IN VIVO EFFICACY OF METHANOLIC EXTRACTS OF ROOT AND LEAF OF *Morinda lucida* IN *Plasmodium berghei* - INFECTED MICE

Therapeutic effects of the methanolic root extract and a combination of leaf and root extracts of *Morinda lucida* were evaluated for antiplasmodial activities. The phytochemical screening of the methanolic root extract showed the presence of saponins, flavonoids, alkaloids, phenolic nucleus and phlobotannins. The LD_{50} of the crude methanolic root extract was calculated to be 3807.89mg/kg and percentage suppression of parasitaemia was 56.30, 59.84, 67.72 and 81.80% for doses of 100, 200, 400mg/kg body weight (b.w) and 5mg/kg chloroquine respectively. Effective dose dependent inhibitions of parasitaemia were also observed in the curative test and the mean survival period in days were 15.00 ± 0.70, 18.75 ± 0.5, 19.75 ± 1.39, 23.25 ± 1.38 and 8.75 ± 1.25, for 100, 200, 400mg/kg body weight of the extract, 5mg/kg chloroquine, and untreated control respectively. Percentage prophylaxis was calculated to be 24.26, 39.41, 43.67 and 84.91% for doses of 100, 200, 400mg/kg body weight of the methanolic root extract, and 5mg/kg chloroquine respectively. The crude methanolic extract of the root was partially purified by Column chromatography to give fractions 1-4. Fractions 3 and 4, exhibited a significant curative effect in established infection. There was no significant difference at $p>0.05$ in antimalarial activity of fraction 4 and the crude extract. Remarkable parasitaemia inhibition by the extracts resulted into longer mouse survival relative to the control, as demonstrated in the mean survival time of the mice (29.25±1.43, 11.25±0.75, 11.75±1.60, 24.25±1.11, 28.50±1.32, 28.00±0.00 and 5.75±2.14, for crude methanolic root extract, fraction 1, fraction 2, fraction 3, fraction 4 of the crude methanolic root extract, chloroquine and control groups respectively). For the chloroquine group, two of the mice out of four used in the experiment, survived beyond 30 days. In the combination study, it was observed that, the antimalarial activity for leaf and root was slightly more, compared to that of each of the extract used singly, as seen in parasite inhibition, after 5days of treatment (26.00, 20.00, 25.28, 21.35, 27.00, 19.50, 8.5 and 85.00), for 100, 200mg/kg leaf extract alone, 100 and 200mg/kg root extract alone, 50, 100mg/kg leaf and root extracts, 5mg/kg chloroquine and control groups respectively. The effect of methanolic root extract on some serum and liver enzymes in mice infected with *Plasmodium berghei* were also studied. Specific activities of Glutamate oxaloacetate transaminase (GOT), Glutamate pyruvate transaminase (GPT), Alkaline Phosphatase (ALP) and Gamma glutamyl transferase (GGT) were significantly ($p<0.05$) high in the infected not treated group, compared to the positive control (not infected, not treated). It is concluded that the methanolic root extract of *Morinda lucida* is potentially useful for the development of antimalarial drug.

CHAPTER ONE

1.0 INTRODUCTION

Malaria is a mosquito-borne infectious disease caused by a eukaryotic protist of the genus *Plasmodium*. It is widespread in the tropical and subtropical regions, including parts of the Americas, Asia, and Africa. According to the World Malaria Report, malaria is prevalent in 108 countries of the tropical and semitropical world, with 35 countries in central Africa bearing the highest burden of cases and deaths. Of the 35 countries that account globally for ~98% of malaria deaths, 30 are located in sub-Saharan Africa, accounting for 98.5% of the deaths in Africa, with four countries alone accounting for ~50% of deaths on the continent (Nigeria, Democratic Republic of Congo, Uganda and Ethiopia) (Rowe, 2006).

In sub-Saharan Africa, approximately 365 million cases occurred in 2002 and 963 thousand deaths in 2000, equating to 71% of worldwide cases and 85.7% of worldwide deaths. Almost 1 out of 5 deaths of children under 5 in Africa is due to malaria. With increased efforts in controlling malaria in Africa in the recent years, it is reported that a total of 11 countries and one area in the African Region showed a reduction of more than 50% in either confirmed malaria cases or malaria admissions and deaths (Algeria, Botswana, Cape Verde, Eritrea, Madagascar, Namibia, Rwanda, Sao Tome and Principe, South Africa, Swaziland, Zambia, and Zanzibar, United Republic of Tanzania), whereas there was evidence of an increase in malaria cases in 3 countries in 2009 (Rwanda, Sao Tome and Principe, and Zambia) (World Malaria Report, 2010 and Rowe, 2006). However, these claims of improved malaria situation in Africa, as presented by the World Health Organisation (WHO), have been challenged (Roberts and Tren (2011) and Rowe, 2009).

Malaria is naturally transmitted by the bite of a female *Anopheles* mosquito. When a mosquito bites an infected person, a small amount of blood is taken, which contains malaria parasites. These develop within the mosquito, and about one week later, when the mosquito takes its next blood meal, the parasites are injected with the mosquito's saliva into the person being bitten. After a period of between two weeks and several months (occasionally years) spent in the liver, the malaria parasites start to multiply within red blood cells, causing symptoms that include fever and headache. In severe cases, the disease worsens, leading to coma and death (Snow *et al*., 2005).

The vast majority of malaria deaths occur in Africa, south of the Sahara, where malaria also presents major obstacles to social and economic development. Each year, there are approximately 350–500 million cases of malaria (CDC), killing between one and three million people, the majority of whom are young children in sub-Saharan Africa (Snow *et al*., 2005).

Malaria is Africa's leading cause of under-five mortality (20%) and constitutes 10% of the continent's overall disease burden. It accounts for 40% of public health expenditure, 30-50% of inpatient admissions, and up to 50% of outpatient visits in areas with high malaria transmission. Malaria is commonly associated with poverty, but is also a cause of poverty and a major hindrance to economic development. Malaria has been estimated to cost Africa more than US$ 12 billion every year in lost Gross Domestic Product (GDP), even though it could be controlled for a fraction of that sum (World Malaria Report, 2010).

There are several reasons why Africa bears an overwhelming proportion of the malaria burden. Most malaria infections in Africa south of the Sahara are caused by *Plasmodium falciparum,* the most severe and life-threatening form of the disease. This region is also home to the most efficient, and therefore deadly, species of the mosquitoes which transmit the disease. Moreover, many countries in Africa lacked the infrastructures and resources necessary to mount sustainable campaigns against malaria and as a result few benefited from historical efforts to eradicate malaria.

One of the greatest challenges facing Africa in the fight against malaria is drug resistance. Resistance to chloroquine, (Wellems, 2002) the cheapest and most widely used antimalarial, is common throughout Africa (particularly in southern and eastern parts of the continent). Resistance to sulfadoxine-pyrimethamine (SP), often seen as the alternative to chloroquine, is also increasing in east and southern Africa. As a result of these trends, many countries have no choice, but to change their treatment policies and use drugs which are more expensive, including combinations of drugs, which is hopefully believed will slow the development of resistance (Wellems, 2002).

Malaria remains a major public health problem in Nigeria, accounting for nearly 110 million clinically diagnosed cases per year, 60 per cent of outpatient health-care visits and 30 per cent of hospitalizations. At least 200,000 children die of malaria each year, and up to 11 per cent of maternal mortality is caused by the disease (Geoffrey and Paula (2009)).

Nation-wide in Nigeria, malaria accounts for one in every five deaths of children and 1 in 10 deaths of pregnant women. In addition to its direct health impact, the disease imposes a heavy social and economic burden: an estimated $900 million is lost to

malaria annually in prevention and treatment costs and productivity loss (UNICEF, 2009).

Growing political commitment by African leaders for action on malaria was given a boost by the founding of the Roll Back Malaria (RBM) global partnership in 1998. Less than two years later African Heads of State and their representatives met in Abuja, Nigeria to translate RBM's goal of halving the malaria burden by 2010 into tangible political action. The Abuja Declaration, signed in April 2000 endorsed a concerted strategy to tackle the problem of malaria across Africa. The Abuja Declaration endorsed RBM's goal and established a series of interim targets for the number of people having access to treatment, protective measures or, in the case of pregnant women, receiving intermittent preventive treatment to ensure that progress would be made towards the goal and malaria-endemic countries and other RBM partners held responsible.

In Nigeria, *Morinda lucida* is one of the 4 most used traditional medicines against fever. It grows in grassland, exposed hillsides, thickets and forests. It is widely known and used, especially the root and leaf parts, in the southern and north-central parts of Nigeria as a remedy against various ailments, including malaria fever (Makinde and Obih (1985)).

Research Aim and Objectives

Aim: To investigate the in vivo efficacy of methanolic root and leaf extracts of *Morinda lucida* in *Plasmodium berghei* infected mice.

Objectives:

i. Screening of crude extracts of *Morinda lucida* against *Plasmodium berghei*

ii. Fractionation of crude extract by Column chromatography

iii. Screening of fractions for antiplasmodial activity

iv. Evaluation of polytherapy for antiplasmodial activity

Justification for this Research

Given the future scenario challenging the health sector such as inadequate health care, cost of producing drugs, additional research is needed in order to realize the full benefits of natural plants and respond to the health needs of people, especially in developing countries like Nigeria. History reveals that plants have always been considered as an important source of medicine against malaria: both quinine and artemisinin have been derived from traditional medicine and plant extracts. Artemisinin derivatives are now recommended by World Health Organization (WHO) worldwide, in combination with other drugs, such as lumefantrine, amodiaquine, mefloquine, sulphadoxine-pyrimethamine, as the first line of treatment of malaria. This fact has encouraged the continuing search for new natural product-derived anti-malarial drugs. One of the strategies in the search for new antimalarial compounds is a study of active constituents of medicinal plants. It is important, therefore, to investigate the antimalarial activities of *Morinda lucida* in order to determine the potential as source of new antimalarial agent. The plant parts (root and leaf) of *Morinda lucida*, "*Ugigo"* (local name in Ebira tribe), have been used as traditional remedy for the treatment of symptomatic malaria by the tribal population of Ebira land, Kogi State North – Central Nigeria, as such, the choice for their antimalarial screening in this work.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Malaria parasite

Plasmodium is a genus of parasitic protists. Infection by these organisms is known as malaria**.** The genus Plasmodium was described in 1885 by Ettore Marchiafava and Angelo Celli. Currently over 200 species of this genus are recognized and new species continue to be described (Chavatte *et al*., 2007 and Perkins and Austin (2008)).

