADP)$$

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

$$2H_2O_2 + 4\text{-}Aminoantipyrine + TOPS \xrightarrow{\text{POD}} Violet\ colored\ complex$$
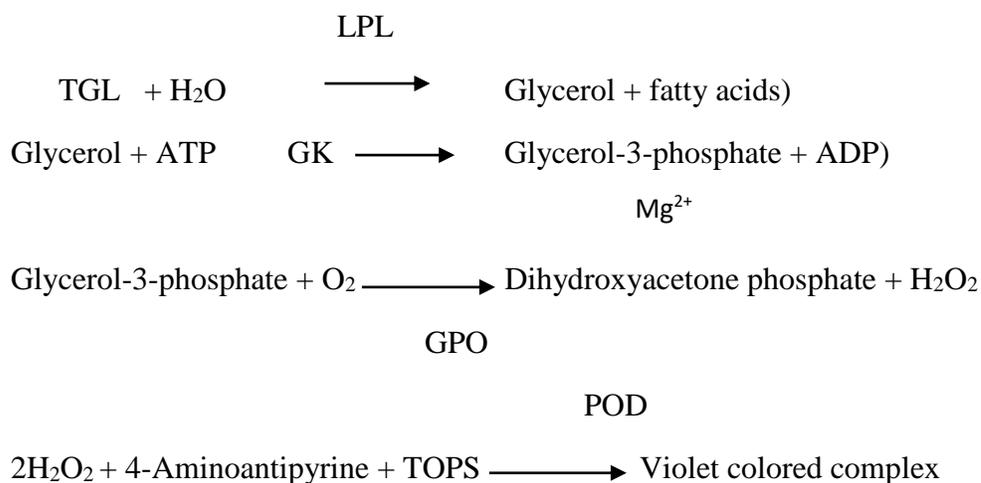
Working reagent: 4 mL of reconstituted free glycerol reagent + 1 mL of the reconstitutedtriglyceride reagent.

Procedure: One thousand microlitres (1000 µL) of working reagent was pipetted into three test tubes labeled sample, standard and blank. Ten microlitres (10 µL) of serum sample, 10 µL of standard and 10 µL of distilled water were pipetted into sample, standard and blank test tubes respectively. The mixture was mixed gently and incubated for 5 minutes at 37 °C. Change in absorbance of standard and sample against reagent blank was measured at 546 nm.

Concentration of triacylglycerol was calculated using the formula below:

Triacylglycerol conc. (mg/dl) = Absorbance of sample $\times$ 200
                                                    Absorbance of standard

Where 200 is triacylglycerol standard concentration (mg/dl)

3.2.9.2 Aspartate aminotransferase activity (AST)

Principle: An Aspartate aminotransferase (AST) test kit (Randox Laboratories Ltd, Crumlin, UK) was used. Aspartate aminotransferase was measured by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4 – dinitrophenylhydrazine.

<div align="center">GOT</div>

α-oxoglutarate + L-aspartate $\xrightarrow{\hspace{4cm}}$ glutamate +oxaloacetate

Procedure: Solution A contained phosphate buffer, L – aspartate, α – oxoglutarate while solution B contained 2, 4 – dinitrophenylhydrazine. 0.05 ml of sample was added to 0.25 mL of solution A. It was mixed and incubated for exactly 30 minutes at 37 $^{\circ}$C. Afterwards, 0.25 mL of solution B was added, mixed and allowed to stand for 20 minutes at 25 $^{\circ}$C. Thereafter 0.25 mL of 0.1 M NaOH was added to the mixture and thoroughly mixed. 0.05 mL of distilled water was added to blank test tube and same procedure as done for sample was repeated. The absorbance was taken at 546 nm after 5 minutes. The activity of AST in the sample was read off the standard curve. This was carried out based on the procedure of Reitman and Frankel (1957) that was modified by Schmidt and Schmidt (1963).

3.2.9.3 Alanine aminotransferase activity (ALT)

Principle: An Alanine aminotransferases test kit (Randox Laboratories Ltd, Crumlin, UK) was used. Alanine transminase (ALT) or Glutamate pyruvate transaminse (GPT) catalyzes the formation of pyruvate and glutamate from alanine and α – oxoglutarate.

$$\text{A-oxoglutarate + alanine} \xrightarrow{\text{GPT}} \text{L-glutamate + pyruvate}$$

Procedure: Solution A contained buffer (phosphate buffer), L – alanine, α – oxoglutarate while solution B contained 2, 4 – dinitrophenylhydrazine. 0.05 mL of sample was pipetted into sample test tube, 0.25 mL of solution A was added to it. It was mixed and incubated for exactly 30 minutes at 37 °C. Afterwards, 0.25 mL of solution B was added, mixed again and allowed to stand for 20 minutes at 25 °C. 0.25 mL of 0.1M NaOH was then added to the mixture. It was mixed, and same procedure was repeated for blank using 0.05 mL of distilled water in place of sample. The absorbance was taken at 540 nm after 5 minutes. The activity of ALT in the sample was read off the standard curve. This was carried out based on the procedure of Reitman and Frankel (1957).

3.2.9.4 Alkaline phosphatase (ALP)

An alkaline phosphatase test kit (Randox Laboratories Ltd, Crumlin, UK) was used for the estimation of alkaline phosphatase (ALP).

The principle of this method is based on the reaction involving serum alkaline phosphatase and colourless substrate of phenolphthalein monophosphate. The ALP hydrolyses phenolphthalein monophosphate to phosphoric acid and phenolphthalein. The mixture turned pink at pH 9.8 and ALP was measured spectrophotometrically at 540 nm.

$$\text{P-nitrophenylphosphate + H}_2\text{O} \xrightarrow{\text{ALP}} \text{PO}_4 + \text{P-nitrophenol (pink, pH= 9.8)}$$

The 0.05 mL of sample and 0.05 mL of distilled water were dispensed into their respective labeled test tubes. Three milliliters (3.0 mL) of substrate was added to each test tube, and

then mixed. The initial absorbance (A1) was taken at 1 min and subsequent absorbances were taken every minute for additional 3 minutes at 405 nm and 37 °C. The difference between absorbances (ΔA) and the average absorbance differences per minute (ΔA / min) were calculated. It was carried out as described by Wright *et al.* (1972).

