IN VIVO ASSESSMENT OF ANTIPLASMODIAL ACTIVITIES OF METHANOLIC LEAF EXTRACT OF *Chromolaena odorata* IN MICE

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M.TECH/SSSE/2008/2021

DEPARTMENT OF BIOCHEMISTRY

FEDERAL UNIVERSITY OF TECHNOLOGY

MINNA

MAY, 2012

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THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL, FEDERAL UNIVERSITY OF TECHNOLOGY, MINNA, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF DEGREE OF MASTER OF TECHNOLOGY (M.TECH) IN BIOCHEMISTRY

MAY, 2012

DECLARATION

I hereby declare that this thesis titled: In vivo assessment of antiplasmodial activities of methanolic leaf extracts of ***Chromolaena odorata*** (siam weed) in mice is a collection of my original research work and it has not been presented for any other qualification anywhere. Information from other sources (published or unpublished) has been duly acknowledged.

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.....I. Ilumi. 15/06/2012.

Date/Signature

CERTIFICATION

The thesis titled: In vivo assessment of antiplasmodial activities of methanolic leaf extracts of ***Chromolaena odorata*** (saim weed) in mice by Ilumi, Josephine Yemisi, (M.TECH/SSSE/08/2021), meets the regulations governing the award of the degree of Master of Technology in Biochemistry, Federal University of Technology, Minna and it is approved for its contribution to scientific knowledge and literary presentation.

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ABSTRACT

The in*vivo* antiplasmodial activity of methanol leaf extract of *Chromolaena odorata* was evaluated in *Plasmodium berghei* infected mice. Different doses of the extract (200, 400, and 800mgkg) were administered to mice infected with *Plasmodium berghei.* The phytochemical components, acute toxicity and liver function test were analysed. The suppressive and curative effects against established infections as well as prophylactic activities were investigated. Combination of extract with *Morinda lucida* extract was also evaluated. Crude extract of the *Chromolaena odorata* as well as the combinated extracts were fractionated. The phytochemical screening showed the presence of flavonoids, alkaloids, saponins, tannins and phenolic nucleus. The median acute toxicity value (LD_{50}) of the extract was determined to be greater than 5000mg/kg body weight. Crude extract (200,400 and 800mg/kg) showed chemosuppression of 50.79, 55.23 and 67.24% respectively which was significantly different from the control and close to the standard drug, chloroqunine 5mg/kg day which showed 81.89%.The curative test of *Chromolaena odorata* (200,400 and 800mg/kg) showed a considerable mean survival days period of 16.5±1.44, 19.0±2.04 and 19.5 ± 0.96 respectively while the percentage prophylactic effect were 33.25 %, 49.7 % and 49.47 % respectively. The survival period of mice increased with the extract dose over the experimental dose range. Alkaline phosphatase and Glutamate oxaloacetate transaminase showed no significant difference in all the groups while a significant decrease in Gamma glutamyl transferase and glutamyl pyruvate transaminase was observed in all the treated group when compared with the infected and not treated. The combination therapy of *chromolaena odorata* and *mucida lucide* showed a significant curative effect with the survival day of 24.00±2.06 at 200mg/kg while the *Chromolaena odorata* alone at 800mg/kg survived 19.50±1.5546.All the fractions showed significant but varying levels of antiplasmodial activity. Fractions 4 and 5 which is the polar fraction showed the best antiplasmodial activity with the survival period of 26.00±1.2247 and 25.50 ±2.662 for combined extract of *Chromolaena odarata and Morinda lucida* and *Chromolaena odorata* alone respectively. These results show that *Chromolaena odorata* has great therapeutic potential as antiplasmodial agent especially when it is been combined with other plants, which can be used in the development of novel drugs.

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ABBREVIATIONS

WHO:	World Health Organisation
GDP:	Gross Domestic Products
UNICEF:	United Nations International Children's Emergency Fund
RBM:	Roll Back Malaria
P. vivax:	*Plasmodium vivax*
P. malariae:	*Plasmodium malariae*
P. ovale:	*Plasmodium ovale*
P. falciparum:	*Plasmodium falciparum*
DNA:	Deoxyribonucleic acid
RNA:	Ribonucleic acid
LDH:	Lactate dehydrogenase
NAD+:	Nicotinamide adenine dinucleotide (oxidized form)
NADH:	Nicotinamide adenine dinucleotide (reduced form)
ATP:	Adenine triphosphate
NADPH:	Nicotinamide adenine dinucleotide phosphate (reduced form)
TCA:	Tricarboxylic acid cycle
MSP:	Merozoite surface protein
CQ:	Chloroquine
ALP:	Alkaline phosphatase
GOT:	Glutamate oxaloacetate transaminase
GPT:	Glutamate pyruvate transaminase

GGT:	Gamma glutamyltransferase
C.O:	*Chromolaena odorata*
BR:	Biuret reagent
N.S:	Normal saline
TLC:	Thin Layer Chromatography
BSA:	Bovine Serum Albumin

CHAPTER ONE

1.0 INTRODUCTION

1.1 General Introduction

It is estimated that 80% of people living in developing countries are almost completely dependent on traditional medicinal practices for their primary health care needs. Higher plants are also known to be the main source of drug therapy in traditional medicine. While the scientific world races to find a new cure for the mosquito-borne disease, a child dies every 3 to 5 minutes of malaria, attributing to the 300 to 500 million clinical cases each year and a staggering 1.5 to 2.7 million deaths Breman, (2001). Malaria is one of the major tropical parasitic diseases responsible for significant morbidity and mortality especially among children and pregnant women. Efforts are now being directed towards obtaining drugs with different structural features, along with new strategies in malaria control and the recognition and validation of traditional medical practices.

According to Okochi *et al.*, (1999), one of the factors that draw back the rate of development of the health care system in Nigeria and probably other developing countries is the prohibitive high cost of importing drugs and producing new ones. Meanwhile as the levels of resistance to chloroquine and mefloquine continue to rise, the future for antimalarial treatment with existing drugs look increasingly bleak eFoley and Tilley, (1998). With this increasing level of chloroquine resistance and fears of toxicity and decreased efficacy of sulphadoxine-pyrimethamine, there is an urgent need for an affordable, effective and safe alternative to chloroquine. Medicinal plants, since times 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, 1991). Records of early civilization in all parts of the world revealed that a considerable number of drugs that are used in modern medicine were in used even in ancient times (Johnson, 2005). The use of medicinal herb in the treatment and prevention of diseases is attracting attention by scientists'worldwid (Sofowora, 1982).

Medicinal plants according to Johnson, (2005) are now widely used in the treatment of microbial infections because of the problem of resistance of modern antimicrobial drugs or agents. Examples of such plant include the extract of Neem tree leaf active against malaria and ring worm infections, (Beardely, 1996). One of the reasons that plants will continue to be of importance as source of drugs, despite the ease with which many drugs can be synthesized in the laboratory, is that traditional medicinal practices are often affordable and accessible to the vast rural population.

New antimalarial drugs and approaches to overcome parasite resistance are needed to deal with the expanding problem of drug resistance which continues to challenge malaria control efforts based on early diagnosis and treatments. Only a limited number of

antimalarial drugs are currently at an advanced stage of clinical development. In line with this, there is a renewed interest in plant products since the identification of sesquiterpene lactone artemisinin (quighaosu). An attractive option for poor countries is the exploitation of the possible therapeutic effects of their local herb sour local herbs.

1.2 Justification

Malaria is still the most dangerous parasitic infectious disease which causes millions of deaths every year. Recently it was estimated that 300 to 500 million cases each year cause 1.5 to 2.7 million deaths, more than 90% in children under 5 years of age in Africa (Good, 2001; Sachs and Malaney, 2002).

Malaria situation has deteriorated recently and mortality from malaria is probably increasing in the whole of sub-Saharan Africa. This is because of the drug resistance to most antimalarial drugs and insecticide resistance in mosquitoes but the main problem of malaria control at present is the antimalarial drug resistance especially of plasmodium falciparium, the most deadly malaria parasite (Kretti, 2009).

Medicinal plants have been used in the treatment and prevention of malaria in various parts of the world. Quinine extracted from the bark of the cinchona tree, was used as an antimalarial agent as early as 1632 (Baird *et al.*, 1996) and by the 19th century, it was still the only known antimalarial agent. Many plants have been used in the treatment of malaria such as *Psidium guajava, Enanthia chlorantha* and scientific information about the antimalarial activity of these plants is very limited. It is important, therefore, to investigate

the antimalarial activities of these medicinal plants in order to determine their potential as sources of new antimalarial agents.

Hence, there is need to conduct an investigation for useful plants such as *Chromolaena* ***odorata*** **(***ewe akintola***)** which is known to have medicinal uses. Extracts of *Chromolaena odorata* when applied to plasmodial parasites using mices would inhibit plasmodium, thereby forming the basis for derivation of potent, safe and affordable for malaria in sub suharan African.

1.3 Research Aim and Obiectives

Aim: To investigate the in vivo antiplasmodial activities of methanolic leaf extracts of *Chromolaena odorata* in mice

Objectives

i. To screen the plants for its toxicity
ii. To fractionate the crude extract and screen the fractions for its activities.
iii. To identify the active ingredients responsible for antiplasmodial effect
iv. To combine the plant with another plant for its efficacy.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History and Epidemiology of Malaria

Malaria, a mosquito-borne, protozoal disease, is older than recorded history, and probably plagued prehistoric man. The first record of a treatment for the disease dates from 1600 A.D. in Peru, and utilized the quinine-rich bark of the Cinchona tree. Scientifically, it is not a newly described disease. The French physician, an army doctor working in the military hospital in Algeria, Charles Louis Alphonse Laveran first identified the parasite inside the red blood cells of people suffering from malaria under the microscope in 1880. Thus he proposed that malaria is caused by a protozoan called *plasmodium.* A year later, Carlos Finlay, a Cuban doctor treating patients with yellow fever in Havana, provided strong evidence that mosquitoes were transmitting disease to and from human. Ronald Ross and Giovanni Grassi recognized the mosquito as the malaria vector in 1897 (Good, 2001).

However, despite enormous and diverse efforts to control this disease, malaria is among the top three most deadly communicable diseases and the most deadly tropical parasitic disease today. (Sachs and Malaney, 2002). Worldwide, great and varied efforts are being made to learn about this disease and to determine how to control it. The official malaria eradication program, run by the World Health Organization (WHO) was cancelled in the late 1960s because of growing difficulties, given that the complex and persistent nature of this disease became increasingly obvious. The discovery of the insecticide DDT in 1942, by Paul Müller the Nobel Prize Laureate in Medicine and its first use in Italy in 1944, made the idea of global eradication of malaria seem possible. Subsequently, widespread systematic

control measures such as spraying with DDT, coating marshes with paraffin (to kill Anopheles mosquito larvae), draining stagnant water, and the widespread use of nets and cheap, effective drugs such as chloroquine were implemented - with impressive results. Despite initial success, there was a complete failure to eradicate malaria in many countries due to a number of factors. Although technical difficulties such as mosquito and parasite drug resistance have played a part, the main failure to reduce the disease is probably due to social and political factors preventing efficient application of control measures.

Management strategies today include the development of vaccines and chemotherapeutic agents, vector control, insecticides, education, bed nets and insecticide treated bed nets. Resistance to drugs by both the mosquito and the parasite is a growing obstacle in the battle against malaria. Combination therapy has been shown to increase the efficacy of combining drugs (Toure *et al.*, 2003).

Though these efforts have been successful to varying degrees, approximately 500 million clinical cases of malaria are reported each year and mortality estimates range between 0.7 and 2.7 million. Most of these deaths are young children. In sub-Saharan Africa, where malaria mortality is highest, 90% of reported malaria-related deaths are children under the age of five (Gardner, 1999). Accuracy is impeded by the facts that most malarial deaths occur at home, many cases are misdiagnosed and functional microscopes are not available to most clinics in the area (Greenwood and Mutabingwa, 2002). Unfortunately, the disease burden is on the rise. Approximately 40% of the world's population lives in regions where malaria transmission is endemic, mainly tropical and sub-tropical regions (Aultman *et al.*, 2002).

2.1.1 Ecology of Malaria

Plasmodium, a parasite which belongs to the family *Plasmodiidae* (Levine, 1981) and order of *haemosporidia* is widely distributed all over the world. Malaria-generating species of p*lasmodium* are generally limited to tropical and sub-tropical location, because it requires warm, humid environnements for replication in the insect vector. Global warming and population migrations do have a bearing on *Plasmodium's* distribution. *Plasmodium falciparum* is the most widespread in tropical and sub-tropical areas. *Plasmodium ovale* is most prevalent in the west coast region of Africa. *Plasmodium malariae* has a widespread distribution area but is fairly scattered within this area. *Plasmodium vivax*, like *falciparum*, ranges over a wide area, but in relatively rare in African countries.

2.1.2 Symptoms of Malaria

The symptoms characteristic of malaria include flulike illness with fever, chills, muscle aches, and headache. Some patients develop nausea, vomiting, cough, and diarrhea. Cycles of chills, fever, and sweating that repeat every one, two, or three days are typical. There can sometimes be vomiting, diarrhea, coughing, and yellowing (jaundice) of the skin and whites of the eyes due to destruction of red blood cells and liver cells.

People with severe *P. falciparum* malaria can develop bleeding problems, shock, liver or kidney failure, central nervous system problems, coma, and can die from the infection or its complications. Cerebral malaria (coma, or altered mental status or seizures) can occur with severe *P. falciparum* infection. It is lethal if not treated quickly; even with treatment, about 15%-20% die.

2.1.3 Burden of Malaria in Africa

Malaria is a disease devastating millions of people each year and has been described for over a century. About 90% of all malaria deaths in the world today occur in Africa south of the Sahara and these occur mostly in young children. This is because the majority of infections in Africa are caused by *Plasmodium falciparum*, the most dangerous of the four human malaria parasites. An increase in population in malarious regions, compounded by weak public health systems in developing countries, climate changes (Hay *et al.*, 2002), new agriculture practices such as irrigation and dam construction. Sachs and Malaney, (2002), increased resistance to antimalarial treatments and insecticides (Bozdech *et al.*, 2003) and the complexity and flexibility of the genetics (Gardner *et al.*, 2002) have all contributed to the increase in malaria.

Poor people are at increased risk both of becoming infected with malaria and of becoming infected more frequently. Child mortality rates are known to be higher in poorer households and malaria is responsible for a substantial proportion of these deaths. In a demographic surveillance system in rural areas of the United Republic of Tanzania, under-5 mortality following acute fever (much of which would be expected to be due to malaria) was 39% higher in the poorest socioeconomic group than in the richest. (Akazili, 2002).

Malaria affects the lives of almost all people living in the area of Africa defined by the southern fringes of the Sahara Desert in the north, and latitude of about 28° in the south. Most people at risk of the disease live in areas of relatively stable malaria transmission infection are common and occur with sufficient frequency that some level of immunity develops. A smaller proportion of people live in areas where risk of malaria is more

seasonal and less predictable, because of either altitude or rainfall patterns. People living in the peripheral areas north or south of the main endemic area or bordering highland areas are vulnerable to highly seasonal transmission and to malaria epidemics (Mwageni, *et al.,* 2002). In areas of stable malaria transmission, very young children and pregnant women are the population groups at highest risk for malaria morbidity and mortality. Most children experience their first malaria infections during the first year or two of life, when they have not yet acquired adequate clinical immunity which makes these early years particularly dangerous. Adult women in areas of stable transmission have a high level of immunity, but this is impaired especially in the first pregnancy, with the result that risk of infection increases (Steketee, 2001).

Malaney and Sachs, (2001) hypothesize that this apparent correlation between poverty and malaria runs both ways. Poverty may increase the incidence of malaria; malaria may also increase the likelihood of poverty. Poor people are at increased risk both of becoming infected with malaria and of becoming infected more frequently. Child mortality rates are known to be higher in poorer households and malaria is responsible for a substantial proportion of these deaths. In a demographic surveillance system in rural areas of the United Republic of Tanzania, under-5 mortality following acute fever (much of which would be expected to be due to malaria) was 39% higher in the poorest socioeconomic group than in the richest (Mwageni *et al.*, 2002).

A survey in Zambia also found a substantially higher prevalence of malaria infection among the poorest population groups. Poor families live in dwellings that offer little

protection against mosquitoes and are less able to afford insecticide-treated nets. Poor people are also less likely to be able to pay either for effective malaria treatment or for transportation to a health facility capable of treating the disease. Both direct and indirect costs associated with a malaria episode represent a substantial burden on the poorer households. A study in northern Ghana found that, while the cost of malaria care was just 1% of the income of the rich, it was 34% of the income of poor households (Akazili, 2002). During the period 1975 to 1996, only 3 of 1,223 drugs developed were antimalarials Greenwood and Mutabingwa, (2002). The need for malarial drugs has long outstripped the supply. This can be explained, in part, by the fact that the large number of people suffering from malaria and other diseases of poverty are not customers Kettler and Marjanovic, (2004). They can not purchase new drugs and therefore do not exist as a viable market for a biotechnology company's investment

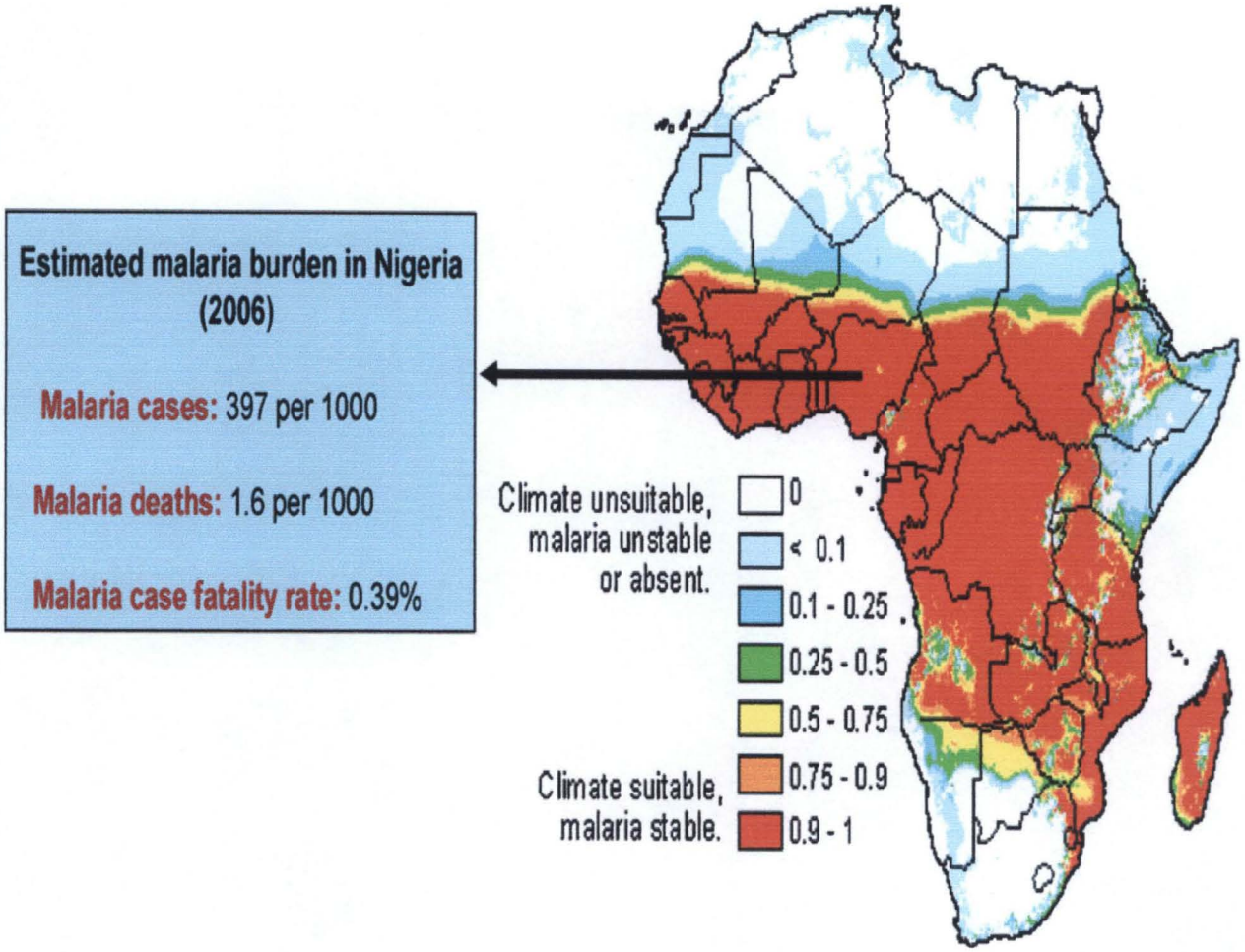


Figure 2.1 Human malaria distribution in Africa and its estimated burden in Nigeria.