Of the over 200 known species of Plasmodium, at least 11 species infect humans. Other species infect other animals, including monkeys, rodents, birds, and reptiles. The parasite always has two hosts in its life cycle: a mosquito vector and a vertebrate host.

2.1.1 Classification of Malaria Parasite

Scientific Classification

Domain	:	Eukaryota
Kingdom	:	Chromalveolata
Superphylum	:	Alveolata
Phylum	:	Apicomplexa
Class	:	Aconoidasida
Order	:	Haemosporida
Famiy	:	Plasmodiidae
Genus	:	Plasmodium

Source: Marchiafava and Celli, 1885.

Subgenera

Asiamoeba	:	(5 species)
Bennetinia	:	(1 specie)
Carinamoeba	:	(7 species)
Giovannolaia	:	(14 species)
Haemamoeba	:	(12 species)
Huffia	:	(2 species)
Lacertamoeba	:	(2 species)
Laverania	:	(5 species)
Ophidiella	:	(3 species)
Novyella	:	(19 species)
Nyssorhynchus:		(1 specie)
Paraplasmodium:		(3 species)
Plasmodium	:	(30 species)
Sauramoeba	:	(15 species)
Vinckeia	:	(32 species)
Incertae sedis	:	(124 species)

2.1.2 Types of Malaria

There are 4 types of malaria that infect humans:

i. *Plasmodium vivax* (*P. vivax*) - has the widest geographic distribution throughout the world and causes much debilitating disease. It is usually found in Central and South America, India and Southeast Asia. Malaria caused by *Plasmodium vivax* is rarely fatal. However, it can still cause significant illness. Since *P. vivax* can tolerate cooler temperatures, it is more geographically widespread than *P. falciparum*, although transmission is usually low because it is season-dependent.

ii. *Plasmodium malariae* (*P. malariae*) - has restricted distribution and is said to be responsible for less than 1% of infections in India. It is found in tropical and subtropical areas of Central and South America, Africa, and Southeast Asia. *Plasmodium malariae* ranks third in prevalence, but has a widespread distribution.

iii. *Plasmodium ovale* (*P. ovale*) - is the rarest of the four species, and is mostly confined to tropical West Africa including countries such as Ghana, Liberia, and Nigeria.

iv. *Plasmodium falciparum* (*P. falciparum*) - is wide spread and found in tropical and subtropical areas of Central and South America, Africa, and Southeast Asia. *P. falciparum* malaria results in the most severe infections and is responsible for nearly 90% of malaria-related deaths in sub Saharan Africa.

2.2 Morphology and Life cycle

2.2.1 Life cycle

A complete understanding of the life cycle of *Plasmodium* is necessary in understanding the methods used in controlling the epidemic nature of the parasite. The malaria parasite is a one-celled organism that belongs to the phylum Apicomplexa, named because it possesses an apical complex. The life cycle of Plasmodium while complex is similar to several other species in the *Haemosporidia*. All the Plasmodium species causing malaria in humans are transmitted by mosquito species of the genus Anopheles. Species of the mosquito genera Aedes, Culex, Culiseta, Mansonia and Theobaldia can also transmit malaria but not to humans.

The malaria parasite life cycle (figure 2.1) involves two hosts. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host (1). Sporozoites infect liver cells (2) and mature into schizonts (3), which rupture and release merozoites (4). (Of note, in *P. vivax* and *P. ovale* a dormant stage [hypnozoites] can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.) After this initial replication in the liver (exo-erythrocytic schizogony {A}), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony {B}). Merozoites infect red blood cells (5). The ring stage trophozoites mature into schizonts, which rupture releasing merozoites (6). Some parasites differentiate into sexual erythrocytic stages (gametocytes) (7). Blood stage parasites are responsible for the clinical manifestation of the disease. The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal (8). The parasites' multiplication in the mosquito is known as the sporogonic cycle {C}. While in the mosquito's stomach, the

microgametes penetrate the macrogametes generating zygotes (9). The zygotes in turn become motile and elongated (ookinetes) (10), which invade the midgut wall of the mosquito where they develop into oocysts (11). The oocysts grow, rupture, and release sporozoites (12), which make their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle (1).

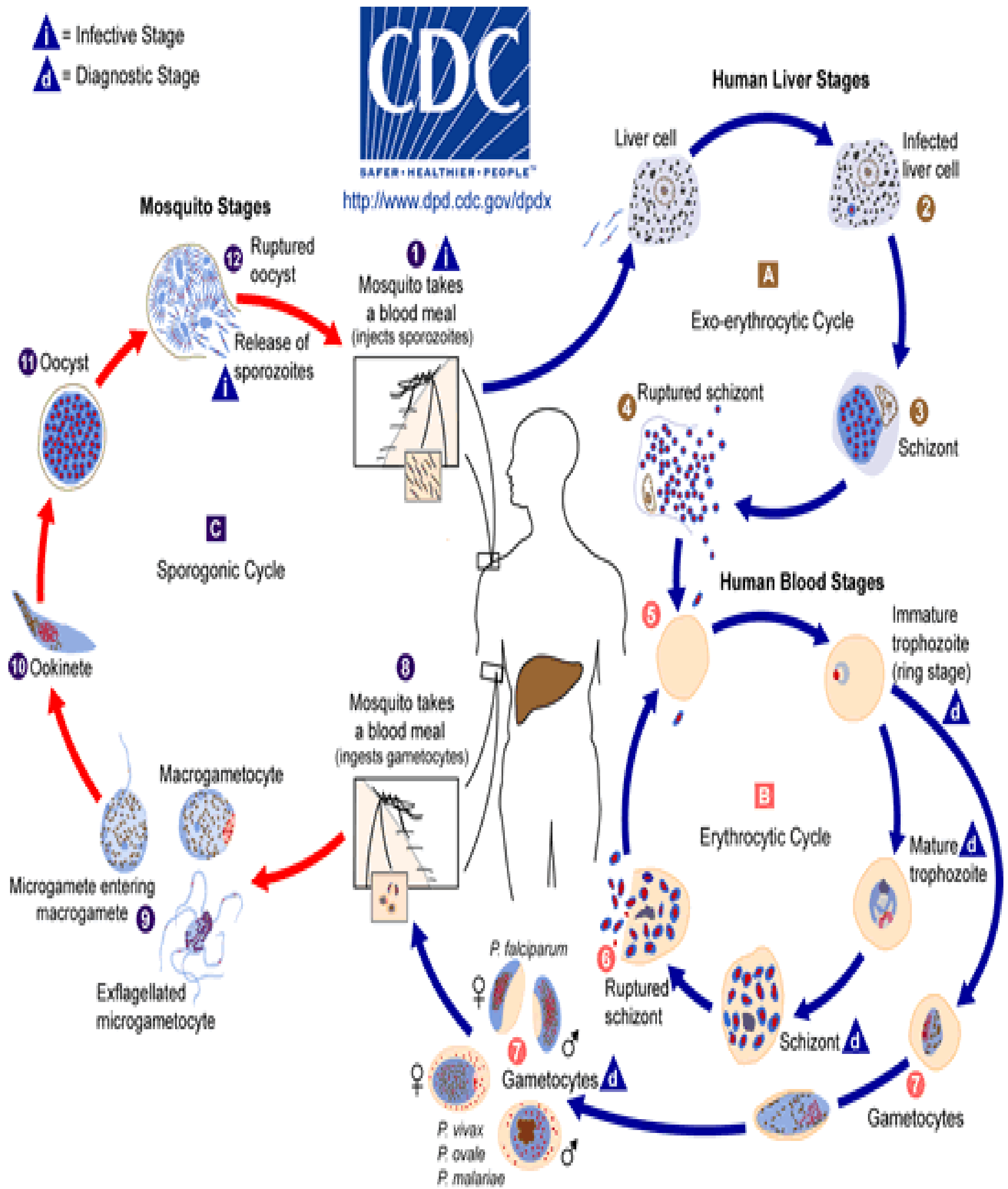


Figure 2.1: Life Cycle of the Plasmodium Parasite

Source: Centres of Disease Control and Prevention (CDC), 2010.

2.2.2 Structure of Malaria Parasite

Figure 2.2: Structure of the malaria parasite (*plasmodium*)

Source: Public library of science journal

2.3 Biochemistry of Plasmodium

The malaria parasite, like all organisms, must acquire nutrients from the environment and convert these nutrients to other molecules or energy (i.e., catabolism). These other molecules and the energy are then used to maintain the homeostasis of the parasite, and in the growth and reproduction of the parasite (i.e., anabolism). Both anabolic and catabolic processes are catalysed by enzymes. Growing and reproducing organisms require high levels of macromolecules and other biochemicals for the maintenance of cellular structure and function. The malaria parasite needs to acquire these biochemicals and precursors from the host. The unique life cycle and resulting microenvironments of the parasite has led to the evolution of metabolic pathways which differ from the human host. It may be possible to exploit these unique pathways and enzymes in the design of therapeutic strategies. For example, many types of anti-malaria are known to affect the food vacuole which is a special organelle for the digestion of host haemoglobin (Ginsburg *et al*., 1999).

2.3.1 Metabolic Pathways

Organisms acquire organic material from their environments and convert this material into energy or their own substance (i.e., biomolecules). Cells are made of distinct classes of biomolecules (DNA, RNA, Protein, Lipid and Carbohydrate) with specific functions (Genetic material, Template for Protein synthesis, Cell structure & function, Membrane Component, and Energy production respectively). These macromolecules are synthesized from small molecular weight precursors, or building blocks. These molecular precursors (Nucleotides, Amino acids, Fatty acids and Sugars) are components of interconnected metabolic pathways.