Calculation: ALP activity (U/L) = (ΔA/min) x 2750.

Where, ΔA is change in absorbance per minute

3.2.9.5 Determination of serum total protein concentration

Serum total protein concentration was calculated using Randox kit from Randox Laboratories Limited, U. K

Principle: Cupric ions in an alkaline medium interact with protein peptide bonds resulting in the formation of a coloured complex.

Procedure: Serum (0.02 mL), 0.02 mL of standard regent and 0.02 mL of distilled water were respectively pipetted into test tubes labeled serum, standard and blank. One milliliter (1 mL) of R1 (Biuret reagent) was added to all test tubes, mixed gently and incubated for 30 minutes at 25 °C. The absorbances of sample and standard were measured at 546 nm against blank (Weichselbaum, 1946). The concentration was calculated using the formula below:

$$\text{Total protein concentration (g/dl)} = \frac{\text{Absorbance of sample} \times \text{Standard protein conc.}}{\text{Absorbance of standard}}$$

(Where standard protein concentration is 5.58 g/dl)

R1 reagent consists of 100 mmol/L of sodium hydroxide, 16 mmol/L of Na-K-tartrate, 15 mmol/L of Potassium iodide and 6 mmol/l of cupric sulphate.

3. 2.9.6   Determination of serum sodium concentration

Sodium ion concentrationwas determined using Randox kit from Randox Laboratories Limited, U.K

Principle: This method is based on the reaction of sodium with a selective chromogen producing a chromophore whose absorbance varies directly as concentration of sodium in the specimen.

Procedure: One thousand milliliters (1000 µL) of sodium reagent was added to each tubes labeled sample, standard and blank. Ten milliliters (10 µL) of serum, 10 µL of standard reagent and 10 µL of blank were respectively added to sample, standard and blank tubes. The mixtures were shaken gently and incubated for 5 minutes at room temperature and absorbance was read at 630 nm using a spectrophotometer. Concentration was calculated using the formula below:

$$\text{Concentration of sodium (mmol/l)} = \frac{\text{Absorbance of sample} \times \text{conc. of standard (180 mEq/l)}}{\text{Absorbance of standard}}$$

3.2.9.7   Determination of serum chloride concentration

Chloride ion concentration was determined using (Randox Laboratory Ltd, Crumlin, UK).

Principle: Chlorideions form a soluble non-ionized compound with mercury ions and displaces thiocyanate ions from non ionized mercury thiocyanate. The released thiocyanate ions react with ferric ions to form a coloured complex which absorbs light at 480 nm. The intensity of coloured produced is directly proportional to the concentration of chloride.

Procedure: Chloride reagent (1.5 mL) was pipetted into each test tube labeled blank, standard and sample. Standard reagent (100 mEq/L of sodium chloride), 0.01 mL of sample and 0.01 mL of distilled water were added to standard, sample and blank tubes respectively. The tubes were mixed thoroughly and incubated at room temperature for 5 minutes and absorbance of standard and sample were read at 480 nm against reagent blank. The chloride concentration was calculated as:

Calculation:

$$\text{Chloride ion conc.} = \frac{\text{Absorbance of sample} \times \mathbf{100}}{\text{Absorbance of standard}}$$

3.2.9.8 Determination of potassium concentration

This was carried out acoording to the method described by Barry and Rowland (1952).

Principle: The potassium is precipitated as potassium sodium cobaltnitrite by adding a sodium cobaltnitrite reagent to serum in a centrifuge tube. After washing with aqueous ethanol, the precipitate is dissolved in water and the cobalt measured from the intensity of the green color produced on adding choline and potassium ferrocyanide solutions.

Procedure: To 1 mL of serum, 3 mL of cobaltnitrite reagent, mixture is allowed to stand for 2 hours at room temperauture and centrifuged at 2500 rpm and absorbance taken at 607 nm.

3.2.10 Gas chromatography mass spectrometry (GC/MS) analysis

The GC/MS analysis of alkaloid extract from *Maytenus senegalensis* was perform using GC-MS clarus 500 per kin Elmer system comprising an AOC-20i auto sampler. "The instrument

is equipped with a VF 5 ms fused silica capillary column of 30 m length, 0.25 mm diameter and 0.25 μm film thickness." The temperatures employed were; column oven temperature 80 °C, injection Temp 250 ºC at a pressure of 108.0 kPa, with total flow and column flow of 6.20 mL/min and 1.58 mL/min, respectively. The linear velocity was 46.3 cm/s and a purge flow of 3.0 mL/min. The GC program ion source and interface temperature were 200 °C and 250 °C, respectively, with solvent cut time of 2.50 min. The MS program starting time was 3 mins which ended at 30 mins with event time of 0.50 s, scan speed of 1666 μL/s, scan range 40-800 u, and an injection volume of 1 L of the propolis extract (split ratio 10:1). The total running time of GC-MS was 30 mins. The relative percentage of the extract was expressed as percentage with peak area normalization as previously reported by Lawal *et al*. (2015).

3.2.11   Statistical analysis and data presentation

Data collected were subjected to statistical analysis using the statistical package for social science version 21.0 and express as mean ± standard error of mean (SEM). Statistical significance of the results between groups was determined using One-way analysis of variance (ANOVA) followed by Duncans multiple range test (DMRT) to check differences between the individual groups. Differences in mean were considered to be significant at $p<0.05$, and values with different superscripts were considered to be significantly different (Adamu and Johnson, 1997).