Source: WHO, 2008a

2.2 The Parasite: *PLASMODIUM*

Human malaria results from infection with *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* or *Plasmodium malariae*. *Plasmodium falciparum* causes a large majority of the clinical cases and mortalities (Bozdech *et al.*, 2003). The protozoan *Plasmodium* is transmitted to humans by mosquitoes of the genus *Anopheles*. The mosquito picks up the parasite during a blood feeding from an animal with parasitaemia.The virulence patterns of malaria were being documented as early as the 1920s. Malaria, specifically the fevers induced by infection with *Plasmodium*, was used at the time as a treatment for syphilis. It was also noted that reproducible virulence patterns were seen with the same isolate and immunity to a specific strain of *one* species of *Plasmodium* was achieved by infecting previously naïve patients (Kyes *et al.*, 2001). Even through these most rudimentary types of experiments it was observed that immunity to a certain strain of *Plasmodium* was not equivalent to immunity to the disease malaria. More recent, controlled studies indicate that immunity to severe malaria is acquired more rapidly than immunity to mild malaria, especially in regions where transmission is high (Gupta *et al.*, 1999).

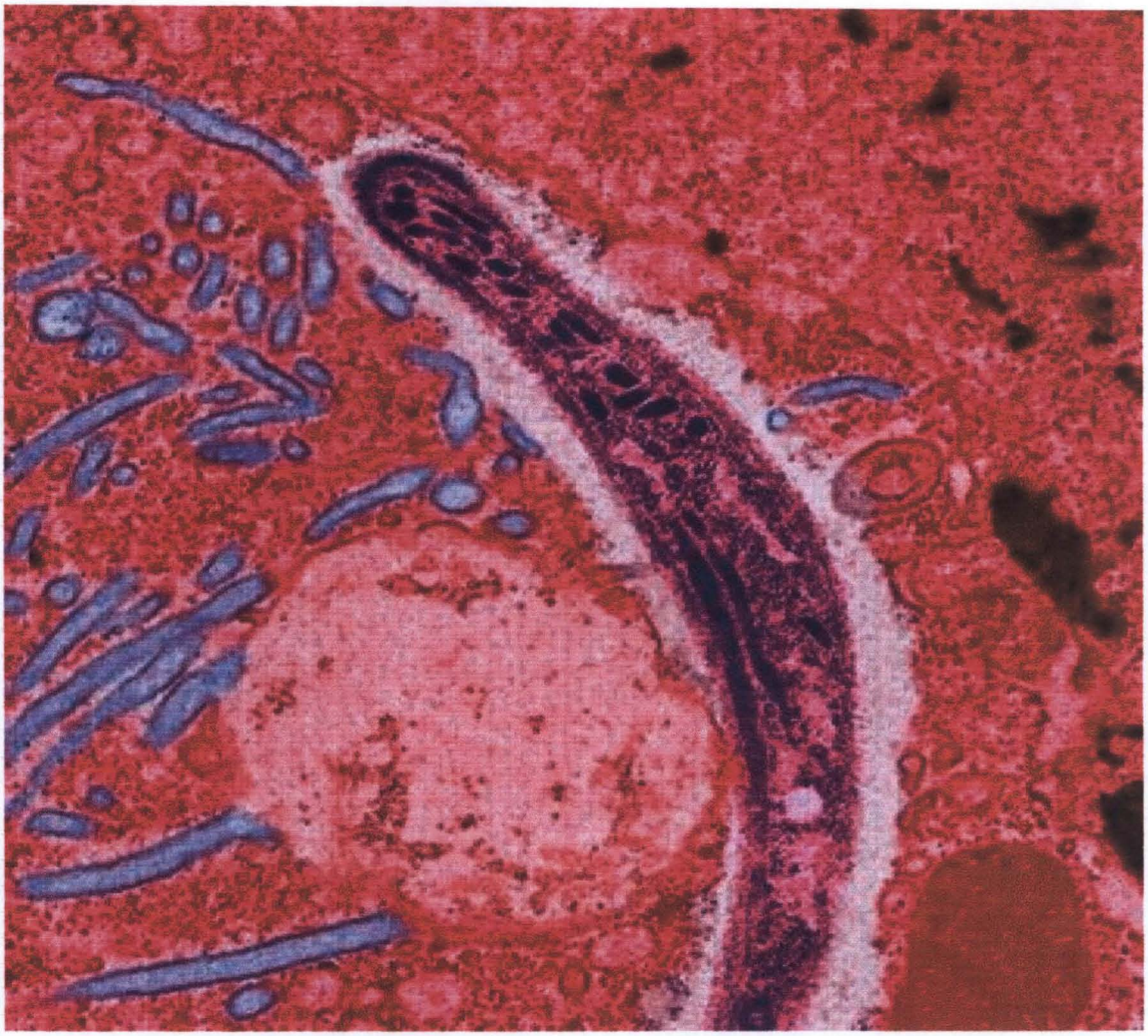


Figure 2.2 The structure of *Plasmodium*

Source: Public library of science journal / Wikimedia commons

2.2.1 Life Cycle of Plasmodium

Plasmodium parasites are protozoa of the phylum *Apicomplexa* (often referred to as sporozoans). These are animal parasites which exist in two hosts, have sexual and asexual stages, alternate between haploid and diploid phases and must be able to survive inside both hosts. The life cycle begins with the bite of an infected female anopheline mosquito. The mosquito takes her blood meal from the vertebrate (in this case a human) and injects both anticoagulant and haploid sporozoites into the human blood stream (Prescott *et al.*, 2002). The sporozoites quickly travel to the hepatocytes (liver cells), where they mature. In the hepatocyte the sporozoites undergo multiple asexual fissions, or schizogony, to produce thousands of infective, haploid merozoites (Kyes *et al.*, 2001). The merozoites are released into the blood stream and rapidly adhere to and invade host erythrocytes (red blood cells). These parasites have high metabolic demands and cannot survive in the compromised red blood cells of a human with sub-optimal oxygen carrying-capacity. At this point, the patient is still in the prepatent period and does not exhibit any clinical symptoms.

Inside the erythrocyte, the merozoites again begin to replicate and divide asexually. Each merozoite gives rise to 6-32 daughter merozoites (Kyes *et al.*, 2001) during 24-72 hours, depending on the species. Ultimately the infected erythrocytes lyse and merozoites are again released into the bloodstream and invade more cells. This cycle continues until the patient dies or the parasite slowed by either the host immune system or chemotherapeutic agents. The destruction of the erythrocyte and release of merozoites corresponds to the hallmark clinical presentation of the disease, the periodic fevers.

The other possible life cycle for the merozoites is differentiation into macrogametocytes and microgametocytes that do not destroy the erythrocyte they inhabit while in the human host. These are ingested by anopheline mosquitoes and develop into male and female gametocytes. Inside the gut of the mosquito the erythrocytes do lyse and the gametes meet and fuse into diploid zygotes (the ookinete). This is the only diploid stage during the life time of the *Plasmodium* parasite. The ookinetes develop into sporozoites and migrate to the salivary gland of the mosquito. The cycle is thus ready to begin again.

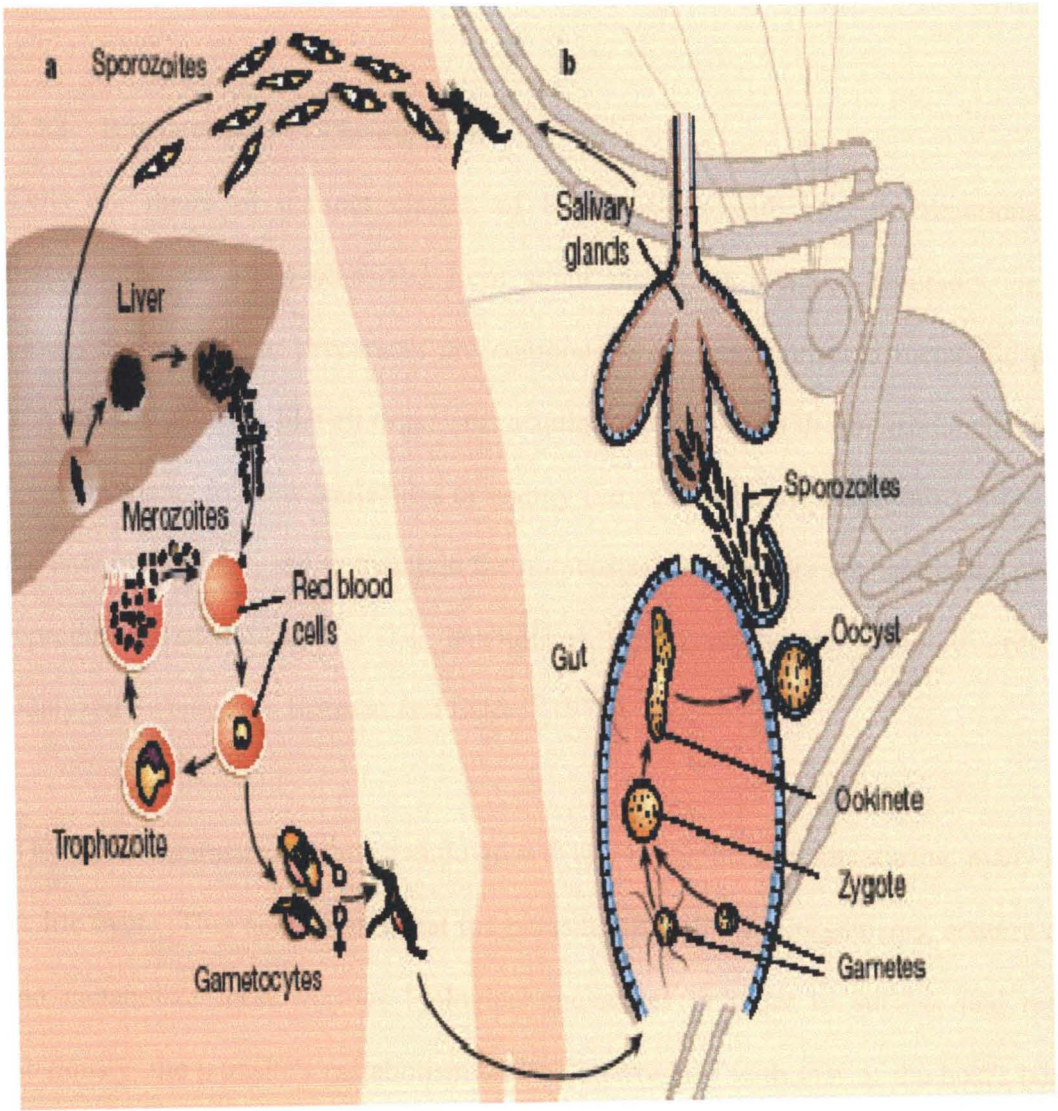


Figure 2.3: Life cycle of malaria parasite

Source: Wirth (2002)

The pentose phosphate pathway of erythrocytes provides ribose sugar which are used for nucleotide metabolism, provides for the regeneration of reduced NADPH to be used in biosynthesis or defense against reactive oxygen intermediates and prevent the accumulation of met haemoglobin.

In *plasmodium,* the pentose pathway is not present due to the absence of glucose -6-phosphate dehydrogenase the first enzyme of the pathway. The activity of the overall pentose pathway in malaria-infected erythrocytes appear to be low and this decreased activity in host cells would impair the function of the erythrocytes by promoting the oxidation of reduced glutathione and contribute to the formation of met haemoglogin Eckman and Eaton, (1979).

2.2.2.2 The Tricarboxylic Cycle

Aerobic metabolism involves further catabolism of pyruvate to carbondioxide and hydrogen atoms via the tricarboxylic acid (TCA) cycle. The hydrogen atoms are captured by the reduction of NAD^+ to NADH. The electrons from the captured hydrogen are then fed into a chain of electron carriers and ultimately transferred to molecular oxygen to form water. ATP is generated by capturing energy during electron transport by a process known as oxidative phosphorylation. The blood-stages of mammalian malaria parasites do not exhibit a complete TCA cycle. An explanation for this inefficiency is the abundance of glucose in the mammalian blood stream. In contrast, the parasite does appear to exhibit a TCA cycle in the glucose-poor environment of the mosquito host (Uyemura *et al,* 2000). The TCA cycle and oxidative phosphorylation are generally carried out in the mitochondria of eukaryotes. These processes are generally assumed to be non-functional in the blood-

stage parasite as evidenced by the acristae mitochondria. However, recently a functional electron transport chain and oxidative phosphorylation have been demonstrated in the blood-stage parasite. In addition, the parasite mitochondrion does have a membrane potential and cytochrome oxidase is present. The antimalarial drug atovaquone has been shown to inhibit electron transport and to collapse the mitochondrial membrane potential in malaria parasite. One possible function of the mitochondrion during the blood stage is for pyrimidine synthesis.(Uyemura *et al,* 2000)

2.2.2.3 Fatty Acid and Lipid Metabolism

Lipids are a major component of membranes. The rapidly growing parasite requires large amounts of lipids for this increase in parasite surface area and volume of internal membranes. This huge demand for lipids makes lipid metabolism an attractive target for anti-malarial drugs and several potential drugs targeting lipid metabolism have been identified Mitamura and Palacpac,(2003). Membrane lipids are composed of a glycerol (3-carbon unit) backbone which has a polar head group and two long chain fatty acids. Historically, the parasite has been considered to be incapable of synthesizing fatty acids de novo and restricted to obtaining preformed fatty acids and lipids from the host. However, several enzymes associated with the type II fatty acid synthesis pathway have been identified in *Plasmodium* and appear to be located in the apicoplast. This type II pathway is found in plants and prokaryotes, whereas the type I fatty acid synthetase is found in yeast and metazoan, Mitamura and Palacpac,(2003).

Apicoplast is non-photosynthetic plastid described in the Apicomplexa. This plastid is most likely of red alga origin and has a long evolutionary history within the apicomplexa.

apicomplexa. Possible functions associated with the apicoplast are biosynthesis of fatty acids, isoprenoid precursors, and heme. *Plasmodium* homologs of enzymes involved in type II fatty acid synthesis have apicoplast-targeting sequences and are sensitive to known inhibitors of type II fatty acid synthesis. Similarly, the synthesis of isoprenoids in *Plasmodium* also appears to involve enzymatic pathways that are found in bacteria and plastids and is distinct from the synthetic pathway found in eukaryotes. Both of these pathways are particularly attractive drug targets since the human host synthesizes fatty acids and isoprenoids via different pathways utilizing different enzymes.

Several parasite enzymes involved in lipid synthesis from glycerides and fatty acids, as well as enzymes involved in the remodeling of lipid polar head groups have been identified Mitamura and Palacpac, (2003).

2.2.2.4 Haemoglobin Degradation and the Food Vacuole

Proteins are composed of linear chains of amino acid which fold three dimensional structures and the malaria parasite requires amino acids for the synthesis of its proteins. The three sources of amino acids are: *de novo* synthesis, import from host plasma, and digestion of host haemoglobin. Haemoglobin is an extremely abundant protein in the erythrocyte cytoplasm and serves as the major source of amino acids for the parasite.

2.2.2.5 Ingestion of Host Cytoplasm

During the early ring stage, the parasite takes up the host cell stroma by pinocytosis resulting in double membrane vesicles. The inner membrane, which corresponds to the PVM, rapidly disappears and the digestion of hemoglobin takes place within these small vesicles during the early trophozoite stage. As the parasite matures, it develops a special organelle, called the cytostome, for the uptake of host cytoplasm and the small pigment-containing vesicles fuse to form a large food vacuole. (Gametocytes do not form the large food vacuole and are characterized by small pigment-containing vesicles found throughout their cytoplasm.) Double-membrane vesicles pinch off from the base of the cytostome and fuse with the food vacuole. The inner membrane (originally the PVM) is lysed and the haemoglobin is released into the food vacuole Goldberg, (2005).

2.2.2.6 Proteases and Food vacuole

The food vacuole is an acidic compartment (pH 5.0-5.4) that contains protease activities. Several distinct protease activities, representing three of the four major classes of proteases, have been identified in the food vacuole, these are plasmepsins, falcipains and facilysin. The digestion of haemoglobin probably occurs by a semi-ordered process involving the sequential action of different proteases Goldberg, (2005).

Plasmespsin genes have been identified in the genome of *P. falciparum* and four of these appear to function in the food vacuole Banerjee, (2002). Plasmepsin-1 and plasmepsin-2 are the best characterized and both are capable of cleaving undenatured haemoglobin between phenylalanine and leucine residues located at positions 33 and 34 on the alpha-

globin chains. These residues are located in a conserved domain known as the hinge region, which is believed to be crucial in stabilizing the overall structure of haemoglobin.

Falcilysin cannot digest either native hemoglobin or denatured globin, but readily cleaves the small polypeptide fragments (up to 20 amino acids) generated by the action of falcipain and plasmepsin. The site specify of falcilysin complements the plasmepsins and falcipains and leads to the formation of peptides 6-8 amino acids in length. Therefore, the digestion of haemoglobin is a semi-ordered process involving the initial degradation to large fragments followed by subsequent degradation to small peptide. The proposed pathway of haemoglobin digestion involves an initial cleavage by plasmepsin-1 (and possibly falcipain-2) followed by the combined actions of several plasmepsins and falcipains. The peptide fragments produced by these digestions are then digested into smaller peptides by falcilysin. Initially no food vacuole associated exopeptidase activity could be identified within the food vacuole (Kolakovich 1997). However, recently two amino peptidases have been found in the food vacuole Dalal and Klemba, (2007) which can convert the peptides into amino acids.