2.3.2 Carbohydrate and Energy production

The blood-stage parasite actively ferments glucose as a primary source of energy. The metabolic steps involved in the conversion of glucose to lactate (referred to as glycolysis) are essentially the same as that found in other organisms. All of the enzyme activities have been identified in *Plasmodium* and some of the genes cloned. The parasite exhibits a high rate of glycolysis and utilizes up to 75 times more glucose than uninfected erythrocytes. Most of the glucose is converted to lactate and the high lactate dehydrogenase (LDH) activity is believed to function in the regeneration of NAD^+ from NADH which is produced earlier in the glycolytic pathway by glyceraldehyde-3-phophate dehydrogenase (Figure 2.3). The net result of glycolysis is to produce ATP which is the energy currency of the cell. In other words, ATP is needed for anabolic and homeostatic processes (Uyemura *et al*., 2000)

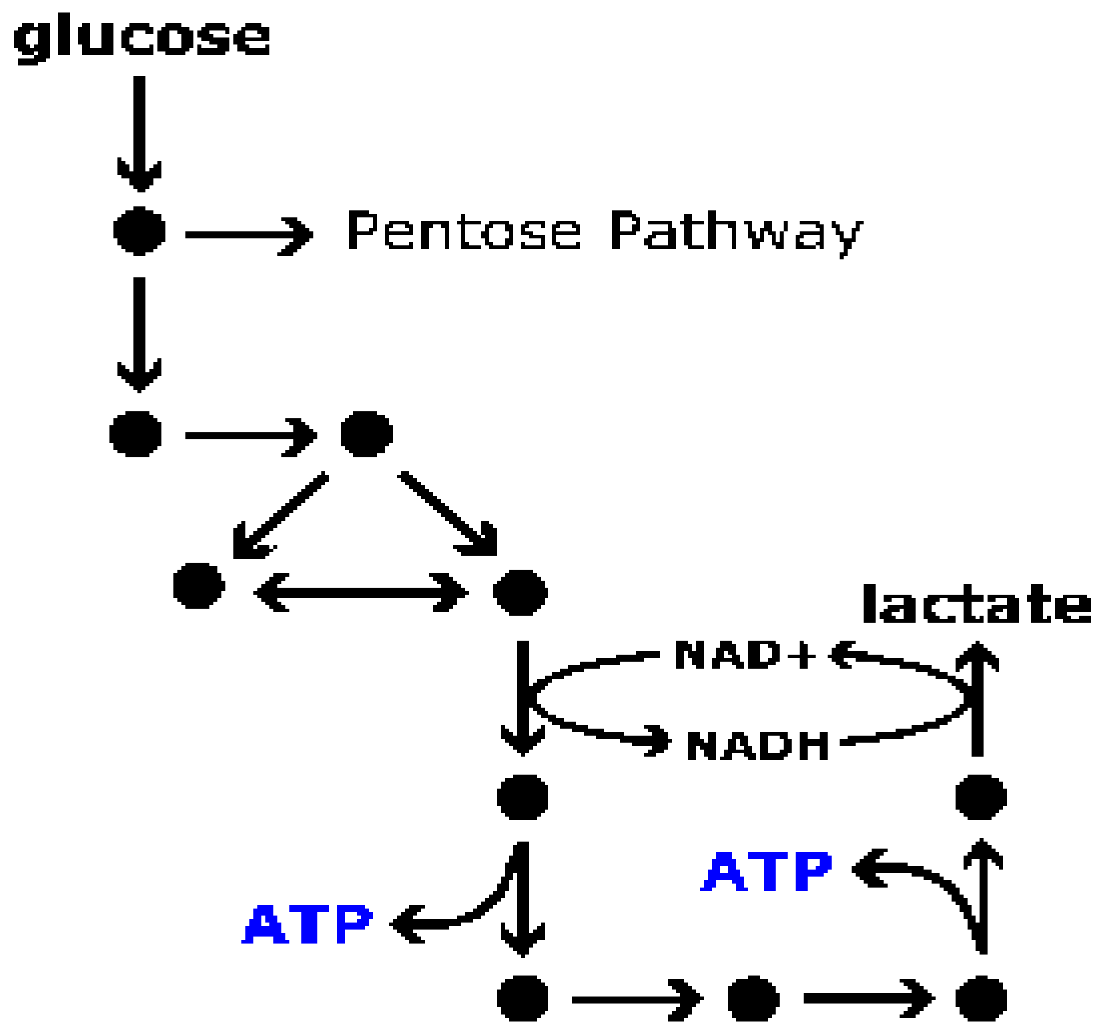


Figure 2.3: Production of ATP in the Glycolytic pathway

Most (approximately 85%) of the glucose utilized by the parasite is converted to lactate. However, some of the glycolytic intermediates may be diverted for synthetic purposes. For example, enzymes of the pentose phosphate pathway have been identified. This pathway probably provides some of the ribose sugars needed for nucleotide metabolism and provides for the regeneration of reduced NADPH to be used in biosynthesis or defence against reactive oxygen intermediates. Similarly, the further metabolism of pyruvate may provide intermediates in several biosynthetic pathways.

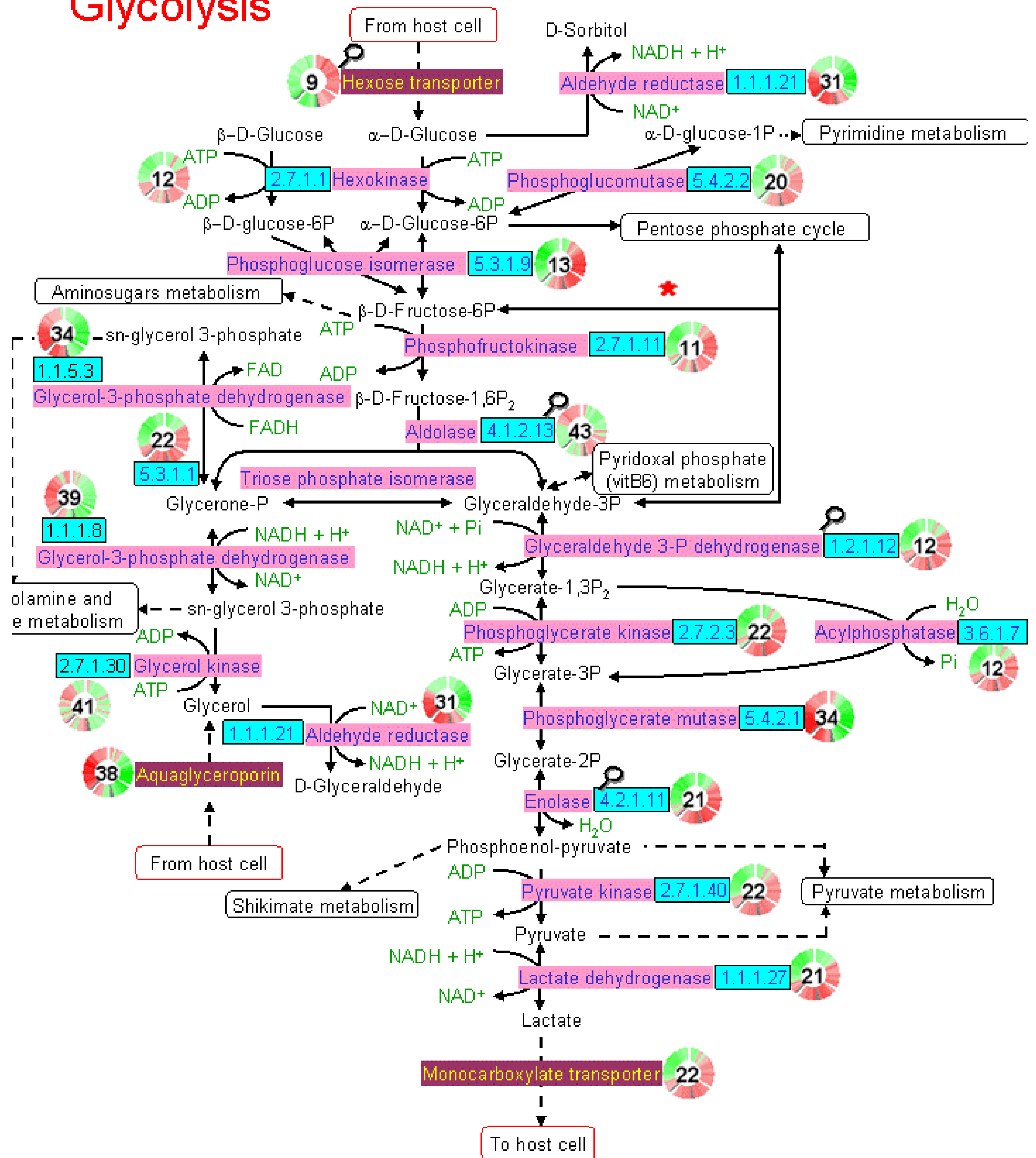


Figure 2.4: Glycolytic Pathway

Aerobic metabolism involves the further catabolism of pyruvate (glycolysis intermediate preceding lactate) to carbon dioxide 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 TCA cycle and oxidative phosphorylation can generate up to 38 molecules of ATP per glucose molecule, whereas glycolysis only produces two molecules of ATP per glucose molecule. Nonetheless, 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 (Uyemura *et al*., 2000). In addition, the parasite mitochondrion does have a membrane potential and cytochrome oxidase is present. One possible function of the mitochondrion during the blood stage is for pyrimidine synthesis.

2.3.3 Fatty Acids and Lipids

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.

Several parasite enzymes involved in lipid synthesis from glycerides and fatty acids, as well as enzymes involved in the remodelling of lipid polar head groups have been identified (Mitamura and Palacpac (2003)). An enzyme capable of activating fatty acids (necessary for incorporation into lipids) has been localized to membranous structures found within the cytoplasm of the infected erythrocyte (Matesanz *et al*., 1999).