CHAPTER FOUR

4.0   RESULTS AND DISCUSSION

4.1   Results

4.1.1   Phytochemical composition

The qualitative phytochemical composition of *Maytenus senegalensis*is shown in Table 4.1. The crude methanol leaf extract of *M. senegalensis* was found to contain alkaloids, flavonoids, tannins, phenol, saponins, anthraquinone, steroids and phlobatannins. Quantitatively, *M. senegalensis* contains 145.56±4.85 mg/g of flavonoids, 198.46±2.56 mg/g alkaloids, 78.65±1.45 mg/g total phenol, 34.56±2.34 mg/g of saponins and 12.45±0.95 mg/g of tannins (Table 4.2)

Table 4.1: Qualitative Phytochemical Composition of Crude Methanol Leaves Extract of *Maytenus senegalensis*

Phytochemicals	Inference
Alkaloids	+
Total Flavonoids	+
Total Phenols	+
Saponins	+
Tannins	+
Anthraquinone	+
Phlobatannins	+

Steroids	+

Table 4.2: Quantitative Phytochemical Composition of Crude Methanol Leaves Extract of *Maytenus senegalensis*

Phytochemicals	Amount present mg/g
Alkaloids	198.46±2.56
Total Flavonoids	145.56±4.85
Total Phenols	78.65±1.45
Saponins	34.56±2.34
Tannins	12.45±0.95

Values are expressed as Mean ± SEM.

4.1.2 Acute oral toxicity of alkaloid extract of *Maytenus senegalensis* leaves in mice

The acute oral toxicity of alkaloid fraction of *Maytenus senegalensis* leaves is presented in Table 4.3. No death was recorded throughout the study period. Animals that were administered 2900 and 5000 mg/kg bw of the alkaloids showed some behavioural changes including; hair erection, accelerated heart rate, hyperactivity, but no death was recorded (Table 4.3). The LD_{50} of the alkaloid fraction of *Maytenus senegalensis* leaves in mice was> 5000 mg/kg bw.

Table 4.3: Acute Oral Toxicity of Alkaloid Fraction of *Maytenus senegalensis* Leaves

Dosage (mg/kgbw)	Mortality	Sign of Toxicity
		Phase1
10	0/3	Normal behaviour of mice after gavage
100	0/3	Normal behaviour of mice after gavage
1000	0/3	Normal behaviour of mice after gavage
		Phase2

1600	0/3	Normal behaviour of mice after gavage
2900	0/3	Hair straightening, Drowsiness, No death
5000	0/3	Hair Straightening, Drowsiness, Slow activity, No death

LD50=> 5000 mg/kg bw

4.1.3 Antiplasmodial activities of alkaloid fraction of *Maytenus senegalensis* leaves in mice

Results of the anti-*plasmodial* screening of alkaloidal fraction of *M. senegalensis* leaf against *Plasmodium berghei* are presented in the Figure 4.1. The alkaloid fraction of *M. senegalensis* leaf shows dose dependent antiplasmodial activity agains*t Plasmodium berghei* (Figure 4.1). The alkaloid % effects of 38.22±3.21, 69.80±6.54 and 79.43±8.32 respectively were recorded against *Plasmodium berghei* (Table 4.4)

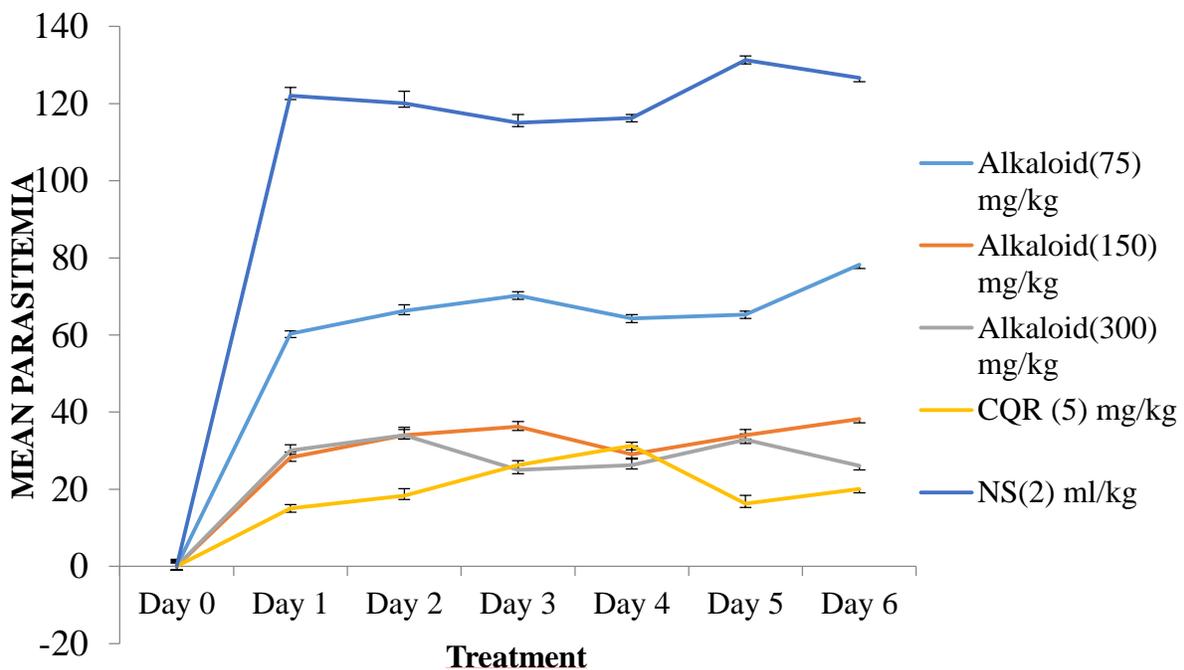**Figure 4.1: Antiplasmodial Activity of Alkaloid Fraction of *M. senegalensis* Leaf against *Plasmodium berghei Infected* Mice**

Table 4.4: **Antiplasmodial Activity of Alkaloid Fraction of** *M. senegalensis* **Leaf against** *Plasmodium berghei* **Infected Mice on Day 6**

	DOSES (mg/kg)	MEAN PARASITEMIA AT DAY 6	% INHIBITION
MS = *M. Senegalensis*			
NS = Normal Saline			
CQR = Chloroquine MS	75	78.25±0.95	38.22±0.53
MS	150	38.25±01.47	69.80±0.28
MS	300	26.05±1.53	79.43±0.42
CQR	5 mg/kg	20.05±2.17	83.82±1.01
NS	2 mL	126.67±1.08	