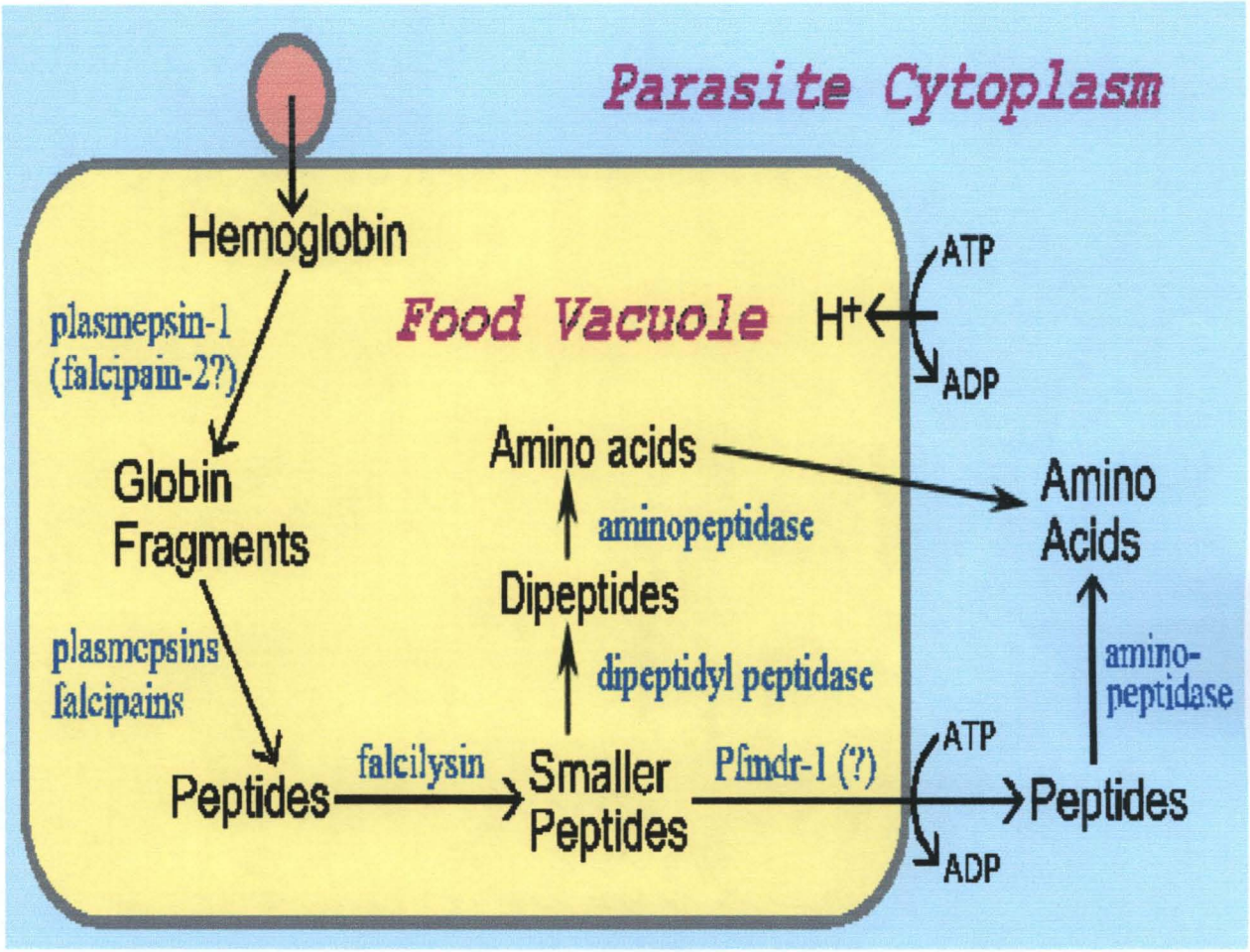


Figure 2.5 Haemoglobin degradation in food vacuole (Ginsburg,1999)

2.2.3. Sequestration

Red blood cells infected with Plasmodium falciparum display protein-rich 'knobs' on their outer surfaces, which cause the cells to adhere to one another and to capillary walls. This adhesion allows parasite-infected cells to remain 'sequestered' in particular organs rather than circulating freely in the bloodstream, helping the parasite to evade the host's immune system. Since almost all of the human deaths attributed to malaria are caused by P. falciparum—the only human-infecting Plasmodium species with the ability to sequester—it is widely believed that that sequestration plays a key role in cerebral malaria and other fatal complications of the disease (Ramasamy,1998). However, there are conflicting theories about how this occurs.

The traditional explanation for cerebral malaria is that sequestration, perhaps combined with the reduction in the deformability of red blood cells that occurs when the cells are infected with Plasmodium, leads to the blockage of capillaries in the brain, depriving the tissue of oxygen (Dondorp *et al.*, 2000). However, measurements made using Near Infrared Spectroscopy and Doppler sonography show that levels of blood flow in the brains of cerebral malaria patients are not abnormally low (Mendis and Carter,1995), and individuals who recover from cerebral malaria do not generally exhibit the permanent brain damage that is typically associated with acute oxygen deprivation. It has therefore been suggested that sequestered cells infected with *Plasmodium falciparum* harm the brain by causing an excessive immune reaction there, rather than by physically blocking capillaries. Nitric oxide (NO), a substance that is manufactured by macrophages to kill parasites but is also toxic to host cells at high concentrations, has been implicated in this damaging immune reaction. It has been reported that a toxin produced by P. falciparum can induce the NO-

synthesising enzyme iNOS in human endothelial cells, and iNOS has been found in samples of brain tissue taken during autopsies of cerebral malaria victims (Clark *et al.*, 1997).

In pregnant women, *P. falciparum* frequently sequesters in the placenta, where rich capillary beds and weakened immune responses create a hospitable environment for the parasite. Placental malaria can have harmful consequences for the foetus, disrupting its supply of oxygen and nutrients, and increasing the risk of premature delivery. (Menendez, 1995).

2.2.4 Anaemia

Plasmodium, like many parasites, can damage its host by causing anaemia—a reduction in the ability of the blood to transport oxygen, which leads to lethargy and (in very extreme cases) can be fatal. The decrease in red blood cell concentration that is responsible for malarial anaemia occurs both through an increase in the rate at which red blood cells are destroyed and a decrease in the rate at which new ones are produced (Menendez *et al.*, 2000). Plasmodium not only causes the rupture of parasitized red blood cells, but stimulates the activity of macrophages in the spleen, which then destroy both parasitized and unparasitized red blood cells. TNF-alpha and other cytokines produced during malaria depress the rate of erythropoiesis (the manufacture of new red blood cells, further contributing to anaemia (Menendez *et al.*, 2000). The health risks due to malaria-related anaemia are particularly severe in pregnant women, and there is some evidence that maternal anaemia is associated with anaemia in the foetus, which increases the risk of infant mortality (Menendez, 1995).

2.2.5 Physiological changes

Parasites can produce physiological abnormalities in their host, which may have harmful consequences throughout the body, not just in the tissues or organs in which the parasite is present. Malaria patients commonly exhibit hypoglycaemia—a particular problem in pregnant women, in whom hypoglycaemia may be a cause of low foetal birth weight (Menendez, 1995)—and metabolic acidosis, which is the cause of a significant number of malaria-related fatalities among young children. Mendis and Carter, (1995). Possible explanations for these physiological changes include anaerobic consumption of glucose by the parasites and hypoxia due to the blockage of capillaries by parasite-infected red blood cells. There is also strong evidence that the cytokines produced during malaria can cause hypoglycaemia and acidosis by inducing changes in the body's carbohydrate metabolism. Hypoglycaemia and acidosis can be induced in the absence of malaria by injecting TNF into animals, and in human malaria patients, TNF levels correlate with hypoglycaemia (Clark *et al.*, 1997), Clark and Cowden, (2003).

2.2.6 Molecular Biology of Malaria Parasite

Members of the genus *Plasmodium* are eukaryotic microbes. Therefore, the cell and molecular biology of *Plasmodium* will be similar to other eukaryotes. A unique feature of the malarial parasite is its intracellular lifestyle. Because of its intracellular location the parasite has an intimate relationship with its host cell which can be described at the cellular and molecular levels. In particular, the parasite must enter the host cell, and once inside, it modifies the host cell. The molecular and cellular biology of host-parasite interactions involved in these two processes will be discussed.

2.2.6.1 Host Cell Invasion

Malaria parasites are members of the Apicomplexa. Apicomplexa are characterized by a set of organelles found in some stages of the parasite's life cycle. These organelles, collectively known as apical organelles because of their localization at one end of the parasite, are involved in interactions between the parasite and host. In particular, the apical organelles have been implicated in the process of host cell invasion. In the case of *Plasmodium*, three distinct invasive forms have been identified: sporozoite, merozoite, and ookinete (Gratzer and Dluzewski, 1993).

2.2.6.2 Merozoites and Erythrocyte Invasion

Merozoites rapidly (approximately 20 seconds) and specifically enter erythrocytes. This specificity is manifested both for erythrocytes as the preferred host cell type and for a particular host species, thus implying receptor-ligand interactions. Erythrocyte invasion is a complicated process which is only partially understood at the molecular and cellular levels Gratzer and Dluzewski, (1993). Four distinct steps in the invasion process can be recognized which involved initial merozite binding, reorientation and erythrocyte deformation, junction formation and parasite entry.

2.2.6.3. Merozoite Surface Proteins and Host-Parasite Interactions

The initial interaction between the merozoite and the erythrocyte is probably a random collision and presumably involves reversible interactions between proteins on the merozoite surface and the host erythrocyte. Several merozoite surface proteins have been described. The best characterized is merozoite surface protein-1 (MSP-1). Circumstantial evidence implicating MSP-1 in erythrocyte invasion includes its uniform distribution over the merozoite surface and the observation that antibodies against MSP-1 inhibit invasion (Holder, 1994). In addition, MSP-1does bind to band 3 (Goel *et al.,* 2003). However, a role for MSP-1 in invasion has not been definitively demonstrated. Similarly, the circumsporozoite protein (CSP) probably plays a role in targeting sporozoites to hepatocytes by interacting with heparin sulfate proteoglycans Sinnis and Sim, (1997).

Another aspect of MSP-1 is the proteolytic processing that is coincident with merozoite maturation and invasion (Cooper, 1993). A primary processing occurs at the time of merozite maturation and results in the formation of several polypeptides held together in a non-covalent complex. A secondary processing occurs coincident with merozoite invasion at a site near the C-terminus. The non-covalent complex of MSP-1 polypeptide fragments is shed from the merozoite surface following proteolysis and only a small C-terminal fragment is carried into the erythrocyte. This loss of the MSP-1 complex may correlate

with the loss of the 'fuzzy' coat during merozoite invasion. The C-terminal fragment is attached to the merozoite surface by a GPI anchor and consists of two EGF-like modules. EGF-like modules are found in a variety of proteins and are usually implicated in protein-protein interactions. One possibility is that the secondary proteolytic processing functions to expose the EGF-like modules which strengthen the interactions between merozoite and erythrocyte.

2.2.6.4 Host Erythrocyte Modification

Once inside of the erythrocyte, the parasite undergoes a trophic phase followed by replicative phase. During this intraerythrocytic period, the parasite modifies the host to make it a more suitable habitat. For example, the erythrocyte membrane becomes more permeable to small molecular weight metabolites, presumably reflecting the needs of an actively growing parasite. Another modification of the host cell concerns the cytoadherence of *P. falciparum*-infected erythrocytes to endothelial cells and the resulting sequestration of the mature parasites in capillaries and post-capillary venules. This sequestration likely leads to microcirculatory alterations and metabolic dysfunctions which could be responsible for many of the manifestations of severe falciparum malaria. The cytoadherence to endothelial cells confers at least two advantages for the parasite which are a microaerophilic environment which is better suited for parasite metabolism, and avoidance of the spleen and subsequent destruction, Gratzer and Dluzewski, (1993).

2.2.7. Reorientation and Secretory Organelles

After the binding of the erythrocyte, parasite reorients itself so that the 'apical end' of the parasite is juxtaposed to the erythrocyte membrane. This merozoite reorientation also coincides with a transient erythrocyte deformation. Apical membrane antigen-1 (AMA-1) has been implicated in this reorientation. AMA-1 is a transmembrane protein localized at the apical end of the merozoite and binds erythrocytes. Antibodies against AMA-1 do not interfer with the initial contact between merozoite and erythrocyte thus suggesting that AMA-1 is not involved in merozoite attachement. But antibodies against AMA-1 prevent the reorientation of the merozoite and thereby block merozoite invasion (Mitchell *et al.*, 2004).

2.2.8 Antigenic Variation in Plasmodium

Plasmodium falciparum, the major etiologic agent of human malaria, has a very complex life cycle that occurs in both humans and mosquitoes. While in the human host, the parasite spends most of its life cycle within erythrocytes (in contrast to *T. brucei* which remains extracellular). As a result of its mainly intracellular niche, parasitized host cells which display parasite proteins must be modified to prevent destruction by the host immune defenses. In the case of *Plasmodium*, this is accomplished via the dual purpose. The encoding of the cytoadherence ligand by a highly polymorphic gene family presents a paradox in that receptor/ligand interactions are generally considered highly specific. Interestingly, selection for different cytoadherent phenotypes result in a concommitant change in the surface antigenic type (Biggs 1992). Similarly, examination of clonal parasite lines revealed that changes in the surface antigenic type correlated with differences in

binding to CD36 and ICAM1. For example, the parental line (A4) adhered equally well to CD36 and ICAM1, whereas one of the A4-derived clones (C28) exhibited a marked preference for CD36. Binding to ICAM1 was then reselected by panning the infected erythrocytes on ICAM1. All three parasite clones (A4, C28, C28-I) exhibited distinct antigenic types as demonstrated by agglutination with hyper-immune sera.

The expression of a particular PfEMP1 will result in a parasite with a distinct cytoadherent phenotype and this may also affect pathogenesis and disease outcome. For example, binding to ICAM-1 is usually implicated in cerebral pathology. Therefore, parasites expressing a PfEMP1 which binds to ICAM1 may be more likely to cause cerebral malaria. In fact, higher levels of transcription of particular *var* genes are found in cases of severe malaria as compared to uncomplicated malaria (Rottmann, 2006). Furthermore, placental malaria is frequently associated with higher levels of transcription of a particular var gene which binds CSA (Duffy, 2006). This tissue specific expression of particular *var* genes implies that different tissues are selecting out different parasite populations based on the particular PfEMP1 being expressed on the surface of the infected erythrocyte.

2.3 Disease Treatment

2.3.1 Quinine

Intravenous quinine is currently the most widely used agent in the treatment of severe falciparium malaria, usually formulated as a dihydrochloride salt. Although Quinidine has a twofold to threefold greater antimalarial activity than quinine. There are four artemisinin formulations : dihydroartemisinin, artesunate (a water-soluble compound for intramuscular injection). Artesunate and artemeter are metabolized to the biologically active metabolite dihyroartemisinin. Artemisinin derviatives are well tolerated. Side effects includes nausea, vomiting, fever and bleeding. Despite reports of brainstem neurotoxicity with high doses in animal studies, it has been observed in humans to date (Trampuz *et al.*, 2003).

2.3.2 Chloroqunine

Chloroqunine is a 4-Aminoquinoline that has marked and rapid schizonticidal activity against all infections of *P. malariae* and *P. ovale* and against chloroquine-sensitive infections of *P. falciparum* and *P. vivax*. It is also gametocytocidal against *P. vivax*, *P. malariae* and *P. ovale* as well as immature gametocytes (stages 1-3) of *P. falciparum*. It is not active against intrahepatic forms, and should therefore be used with primaquine to effect radical cure of *P. vivax*. and *P. ovale* Hatz, (2001). The use of chloroqunine as a single first-line drug treatment is now increasingly limited following the evolution of chloroquine-resistant *P. falciparum*, but chloroqunine remains the first-line drug of choice in most African countries south of the Sahara where acceptable clinical cure rates can be

obtained. In areas where it is still used as a first-line drug, persistent parasitaemia and lack of haematological recovery in children may be one of the early signs of chloroqunine resistance. Even if the frequency of clinical failures is acceptable in the general population, a more effective first-line treatment may be required for vulnerable groups such as young children and pregnant women. However, the possible desirability of giving different drugs to different population groups must be balanced against logistic and acceptability problems. In some areas chloroqunine use could potentially be extended by its combination with other antimalarial drugs, in order to take continuing advantage of its antipyretic and anti-inflammatory effect and for its action against vivax malaria (Phillips, *et al.*, 1996)

2.3.3 Amodiaquine

Amodiaquine is a 4-aminoquinoline antimalarial drug similar in structure and activity to chloroquine. Like chloroquine, it also possesses antipyretic and anti-inflammatory properties. The efficacy of amodiaquine in the treatment of chloroquine-resistant vivax malaria has not been adequately investigated although a report from Papua New Guinea showed that amodiaquine was more effective than chloroquine for this purpose . (Rieckman, 1990). Although global use of amodiaquine has declined owing to the reports of adverse reactions.

2.3.4 Artemisinin

Artemisinin *(qinghaosu)* is the antimalarial principle isolated by Chinese scientists from *Artemisia annua* L. It is a sesquiterpene lactone with a peroxide bridge linkage.

Artemisinin is poorly soluble in oils or water but the parent compound has yielded dihydroartemisinin, the oil-soluble derivatives artemether and arteether, and the more water-soluble derivatives sodium artesunate and artelinic acid. These derivatives have more potent blood schizonticidal activity than the parent compound and are the most rapidly effective antimalarial drugs known. They are used for the treatment of severe and uncomplicated malaria. Mclntosh and Ollijaro, (2000). They are not hypnozoiticidal but gametocytocidal activity has been observed (Price , *et al.*,1996)

The antimalarial activity of artemisinin and its derivatives is extremely rapid and most patients show clinical improvement within 1-3 days after treatment. However, the recrudescence rate is high when the drugs are used in monotherapy, depending on the drug dose administered, the duration of treatment and the severity of disease, but not at present on parasite resistance . (Hien,1994). So far there is no confirmed *in vivo* evidence of resistance of *P. falciparum* to artemisinin and its derivatives.. The results have indicated declining susceptibility of *P. falciparum* to artemisinin derivatives (Yang, *et al,* 1997). Artemether is an oil-soluble methyl ether derivative of dihydroartemisinin. As with artemisinin, it is effective against *P. falciparum* resistant to all other operationally used antimalarial drugs. As with artemisinin, when artemether is used for the treatment of uncomplicated *P. falciparum* malaria, it should always be administered in combination with another effective blood schizonticide to prevent recrudescence and delay the selection of resistant strains. Monotherapy with oral or intramuscular artemether with a dose of 1-4 mg/kg per day for 3-5 days results in an unacceptable rate of recrudescence (Karbwang, *et al*, 1994)).

Other drugs in the treatment of this disease includes Sulfadoxine/pyrimethamine, Primaquine , Mefloquine, Halofantrine and *Artemisinin-based combination therapy (ACT).*

2.4 Animal Plasmodium

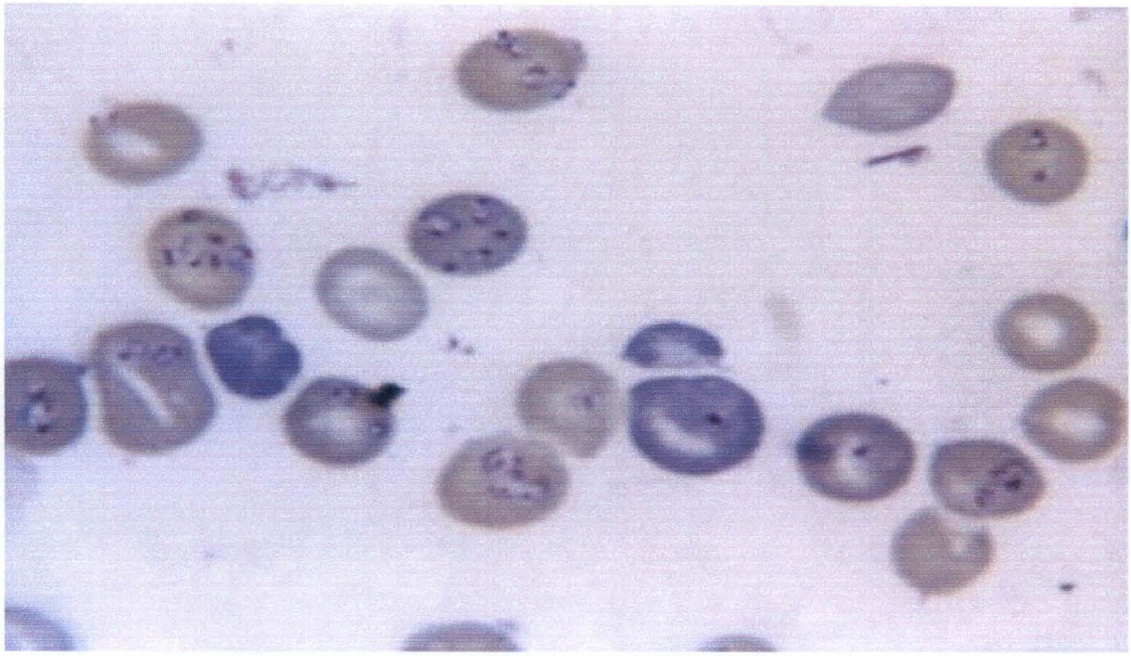
2.4.1 *Plasmodium berghei*

Plasmodium berghei is a unicellular parasite (protozoan) and it is one of the many species of malaria that infect mammals other than humans. It was first described by Vincke and Lips in 1948 in the Belgian C. *Plasmodium berghei* is one of the four *Plasmodium* species that have been described in African murine rodents and it is found in the forests of Central Africa, where its natural cyclic hosts are the thicket rat (*Grammomys surdaster*) and the mosquito (*Anopheles dureni*). The other three includes *Plasmodium chabaudi, Plasmodium vinckei* and *Plasmodium yoelii* (Vincke and Lips, 1948)

Malaria parasites of rodents are not of direct practical concern to man or domestic animals. The interest in these parasites is because they are used as practical model organisms in the laboratory for the experimental study of human malaria. Many research institutes used these rodent malaria parasite for studies aiming at the development of new drugs or a vaccine against malaria since they are recognised as valuable model organisms for the investigation of human malaria because they are similar in most essential aspects of morphology, physiology and life cycle and the manipulation of the complete lifecycle of these parasites, including mosquito infections, is simple and safe. (Janse *et al.*,2006). The use of this model malaria parasite has provided biologists and medical researchers with

more insight into: the interactions of malaria parasites with the immune system., The process of infection of the liver by malaria parasites, the cause of severe pathology, such as cerebral complications in malaria patients and the infection of the mosquito and transmission of the parasite by the mosquito.