2.3.4 Proteins and Amino acids

Proteins are composed of linear chains of amino acids which fold into 3-dimensional structures. Through their roles as enzymes or structural proteins, proteins are responsible for cellular structure and function. The blood-stage parasite obtains amino acids for protein synthesis from three sources: 1) degradation of ingested haemoglobin, 2) uptake of free amino acids from the host plasma (or cells), and 3) de novo synthesis. The most abundant source of amino acids is the ordered degradation of haemoglobin. The parasite digests up to 65% of the total host haemoglobin into amino acids. However, most of these amino acids are effluxed from the infected erythrocyte and only

16% of the digested haemoglobin is incorporated into parasite proteins (Krugliak *et al*., 2002).

Several amino acids are taken up by infected erythrocytes at accelerated rates (Ginsburg, 1994) and in vitro culture studies indicate that *P. falciparum* requires seven exogenously supplied amino acids: isoleucine, methionine, cysteine, glutamate, glutamine, proline, tyrosine (Divo *et al*., 1985). The parasite is also able to fix carbon dioxide and thereby synthesize alanine, aspartate and glutamate. However, the amino acids formed via carbon dioxide fixation and some of the exogenously added amino acids are not readily incorporated into proteins. Many of these amino acids (through transamination reactions) can interact with pathways involved in energy production and possibly serve as fuel sources. In addition, some amino acids serve as precursors or components of biosynthetic or other metabolic pathways. Of particular note is the proposal that glutamate dehydrogenase provides the reduced NADPH needed for glutathione reductase (Krauth-Siegel *et al*., 1996) which presumably functions in redox metabolism.

Ribosomes are supramolecular complexes composed of ribosomal RNA and proteins. Their function is to translate mRNA into protein. The mechanism of protein synthesis is presumably typical of other eukaryotes. Interestingly, different rRNA molecules are expressed during the vertebrate and invertebrate stages of the parasite's life cycle (McCutchan *et al*., 1995). The functional significance of stage specific ribosomes is not known.

2.4 Plasmodium Damage and Host Responses

The plasmodium parasite, a protozoan which causes malaria, is an organism that lives on or inside another organism and depends upon its host's resources. The symptoms of malaria are the result of complex interactions between Plasmodium and the immune system of its host (Clark and Cowden (2003)). Sometimes this relationship is benign, but often parasites cause severe damage to their hosts. Below, illustrates the varied and complex ways in which such damage can arise, and the role that the host's immune system plays in the process.

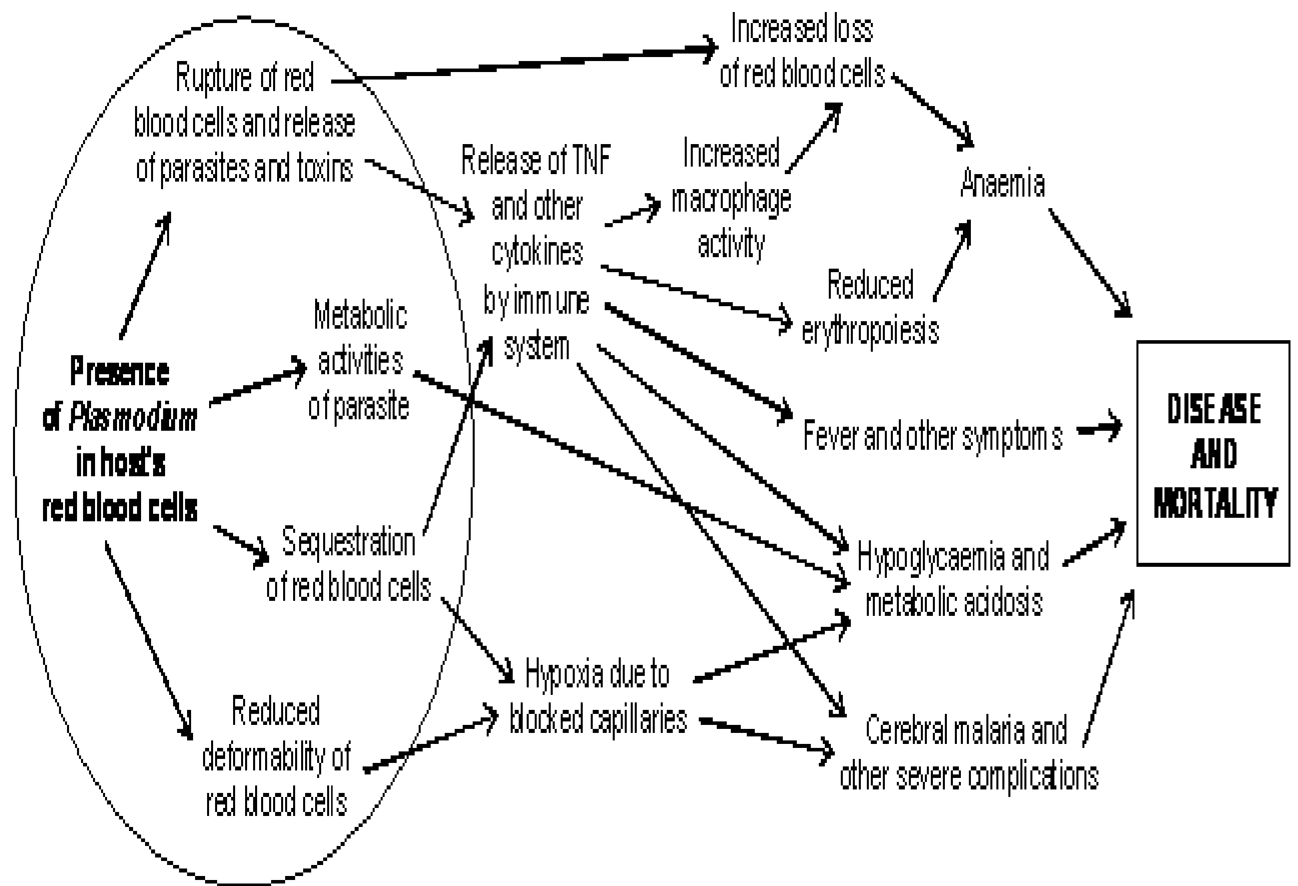


Figure 2.5: Possible mechanisms by which Plasmodium causes disease

Source: andrewgrey.com, 2005

2.4.1 The immune response

The periodic bouts of fever that occur in malaria coincide with the synchronised rupture of Plasmodium-infected red blood cells. This causes the release of parasites en masse into the blood stream, along with pigments and toxins that have accumulated inside the red blood cells as a result of the parasites' metabolic activities. The presence of large quantities of parasite material in the blood triggers a dramatic immune response, mediated by the secretion of cytokine molecules by the cells of the immune system (Hommel and Gilles (1998)). Some cytokines—such as 'tumour necrosis factor' (TNF), interferon-gamma, interleukin-12 and interleukin-18—enhance the immune response, stimulating macrophages and other immune cells to destroy parasites by phagocytosis and by the production of toxins. Other cytokines—including interleukin-4, interleukin-10 and TGF-beta—help to regulate the immune response by dampening these effects (Malaguarnera and Musumeci (2002)).

Although the immune response stimulated by cytokines undoubtedly plays an important role in suppressing and killing malaria parasites within the body, excessive production of cytokines can have pathological consequences (Richards, 1997).

2.4.2 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).

2.4.3 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. 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 un-parasitized red blood cells (Menendez *et al*., 2000). (During malaria infection, un-parasitized red blood cells may be targeted because they have abnormally rigid membranes, or because malarial antigens present in the bloodstream bind to their surfaces).

2.4.4 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. (Clark *et al*., 1997; Clark and Cowden (2003)).

2.4.5 Factors affecting the severity of the disease

A given variety of parasite will not affect all hosts in an identical way. The likelihood of parasite infection being established and the severity of the damage caused depend upon many factors, included the host's genotype, age, nutritional status and immunological history. It has been known for a long time that individuals with certain genotypes, such as those carrying the famous 'sickle-cell' haemoglobin allele, are protected against infection by Plasmodium. More recently, researchers have also discovered genetic variations among humans that affect the severity of the damage caused when Plasmodium infection does become established (Mendis and Carter (1995)). The outcome of an infection also depends upon the genotype of the parasite. An obvious (albeit somewhat unnatural) illustration of this is provided by the existence of localised varieties of *P. falciparum* that show an inherited resistance to certain anti-malarial drugs (Mendis and Carter (1995)).

Factors that weaken the immune system—such as pregnancy (Shulman and Dorman (2003)), micronutrient malnutrition (Nussenblatt and Semba (2002)), and HIV infection (Rowland-Jones and Lohman (2002)—have been associated with an increased incidence of Plasmodium infection, and with a resurgence of symptoms in those chronically infected with the parasite.

2.5 Cellular and molecular biology of Plasmodium

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 malaria 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.

2.5.1 Host Erythrocyte Invasion

Malaria parasites are members of the Apicomplexa, 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. 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 (figure 2.6).