4.1.4   Effect of alkaloid fraction of *M. senegalensis* leaf on packed cell volume (PCV)

   of *Plasmodium berghei* infected mice

Effect of alkaloid fraction of *M. senegalensis* leaf on PCV of *Plasmodium berghei* infected

mice is seen in the Figure 4.2, PCV of all the experimental animals decreases 3 days after *P.*

berghei infection. Infected untreated mice show further loss of PCV as the diseases progress,

however, treatments of the infected mice with 75,150 and 300 mg/kg bw of the alkaloid cause

significant increase in PCV after treatments. The chloroquine treatments also significantly

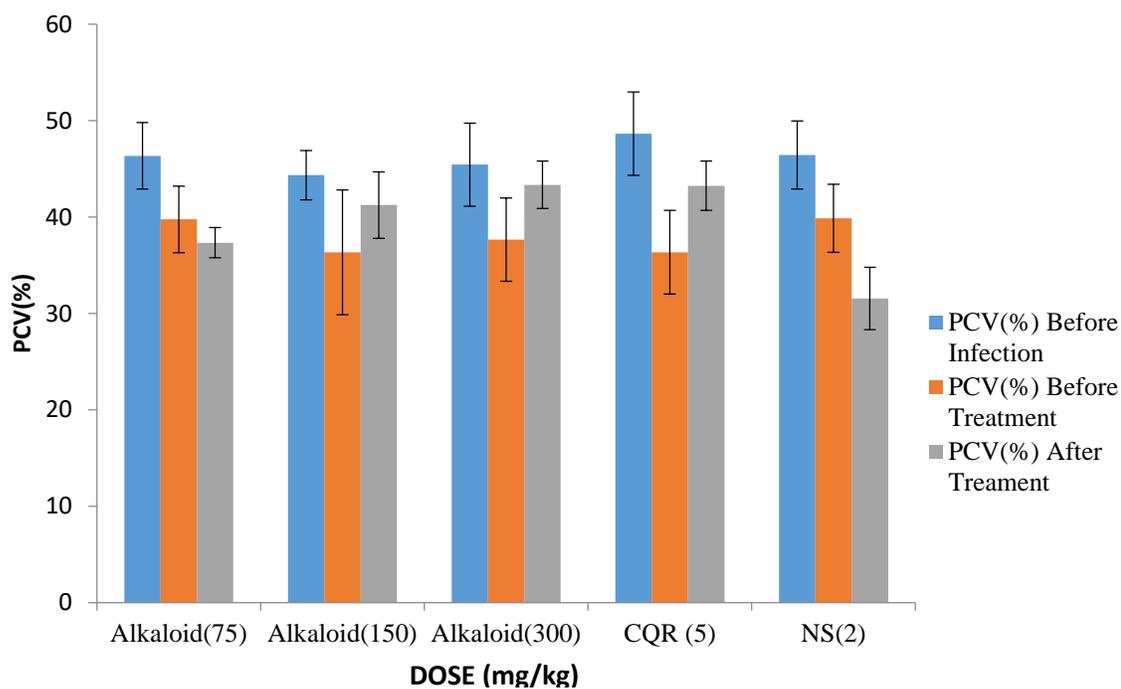restored the PCV compared to the untreated mice.

Figure 4.2: Effect of Alkaloid Fraction of *M. senegalensis* Leaf on PCV of *Plasmodium*

   ***berghei* Infected Mice**

4.1.5 Anti-inflammatory activities of alkaloid fraction of *Maytenus senegalensis* leaf in mice

Results of the anti-inflammatory effects of the of the alkaloid fraction of *M. senegalensis* are shown in Figure 4.3. Alkaloid fraction of *M. senegalensis* leaf exhibited dose dependent inhibition egg albumin-induced paw oedema with percentage inhibition of 53.16±4.09 and 60.76±7.54 at 75 and 150 mg/kg bw respectively while ASA exhibited 63.29±5.98 inhibition of paw oedema (Figure 4.3).

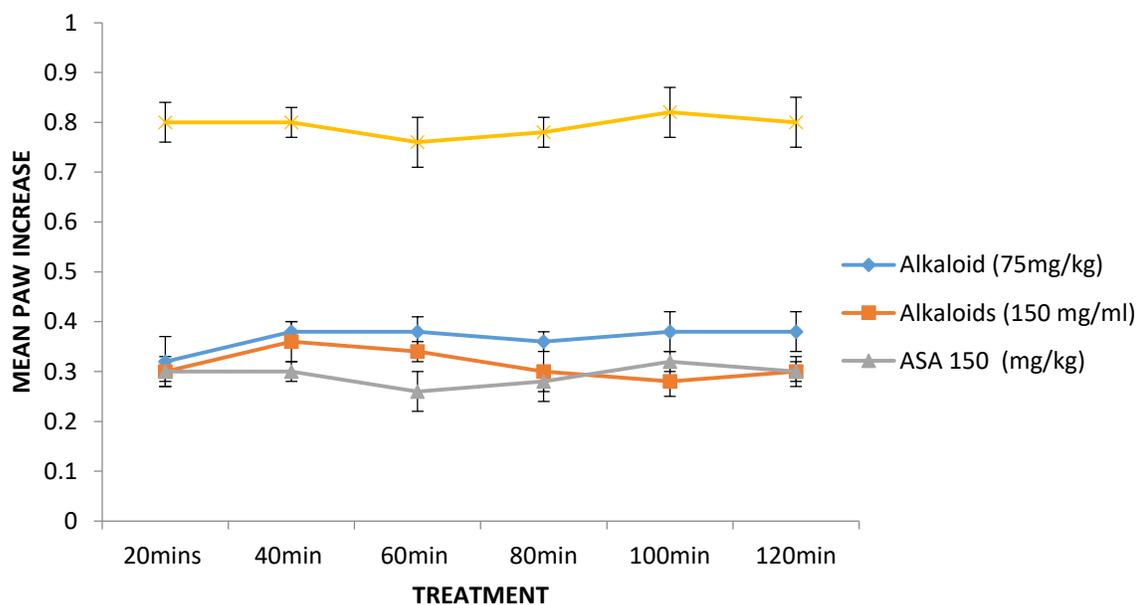**Figure 4.3: Effect of Alkaloid Fraction of *M. senegalensis* Leaf on Oedema in Mice**