Like all malaria parasites of mammals, including the four human malaria parasites, *P. berghei* is transmitted by *Anopheles* mosquitoes and it infects the liver after being injected into the bloodstream by a bite of an infected female mosquito. After a short period (a few days) of development and multiplication, these parasites leave the liver and invade erythrocytes (red blood cells). The multiplication of the parasite in the blood causes the pathology such as anaemia and damage of essential organs of the host such as lungs, liver, spleen. *P. berghei* infections may also affect the brain and can be the cause of cerebral complications in laboratory mice. These symptoms are to a certain degree comparable to symptoms of cerebral malaria in patients infected with the human malaria parasite *Plasmodium falciparum*. (Amino, *et al.*, 2005)



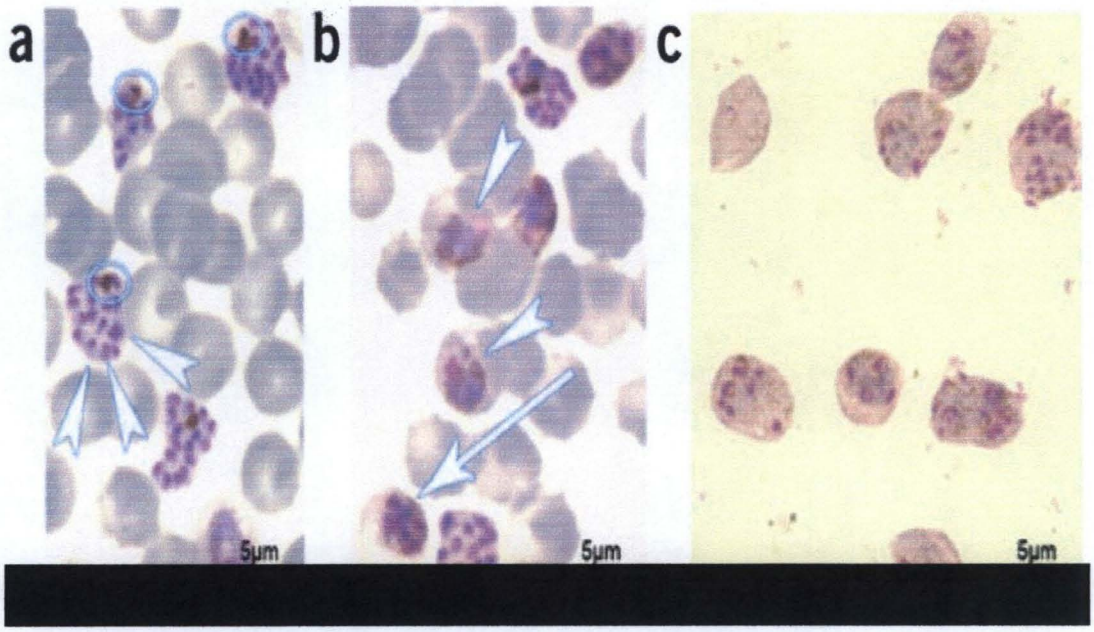


Figure 2.6 Structure of *plasmodium berghei* under the microscope

Source : Janse*et al.*, (2006)

2.5 Strategies of Treatment of *Plasmodium*

Considering all the side effects associated with the present Malaria treatment and with the problems of increasing levels of drug resistance , a vaccine proves to be the way forward but for decades the public health community has tried to devise a vaccine that would confer lifetime immunity against the malaria parasite and help stamp out disease. Yet the effort has always been an exercise in frustration. The complex life cycle of the parasite makes it challenging to know the best way to create an effective vaccine. But the advent of new funding and a spate of innovative ideas have changed the outlook dramatically in recent years. Vaccines for malaria are presently under development with no completely effective vaccine yet. Hoffman *et al.*,2002). The first promising studies demonstrating the potential for a malaria vaccine were performed in 1967 by immunizing mice with live, radiation attenuated sporozites, which provided about 605 protection of the mice upon subsequent injection with normal, viable sporozites (Nussenzweig, *et al.*,1967).

2.6 Traditional Medicines

Traditional medicines have been used to treat malaria for thousands of year and are the source of two main groups (artemisinin and quinine derivatives) of modern antimalarial drugs.Although herbs had been priced for their medicinal, flavouring and aromatic qualities for centuries, the synthetic products of the modern age surpassed their importance, for a while. However, the blind dependence on synthetics is over and people are returning to the naturals with hope of safety and security. Over three-quarters of the world population relies mainly on plants and plant extracts for health care. More than 30% of the entire plant species, at one time or otherwas used for medicinal purposes (Joy, *et al.*,2001).

Traditional systems of medicine continue to be widely practised on many accounts. Population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of several allopathic drugs and development of resistance to currently used drugs for infectious diseases have led to increased emphasis on the use of plant materials as a source of medicines for a wide variety of human ailments. According to WHO, 2001, around 21,000 plant species have the potential for being used as medicinal plants.

Medicinal plants have been used in the treatment and prevention of malaria in various parts of the world. Quinine extracted from the bark of the cinchona tree, was used as an antimalarial agent as early as 1632 (Baird *et al.*, 1996) and by the 19th century, it was still the only known antimalarial agent. Cinchona tree has been cultivated for this purpose all over the world. Primaquine and quinacrine were produced after the first World War. Chloroquine followed shortly thereafter in 1934. Thomson and Werbel, (1972), in 1946 it

was designated the drug of choice for treatment of malaria (Coatney, 1963). It is known as the cheapest, and drug of choice for malaria treatment in Nigeria.

2.6.1 Efficacy and Safety of Herbal Medicines

The use of herbal remedies is often justified bytheir long history of usage—from prehistoric time in some cases. But, age-old wisdom does not necessarily guarantee that the product in question is efficacious with reasonable specificity. The term "efficacious", however, has a relative meaning as it may be interpreted differently by the practitioners of traditional medicine (of which herbal medicine is a type) and the proponents of so-called modern medicine (conventional medicine). However, there are case reports of serious adverse events after administration of herbal products. In alot of cases, the toxicity has been traced to contaminants and adulteration. However, some of the plantsused in herbal medicines can also be highly toxic. As a whole, herbal medicines can have a risk of adverseeffects and drug–drug and drug–food interactions if not properly assessed. Often times, studies reported by researchers provide limited information on the methods used to prepare the remedies, making it difficult to replicate them. Only few studies provide data on side effects or postulate any likely side effect while some protect intellectual propertyrights deliberately. (Willcox, 1999). Few studies or controlled trials reported effects on biochemical variables (most commonly liver function tests), and hardly any study on monitored electrocardiograms or acute toxicity were reported. Minor side effects can, however, be important for example, some herbal antimalarials have a bitter taste, making it difficult to give them to children. Doses often need to be taken repeatedly, and the volume may be larger than with conventional drugs (Valecha *et al.*, 2000).

2.6.2 Some Nigerian Medicinal Plants used to treat Malaria

A larger number of medicinal plants are used to treat malaria in the Southern part of the Nigeria where rain forests exist and originate a humid tropical climate, with ideal conditions for persistent malaria transmission all year round. A review of studies into medicinal plants used to treat malaria across all ethnic and cultural groups in the country showed that there were more than 110 plants varieties.Some plants used in the treatment of malaria in Nigeria includes *Sphenocentrum jollyanum* (root) (Akerejupon in Yoruba), *Enatia chlorantha* (African yellow wood, Iyani or Awopa in Yoruba), *Eupatorium odoratum* (ogbogbo or Ibo- ofo in Yoruba), *Khaya grandifoliola (*Oganwo in Yoruba,Mahogany), *Senna siamea (*Kasia in Yoruba, Senna), *Tithonia diversifolia* Leave (Jogbo In Yoruba, Tree marigold) *Xylopia aethiopica* (Eeru), *Alstonia boonei* (Ahun in Yoruba and stood wood), *Funtumia africana* (Ako-ire in Yoruba and Funtumia) *Bridelia micrantha* (abere-aluko or akisan in Yoruba), *Cajanus Cajan* (Pigeon pea in English, olele in Edo, shingwazo in Gwari), *Carica papaya* (pawpaw) (Odugbemi et al., 2007).

In addition, were stem bark of plants such as Abrus precatorius (omisinmisin in Yoruba), Afzelia Africana (Mohogany bean tree), Psidium guajava (guava) Anacardium occidentale (cashew plant), Blighia sapida (Achee, Akee, Akee or Apple Tree), Alchornea cordifolia (baushe in Hausa, edo in Igbo and idi-odan in Yoruba). Also include were stems of Citrus sinensis (sweet orange), Cymbopogon giganteus (Oka eye in Yoruba), Khaya senegalensis (African mahogany, madachi in Hausa and ono in Igbo), Mangifera indica (mango) as well as onion bulbs and ginger to mention a few (Odugbemi et al., 2007).

Table 2.1: SOME MEDICINAL PLANTS THAT ARE USED IN COMBINATION

Combination of two plants	Combination of three plants	Combination of four plants	Group of five or more plants
A *Alstonia boonei* (bark) *Capsicum fructescens* (fruit) B Gossypium barbadense *(leaves)* *Citrus aurantium* (fruit) C *Vernonia amygdalina* (leaves) *Citrus aurantium* (fruit) D *Enantia Chlorantha* (bark) *Alstonia boonei* (bark) E *Chrysophyllum albidum* (leaves, bark) *Citrus aurantifolia* (leaves, fruit) F *Lecaniosdiscus cupanoides* (root) *Citrus aurantium* (fruit)	A *Chromolaena Odorata* (leaves) *Physallis angulata* (leaves) *Tithonia diversifolia* (leaves) B *Funtumia africana* (root) *Enantia chlorantha* (bark) *Zanthoxylum zanthoxyloides* (root) C *Gossypium barbadense* (leaves) *Ocimum gratissimum* (Leaves) *Citrus aurantium* (fruit) D *Enantia Chlorantha* (bark) *Alstonia boonei* (bark) *Diospyros mespiliformis* (bark) E ***Curcuma longa* (rhizome)** *Cymbopogon citratus* (leaves) *Citrus aurantifolia* (leaves)	A *Vernonia amygdalina* (leaves) *Ocimum gratissimum* (Leaves) *Azadirachta indica* (bark, leaves) *Cymbopogon citratus* (leaves) B *Canna indica* (leaves) *Citrus paradisi* (fruit) Ananas comosus *(fruit)* *Citrus aurantifolia* (fruit) C *Alstonia boonei* (bark) *Mangifera indica* (bark, leaves) *Psidium guajava* (leaves) *Carica papaya* (leaves)	A *Curcuma longa* (rhizome) *Harungana madagascariensis* (bark, leaves) *Rauvolfia vomitoria* (bark, leaves) *Mangifera indica* (bark, leaves) *Psidium guajava* (bark, leaves) *Enanthia chlorantha* (bark) B Sphenocentrum jollyanum *(root)* *Citrus aurantifolia* (twigs, leaves, fruit) *Cymbopogon citratus* (leaves) *Lawsonia guineensis* (leaves) *Carica papaya* (root) *Citrus aurantium* (fruit) C *Citrus aurantifolia* (leaves) *Chrysophyllum albidum* (leaves) *Mangifera indica* (bark, foliage leaves) Anarcadium occidentale (bark) *Sorghum bicolor* (leaves, stem) D *Ocimum gratissimum* (Leaves) *Anarcadium occidentale* (foliage leaves) *Lecaniodiscus cupanioides* (foliage leaves) *Curcuma longa* (foliage leaves)

Source: Odugbemi, *et al.*,2007

2.6.4 *Chromolaena odorata*

Taxonomic Position

Chromolaena was known as *Eupatorium odoratum* before the genus was split up by King and Robinson in the 1970s. *Eupatorium* contained over 1200 species and the genus *chromolaena* contained 129species all from South and Central Ameria and the West indies. Of these, *Chromolaena ivaefolia* and *Chromolaena laevigata* are widespread and occasionally weedy in the Americas, but only *Chromolaena odorata* has spread beyond the New World. Its common names include ewu akintolu, "ewu Awolowo", "Independence weed", siam weed, triffid weed, bitter bush or jack in the bush. Okon and Amalu, (2003) *Chromolaena odorata* belong to the family Asteraceae (Compositae). It is a native of Central and South America which has spread throughout the tropical and subtropical areas of the world. It is a perennial, diffuse and scrambling shrub which grows to 3-7m in height when growing in the open. It is now a major weed that is widespread in central and western Africa, tropical America, West India and Southeast Asia and western part in Nigeria. The economic value of *C. odorata* is low. (Ling *et al.*, 2007, Akinmoladun *et al.*, 2007, Phan *et al.*, 2001).

It suppresses neighbouring vegetation because of its allelopathic properities.Ambika and Jayachandra, (1980). In addition during dry season, the aerial stems dry up and readilt burn, but the strubbles remains alive and grow rapidily over the area in the succeding rainy season.

2.6.4.1 Natural Distribution of *Chromolaena odorata:*

The first African country to be affected was Nigeria, where the weed appeared in the 1940s. The original introduction was probably via contaminated seeds of Gmelina arboreal a fast-growing forestry tree from Ceylon. By the late 1960s, *C. odorata* was a major weed in Nigeria and since then, has spread to Ghana, Ivory Coast and Cameroon. *C. odorata* also appeared near Durban, in South Africa in the late 1940s, from where it spread till it is now a problem throughout the coastal region of Natal, and recently has been found inland in the Transvaal.

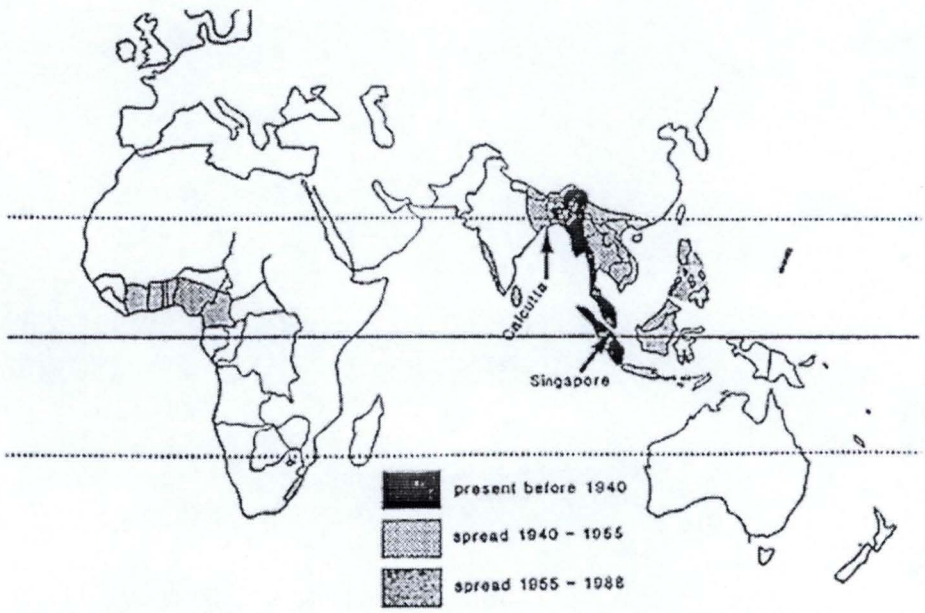


Figure 2.7 : Distribution of C*hromolaena odorata*

Source: Bennett and Rao (1968).

2.6.4.2 Chemical Constituents and Medicinal uses of *Chromolaena odorata*

Despite the negative sides to the plant, it still has patronage from practitioners of traditional medicine. *Chromolaena odorata*have been reported to be used in traditional medicine as an antispasmodic, antiprotozoal, antitrypanosomal, antibacterial, antifungal, antihypertensive, antiinflammatory, astringent, diuretic and hepatotropic agent (Iwu, 1993; Phan *et al.*, 2001; Akinmoladun *et al.*, 2007).

The phytochemical studies of Igboh, *et al*, 2009 reveals that the plant C*hromolaena odorata* contains alkaloids, cyanogenic glycosides, flavonoids, phytates, saponins. Also the chemical composition and bioactivity of the essential oil studies of *Chromolaena odorata* revealed that *Chromolaena odorata* contains alpha and ß-pinene, germacrene D, ß-copaen-4-alpha-ol and caryophyllene. They also screened the oil for its antimicrobial activity and was revealed that C.*odorata* showed antibacterial activity against *bacillus cereus* and antifungal activity against *Aspergillus niger* but these result was in contrast to those previously reported by Inya-Agha *et al*, 1987 and Bamba *et al*, 1993, who did observe activity against *S. aureus* , *E. coli* , and *P. aeruginosa.*

Idowu, *et* al,.2010,also reported that antimalarial activity was slightly significant in *Chromolaena odorata (aerial part)* but not toxic after an ethnobotanical survey carried out on plants used in curing malaria in Ogun State. The method used in preparing the herbal antimalarial remedies was either by boiling in water or aqueous extracts from fermented maize and steeping in the solvent. Also the treatment regimen was reported to include drinking, bathing and steam inhalation of the aqueous herbal prapation for 4-10days.

Chromolaena odoratum L. has been the subject of chemical examinations by several groups of researchers. The chemical group has been found is monoterpene, sesquiterpene hydrocarbons, triterpene/steroid, alkaloids and flavonoids. *Chromolaena odorata is* found to be a rich source of flavonoids, of which quercetin, sinensetin, sakuranetin, padmatin, kaempferol, salvagenin were isolated and identified. In traditional medicine, a decoction of the leaf is used as a cough remedy and as an ingredient with lemon grass and guava leaves for the treatment of malaria. Medicinally, the plant decoction is taken as a remedy for coughs and colds or in baths to treat skin diseases (Morton., 1981). The plant, locally called 'ewe akintola', is used in West African traditional medicine as a wound healing and a local antiseptic agent (Inya-Agha *et al.,*1987).

The diuretic activity for the infusions of *Chromolaena odorata* Linn at 10, 20 and 30 % was evaluated in albino rats. Urinary excretion of water, pH, density, conductivity and Na+, K+ and Cl- content were investigated in saline-loaded rats. The extract showed a dose-dependent decrease diuretic effect, but augmented significantly with respect to the control group for the urinary excretion of water and sodium. These results justify the use of *E. odoratum* as diuretic agent by the Malaysian traditional medicine, but furthermore, this species could be indicated for the urinary infections, through the so called "therapeutic washing" while simultaneously taking sufficient amounts of liquid.*Chromolaena odorata* essential oil has exhibited insecticidal (Bouda *et al.*1987), and antibacterial activities. (Bamba *et al.,* 1993) .

The plant is locally used in yourba land as malarial remedy. The crude ethanol extract of the plant had been demonstrated to be a powerful antioxidant to protect fibroblasts and keratinocytes *in vitro*. The results showed that the phenolic acids present (protocatechuic, *p*-hydroxybenzoic, *p*-coumaric, ferulic and vanillic acids) and complex mixtures of lipophilic flavonoid aglycones (flavanones, flavonols, flavones and chalcones) were major and powerful antioxidants to protect cultured skin cells against oxidative damage.(Phan *et al.*, 2001).

2.7 Medicinal uses of *Morinda lucida*

In West Africa *Morinda lucida* is an important plant in traditional medicine. In Nigeria, *Morinda lucida* is one of the 4 most used traditional medicines against fever. In Côte d'Ivoire a bark or leaf decoction is applied against jaundice and in DR Congo it is combined with a dressing of powdered root bark against itch and ringworm.