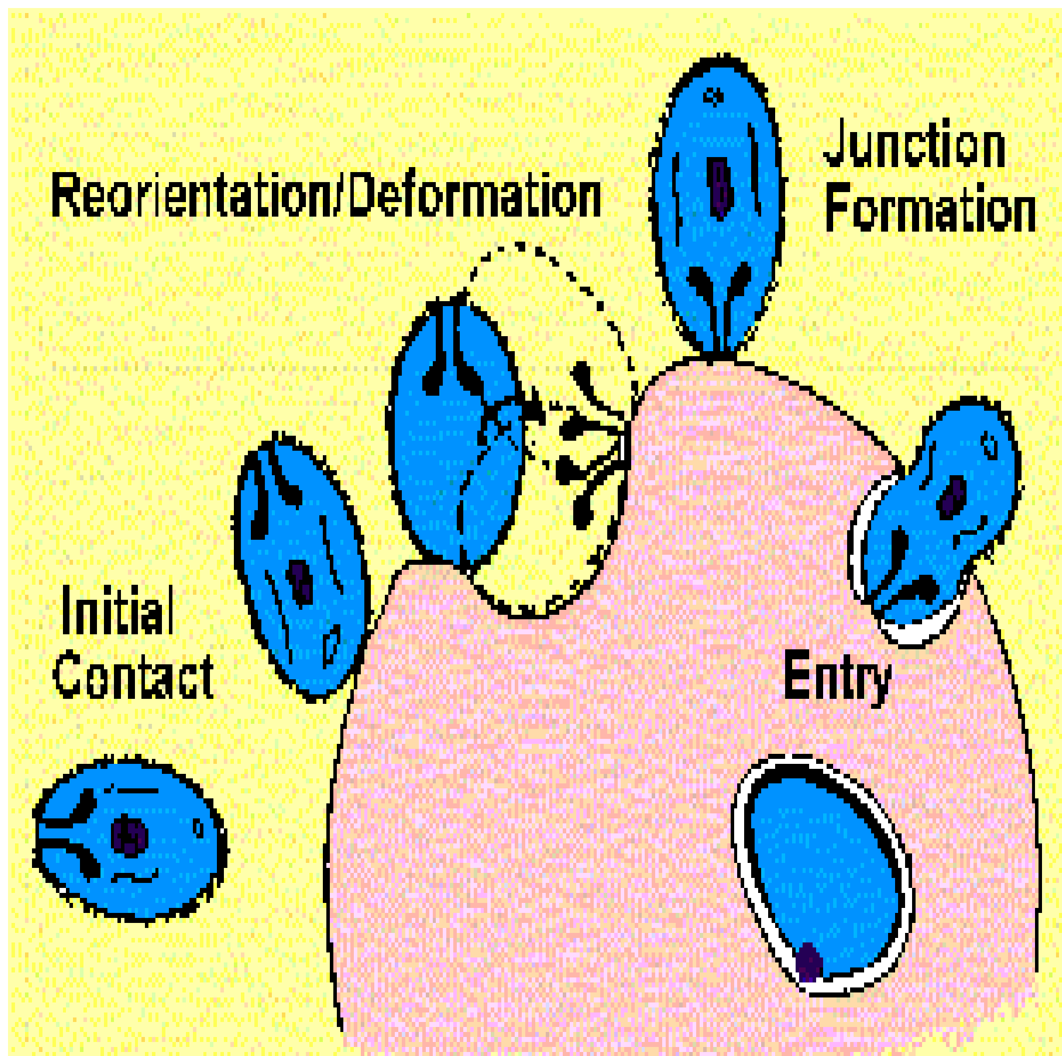


Figure 2.6: Erythrocyte Invasion

2.5.1.1 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 *et al.*, 1994). In addition, MSP-1does bind to band 3 (Goel *et al.*, 2003). Another interesting aspect of MSP-1 is the proteolytic processing that is coincident with merozoite maturation and invasion (Cooper, 1993).

2.5.1.2 Reorientation and Secretory Organelles

After binding to the erythrocyte, the 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 (Mitchell *et al.*, 2004). AMA-1 is a transmembrane protein localized at the apical end of the merozoite and binds erythrocytes.

Specialized secretory organelles are located at the apical end of the invasive stages of apicomplexan parasites. Three morphologically distinct apical organelles are detected by electron microscopy: micronemes, rhoptries, and dense granules. Dense granules are not always included with the apical organelles and probably represent a heterogeneous population of secretory vesicles**.** The contents of the apical organelles are expelled as the parasite invades, thus suggesting that these organelles play some role in invasion (Holder *et al.*, 1994).

2.5.1.3 Specific Interactions and Junction Formation

Following merozoite reorientation and microneme discharge a junction forms between the parasite and host cell. Presumably, microneme proteins are important for junction formation. Proteins localized to the micromenes include:

- EBA-175, a 175 kDa 'erythrocyte binding antigen' from *P. falciparum*
- DBP, Duffy-binding protein from *P. vivax* and *P. knowlesi*
- SSP2, Plasmodium sporozoite surface protein-2. Also known as TRAP (thrombospondin-related adhesive protein).
- Proteins with homology to SSP2/TRAP from Toxoplasma (MIC2), Eimeria (Etp100), and Cryptosporidium
- CTRP, circumsporozoite- and TRAP-related protein of Plasmodium found in the ookinete stage.

Of particular note are EBA-175 and DBP which recognize sialic acid residues of the glycophorins and the Duffy antigen, respectively. The other microneme proteins in the 'TRAP' family have also been implicated in locomotion and/or cell invasion (Tomley and Soldati (2001)). All of these proteins have domains that are presumably involved in cell-cell adhesion, as well as N-terminal signal sequences and trans-membrane domains at their C-termini.

2.5.1.4 Parasite Entry

Apicomplexan parasites actively invade host cells and entry is not due to uptake or phagocytosis by the host cell. In addition, the zoites are often motile forms that that crawl along the substratum by a type of motility referred to as ‘gliding motility’. Gliding motility, like invasion, also involves the release of micronemal adhesins, attachment to the substratum, and a capping of the adhesins at the posterior end of the

zoite. One difference between gliding motility and invasion is that the micronemes must be continuously released as the organism is moving. Thus, gliding motility does not involve this relatively small moving junction, but a continuous formation of new junctions between the zoite and the substratum. In addition, the adhesins are cleaved from the surface of the zoite as the adhesions reach the posterior of the zoite and a trail of the adhesive molecules are left behind the moving zoite on the substratum. However, the mechanism of motility and invasion are quite similar and thus, during invasion the parasite literally crawls into the host cell through the moving junction. In addition, some apicomplexans use this type of motility to escape from cells and can traverse biological barriers by entering and exiting cells.

Many proteins that are involved in the invasion process have been identified. However, much still remains to be learned about the cellular and molecular biology of merozoite invasion (Iyer *et al*., 2007; Baum *et al*., 2009).

2.5.2 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: 1) a microaerophilic environment which is better suited for parasite metabolism, and 2) avoidance of the spleen and subsequent destruction.

2.5.2.1 Knobs and Cytoadherence

A major structural alteration of the host erythrocyte are electron-dense protrusions, or 'knobs', on the erythrocyte membrane of *P. falciparum*-infected cells. The knobs are induced by the parasite and several parasite proteins are associated with the knobs (Deitsch and Wellems (1996)). Two proteins which might participate in knob formation or affect the host erythrocyte submembrane cytoskeleton and indirectly induce knob formation are the knob-associated histidine rich protein (KAHRP) and erythrocyte membrane protein-2 (*Pf*EMP2), also called MESA. Neither KAHRP nor *Pf*EMP2 are exposed on the outer surface of the erythrocyte, but are localized to the cytoplasmic face of the host membrane. Their exact roles in knob formation are not known, but may involve reorganizing the submembrane cytoskeleton.

2.5.2.2 Endothelial Cell Receptors

Several possible endothelial receptors have been identified by testing the ability of infected erythrocytes to bind in static adherence assays (Beeson and Brown (2002)). One of the best characterized among these is CD36, an 88 kDa integral membrane protein found on monocytes, platelets and endothelial cells. Infected erythrocytes from most parasite isolates bind to CD36 and the binding domain has been mapped to the CIDR of *Pf*EMP1. However, CD36 has not been detected on endothelial cells of the cerebral blood vessels and parasites from clinical isolates tend to adhere to both CD36 and intracellular adhesion molecule-1 (ICAM1). ICAM1 is a member of the immunoglobulin superfamily and functions in cell-cell adhesion. In addition,

sequestration of infected erythrocytes and ICAM1 expression has been co-localized in the brain (Turner *et al*., 1994).

2.5.2.3 Antigenic Variation

The encoding of the cytoadherence ligand by a highly polymorphic gene family presents a paradox in receptor/ligand interactions are generally considered highly specific. Interestingly, selection for different cytoadherent phenotypes result in a concomitant change in the surface antigenic type (Biggs *et al*., 1992). Similarly, examination of clonal parasite lines revealed that changes in the surface antigenic type correlated with differences in binding to CD36 and ICAM1 (Roberts *et al*., 1992).

2.6 *Plasmodium berghei berghei*

Plasmodium berghei is a unicellular parasite (protozoan) and it infects mammals other than humans. It is one of the four *Plasmodium* species that have been described in African murine rodents. The four malaria parasites of African murine rodents are:

- *Plasmodium berghei*
- *Plasmodium chabaudi*
- *Plasmodium vinckei*
- *Plasmodium yoelii*

These are not of direct practical concern to man or his domestic animals. The interest of these parasites is that they are practical model organisms in the laboratory for the experimental study of human malaria.

2.6.1 Classification

- **Scientific Classification**

Kingdom	:	Protista
Phylum	:	Apicomplexa
Class	:	Aconoidasida
Order	:	Haemosporida
Family	:	Plasmodiidae
Genus	:	Plasmodium
Species	:	P. berghei

- Binomial name : *Plasmodium berghei*

2.6.2 Description

This species was first described by Vincke and Lips in 1948 in the Belgian Congo (Vincke and Lips (1948)).

2.6.3 Geographical occurrence

P. berghei is found in the forests of Central Africa, where its natural cyclic hosts are the thicket rat (*Grammomys surdaster*) and the mosquito (*Anopheles dureni*).

2.6.4 Mammalian hosts

Thicket rat (*Grammomys surdaster*), *Leggada bella*, *Praomys jacksoni* and *Thamnomys surdaster*, and the vectors are *Anopheles dureni*.

2.6.5 Research

Rodent parasites are recognized 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.

2.6.6 Transmission

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*.

The use of this model malaria parasite has provided biologists and medical researchers with more insight into:

i. The interactions of malaria parasites with the immune system.
ii. The process of infection of the liver by malaria parasites.
iii. The cause of severe pathology, such as cerebral complications in malaria patients.
iv. The infection of the mosquito and transmission of the parasite by the mosquito.

Moreover, *P. berghei* is used in research programs for development and screening of anti-malarial drugs and for the development of an effective vaccine against malaria.