ASA= Acetyl Salicylic Acid, NS=Normal Saline

4.1.6 Analgesic activities of alkaloid fraction of *Maytenus senegalensis* leaf in mice

Results of the analgesic effects of the of the alkaloid fraction of *M. senegalensis* are shown in Figure 4.4. Alkaloid fraction of *M. senegalensis* leaf exhibited dose dependent thermal stimuli with percentage inhibition of 43.35±4.98and 44.83±3.86 at 75 and 150 mg/kg bw respectively while ASA exhibited 74.88±6.87 inhibition of paw oedema (Figure 4.4)
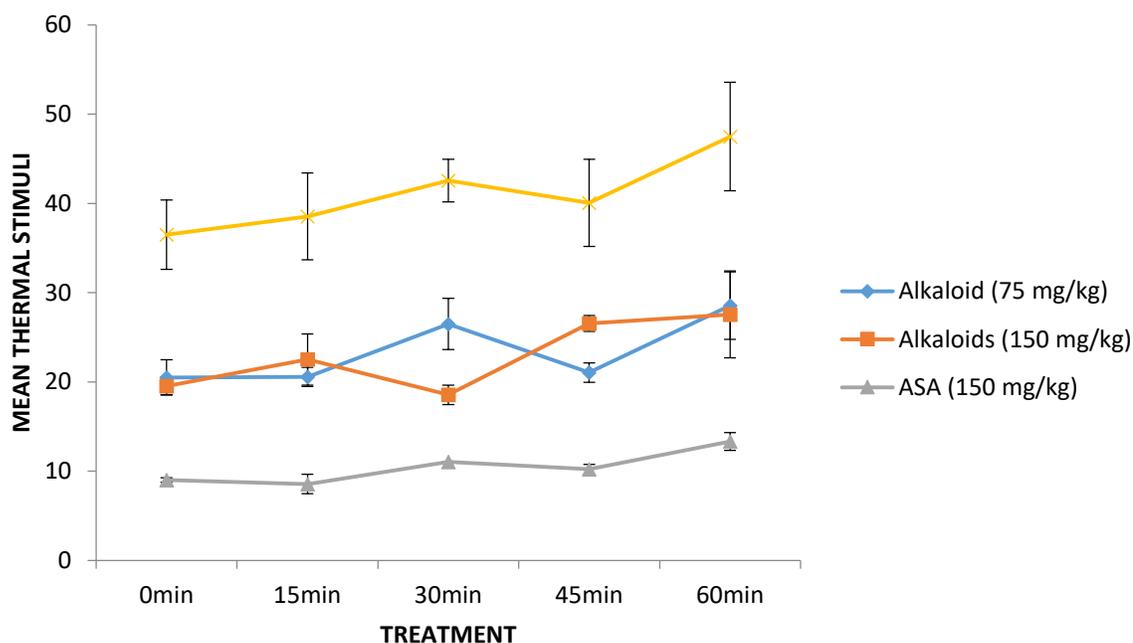

Figure 4.4: Effect of Alkaloid Fraction of *M. senegalensis* Leaf on Thermal Stimuli in
            Mice

ASA= Acetyl Salicylic Acid, NS=Normal Saline

4.1.7 Effect of sub-acute administration of alkaloid fraction of *Maytenus senegalensis* serum biochemical parameters in mice

Sub-chronic administration of the alkaloid fraction of *M. senegalensis* significantly ($p<0.05$) increase the concentrations of transaminases (aspartate transaminase and alanine transaminase), and proteins when compared with the untreated control. However, sodium, potassium, chloride, alkaline phosphatase, triglyceride and glucose concentrations were not ($p<0.05$) significantly altered by treatment with alkaloid extract of *M. senegalensis* (Table 4.5)

Table 4.5: Effect of Alkaloid Fractions of *M. senegalensis* Leaf on Biochemical Parameters in Mice

	Control (normal saline)	75 mg/kg bw	150 mg/kg bw
Weight gain (g)	16.51±0.78[a]	17.85±1.23[a]	18.23±0.98[a]
Glucose (mg/dL)	107.64±2.90[a]	109.03±3.87[a]	117.36±5.98[a]
Tag	170.97±3.89[a]	170.93±5.98[a]	173.70±7.09[a]
Prot (g/dL)	4.66±0.04[a]	5.20±0.13[b]	5.41±0.43[b]
AST	9.50±0.67[a]	13.50±1.03[b]	21.75±2.98[c]
ALT	6.25±0.56[a]	11.00±0.76[b]	18.00±0.65[c]
ALP	34.5±2.90[a]	37.25±2.90[a]	36.09±3.98[a]

Sodium	134±5.09 [a]	132.5±7.97 [a]	135.97±9.67 [a]
Potassium	3.35±0.54 [a]	3.60±0.43 [a]	3.24±0.21 [a]
Chloride	101.5±6.98 [a]	103.25±4.89 [a]	108.78±5.89 [a]

Data are Mean ± SEM of five determinations. Value followed by different superscript alphab*et al*ong the row were significantly different ($p<0.05$).