Morinda Lucida stem bark infusion is used as an antimalarial and antidiabetic (Burkill, 1997), Antimalarial activity (Koumaglo *et al.*, 1992), Anti-salmonella typhi activity effect on contractility of isolated uterine smooth muscle of pregnant and anti-diabetic property (Olajide *et al.*, 1999) of *Morinda Lucida* extracts have all been reported.

The petroleum ether ectract and fractions of the leaf samples of *Morinda Lucida* were evaluated for antimalarial effects against *Plasmodium falciparum* using the Rabbit in vivo technique (Awe and Makinde, 1998). It was observed that the extract and some fractions inhibited the maturation of a drug sensitive strain of *plasmodium falciparum*. Active anthraquinones were isolated from the plant, the most active being damnacanthal.

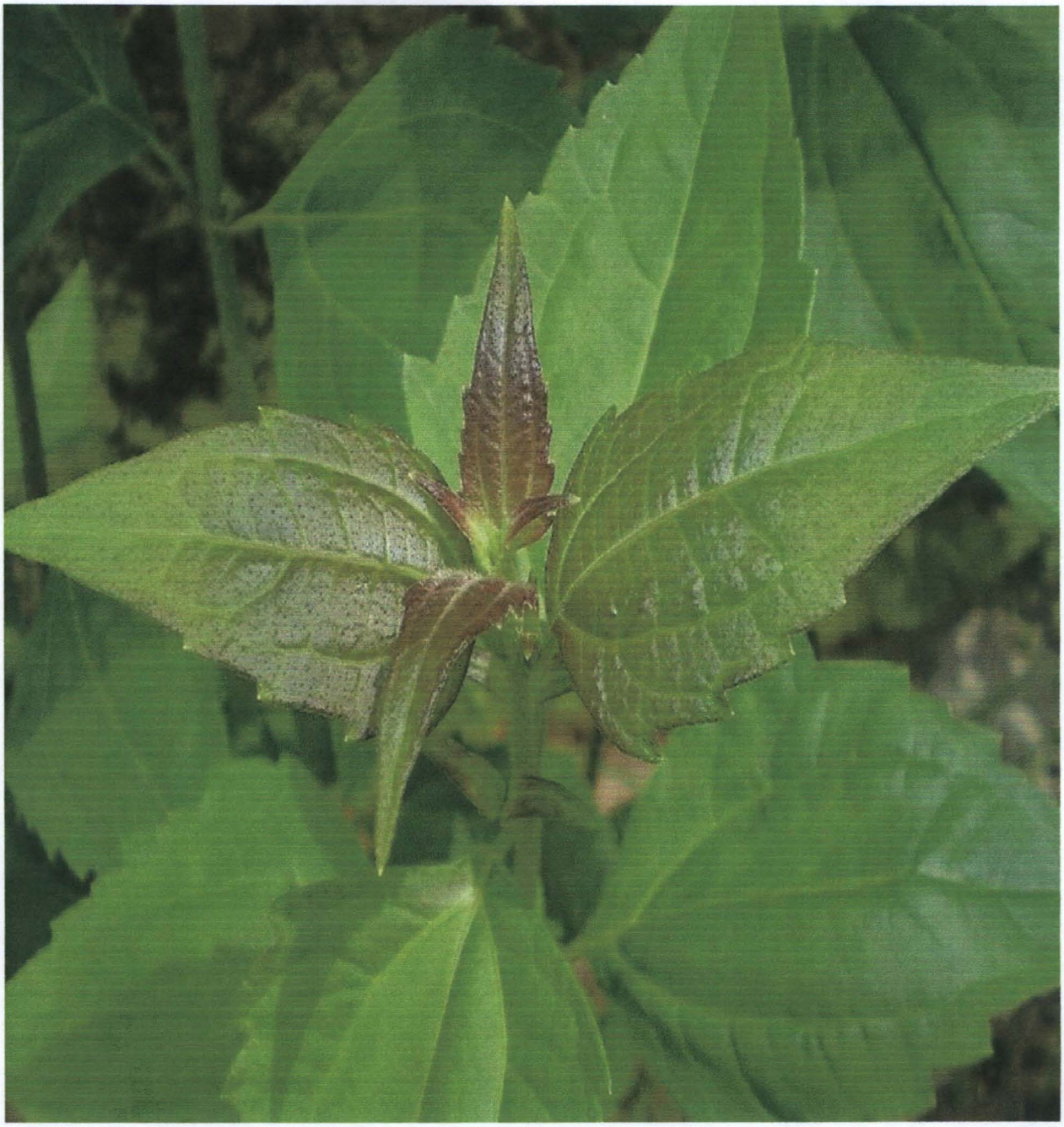


Figure 2.8: The picture ofChromolanae odorata

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 PlantSampleCollection

The fresh leaf of *Chromolaena odorata*(*ewu akintolu*)plant*s were* obtained from Federal College of Education, Okene, Kogi State (North Central) in the month of July, 2010 and was identified by Mrs Jamila Ibrahim, Department of Medicinal Plant Research and Traditional Medicine, National Institute for Pharmaceutical Research and Development, (NIPRD), Idu Abuja, Nigeria .

3.1.2 Experimental Animals and Malaria Parasite

Both sexes of albino mice (18-32g) were obtained from Department of Veterinary Medicine of Ahamdu Bello University, Zaria and National Veterinary Research Institute, Vom, near Jos. They were kept in aerated room and maintain on standard pelleted diet and water ad libitum during the whole period of the study. Chloroquine resistance*Plasmodium beighei berghei* (NK65 Strain) was sourced from the Department of Biochemistry, Ahmadu Bello University, Zaria and maintained in the Laboratory by serial passaging in mice.

3.1.3 Inoculums

Parasitized erythrocytes were obtained from a donor- infected mouse by cardiac puncture in heparin and made up to 20 ml with normal saline. Animals were inoculated intraperitoneally with infected blood suspension (0.2 ml) containing $1x10^7$ parasitized erythrocytes.

3.2 Methods

3.2.1 Plant Preparation and Extraction

The fresh leave of the plant were washed and air-dried at room temperature and polverized using mortar and pestles and later an electric blender. 70 g was extracted exhaustively for two hours using Soxlet extractor with 400ml of hexane, ethyl acetate and methanol respectively.

3.2.2 Phytochemical Screening of the Plant Extract

Phytochemical analysis to determine the presence of alkaloids, flavonoids, tannins, saponins, glycosides and carbohydrates was, carried out according to the method described by (Odebiyi and Sofowora, 1979; Trease and Evans, 1989). The tests for the different groups of phytochemicals are briefly described below:

3.2.2.1 Test for Saponins

A small quantity of the methanol crude extract was boiled. The mixture was filtered and 2.5ml of the filtrate was added to 10ml of the distilled water in the test tube. The test tube was shaken vigorously for 30sec and allows standing for 30second; them it was allowed to stand for 30minutes. A honeycomb forth was an indicator of the presence of saponins . (Sofowora,1993).

3.2.3 Acute Toxicity Studies

3.2.3.1 Determination of LD_{50}

The acute toxicity of the extract was evaluated by determining its median lethal dose (LD_{50})(Lorke1983).

Procedure

The test was carried out in two phases. In the first phase, Nine mice, randomly divided into three groups (A,B, and C) of three mice each, were given 10, 100 and 1000mg extract / kg/body weight orally respectively after 24hour of fasting. Signs of adverse effect and death were observed for 24hours. More specific doses of 1600, 2900 and 5000mg/kg were administered to another nine mice of three groups each orally and were also observed for twenty-four hour.

3.2.3.2 Effect of Extract on some Serum Biochemical Parameters

The test was carried out only on the serum and liver. Twelve mice were used for this analysis. The mice were divided into four groups of three mice each. The first group was infected with *plasmodium berghei* and was not treated, the second group was infected and given 800 mg/kg/day crude hot methanolic extract of *chromolaena odorata,* the third group was not infected but treated with 800mg/kg of the extract while the last group was given only normal saline without infected. All the Mice in each group were treated for five day and after they were sacrificed.

3.2.3.3 Sample Collection, Preparation and Biochemical assay

Collection of sample for biochemical analyses was as described previously (Yakubu, *et al.*, 2005). Mice were anaesthetized in slight chloroform and blood sample collected into a clean, dry centrifuge tubes. The blood sample was allowed to stand for 10minutes at room temperature and then centrifuged at 1000rpm for 15minutes. The supernatant (serum) was carefully removed with Pasteur pipette and stored in fridge until needed for further analysis. The liver were weighed, excised and transferred into 0.25M sucrose solution. 1g of the liver was homogenized in 4ml of ice cold 0.25 sucrose solution using mortar and pestle. Then transferred into clean centrifuge tube and centrifuged at 1000rpm for 10minute too. The supernatant were also stored in the fridge. The total protein concentrations of serum and Liver were determined using biuret method (Gornall *et al.*, 1946). Serum and Liver collection as well as Enzyme assays were carried out using AGAPPE Diagnostic kit, Switzerland GmbH.

3.2.3.4 Determination of Total Protein

Principle; Compounds containing two or more peptide linkages, complex with cupric ions to give a purple coloured complex, the intensity of the colour varies with the concentration of the peptide linkages.

Protein Standard

BSA	0.00	0.10	0.20	0.30	0.40	0.50	0.60	0.70	0.80	0.90	1.00
H_20(ml)1		0.90	0.80	0.70	0.60	0.50	0.40	0.30	0.20	0.10	0.00
BR(ml)4		4	4	4	4	4	4	4	4	4	4
ABS	0.00	0.01	0.02	0.04	0.04	0.06	0.07	0.08	0.09	0.10	0.11

BSA – Bovine Serum Albumin

BR- Buret reagent

Wavelenght used – 580nm

After a graph of absorbance against BSA was plotted. 0.1ml of each of the sample with 0.9ml of distilled water and 4ml of buriet reagent was added together in a test tube. This was allowed to stand for 30minutes and the absorbance taken. The total protein for each sample was extrapolated from the standard graph plotted.

3.2.3.5 Alkaline Phosphatase

Alkaline phosphatase was assayed as described by Schlebusch, *et al* (1974) using the AGAPE kit containing alkaline phosphatase (R1) and (R2)

The working reagent was prepared by mixing 4 volumes of reagent 1 (R1) with 1 volume of reagent 2 (R2). 1000µl of the working reagent was mixed with 20µl of the serum,

incubate for one minute and measure the change in absorbance per minute during three minute.

Principle

Paranitrophenyl phosphate $+H_2O$ $\xrightarrow{ALP}$ p-nitrophenol + inorganic phosphate

Note

Wavelenght used	405nm
Linearity	700U/L
Blank	distilled water

3.2.3.6 Gamma Glutamyl Transferase

The working reagent was prepared by mixing Four volume of reagent 1 (R1) with one volume of R2. 1000 µl of the working reagent was mixed with 100 µl of the serum, incubated for one minute and change in absorbance was read for three minutes.

Principle

GLUPA-C + Glycglycine $\xrightarrow{Gamma\ GT}$ L-Gamma-Glutamy-GlycLglycine + 5-Amino-2-nitrobenzoic acidGLUPA-C: L-Gamma-Glutamyl -3-carboxyl-p-nitroanilide

Note

Wavelenght used	405nm
Factor	1158
Linearity	232U/L

3.2.3.7 Serum Glutamate Oxoloacetate Transaminase

The working reagent was prepared by mixing Four volume of reagent 1 (R1) with one volume of R2. 1000 µl of the working reagent was mixed with 100 µl of the serum, incubated for one minute and change in absorbance was read for three minutes.

Principle

Kinetic determination of Aspartate Aminotransferase (AST)

L-Aspartate + α – ketoglutarate $\xrightarrow{\text{AST}}$ Oxaloacetate + L-glutamate

Oxaloacetate + NADH + H^+ $\xrightarrow{\text{MDH}}$ L- Malate + NAD^+

MDH- Malate dehydrogenase

Note

Wavelenght used	340nm
Factor	1768
Linearity	350U/L

3.2.3.8 Serum Glutamate Pyruvate Transaminase

Principle

Kinetic determination of Alanine Aminotransaminase (ALT)

L- Alanine + α-Ketoglutatrate $\xrightarrow{\text{ALT}}$ Pyruvate + Glutamate

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

Procedure

The working reagent was prepared by mixing Four volume of reagent 1 (R1) with one volume of R2. 1000 µl of the working reagent was mixed with 100 µl of the serum, incubated for one minute and change in absorbance was read for three minutes.

Note

Wavelenght	340nm
Factor	1768
Blank	Distilled water
Linearity	350U/L

3.4 *Invivo* Antiplasmodial Study

3.4.1 Four day Suppressive Test

This is a procedure whereby treatment with the extract is started immediately after the mice have been inoculated (Knight and Peters , 1980).

Procedure

A total of twenty mice were used for the study. Each mouse was given standard intra-peritoneal inoculums of 1.0 $x10^7$ *Plasmodium berghei* parasites with the aid of a 1ml disposable syringe. The animals were divided into five groups of four each. Different doses of 200, 400 and 800mg/Kg/ day) were administered orally to these groups.

Chloroquine (5mg/Kg/day) was given as positive control and 0.2ml of normal saline as negative control for four consecutive days. (D_o to D_3). On the fifth day (D_4), thick blood and thin blood films (fixed with methanol) were made from tail blood, stained with 10% Giemsa stain for 10minutes and washed off with a phosphate buffer (pH 7.0). The slides were allowed to dry and examined microscopically with 100-x magnification to access the parasitaemia. The percentage suppression of parasitaemia was calculated for each dose level by comparing the parasitaemia in infected controls with those of the treated mice (Knight and peter, 1980). Variation in weight was monitored in the course of the study. The mean survival time for each group was determined arithmetically by finding the average survival time (days) of the mice (post-inoculation) in each group.

administered with 5mg/kg/day of chloroquine and 0.2ml/day of normal saline orally. The mice were dosed for four consecutive days

(Do-D_3). On the fifth day (D4), the mice were inoculated with plasmodium berghei infected red blood cells. Seventy two (72) hours later, the parasitaemia level was assessed by studying giemsa stained blood smears (Peters,1965, Okokon *et al.,2005*).

$$\text{Precentage prophylactic} = \frac{\text{average parasitaemia in control} - \text{Average parasitaemia in test}}{\text{Average parasitaemia in control}}$$

3.2.4.4 *In vivo* Antiplasmodial test on Combination of Extracts

Antiplasmodial test was carried out on combination of crude methanol root extract of *morinda lucida* and leaf extract of *chromolaena odorata*, in the ratio 1:2.

A total of twenty mice were used for this study. On the first day (D_0), standard inoculums of $1x10^7$ *P. berghei berghei* infected red blood cells were injected intraperitoneally. Seventy-two hours later, the mice were divided into five groups of four mice each. Three groups were administered a combination of hot methanolic root extract of *morinda lucida* and hot methanolic leaf extract of *chromolaena odorata*, in the ratio 1:2, with doses 50, 100 and 200 mg/kg/day. Chloroqunine (5mg/kg/day) was given to the positive control group and 0.2 mL of normal saline to the negative control group. The extract was given once daily for 5 days. Thin blood smears were prepared from tail of each mouse for 5 days to monitor the parasitaemia level. The mean survival time for each group was determined arithmetically by finding the average survival time (days) of the mice (post-inoculation) in each group over a

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Results

4.1.1 Antiplasmodial Activity of Crude Methanol Extract of *Chromolaena odorata* during four day Suppressive test

Different doses of the crude methanolic extract were administered orally to mice infected with *Plasmodium berghei*. The four days suppressive test of the crude extract showed a chemosuppression of 50.79, 55.23 and 67.24% for the different doses (200, 400, 800mg/kg) respectively. There were significant difference ($P<0.05$) in the chemosuppression when compared with the control and it is comparable to the Standard drug (Chloroqunine) which was 81.89%. (Table 4.1,) Mice treated with the extract (200, 400 and 800mg/kg) survived 18.4±0.9273, 20.00±1.3784 and 23.600±1.1225 respectively while the standard drug (Chloroqunine) survived 24.800±1.9339 (Figure 4.1).

Table 4.1 Antiplasmodial activity of crude methanolic extract of *Chromolaena odorata* during four days Suppressive test.

Drug/Extract	Dose (mg/kg/day)	Average % Parasitaemia	Average %suppression
Normal saline	0.2ml	5.08± 0.7067	–
C. *odorata*	200	2.50 ± 0.010*	50.79±5.9130
	400	2.16 ± 0.051*	55.23±4.0776
	800	1.56 ± 0.093*	67.24±4.3315
Chloroquine	5	0.92 ± 0.733*	81.89±2.8835

Data are expressed as mean ± SE for five mice per group.*($P < 0.05$) when compared to control

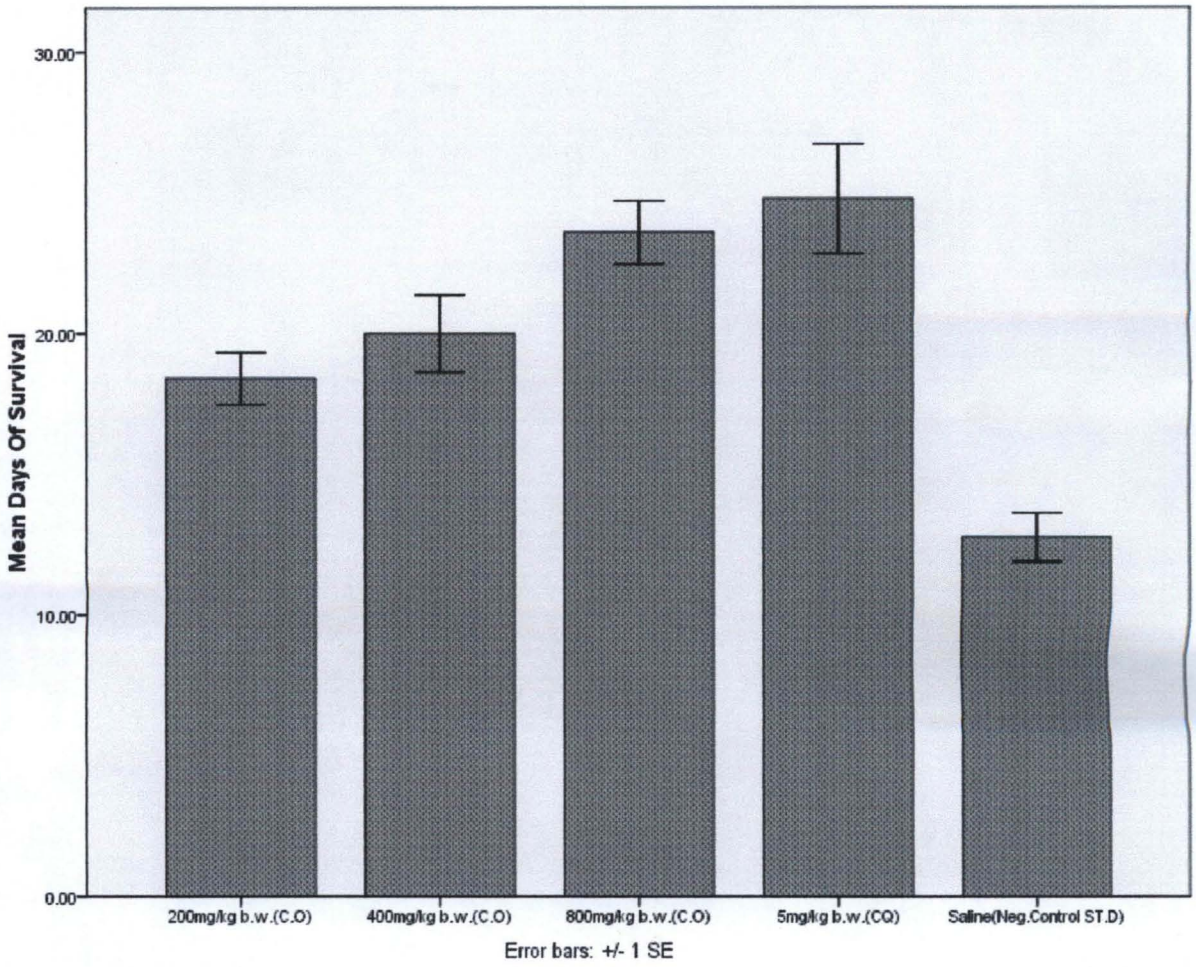


Figure 4.1 Mean Survival period of mice treated with crude Methanol extract of *Chromolaena odorata during* suppressive test

4.1.2 Antiplasmodial Activity of Crude Methanol Extract of *Chromolaena odorata and* Combine Extracts of *Chromolaena odorata* (leaf) with *Morinda lucida* (root) during the Curative test

During the curative test, it was observed that there was a daily increase in the parasitaemia level of the control group and daily reduction in the parasitaemia level of the groups treated with the extract alone, the combination and in the positive control (Standard drugs).