2.6.7 Structures of *Plasmodium berghei* parasite

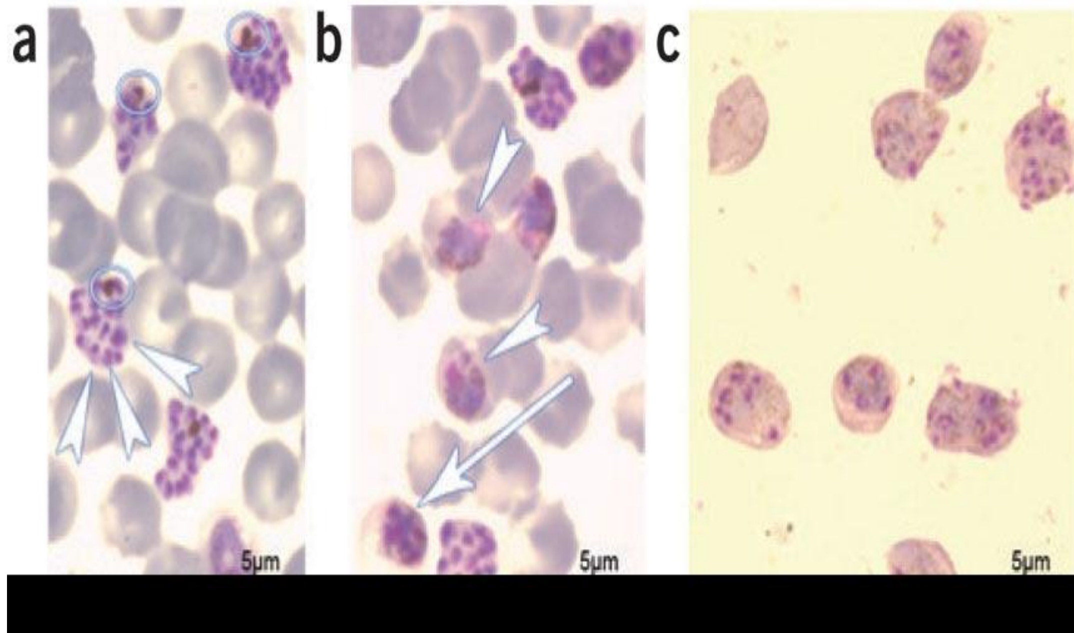


Figure 2.7: Structures of *Plasmodium berghei* parasite

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

2.7 Distribution and Burden of Malaria Disease in Africa

2.7.1 Geographical Distribution

Over 250 million people suffer from malaria every year, of which about one in twenty five die. Of these large numbers, almost 80 to 90 per cent are from sub-Saharan Africa alone (figure 2.8). The rest of the deaths are evenly distributed among countries of Central America and Asia. Thirty-five countries are responsible for 98% of the total malaria deaths world-wide. They also contribute to ~96% of the total number of malaria cases. To achieve the 2010 and 2015 targets, support for these countries is essential: 30 countries in Africa and 5 countries in Asia-Pacific.

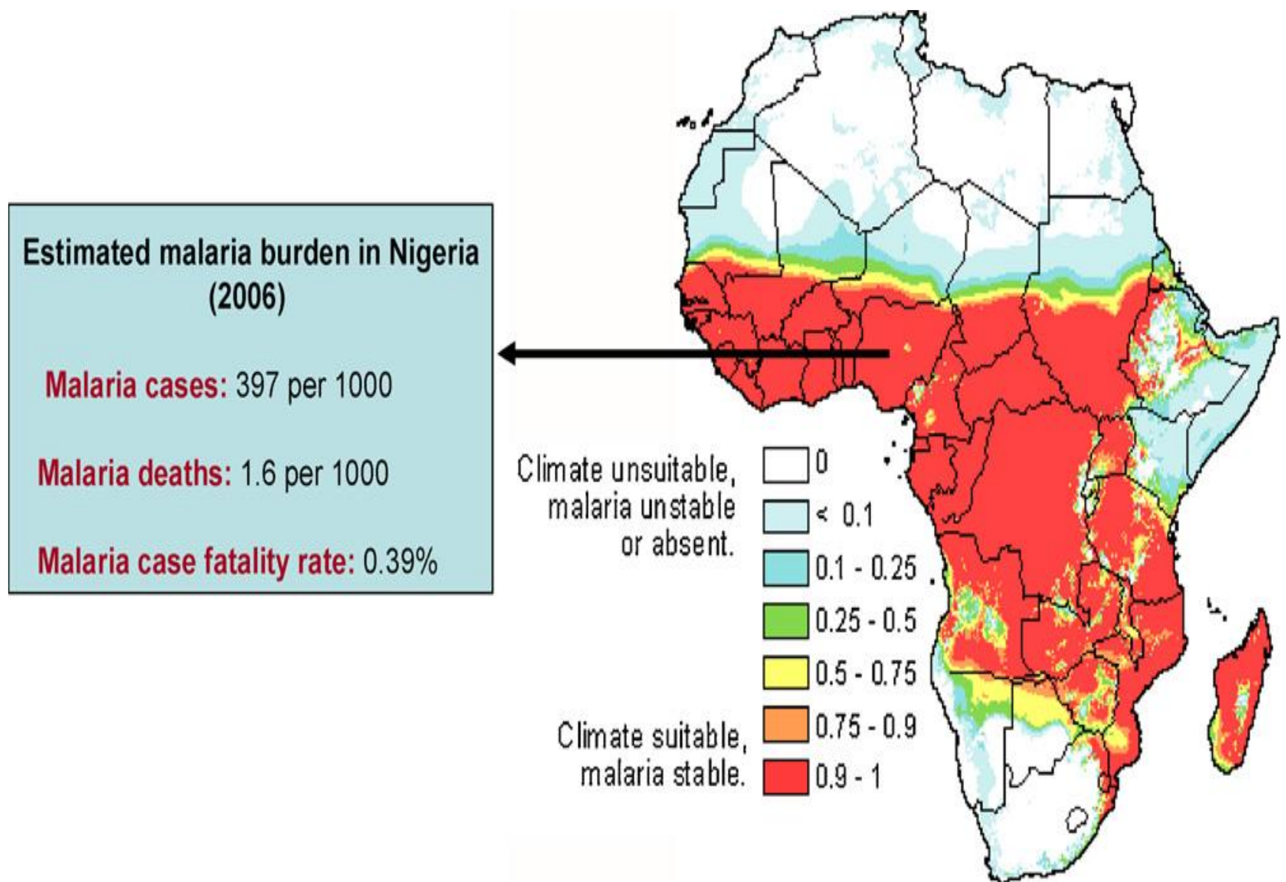


Figure 2.8: Human malaria distribution in Africa and its estimated burden in Nigeria.

Source: WHO, 2008a.

2.7.2 Where Malaria Occurs

Where malaria is found depends mainly on climatic factors such as temperature, humidity, and rainfall. Malaria is transmitted in tropical and subtropical areas, where

i. *Anopheles* mosquitoes can survive and multiply
ii. Malaria parasites can complete their growth cycle in the mosquitoes ("extrinsic incubation period").

Temperature is particularly critical. For example, at temperatures below 20°C (68°F), *Plasmodium falciparum* (which causes severe malaria) cannot complete its growth cycle in the *Anopheles* mosquito, and thus cannot be transmitted. In many malaria-endemic countries, malaria transmission does not occur in all parts of the country. Even within tropical and subtropical areas, transmission will not occur at very high altitudes, during colder seasons in some areas, in deserts (excluding the oases), in some countries where transmission has been interrupted through successful control/elimination programs. Generally, in warmer regions closer to the equator, transmission will be more intense and malaria is transmitted year-round.

2.7.3 The burden of Malaria

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. The disease primarily affects poor populations in tropical and subtropical areas, where the temperature and rainfall are suitable for the development of vectors and parasites (Greenwood *et al*., 2008). More than 40% of the world population is at risk of the disease (Snow *et al*., 2005). An estimated one million people in Africa die from malaria each year and most of these are children under 5 years old. An estimated 1.2 billion are

at high risk of transmission (≥1 case per 1000 population), half of which live in the African regions; 80% of such cases are concentrated in 13 countries, and over half in Nigeria, Congo, Ethiopia, Tanzania and Kenya (WHO, 2008a). Nigeria accounts for a quarter of all malaria cases in Africa (WHO, 2008a). In the southern part of the country, transmission occurs all year round while in the north it is more seasonal. Almost all malaria cases in the country are caused by *Plasmodium falciparum*, considered to be the leading cause of death worldwide in 2004, from a single infectious agent (WHO, 2008b). Malaria is the most common disease in Nigeria; according to the Federal Ministry of Health (2004), half of its population will have one or more malaria attacks annually.

The continuous spread of *Plasmodium falciparum* resistance to antimalarial drugs poses a serious threat to malaria control programs. In Nigeria, a nationwide surveillance data on drug efficacy showed that chloroquine (CQ) and sulphadoxine-pyrimethamine (SP) are no longer viable therapeutic options for the effective treatment of human malaria. Although vaccines could be the best long term control option, they are still undergoing clinical trials (Alonso *et al*., 2005; Aponte *et al*., 2007; Bejon *et al*., 2008; Guinovart *et al*., 2009). This, in addition to the increased number of drug-resistant parasites, makes the development of novel antimalarials urgent. The high cost of malaria treatment has left the poor masses of Nigeria heavily reliant on traditional practitioners and medicinal plants for the treatment of the disease.