4.1.8 GC-MS of the alkaloid fraction of *Maytenus senegalensis*

The results of gas chromatography and mass spectroscopy (GC-MS) analysis led to the identification of 13 compounds from the gas chromatography (GC) fractionations. The mass spectrum of alkaloid fraction of *Maytenus senegalensis* is shown in Figure 4.5. The results were tabulated in Table 4.6. The results revealed the presence of 4 major antimalarial compounds including 3-hydroxy-20(29)-lupen-28-ol (12.95 %), 20α)-3-hydroxy-2-oxo-24-nor-friedela-1(10),3,5,7-tetraen-carboxylic acid-(29)-methylester (6.0 %) and 5,6,7,7a-tetrahydro- 2(4H)-Benzofuranone, (7.0 %), and phytol (1.44 %). Other compounds identified in minute amounts include n-Hexadecanoic acid (0.207 %), 9, 12-Octadecadienoic acid, methyl ester (1.67 %), cis-Vaccenic acid (0.4.90 %), 6 -Methyl-cyclodec-5-enol (0.66 %) each with different biological activities (Table 4.6).
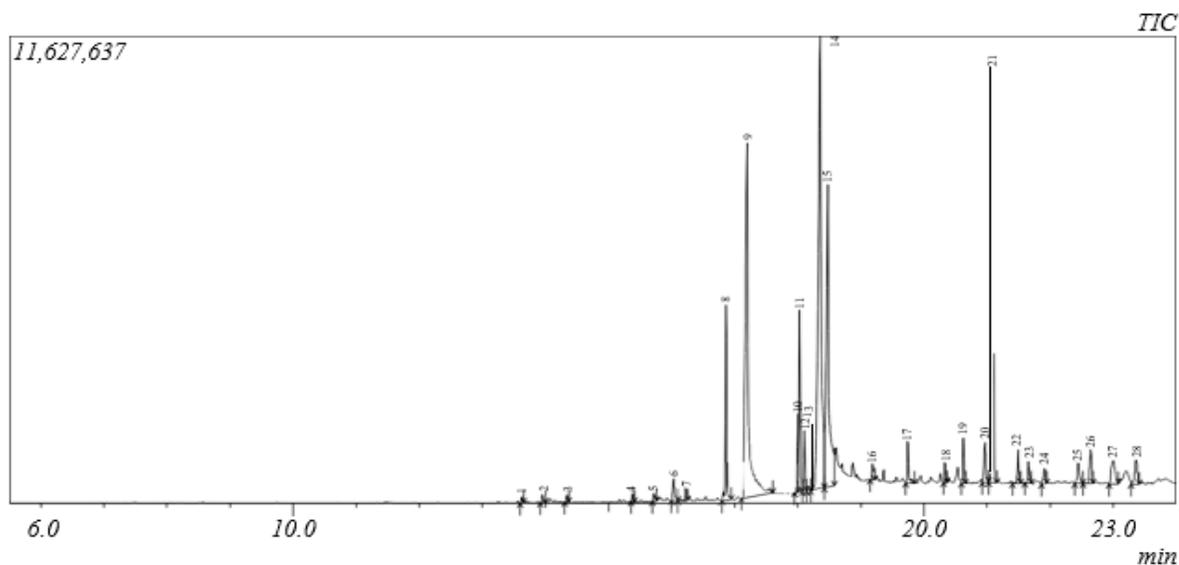
Figure 4.5: GC-MS Mass Spectrum of Alkaloid Fraction of *Maytenus senegalensis*

Table 4.6: Phyto-Components Identified in Alkaloid Fraction of *Maytenus senegalensis*

Peak #	R.Time	Peak Area%	Height%	Name
1	13.624	0.13	0.26	Dodecanoic acid, methyl ester
2	13.953	7.0	0.33	5,6,7,7a-tetrahydro-2(4H)-Benzofuranone,
3	15.379	6.0	0.31	20α)-3-hydroxy-2-oxo-24-nor-friedela-1(10),3,5,7-tetraen-carboxylic acid-(29)-methylester
4	16.031	0.66	0.89	6-Methyl-cyclodec-5-enol
5	16.227	0.38	0.50	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
6	17.197	0.207	14.07	n-Hexadecanoic acid
7	17.992	1.67	3.13	9,12-Octadecadienoic acid, methyl ester
8	18.101	1.44	2.43	Phytol
9	18.170	1.52	2.80	Methyl stearate
10	18.355	0.490	17.94	cis-Vaccenic acid
11	20.336	0.47	0.79	2-methyltetracosane

12	21.095	12.95	16.42	3-hydroxy-20(29)-lupen-28-ol
13	21.493	0.88	1.33	d-Xylose, diheptylmercaptal

4.2 Discussion

Phytochemical constituents are integral part of medicinal plants, and are responsible for their numerous bioactivities. The phytochemical screening of *M. senegalensis* leaf showed the presence of alkaloids, flavonoids, tannins, phenol, saponins, anthraquinone, steroids and phlobatannins. This agrees with phytochemical analysis of other *Maytenus* species which also reports the presence of Alkaloids, Flavonoids, Tannins, Phenols (Shabir *et al*., 2013; Pooja *et al*., 2014).

The traditional use of *M. senegalensis* for the treatment of malaria can be attributed to the presence of certain phytochemicals that constitute the bioactive principles in the plant; some of the secondary metabolites detected in this study have been implicated in anti-*plasmodium* activities. The anti-*plasmodium* activities of some plants have been attributed to the presence of alkaloids, flavonoids and terpenes all of which are contained in this plant. Alkaloids have been widely used in the treatment of malaria; an example is quinine, which is a cinchona

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alkaloid that belongs to the aryl amino alcohol group of drugs. It is an extremely basic compound and therefore, always present as a salt, because it is a base, it is concentrated in the food vacuoles of *P. falciparum*. The theorized mechanism of action for quinine and related anti-malarial drugs is that these drugs are toxic to the malaria parasite. Specifically, the drugs interfere with the parasite's ability to break down and digest hemoglobin. Quinine has rapid schizonticidal action against intra-erythrocytic malaria parasites. It also has analgesic properties (Achan *et al.*, 2011). The anti-oxidant flavonoid and phenolic compounds have also been shown to exert anti-*plasmodium* activity by elevating the red blood cell oxidation and inhibiting the parasites protein synthesis (Philip and Wright, 1990). This activity counteracts the oxidative damage induced by the malaria parasite (Ayeola *et al.*, 2008)

Based on the acute toxicity study, the LD_{50} of alkaloid fraction of *M. senegalensis* were found to be greater than 5000 mg/kg, indicating their wide safety margin. The present result is in line with the finding of (Murjanatu *et al.*, 2015). Generally, if LD_{50} value of the test chemical is more than three times the minimum effective dose, the substance is considered to be a good candidate for further studies in vivo assays (OECD, 2008). The LD_{50} has also been used for classification of chemicals. Based on world health organization (WHO) hazard classification system, alkaloid fraction of *M. senegalensis*, to which the LD_{50} was greater than 5000 mg/kg, are designated as "unlikely to be hazardous (WHO, 2009). Therefore, the alkaloid fraction of *M. senegalensis* is considered to be safe at the tested doses.