Comparing the parasitaemia at different doses of the crude extract of *chromolana odorata*, there were no significant difference ($P > 0.05$) observed on day 1 and day 2 of treatment in the infected mice administered 200, 400 and 800mg/kg when compared with the control but on days 3,4, and 5, a significant difference ($P<0.05$) were observed (Figure 4.2).

The mean survival period of the groups treated with the extract was significant ($P< 0.05$) compared to the control. All the control died within 12days whereas the infected mice treated with the extract survived for 16 to 24 days, though they were not clear of the parasites (Table 4.2)..

Mice treated with the combined extracts (50,100 and 200mg/Kg) showed significant difference ($P<0.05$) when compared with the control (Figure 4.3). The mean survival period of the mice treated with the combined extracts are 10.25±1.25, 12.25±2.02, and 24.00±2.06 for the doses of 50, 100 and 200mg/kg/day respectively (Table 4.3).

The packed cell volume (PCV) and the weight were also observed. The weight of mice treated with the extract (200, 400 and 800mg/kg) before inoculation and after treatment were significantly not different (Figure 4.4). No significant difference was observed in the PCV of mice before innoculation within the groups but after five days of treatment, a significant difference was observed with the group treated with 200mg/kg (Figure 4.5).

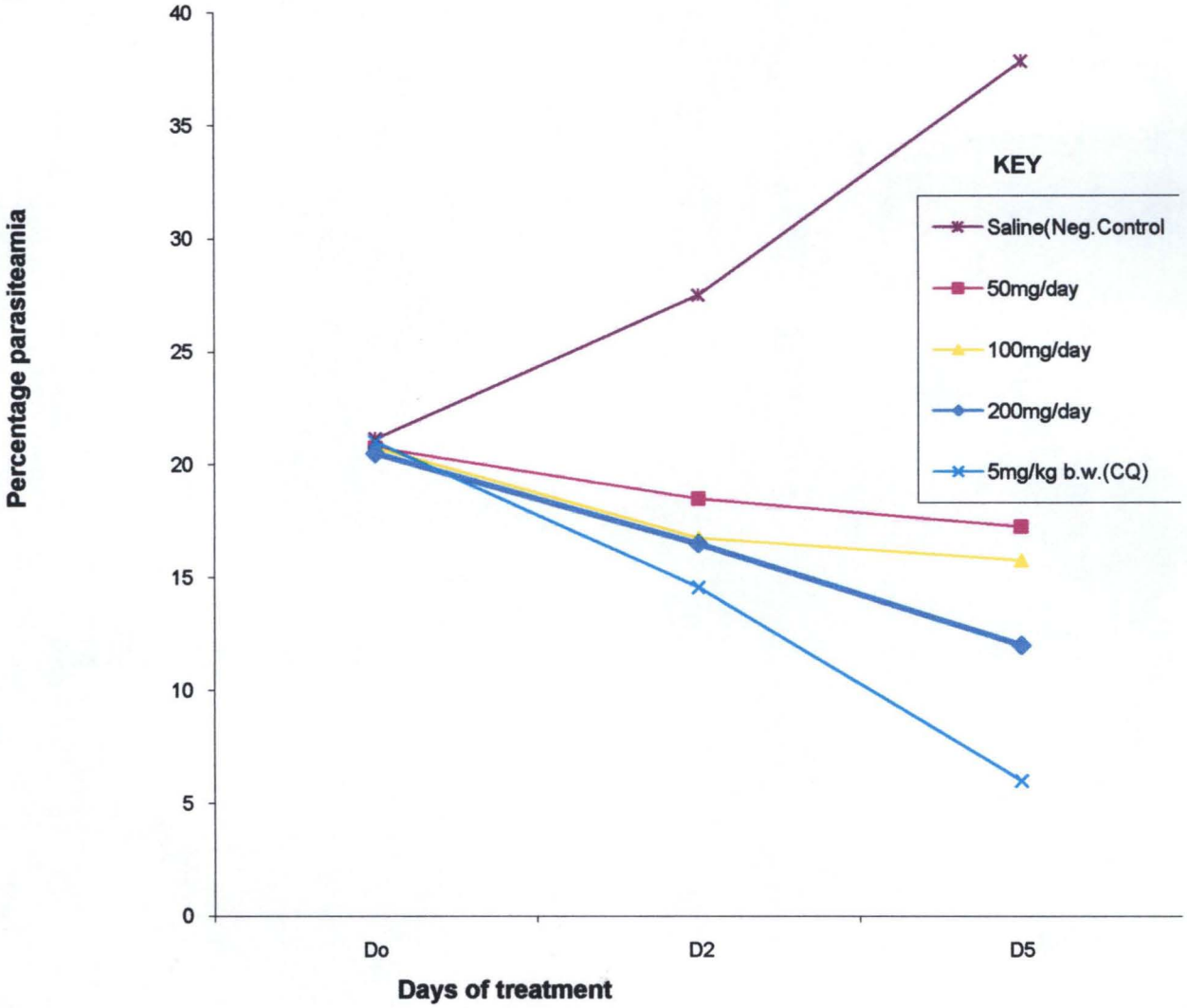


Figure 4.3: Antiplasmodial activity of combination therapy of *Morinda lucida* and *Chromolaena odorata*

Table 4.2 Survival of *Plasmodium berghe*i Infected Mice during the Curative test of the Crude Methanol Extract of *Chromolaena odorata*

Dose of extract (mg/Kg/day)	Mean Survival time (day)
0	8.750 ± 1.2550
200	16.500 ± 1.4434*
400	19.000 ± 2.0412*
800	19.500 ± 1.5546*
5(CQ)	26.250± 1.3769*

Data are expressed as ± standard error of mean for four mice per group (* $P<0.05$) when compared with the control.

Table 4.3 Survival of *Plasmodial berghei* infected mice during the Combination Therapy of *Morinda lucida* and *Chromolaena odorata*

Dose of extract (mg/kg/day)	Mean survival time (days)
0	5.000 ± 0.41
50	10.25± 1.25*
100	12.25± 2.02*
200	24.00 ± 2.06*
5CQ	28.00± 0.00*

Data are expressed as mean ± standard error for four mice per group (*P<0.05) when compared with the control

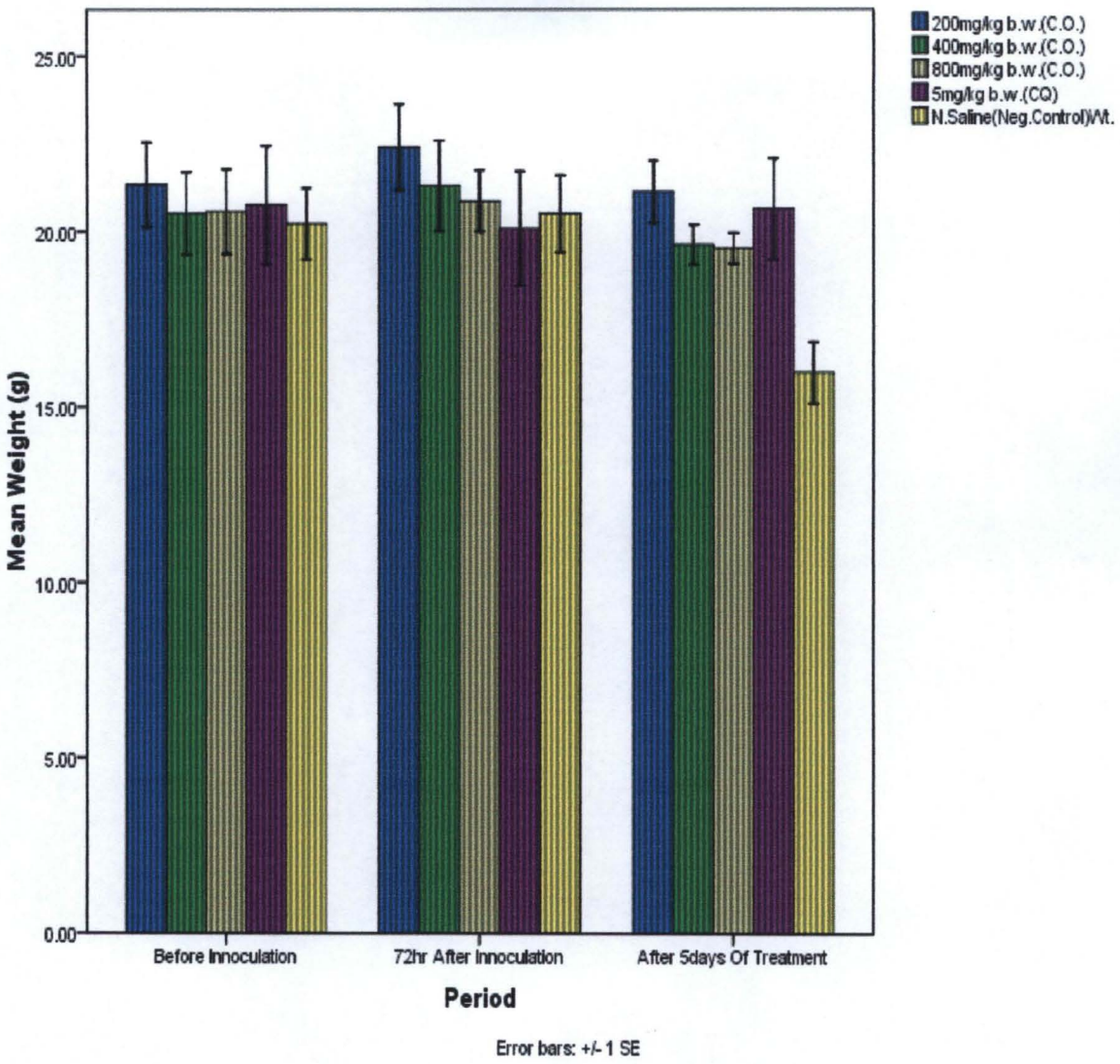


Figure 4.4 Average weight of mice treated with *chromolaena odorata* during curative test

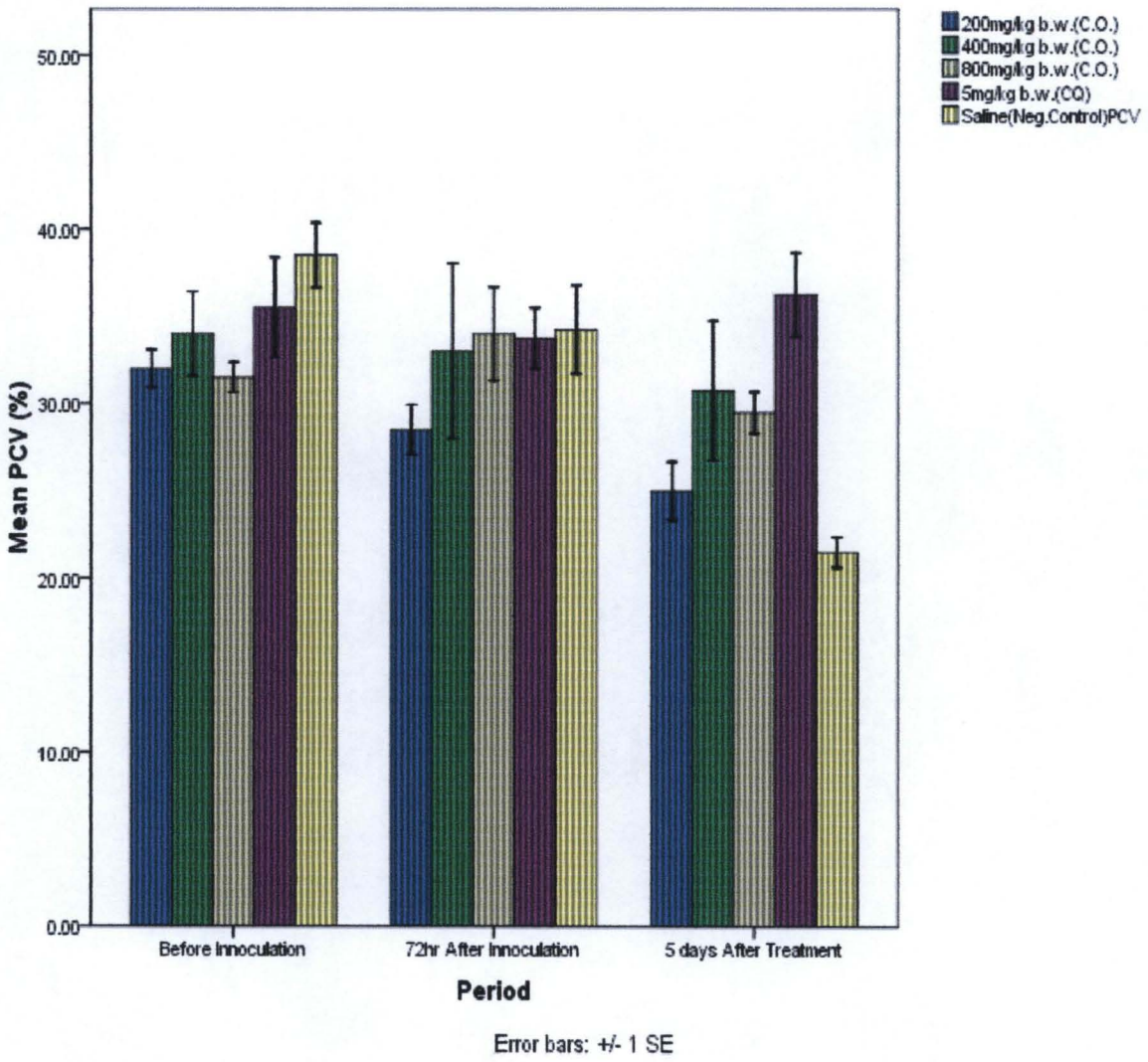


Figure 4.5 Average PCV of mice treated with *chromolaena odorata* (alone)during curative test

Table 4.4 Average Packed Cell Volume (PCV) of infected Mice treated with Combination Therapy of *Chromolaena odorata* (leaf) and *Morinda lucida* (root)

Dose Mg/Kg/bw	Days after infection		
	Before inoculation	3	7
0	46.25±2.6260	34.00±5.1960	27.55±1.056
50	49.75±4.871	39.25±5.089	35.75±2.529*
100	39.75±0.250	39.00±4.223	37.75±3.065*
200	47.75±2.750	46.00±6.164	44.75±2.25*
5CQ	52.50±3.069	34.75±3.092	44.75±8.25*

Data are expressed as mean ± standard error for four mice per group (*P< 0.05) when compared with the control

Table 4.5 Average Weight of Infected mice treated with Combination Therapy of *Chromolaena Odorata* (leaf) and *Morinda lucida* (root)

Dose Mg/Kg/bw	Days after infection		
	Before inoculation	3	7
0	24.52±0.278	23.23±0.720	19.35±1.547
50	25.25 ±0.202	24.72±0.481	21.22±1.361*
100	24.30±1.531	23.11±1.679	20.49±1.5488*
200	27.84±0.9615	27.84±1.126	25.29±1.686*
5CQ	27.00±1.450	25.82±1.442	26.70±1.196*

Data are expressed as mean ± standard error for four mice per group

*means there was significant different ($P<0.05$) when compared with the control

4.2.3 Prophylactic activity of infected mice treated with crude methanol extract of *Chromolaena odorata*

The extract exhibited a high prophylactic effect after 72hours of infection at the three different doses 200, 400, and 800 of 33.25 %, 49.7 % and 49.47 % respectively (Figure 4.5) which was comparable to the standard drug (CQ). The average weight and the PCV were also observed and there were no significant different after the 72hours of infection but there was significant increased in the weight of mice treated with the Standard drug after 72hours of innoculation. (Table 4.6 and 4.7)

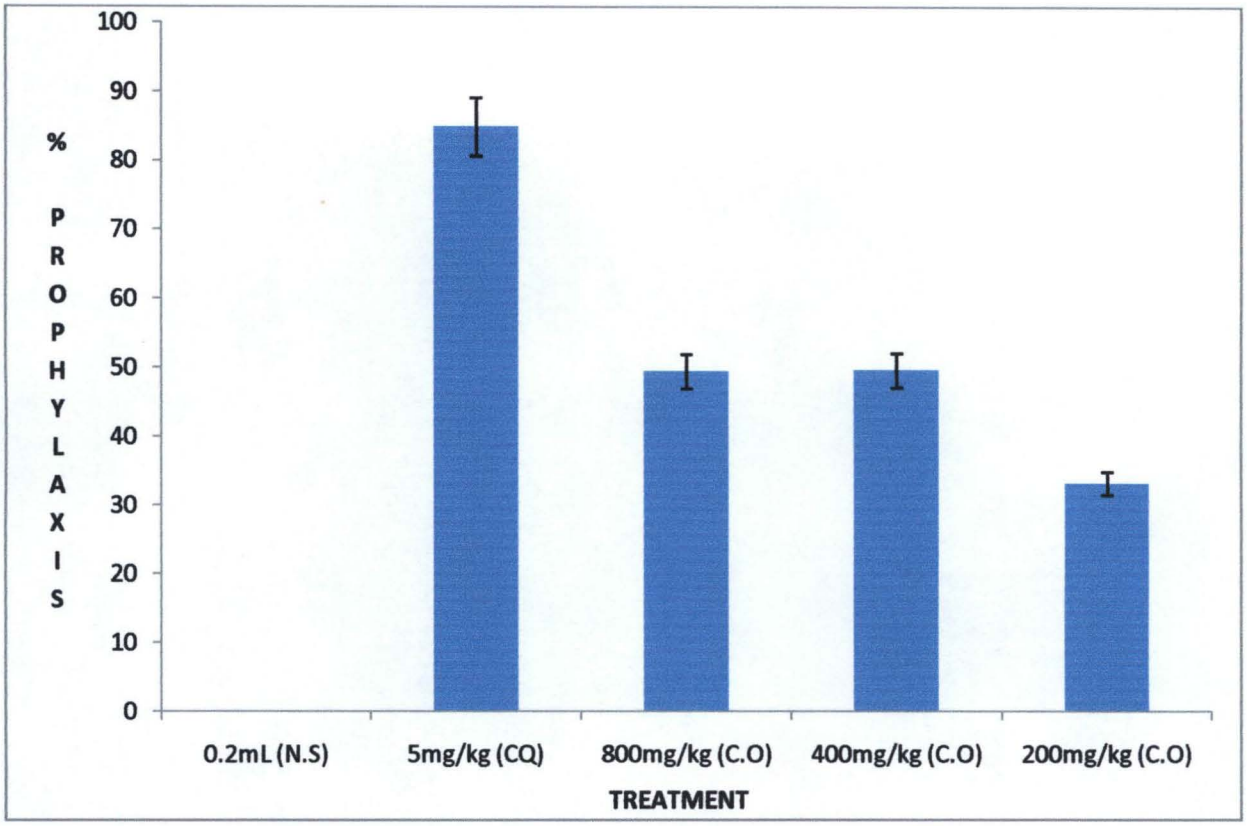


Figure 4.6 Percentage Prophylaxis of the crude methanol Extract of *Chromolaena odorata*with Chloroquine as Positive Control and Normasaline (Nms) as Negative Control on Parasitaemiain mice

Table 4.6 Average Weight (g) of Infected Mice treated with Crude Methanolic Extract of *Chromolaena odorata* (Prophylactic test)

Dose Mg/Kg/bw	Days after infection		
	Before treatment	After treatment	72hrs after innoculation
0	26.6±0.1683	27.15±1.0712	24.74.±0.636**
200	24.63±0.6540	25.60±0.9970	23.54±1.034*
400	26.42±1.5987	27.88±1.4100	25.34±1.3350*
800	26.54±1.5070	26.63±1.6540	26.29±1.3200*
5CQ	26.47±1.6430	28.88±1.3550	30.26±1.5030**

Data are mean± standard error for four mice per group

(*P>0.05) when compared before treatment

(**P< 0.05) when compared before treatment

Table 4.7 Average Packed Cell Vollume of infected mice treated with crude methanolic extract of *Chromolaena odorata* (Prophylactic test)

Dose Mg/Kg/bw	Days after infection		
	Before treatment	After treatment	72hrs after innoculation
0	36.25±1.0310[a]	38.00±1.4720	27.25.±3.9025*
200	37.50±1.5550[a]	37.25±2.3230	38.25±3.5440**
400	41.75±2.4960[a]	41.25±3.1980	48.75±1.4930**
800	35.75±1.9310[a]	36.00±3.2914	46.00±6.9160**
5CQ	30.25±1.8428[a]	33.25±1.7910	43.00±4.1028**

Data are mean± standard error for four mice per group

Data with the same superscript were significantly not different when compared with infected but not treated (control) at $P > 0.05$ before the treatment.