2.8 The Malaria Disease

2.8.1 Ecology of Malaria

Factors that determine the occurrence of malaria are those that influence the three components of the malaria life cycle**:**

i. Anopheles mosquitoes must be present

ii. Humans must be present, who are in contact with Anopheles mosquitoes, and in whom the parasites can complete the "vertebrate host" half of their life cycle

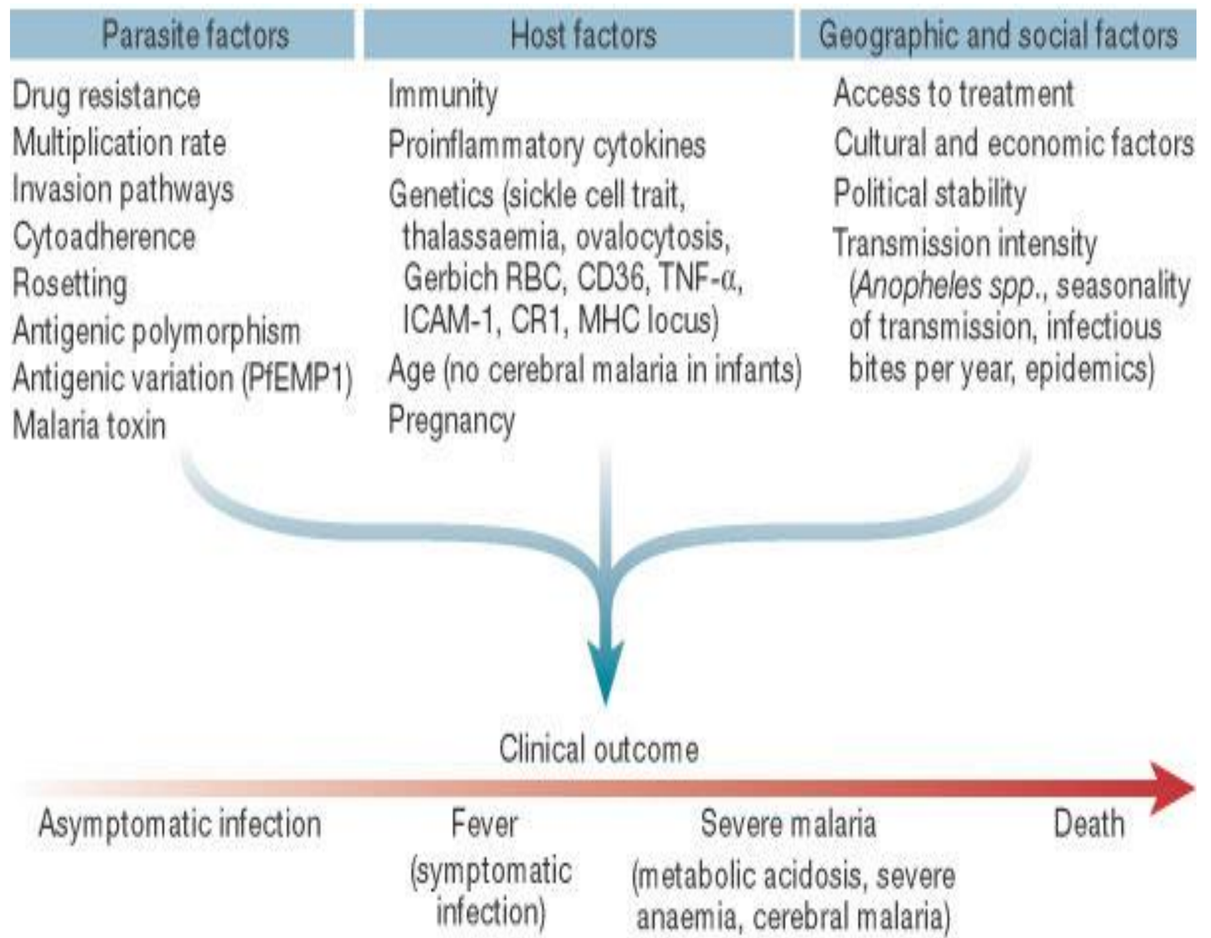
iii. Malaria parasites must be present

iv. Climate can influence all three components of the life cycle. It is thus a key determinant in the geographic distribution and the seasonality of malaria. Rainfall can create collections of water ("breeding sites") where Anopheles eggs are deposited, and larvae and pupae develop into adulthood, a process that takes approximately 9-12 days in tropical areas. Such breeding sites may dry up prematurely in the absence of further rainfall, or conversely they can be flushed and destroyed by excessive rains.

It should be noted that, in rare cases malaria parasites can be transmitted from one person to another without requiring passage through a mosquito (from mother to child in "congenital malaria" or through transfusion, organ transplantation, or shared needles.

2.8.2 Clinical presentation of the Disease

Table 2.1: Clinical presentation of the Disease



Source: Miller and Greenwood (2002)

2.8.3 Transmission of the Disease

Malaria transmission most often occurs through the bite of an *Anopheles* mosquito. No other types of mosquitoes are known to transmit this disease. This type of mosquito becomes infected with one of the four *Plasmodium* parasites that cause malaria in humans, through a previous blood meal from an infected person.

Other modes of transmission include:

i. Rarely malaria can spread by the inoculation of blood from an infected person to a healthy person. In this type of malaria, asexual forms are directly inoculated into the blood and pre-erythrocytic development of the parasite in the liver does not occur. Therefore, this type of malaria has a shorter incubation period and relapses due to persisting exoerythrocytic forms do not occur.
ii. Transfer of parasitized red cells from infected mother to the child either transplacentally or during labor can lead to in malaria in the newborn, called as congenital malaria (Neena *et al*., 2007).
iii. Malaria can be transmitted by transfusion of blood from infected donors.
iv. Cases of malaria transmission through needle-stick injuries, accidentally among health care professionals (some even fatal) or due to needle sharing among drug addicts, have also been reported (Chauhan *et al*., 2009; Weir, 1997).

2.8.4 Diagnosis of the Disease

The preferred and most reliable diagnosis of malaria is microscopic examination of blood films, because each of the four major parasite species has distinguishing characteristics. Two sorts of blood film are traditionally used. Thin films are similar to usual blood films and allow species identification, because the parasite's appearance is

best preserved in this preparation. Thick films allow the microscopist to screen a larger volume of blood and are about eleven times more sensitive than the thin film, so picking up low levels of infection is easier on the thick film, but the appearance of the parasite is much more distorted and therefore distinguishing between the different species can be much more difficult (Warhurst and Williams (1996)).

2.8.5 Disease management/Treatment

Active malaria infection with *P. falciparum* is a medical emergency requiring hospitalization. Infection with *P. vivax*, *P. ovale* or *P. malariae* can often be treated on an outpatient basis. Treatment of malaria involves supportive measures as well as specific antimalarial drugs. Most antimalarial drugs are produced industrially and are sold at pharmacies.

Some of the drugs used in the treatment of malaria include: Amodiaquine (CAMOQUINE), Chloroquine, Sulfadoxine/pyrimethamine (FANSIDAR), Quinine, Primaquine, Mefloquine, Metakalfin, Halofantrin (HALFAN), Artemether (ARTENAM), Arsumax. There are other drugs which have antimalarial activity but are not primarily used as antimalarials. These include: Azithromycin, Clindamycin, Doxycycline, Proguanil, Tetracycline and Septrine (Cotrimoxazole). The use of combination therapy/treatment is the recommended approach and especially atemisinin combined therapy (ACTs)

2.8.6 Prevention and Control

2.8.6.1 Prevention

Methods used to prevent the spread of disease, or to protect individuals in areas where malaria is endemic, include prophylactic drugs, mosquito eradication through In-door

Residual Spraying (IRS) and the prevention of mosquito bites, through the use of Mosquito nets and bed clothes. Vaccine strategies are under investigation. The continued existence of malaria in an area requires a combination of high human population density, high mosquito population density, and high rates of transmission from humans to mosquitoes and from mosquitoes to humans. If any of these is lowered sufficiently, the parasite will sooner or later disappear from that area.

2.8.6.2 Vector control

Efforts to eradicate malaria by eliminating mosquitoes have been successful in some areas. Before DDT (a pesticide), malaria was successfully eradicated or controlled also in several tropical areas by removing or poisoning the breeding grounds of the mosquitoes or the aquatic habitats of the larva stages, for example by filling or applying oil to places with standing water. These methods have seen little application in Africa for more than half a century (Killeen *et al*., 2002).

Sterile insect technique is emerging as a potential mosquito control method. Progress towards transgenic, or genetically modified, insects suggest that wild mosquito populations could be made malaria-resistant. Researchers at Imperial College London created the world's first transgenic malaria mosquito, with the first plasmodium-resistant species announced by a team at Case Western Reserve University in Ohio in 2002 (Ito *et al*., 2002).

2.8.6.3 Other methods

Education in recognizing the symptoms of malaria has reduced the number of cases in some areas of the developing world by as much as 20%. Recognizing the disease in the early stages can also stop the disease from becoming a killer. Education can also inform

people to cover over areas of stagnant, still water e.g. Water Tanks which are ideal breeding grounds for the parasite and mosquito, thus cutting down the risk of the transmission between people. This is most put in practice in urban areas where there are large centres of population in a confined space and transmission would be most likely in these areas.

2.9 Drug Targets

New safe and effective drugs are desperately needed. The majority of drugs are aimed at the fast-replicating stages of the parasite in the blood; only a few drugs kill the liver forms. The parasite has become resistant to most of the drugs used to treat it, the only exception being the most recently developed drug, artemisinine and its derivatives (Bathurst and Hentschel (2006)).

An ideal drug needs to be targeted at a biochemical pathway in the parasite which is not present in the human host. In this regard, the parasite contains a number of unique features in its cells, such as food vacuoles and a body called the apicoplast. These are of much current interest in the search for new drugs. For example, a promising new drug, fosmidomycin, is known to affect an enzyme pathway in the apicoplast (Jomaa *et al.*, 1999). A new antibiotic, azithromycin, has been shown to be effective in mice and monkeys, and has been successfully trialled as a malaria prophylaxis in humans (Taylor *et al.*, 2003). An antibiotic called triclosan, used in mouthwashes, anti-acne preparations and deodorants, could be an effective treatment. It completely clears the parasite from infected mice (McLeod *et al.*, 2001) by blocking a parasitic enzyme called Fab I. New antimalarial drugs may target the gene that produces this enzyme.