The anti-*plasmodial* potency of some plants has been associated with the presence of some secondary metabolites such as alkaloids (Lawal *et al.*, 2015). Alkaloids have been reported in literature of different plant species as having different extents of antimalarial activity (El

Tahir *et al.,* 1998; Khalid *et al*., 2007; Malebo *et al*., 2015). Findings presented in Table 4.4 shows that the alkaloid fraction of *M. senegalensis* demonstrated a good antimalarial activities, this is in concordance with the classification of Munoz *et al.* (2000), antiplasmodial agent are classified on the basis of the percentages parasite inhibition as moderate", "good", and "very good when there is percentage inhibition of above 50 % at metabolite concentration of 500, 250 and 100 mg/kg bwt. The proposed mechanism of antiplasmodial effect of alkaloids is by elevation of erythrocytes oxidation and inhibition of the plasmodiun protein synthesis (Pérez-Amador *et al*., 2017).

Haematological parameters can be used to determine extent of infection and effects of medicinal plant extracts on the blood constituents of an animal (Berinyuy *et al*., 2015). Such analysis is relevant to risk evaluation because changes in the haematological system are highly predictive for human toxicity, when data are translated from animal studies (Umar *et al*., 2019). In the present study, the continuous loss of PCV in *P. berghei*-infected mice could be attributed to RBC destruction, due to either parasite multiplication or spleen reticuloendothelial cell action as the presence of many abnormal RBC stimulates the spleen to produce many phagocytes (Chinchila *et al*., 1998). This massive destruction leads to a decrease in erythroid precursors and erythropoiesis inhibition, usually resulting in the death of the patient (Coronado *et al*., 2013). Anemia is a preventable cause of death in malaria-infected children under five years and pregnant women (WHO, 2006). Treatment of the infected mice with the crude alkaloid fraction of *M. senegalensis* caused a significant (P<0.05) increase in the PCV when compared with the untreated control. This could be due to antiplasmodial effect of the alkaloids and as a result of sustaining the availability of new

RBCs produced in the bone marrow. It could also be an indication of haematopoietic properties of the alkaloid (Marelli *et al.,* 2016).

Natural products have been established to assuage inflammation in *in vivo* and *in vitro* models. Evidence for the anti-inflammatory properties of flavonoids and alkaloids have been reported by several studies using different models of inflammation (Lamikanra *et al*., 2009; Capra, 2009; Chandel and Rastogi, 2012). The significant anti-inflammatory effects demonstrated by the alkaloid fraction of *M. senegalensis* root bark could be mechanistically explained by the fact that alkaloids are known to inhibit the enzymes involved in the production of inflammatory mediator including cyclooxygenase and 5-lipoxygenase pathways (Singh *et al*., 2012).

Although the mechanism by which this alkaloid exhibited analgesic and anti-inflammatory effect were not evaluated, natural products have been reported to exert anti-inflammatory activity by inhibiting the release or synthesis of inflammatory mediators, prostaglandins and polypeptide kinins. Alkaloids have been reported to exert anti-inflammatory activity through the inhibition of activation of nuclear factor-kappa B and down-regulation of the expression of inflammatory enzymes *e.g.* COX-2, 5-LOX and MMP-9 (Okokon and Nwafor, 2010). Therefore, the anti-inflammatory activity demonstrated by this plant could be through this mechanism.

It has been widely reported that many analgesic agents abound but the rationale in the preference of natural products to synthetic compounds is predicated on their reduced adverse effects (Salawu *et al*., 2008). Acetic acid-induced writhing is a common test for analgesic drug development. The writhing response of rodent to administration of toxic chemical is

used to screen for both peripherally and centrally acting analgesic potency of a test compound. In the present study, alkaloid fraction of *M. senegalensis* leaf significantly decreased the number of writhes in test animals. This study showed that alkaloids are the active analgesic component of *M. senegalensis* leaf (Adebayo *et al*., 2014). This finding is in concordance with previous studies on morphine alkaloid fraction of *Stephiaglabra* known as Gindarudine, which showed significant analgesic effect when tested by the same method (Turner, 2014). The significant analgesic and anti-inflammatory effects of alkaloid fractions of *M. senegalensis* root bark extracts *in vivo* is noteworthy. Plants with these added pharmacological phenomena in conjunction with antiplasmodial effects are better antimalarials than plants with the later potential only (Pascual *et al*., 2001; Semwal *et al*., 2011).

Serum biochemical parameters have been widely used as an indicator of pathological condition, toxicology or safety of a test substance, treatment outcome and general health status of animals (Yusuf *et al*., 2018a; Umar *et al*., 2019). Among these biochemical parameters, transaminases, alkaline phosphatases, proteins, lipid profile and electrolyte are the most widely employed in assessing the integrity of liver and kidney following plant extract administration to animals (Bashir *et al*., 2015). Alterations in the normal activities or concentrations of these biochemical parameters are conventional indicators of any of the following conditions; renal or nephrotic impairments, hepatocellular injury, cellular leakage, loss of functional integrity of cell membrane, biliary cirrhosis or liver hepatitis (Yusuf *et al*., 2018b). Consequently, the concentrations of triglyceride, sodium, potassium, chloride, alkaline phosphatase and glucose concentrations were not significantly ($p < 0.05$) altered by treatment with 75 and 150 mg/kg bw *M*. *senegalensis* alkaloidal fraction. This is an indication

that the functional integrity of kidney is well preserved and that the extract does not induced any form of pathological conditions to the kidney. The increases in transaminases (aspartate transaminase and alanine transaminase), and proteins concentration is an indication that the integrity of the liver is not well preserved. The extract might have interfered with the equilibrium in protein metabolism in favor of anabolism. Such drastic increase in protein levels could, negatively affect cellular shomeostasis and consequently affect the health of the animals (Lawal *et al*., 2016).