Data with ** superscript were significantly different when compared with infected but not treated at $P<0.05$ after treatment

Table 4.8 Days of Survival of Mice Treated with *Chromolaena odorata* during the Prophylactic test

Dose of Extract (mg/kg/day)	Mean survival time (days
0	9.00±0.8165
200	15.00±2.6771[a]
400	15.75±2.4632[a]
800	13.00±1.6330[a]
5CQ	20.54±2.6509[a]

Data are expressed as mean ± standard error for four mice per group ([a]P <0.05) when compared with control

.4.1.4 Antiplasmodial activity of partially purified methanol extract of *Chromolaena odoarata* and partially purified combination therapy of *Chromolaena odorata and Morinda lucida*

From the spots on thin layer plate (Plates ii, iii, iv), the 18 eluents which were gotten from the Column chromatography were pulled together to obtain five fractions, on the basis of those with similar Refractive index (1, 2 and 3, 4-6, 7-13 and 14-18). Each fraction was tested for its activity. 800mg/kg of each of fraction was given to each group. None of the fractions were able to clear the parasite from the infected mice during the period of study. Fraction 1 shows just slight activity with the survival time of 8days. Mice treated with fraction 2 and 3 survived between 16 to 18 day. Fraction 4and 5 showed the best activity. The mean surviving days of the fractions were 24 to 28 day. 25% of the mice treated with fraction 5 survived beyond 30days but later died after about 33days. (Figure 4.7 and Table 4.9)

Also five fractions were obtained in the combination therapy of *C.odorata* and *M.lucida*. 200mg/kg of each of the fraction was give to each group. All the fractions showed significant reduction in the parasitized level of infected mice treated. Mice treated with fractions 2 and 5 survived for 16days while fractions 1,3 and 4 survived 21, 19 and 23 days respectively. One of the mouse treated with fraction 4 survived above 30 days but later die. Fraction 4 showed more activity when compared with other fractions. The fractions did not provide a complete protection as all the treated animals eventually died from infection (Figure 4.8 and Table 4.10)

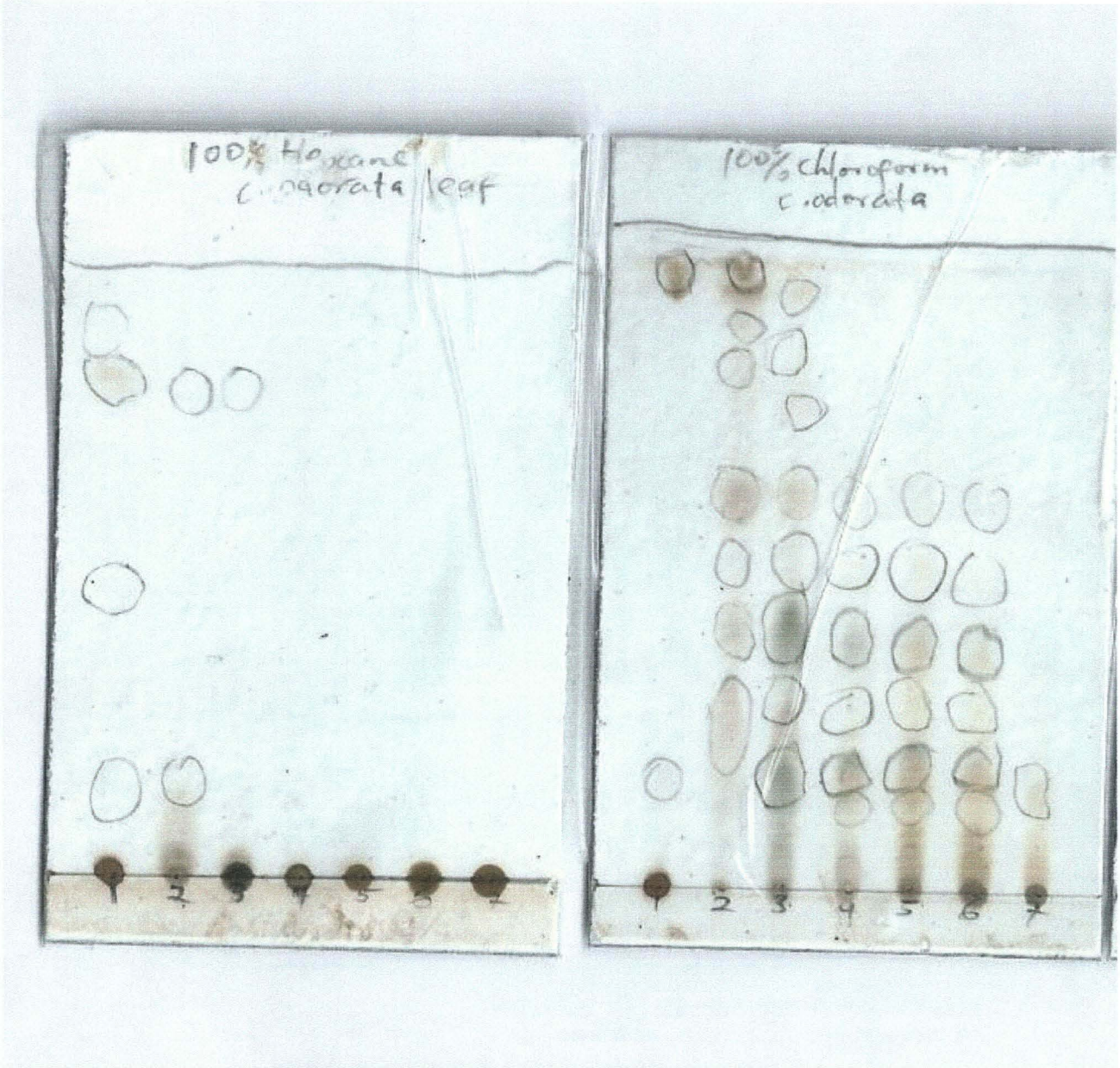


Plate i: Thin Layer Chromatography plate of*Chromolaena odorata*extract

Key

First plate 100% hexane plotted on it Fractions 1-7

Second plate 100%chloroform plotted on it Fractions 1-7

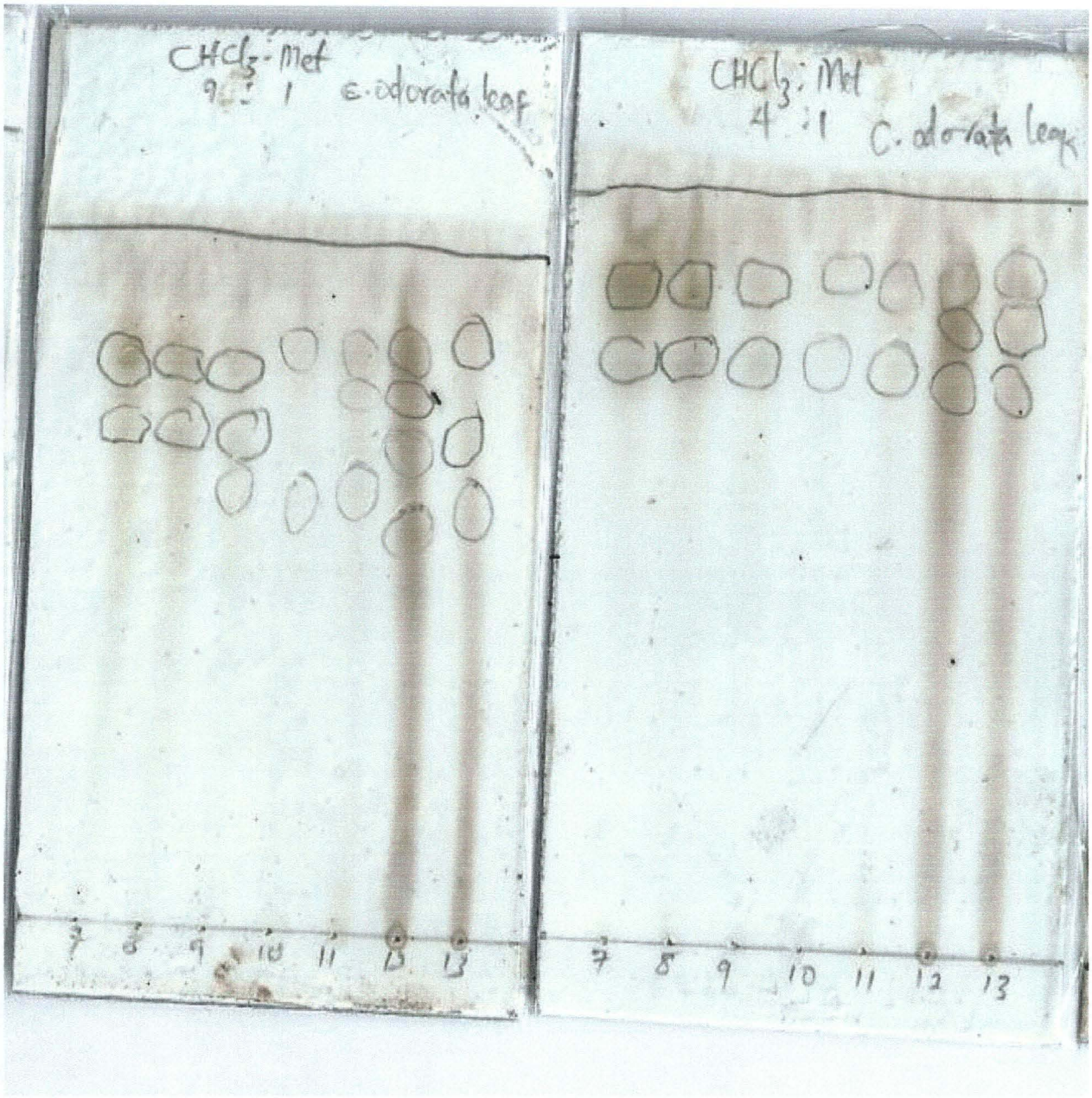


Plate ii: Thin Layer Chromatography plate of*Chromolaena odorata* extract

Key

Third plate 9:1 chloroform/methanol plotted on it Fraction 7-13

Fourth plate 4:1 chloroform/methanol plotted on it Fraction 7-13

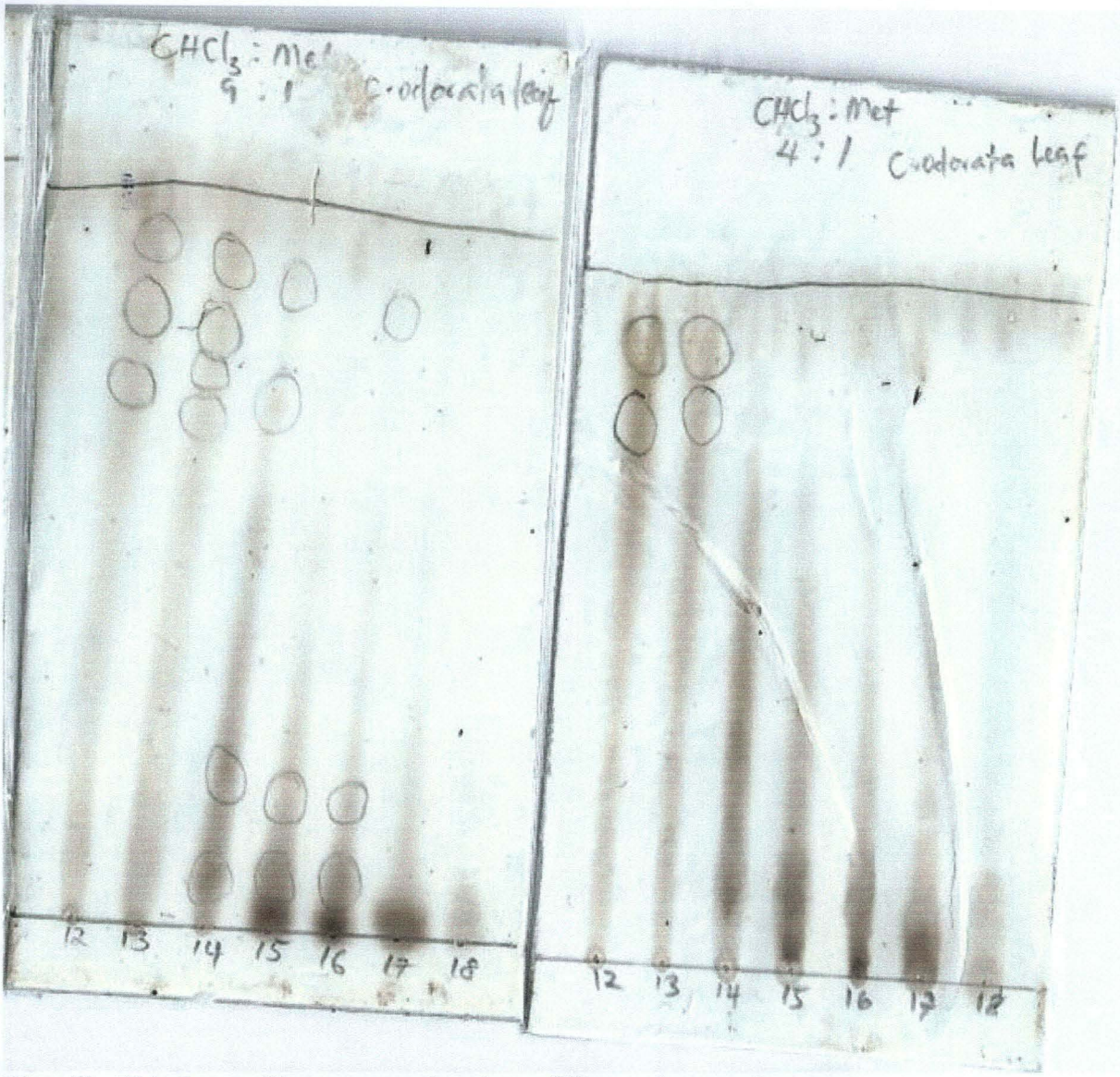


Plate iii: Thin Layer Chromatography plate of*Chromolaena odorata* extract

Key

Fifth plate 9:1 chloroform/methanol plotted on it Fraction 12-18

Sixth plate 4:1 chloroform/methanol plotted on it Fraction 12-18

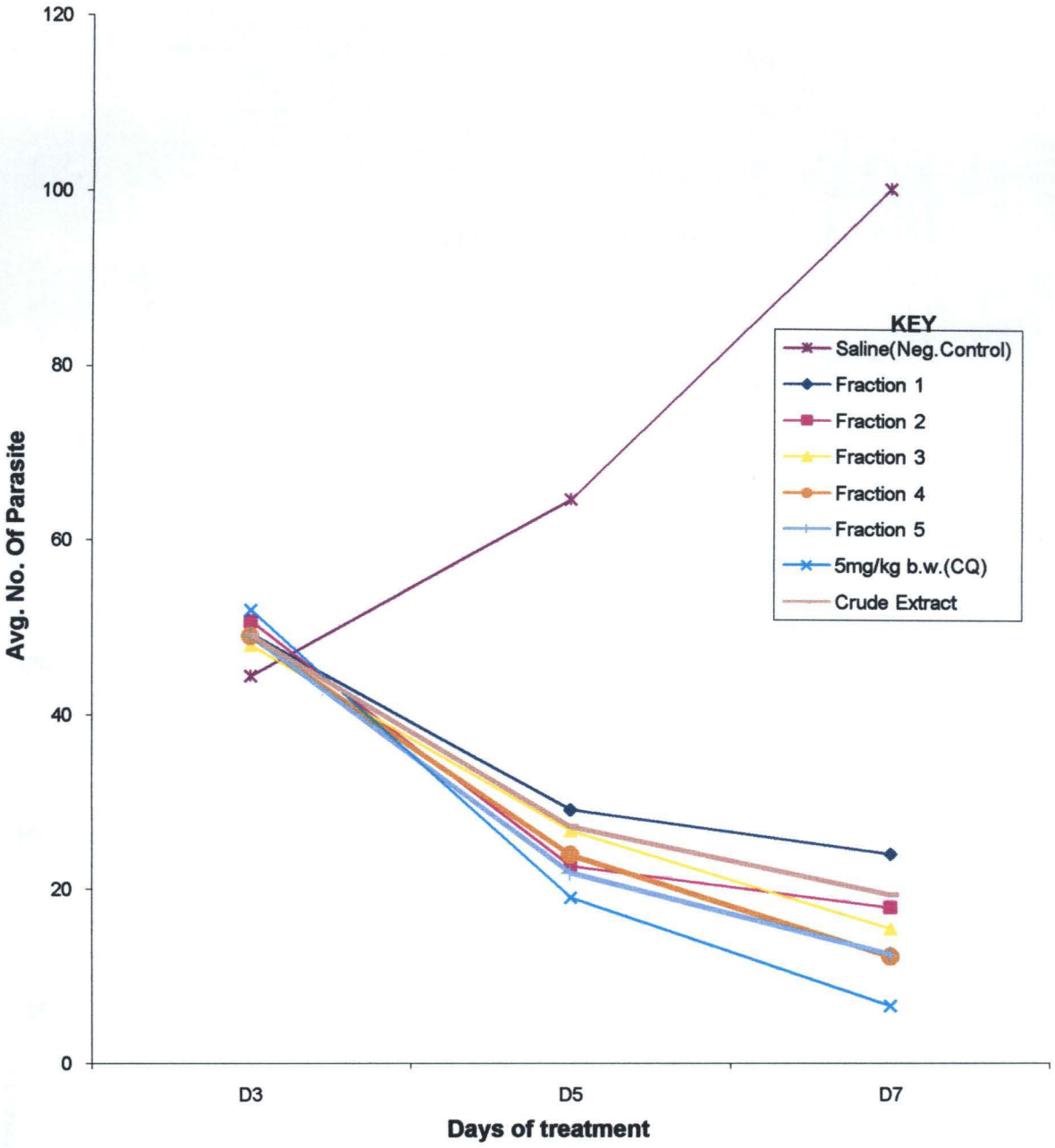


Figure 4.7 Effect of curative test with the fractions of *Chromolaenaodorata*

Table 4.14 Specific activities of Glutamate PyruvateTransaminase in the Liver and Serum of *Plasmodium berghei* infected mice.

Mice grouping	Specific enzyme activity (U/L/mg protein	
	Serum	**Liver**
Infected not treated	3490.41±438.62[a]	24009.63±3304.22[a]
Infected, treated	1391.25±236.56[b]	9093.39±7267.98[b]
Not infected, treated	728.76±335.240[b]	260.36±109.0900[b]
Not infected, not treated	955.73±157.250[b]	79.64±19.62000[b]

Each data are ± standard error of mean for three replicate values (n)

Data with the different superscript were significantly different when compared with infected but not treated (control) at $P < 0.05$.

4.4 Phytochemical screening

Phytochemical screening of ***Chromolaena odorata*** revealed the presence of Alkaloids, saponins,Tannins and flavonoids.