GCMS analysis of the total alkaloid fraction confirmed the presence of 3 different alkaloids; 20α)-3-hydroxy-2-oxo-24-nor-friedela-1(10),3,5,7-tetraen-carboxylic acid-(29)-methylester, 2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro- and 3-hydroxy-20(29)-lupen-28-ol and a terpenes (phytol) as the major constituents of the fraction. The antimalarial activities of these compounds have been previously documented (Khalid *et al*., 2007; Olofsson *et al*., 2014). In addition, phytol has also been reported for anti-inflammatory activities (Olofsson *et al*., 2014)

CHAPTER FIVE

5.0   CONCLUSION AND RECOMMENDATIONS

5.1   Conclusion

The alkaloidal fraction of *M*. *senegalensis* leaf exhibited promising antimalarial, analgesic and anti-inflammatory activity, it is relatively safe upon oral acute and sub-acute doses in mice, thus, could serve as a template for the synthesis of new drug. This study also justifies the use of *M. senegalensis* in traditional medicine as an antimalarial agent. As such, the plants alkaloidal fraction can be formulated into supplements to enable more people benefit from it and as well commercialize the drug since it is already being used for treatment albeit in the crude form.

5.2   Recommendations

i.   The *M. senegalensis* leaf fraction has shown promising anti-malaria, anti-inflamatory and analgesic activity, the next step would be to further identify and characterize the active alkaloid for structural elucidation. The mechanism by which this alkaloid exerts its effect can also be determined, this could be of help even in other areas of research and to formulate synthetic analogues of the compound.

ii.   Other phytochemical components of the plant can also be further analysed to ascertain the extent of their therapeutic activity. This might bring insight into the role of these components in the effect that the plant exerts in its various pharmacological roles.

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Anti-plasmodial Activity of Alkaloid Fraction of *M senegalensis* Leaf against *Plasmodium berghei* Infected Mice

	DOSES	Day1	Day2	Day3	Day4	Day5	Day6	Par a
MS	DOSES	60.36±1.85	66.25±0.71	70.25±1.60	64.25±0.96	65.25±1.01	78.25±0.95	78.2
	75	28.25±0.89	34.05±1.37	36.25±1.44	29.05±1.31	34.05±2.18	38.25±01.47	38.2
	150	30.05±1.59	34.05±1.52	25.05±1.99	26.25±1.10	32.25±1.55	26.05±1.53	26.0
CQ	300	15.05±0.91	18.36±0.95	26.25±1.80	31.25±1.18	16.25±0.91	20.05±2.17	20.0
NS	5	122.05±1.31	120.07±2.16	115±3.11	116.25±2.14	131.25±0.94	126.67±1.08	126.

Data are MEAN±SEM of five determinations. Values followed by different superscript are significantly different ($p<0.05$)

MS= *M senegalensis*

NS= Normal Saline

CQ = Chloroquine

APPENDIX B

Effect of Alkaloid Fractionof *M senegalensis* Leaf on PCV of *Plasmodium berghei* Infected

Mice

Extract	Doses	PCV (%) Of non-infected mice	PCV (%) Of mice 24 hours after infection	PCV (%) After24hours after treatment
Alkaloid	75	46.34±3.45[a]	39.76±3.45[a]	37.34±1.56 [ab]
Alkaloid	150	44.35±2.56[a]	36.34±6.46[a]	41.24±3.45 [b]
Alkaloids	300	45.43±4.32[a]	37.65±4.32[a]	43.34±2.46 [b]
CQ	5	48.64±4.33[a]	36.35±4.33[a]	43.24±2.56 [b]
NS	20 mL/kg	46.43±3.54[a]	39.87±3.54[a]	31.54±3.24 [a]

Values are mean ± SEM of 3 determinations. Values along the same row with different superscripts

are significantly different.

NS = Normal saline

CQ= Chloroquine

APPENDIX C

Effect of Alkaloid Fraction of *M senegalensis* Leaf on Oedema

Extract	Doses	20minutes after injection	40minutes after injection	60minutes after injection	80minutes after injection	100minutes after injection	120minutes after injection	Mean increase in paw

Alkaloid	75	0.32±0.05	0.38±0.02	0.38±0.03	0.36±0.02	0.38±0.04	0.38±0.04	0.37±0.04
Alkaloids	150	0.30±0.03	0.36±0.04	0.34±0.02	0.30±0.06	0.28±0.03	0.30±0.02	0.31±0.02
ASA	150	0.30±0.02	0.30±0.02	0.26±0.04	0.28±0.02	0.32±0.02	0.30±0.03	0.29±0.06
NS	20 mL/kg	0.80±0.04	0.80±0.03	0.76±0.05	0.78±0.03	0.82±0.05	0.80±0.05	0.79±0.03

Data are MEAN±SEM of five determinations. Values followed by different superscript are significantly different ($p < 0.05$)

ASA = Acetyl Salicylic Acid

NS = Normal Saline

APPENDIX D

Effect of Alkaloid Fraction of *M senegalensis* Leaf on Thermal Stimuli in Mice

Extract	Doses	0min	15min	30min	45min	60min	Mean	
Alkaloid	75	20.50±1.98	20.56±1.07	26.50±2.87	21.05±1.08	28.56±3.78	23.00±1.89	4
Alkaloids	150	19.55±0.98	22.51±2.87	18.56±1.09	26.55±0.89	27.56±4.87	22.40±0.97	4

ASA	150	9.00±0.25	8.56±1.09	11.05±0.05	10.21±0.56	13.32±0.99	10.20±0.78
NS	20 mL/kg	36.50±3.89	38.55±4.86	42.55±387	40.06±4.89	47.50±6.07	40.65±8.09

Values are mean ± SEM of 3 determinations. Values along the same row with different superscripts are significantly different.

ASA = Acetyl Salicylic Acid

NS = Normal Saline