Bioactive agent	**Indication**
Alkaloids	+
Saponins	+
Glycosides	ND
Terpenoids	ND
Tannins	+
Flavonoids	+
Phlobotannins	-
Phenolic nucleus	

ND means Not determined

4.2 DISCUSSION

Medicinal values of plant lies in their component phytochemical which produce a definite physiological action on the human body and responsible for their numerous bioactivities. (Hill, 1952). The phytochemical screening of crude methanol of *chromolaena odorata* showed the presence of Alkaloids, saponins, tannins, phenolic nucleus and flavoniods this is similar to that reported by Igboh *et al*, 2009 but in contrast to that reported by Akinmoladum *et al.*,2007 who did report absent of saponins but presence of the alkaloid, tannins, phlobatannins, steroids, flavonoids and Terpenoids in the methanolic extract of *chromolaena odorata*. This difference may be attributed to certain critical factors, which include species, time, place of plant collection and extraction technique. Three examples of flavonoid have been isolated from *Chromolaena odarata* . (Nisit, *et al.*, 2005). Therefore the antiplasmodial activities of *chromolaena odorata* could be attributed to the presence of certain phytochemicals that constitute the bioactive principles in the plant since numerous plants containing a wide variety of phytochemicals as their bioactive principle have shown antiplasmodial activities (Matur *et al.*, 2009)

Experimental screening method is important for ascertaining the safety and efficacy of herbal products as well as to establish their active components.

The acute toxicity study of *Chromolaena odorata has* been investigated to determine any adverse effect that may arise as a result of a single contact or multiple exposures in a short time within 24 h period. Thus, the acute toxicity study of *Chromolaena odorata methanol* extract indicated no changes in the behaviour and no adverse effects were observed in the male and female mice used in the experiment except for the group that received 5000mg /kg which

showed signs of toxicity within 24hrs but were normal again after 24hrs. Thus, *Chromolaena odorata* extract exhibited LD_{50} above 5000mg/kg having shown no mortality at all the doses tested which implies that the experimental doses are relatively safe (Lorke, 1983). This report is in agreement with that reported by Idowu *et al.,* 2010. Also based on Ghosh (1984) and Klaasen *et al.* (1995) recommendation, the extract could be classified as being non toxic, since the LD_{50} was found in the range of greater 15.0g/kg.

The crude methanolic extract of *chromolaena odorata,* the combined extract of *Chromolaena odorata* with *Morinda lucida* and the fractions exhibited antiplasmodial activity and these conform to the claims reported by Odegbemi, *et al.*, 2007 and Kayode, 2006 that *chromolaena odorata* leave and root are used in treating fever and /or malaria in the southern of Nigeria alone or in combination with other plant.

The crude methanolic extract possesses a significant ($P<0.05$) antiplasmodial activity as evident from the chemosuppression and a significant curative effect was also exhibited which is comparable to that of the standard drug. Chloroquine was used as a standard antimalarial drug in this study and it has been used for curative, suppressive and prophylactic antiplasmodial activities. No significant decrease ($P >0.05$) in the weight of infected mice treated with 200, 400, and 800mg/kg before inoculation when compared with the weight after infection (after treatment) but the effect of the extract on the body weight variation of mice treated with the high doses (400 and 800mg/kg) is significant ($P<0.01$) after treatment this may be attributed to loss of appetite in the mice. Comparing all the doses with the control, a significant decrease ($P<0.05$) was observed in the weight. The Packed cell volume (PCV) of infected mice treated with 200mg showed a significant decreased after the treatment when compared with the PCV before inoculation whereas no significant decreased was observed

with 400 and 800mg/kg doses but the PCV of the infected mice not treated showed a significant ($P<0.05$) decreased this could be as a result of increase in the rate at which red blood cells are destroyed and decrease in the rate at which new ones are produced. The crude extract also exhibited a prophylactic activity of 33.25 %, 49.7 % and 49.47 % respectively for the different doses of 200, 400 and 800mg/Kg which are significant when compared with the control. After four days of administering the extract (200,400 and 800mg/kg) to all the mice, no effect on the mean weight of mice was observed, also after 72hours of innoculation, no significant different ($P>0.05$) was observed but there was steady reduction with the weight of all the mice after four days until the mice later die. With the control group, a significant decreased was observed while on the other hand significant increased was observed with the group treated with 5mg/kg chloroqunine. The packed cell volume of mice treated with extract showed a slight increased while the control group showed significant reduction. The mean survival time of infected mice lengthened with those given the smaller doses (200 and 400). In early and established infection chloroquine interrupts with heme polymerization by forming a Iron II protoporphyrin IX (FP) chloroquine complex, this complex is responsible for the disruption of the parasite's cell membrane function and ultimately leads to auto digestion. Chloroqunine exerted higher and better suppressive, curative and prophylactic antiplasmodial activities by the extent of inhibition of parasitamea, *chromolaena odorata* extract also exerted antiplasmodial activities however to a lower potency and this could be used as bases for drug development.

The combination therapy of *Chromolaena odorata* and root of *Morinda lucida* showed significant curative effect compared with the crude extract alone. There was significant decrease in the weight and PCV of mice treated with the combination therapy. This agrees with

the finding reported by Madam Aina Omole from Northern central who did say that the plant is more used traditionally in combination with other plants. The highest dose of crude methanol *Chromolaena odorata* (800mg/kg) *and* combined extract of *Chromolaena odorata* with *Morinda lucida* (200mg/kg) for curative test was observed to sustain the mice for 20 and 24 day respectively out of the 28 days of the study, though lower than that of the standard drug chloroqunine, Thus demonstrating a considerable antiplasmsodial activity. The lower activity could have resulted from the crude nature of the extract.

Although the mechanism of action of this extract has not been known, some plants are known to exert antantiplasmodial action either by causing elevation of red blood cell oxidation (Etkin, 1997) or by inhibiting protein synthnesis (Kirby, *et al.*, 1989) the extract could have elicited its action through either of the two mechanism mentioned or by some other unknown mechanism.

The partially purified fractions of *Chromolaena odorata* reduced the parasitaemia in the infected mice. Comparing the reduction in the parasitaemia of all the fractions, fraction 5 showed significant and dose dependent antiplasmodial activity against *Plasmodium berghei* in mice at the dose of 800mg/kg which was the highest among the five fractions. Fraction 4 at the same dose level exhibited significant antiplasmodial activity closely similar to that of fraction 5 while fractions 1, 2 and 3 exhibited the least activity, though significant activity compared to the untreated mice (control). Mice treated with fraction 5 survived 25 days while fraction 4 survived 23 days. 25% of the mice treated with fraction 5 survived beyond 28day but later die after 32days. The varying degrees of antimalarial activity exhibited by the Fractions may be attributed to the various phytochemical constituent which may singly or incombination account for the pharmacological actions of the extracts. Therefore, the active constitituent(s)

responsible for this antimalarial activity may lie more in the polar solvent fraction and more studies is needed for complete isolation, identification and characterization

The partially purified fraction of combined extract of *Chromolaena odorata* and *Morinda lucida* exhibited a curative activity and ehanced the mean survival period of mice infected with *plasmodium berghei.* Fraction 1 and 4 showed the highest antiplasmodial activity against *plasmodium berghei* at a dose of 200mg/kg. Fraction 2, 3, and 5 also exhibited antiplasmodial activity but to a lower potency, yet significant compared to the control and comparable to the standard drug Chloroqunine. The ability of both the polar and non polar fractions to show activity indicates that there is more than one active constitutent response for the antimalarial activity.

Enzymes activities such as alkaline phosphatase, gamma glutamyl phosphatase, glutamyl pyruvate transaminase and glutamyl oxaloacetate transaminase can be important in the diagnosis of diseases as well as in the investigation and thorough assessment of drugs and extracts used in the treatment of diseases as these could give indications of progressive toxicity long before the actual manifestation of the toxic effects (Hanley, *et al*, 1986). Damage done to tissues or organs often result in increased level of these enzymes in the blood. A significant decrease ($P<0.05$) in the GPT and GGT activities were observed with the treated groups when compared with the control but no significant difference ($P>0.05$) was observed in the activities of ALP and GOT when compared with the control, this is similar to that reported by Ogbonnia *etal.,* 2010 who reported a significant decrease ($P<0.05$) in ALT and significant increase in AST in the treated group when compared with the control. The general significant increase observed in the enzyme activities of the infected mice not treated (control) could be as a result

of progressive damage to organs not necessarily the liver only. It is however possible that the observed results were due to tissue destruction by the parasite. Pentreath and Kennedy,(2004).

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The study revealed that the crude methanolic leaf extract of *Chromolaena odorata* as well as the fractions have antiplasmodial activity against antiplasmodial in Laboratory animal and the high value of the LD_{50} could partly explain its safety for utilization, effectiveness and traditional use for the treatment of malaria. Also *Chromolaena odorata* extract is more effective when combined with *Mucida lucida* extract. The constituent(s) responsible for the antimalarial activity of *Chromolaena odorata* appear to lie more in the polar solvent fraction.

5.2 Recommendations

Chromolaena odorata has been described as plant of low economic values; still it is use in traditional medicine. Since the control of this invasive weed has been unsuccessful, ways of profitably utilizing it may be the best option. The leaf extract of *Chromolaena odorata* showed great promises as potent candidates for antimalarial drugs since it exhibited significant curative and prophylactic effect and more studies is needed for complete isolation, identification and characterization

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APPENDIX

Preparation of extract

800mg/kg

800mg ⟶ 1kg

1kg ⟶ 1000g

800mg (0.8g) was dissolved in 10ml Normal saline so that 1ml will contain 80mg.

For mouse weighing 32g

Therefore, 800mg contains 1000g, what mg will contain 32g

1000xmg= 800mg x 32g

$$Xmg = \frac{800mg \times 32g}{1000} = 25.6mg$$

32g of mouse will take 25.6mg of the extract

And 1ml contains 80mg,

25.6mg of the extract contain 0.32ml

Phosphate buffer preparation

Sodium dihydrogen orthophosphate with molecular weight 156g/mol was used to prepare 0.2M stock A and di-sodium hydrogen orthophosphate with molecular weight 142g/mol was used to prepare 0.2M of stock B.

Stock A: About 3.12g of Sodium dihydrogen orthophosphate was weighed and dissolved in 100ml distilled water

Stock B: About 2.83g of di-sodium hydrogen orthophosphate was weighed and dissolved in 100ml distilled water

For pH of 6.8, 25.5ml of stock A was added to 24.5ml of stock B and made up to 100ml distilled water, the pH was then confirmed using a pH meter.

Enzyme activity

Reagent composition

Alkaline Phosphatase (S.L) R1	2x24ml/2x40ml
➢ Diethanolamine buffer (pH 10.2)	125mmol/l
➢ Magnesium Chloride	0.65mmol/L
Alkaline Phosphatase (S.L) R2	2X6ml/2x 10ml
➢ P-Nitrophenyl phosphatise	50mmol/L

Reagent composition

Gamma GT (SL) R1	2 x24mL
➢ Tris buffer pH (8.25)	133mmol/l
➢ Glycylglycine	138mmol/l
Gamma GT (SL) R2	2x24ml

Glupa-C 23mmol/l

Reagent composition

SGOT R1

- Tris Buffer (pH7.8) 88mmol/L
- L-Aspartate 260mmol/L

SGOT R2

- Malate dehydrogenase ≥600U/L
- Lactate dehydrogenase ≥ 900U/L
- NADH 0.20MMOL/L

α - ketoglutarate 12mmol/L

Reagent composition

SGPT R1 2x63ml/4 x 50ml/2x205ml

Tri Buffer (pH 7.5) 110mmol/L

L-Alanine 550mmol

SGPT R2 6x20ml/4x50mL/8x50

Lactate dehydrogenase ≥ 200U/L

NADH 0.20mmol/L

α-Ketoglutatrate 16mmol/L

CALCULATION

Enzyme activity (U/L) = change in absorbance per minute x factor

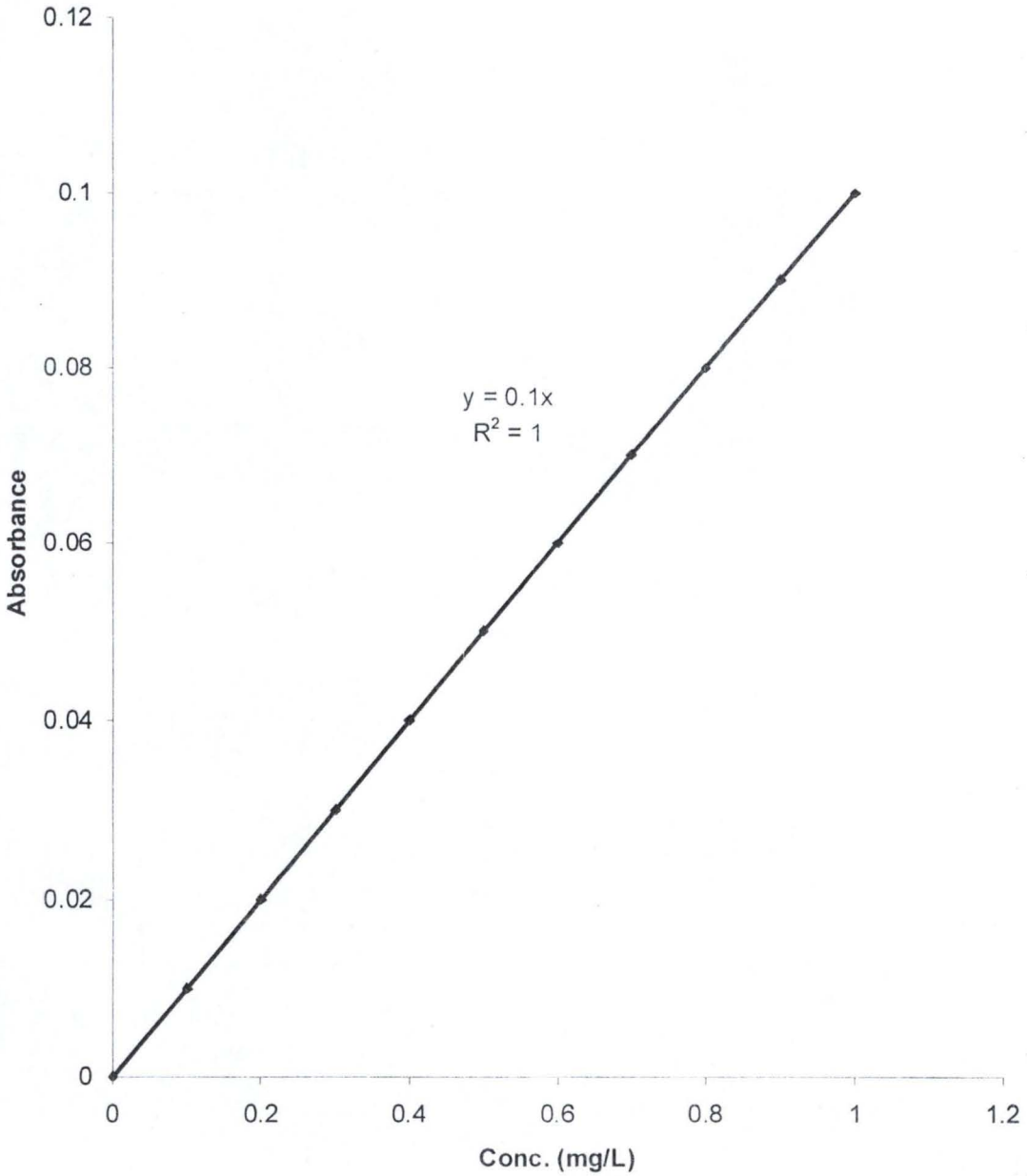
$$\text{Specific enzyme activity} = \frac{\text{Enzyme activity}}{\text{Total protein}} \times \text{dilution factor}$$

Totai protein

Reagent (Biuret) and Albumin standard preparation:

0.75g CuSO4 (Copper sulphate) and 3g of sodium, potassium tartrate were dissolved in 250ml of water. 10% 150ml sodium hydroxide was added to this solution, with good afitation. 1g of potassium iodide (KI) was then added to prevent autoreduction. This solution was then made up to 1litre with distilled water.

The standard was prepared by dissolving 1g of Albumin standard in 100mL of water. It was serial diluted and then absorbance was taken at 450nm wavelength, after adding 4ml of biuret reagent to each test tube, shaken and allowed to stand for 30minutes at room temperature. A calibration curve of absorbance against concentration was plotted which was used to extrapolate.



Bovine serum albumin standard

Table 1: Average parasite count of mice treated with crude methanol extract of *Chromolaena odorata*(suppressive test)

Doses (mg/Kg/day)	Mean parasite count
0	10.1600 ± 1.4134
200	5.000 ± 0.2000
400	4.3200 ± 0.1020
800	3.1200 ± 0.1847
5 CQ	1.8400 ± 0.1470

Table 2 **Antiplasmodial activity of combination therapy of partially purified leaf of *chromolaena Odorata* and root of *Morinda lucida***

Extract	Days of treatment		
	Do	D2	D5
Control	44.55±0.8617	64.95±1.0210	100.87± 2.656
Fraction 1	49.90±0.4726	20.05±1.1442	12.95±1.1843
Fraction 2	50.25±0.8958	24.34±2.5695	16.35±0.6185
Fraction 3	48.75±0.0957	27.20±1.6673	15.80±0.9055
Fraction 4	49.35±1.3623	20.85±0.3367	11.40±1.1576
Fraction 5	48.35±0.5657	21.05±1.0210	16.95±0.2872
Crude	48.35±0.5188	25.35±1.6700	11.50±0.4700
5mgCQ	52.15±0.6021	19.15±0.9142	6.65±0.2062

Table3 **Antiplasmodial activity of partially purified methanol extract of chromolaena odoarata**

Extract	Days of treatment		
	Do	D2	D5
Control	44.55±0.8617	64.95±1.0210	100.87±2.6558
Fraction 1	49.40±2.0753	39.25±2.5184	30.20±1.4091
Fraction 2	50.85±0.8382	22.80±1.2083	18.05±0.2500
Fraction 3	48.13±0.5774	26.85±0.4272	15.60±0.9238
Fraction 4	49.15±1.0689	24.05±2.4945 1	2.35±0.2500
Fraction 5	49.25±0.7228	22.00±0.7439	12.60±0.3559
Crude	49.25±1.2920	27.35±1.6700	19.50±0.5500
5mgCQ	52.15±0.6021	19.15±0.9142	6.65±0.2062

Table 4 Average Weight (g) of Infected Mice treated with Crude Methanol Extract of *Chromolaena odorata*

Dose Mg/Kg/bw	Days after infection		
	Before inoculation	3	7
0	20.23±1.0094	20.51±1.0985	16.00±0.8720[a]
200	21.350±1.2059	22.41±1.2257	21.13±0.8898[b]
400	20.53±1.1600	21.31±1.2899	19.63±0.5625[b]
800	20.58±1.2003	20.87±0.8677	19.53±0.4360[b]
5CQ	20.76±1.6840	20.09±1.6269	20.65±1.4459[b]

Each value are ± standard error of mean for four replicate values (n)

Values with the different superscript were significantly different when compared with infected but not treated (control) at $P > 0.05$ after the treatment.

Table 5 **Average Parasitaemia of Mice Treated with Crude Methanol Extract of *Chromolaena odoarata***

Dose	Days of treatment					
(mg/kg/bw	D3	D4	D5	D6	D7	D8
0	58.85 ±3.003	61.373±2.7732	70.00±3.170	78.23±2.753	91.35±1.6903	115.5±5.6559
200	55.70±1.560	53.425±1.3919	51.33±0.9169	48.75±0.7599	41.823±0.4479	38.25±0.6316
400	55.30±2.3288	52.68±0.9013	50.03±0.6129	46.33 ±0.4171	42.25±0.9887	34.13±2.1983
800	55.15±0.7136	52.85±0.2598	47.83±0.5543	39.68± 0.9375	34.30±0.5066	27.35±1.6660
5CQ	58.25±1.8099	55.05±1.5707	42.33±0.9612	35.35±2.3574	26.18±1.690	8.775±0.78