2.10 Medicinal Plants

2.10.1 Antiplasmodial potentials of some Medicinal Plants

Traditional herbal medicines have been used to treat malaria for thousands of years in various parts of the world. The first antimalarial drug used in the Occident was extracted from the bark of the Cinchona (Rubiaceae) species, the alkaloid quinine, still largely used. Infusions of the plant bark were used to treat human malaria as early as 1632 (Baird *et al*., 1996). Years later quinine was isolated and characterized (Saxena *et al*., 2003), thus becoming the oldest and most important antimalarial drug. Another ancient medicinal plant of millennium use in the West is *Artemisia annua*, rediscovered in China in the seventies as an important source of the antimalarial artemisinin (Klayman, 1985). Artemisinin-combined therapies (ACT) were formally adopted as first-line treatment of uncomplicated malaria in Nigeria from 2005 onwards (Mokuolu *et al*., 2007). However, ACT use is limited due to its high costs, limited production of artemisinin derivatives to Good Manufacturing Practices (GMP) standards and toxicity (Haynes, 2001; Malomo *et al*., 2001; Borstnik *et al*., 2002; Adebayo and Malomo, (2002); Afonso *et al*., 2006; Boareto *et al*., 2008).

The Research Initiative on Traditional Antimalarial Methods (RITAM) was founded in 1999 with the aim of furthering research on traditional medicines for malaria. This initiative now has in excess of 200 members from over 30 countries. It has conducted systematic literature reviews and prepared guidelines aiming to standardize and improve the quality of ethnobotanical, pharmacological, and clinical studies on herbal antimalarials and on plant based methods of insect repellence and vector control (Bodeker and Willcox (2000)). To date, 1277 plant species from 160 families used to treat malaria or fever have been listed on a database known as IVmal (importance value

for the treatment of malaria). No doubt, emergence of chloroquine-resistant Plasmodium falciparum (CRPF) has further made treatment of malaria in children more complicated, so contributing immensely to the deterioration of malaria therapy and control in Nigeria.

2.10.2 Nigerian medicinal plants used for malaria treatment

Neither the Cinchona plants nor Artemisia annua, from which the most potent drugs (quinine and artemisinin) were isolated, are indigenous to sub-Saharan Africa. Tropical rainforest plants are known to have higher concentrations of natural chemical defences and a greater diversity than plants from any other biome, thus they are potential sources of new medicines (Balick *et al*., 1996). It seems logical then to encourage studies on plants from these regions, especially since the major proportion of malaria attributable deaths occur in sub-Saharan African regions.

Nigeria has rich flora diversity and many of the plant species are used by some indigenous people for medicinal purposes. A larger number of medicinal plants are used to treat malaria in the Southern part of the country 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 (Adebayo and Krettlia (2011)). They included plants such as leaves of *Newbouldia laevis* (Akoko leaf in Yoruba), *Enatia chlorantha* (African yellow wood, Iyani or Awopa in Yoruba), *Eupatorium odoratum* (ogbogbo or Ibo- ofo in Yoruba), *Bridelia micrantha* (abere-aluko or akisan in Yoruba), *Cajanus Cajan* (Pigeon pea in English, olele in Edo, shingwazo in Gwari), *Carica papaya* (pawpaw). In addition, the 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 included 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.

Alstonia boonei plant stem bark or leaves are administered as decoction or "teas" and sometimes as an ingredient in malaria "steam therapy". Recently, the stem bark extract was formulated into tablets, and made available as an antimalarial remedy (Majekodunmi *et al*., 2008). Plants of the Meliaceae family are also commonly used for malaria treatment in Nigeria, such as *Azadirachta indica*, *Khaya senegalensis* and *Khaya grandifoliola. Azadirachta indica* is called "neem tree" and is also used in other African countries as a decoction against fever and/or malaria. It is used in traditional medical practice in form of an aqueous decoction of the leaves, stem bark and root (Obih *et al*., 1985). Due to the importance of this plant as an antimalarial remedy in Nigeria, efforts were made to produce the tablet suspensions of the bark and leaf which exhibited high prophylactic, moderate suppressive and a very minimal curative schizonticidal effect in mouse model of malaria (Isah *et al*., 2003).

Morinda lucida (Rubiaceae) is largely used in malaria treatment in Nigeria. The aerial parts, stem bark or root bark of *Morinda lucida* are widely used in West Africa to treat malaria and other tropical diseases. A seasonal variation in its antimalarial activity has been reported (Makinde and Obih (1985)). *Nauclea latifolia* (Rubiaceae) is used as aqueous decoction of the root bark against malaria. Several other plants are used for malaria treatment in Nigeria, like *Quassia amara* and *Quassia undulata* (Simaroubaceae), largely used in the southwestern part of the country (Phillipson and Wright (1991)). *Quassia amara*, called bitterwood tree, with 2–6m in height, has the

highest antimalarial reputation for curative and preventive purposes in the Simaroubaceae family. *Enantia chlorantha* (Annonaceae) stem bark is used against fever/malaria by traditional medicine practitioners in the forest regions. *Fagara zanthoxyloides* (Rutaceae) has its root widely used as chewing stick in Nigeria and West Africa in general (Odebiyi and Sofowora (1979)). The aqueous extract of the root is used for malaria treatment by the indigenous people. *Spathodea campanulata* (Bignoniaceae), popularly known as African tulip tree is native to tropical Africa, though it has now been adapted to other tropical regions around the world, mostly because of its ornamental value. It is used in southwestern Nigeria for malaria treatment by drinking the decoction of its stem bark.

2.10.3 Combination of Herbal Drugs

Most of the antimalarial plants mentioned above, are used in form of monotherapy, and only a few plants are taken together in combined therapies. An example is the multi-herbal extract referred to as 'Agbo-Iba' madeup of *Cajanus cajan* (pigeon pea) leaf, *Euphorbia lateriflora* leaf, *Mangifera indica* leaf and bark, *Cassa alata* leaf, *Cymbopogon giganteus* leaf, *Nauclea latifolia* leaf, and *Uvaria chamae* bark (Nwabuisi, 2002). Another multi-herbal combination is the mixture of *Carica papaya* leaves, *Cymbopogon citratus* leaves, *Anacardium occidentale* leaves and *Azadirachta indica* leaves used in 'steam therapy', in which the patients are covered with a thick blanket and made to inhale the vapour from the cooking pot.

2.10.4 Safety and efficacy of Herbal drugs

There are several problems in the usefulness of these traditional remedies for the treatment of Malaria especially with regards to safety and efficacy of the herbs used. One of such problems is the fact that often times, studies reported by researchers provides limited information on the methods used to prepare the remedies, making it

difficult to replicate them. In some cases, this was deliberate, to protect intellectual propertyrights (Willcox, 1999). Furthermore, only few studies provide data on side effects or postulate any likely side effect (Willcox, 1999). For example, almost half of the patients taking the Uganda herbal remedy “AM” experienced one or more minor side effects (Willcox and Bordeker (2004)). These were sufficiently unpleasant in some cases to deter patients from continuing treatment (for example, diarrhea, and bitter taste). Only 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).

2.10.5 General Profile of *Morinda lucida*

2.10.5.1 Taxonomy

Kingdom : Angiosperm

Phylum : Eudicots

Order : Gentianales

Family : Rubiaceae

Genus : Morinda

Species ; Morinda Lucida

Name: *Morinda lucida* Benth.

2.10.5.2 Ecology

Morinda lucida grows in grassland, exposed hillsides, thickets, forests, often on termite mounds, sometimes in areas which are regularly flooded, from sea-level up to 1300m altitude.

2.10.5.3 Botanic Description

Evergreen shrub or small to medium-sized tree up to 18 (–25) m tall, with bole and branches often crooked or gnarled; bark smooth to roughly scaly, grey to brown. Leaves opposite, simple and entire; stipules ovate or triangular, 1–7 mm long, falling early; petiole up to 1.5 cm long; blade elliptical, 6–18 cm × 2–9 cm, base rounded to cuneate, apex acute to acuminate, shiny above, sometimes finely pubescent when young, later only tufts of hairs in vein axils beneath and some hairs on the midrib. Inflorescence a stalked head 4–7 mm in diameter, 1–3 at the nodes opposite a single leaf; peduncle up to 8 cm long bearing at base a stalked cup-shaped gland.

2.10.5.4 Properties

From the wood and bark of *Morinda lucida* 18 anthraquinones have been isolated, including the red colorants 1-methylether-alizarin, rubiadin and derivatives, lucidin, soranjidiol, damnacanthal, nordamnacanthal, morindin, munjistin and purpuroxanthin. Two anthraquinols, oruwal and oruwalol, have also been found; these give a yellow colour and possibly are intermediates in the formation of anthraquinones. In addition to anthraquinones, tannins, flavonoids and saponosides have been isolated. The wood is yellow (hence the name brimstone tree), darkening to yellow-brown in the sapwood and to dark brown in the heartwood. It is medium-weight and hard; it works and finishes well, and it is durable, being resistant to fungi, termites and other insects.

2.10.5.5 Functional Uses

The wood of *Morinda lucida* yields yellow to red dyes. In Nigeria and Gabon the root bark is used to dye textiles into scarlet red. On occasions of national grief or the death of a chief, the Ashanti people of Ghana dye cotton cloths red with the root bark of *Morinda lucida*. These cloths, called ‘kobene’, are worn as mourning dress by official people and by the family of the deceased. The root is the most important traditional source of yellow dye for textiles in the Kasai Province of DR Congo. It can be used without a mordant. The root is also added to indigo vats in Côte d’Ivoire, to contribute both to the fermentation and reduction process necessary for dyeing with indigo and to get darker blues. In this process it is often combined with leafy twigs of *Saba comorensis* (Bojer) Pichon (synonym: *Saba florida* (Benth.) Bullock). In the region of Kasongo in north-eastern DR Congo, young leaves of *Morinda lucida* are combined with leaves of a *Philenoptera* species (a source of indigo) to obtain a pale green dye used in basket weaving. The bitter-tasting roots are used as flavouring for food and alcoholic beverages and in Nigeria they are popular as chewing sticks. The wood is excellent for making charcoal, but is also used for construction, mining props, furniture, canoes, poles and fuel wood. The leaves are used for cleaning and scouring, e.g. of calabashes.

2.10.5.6 Medicinal uses

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 (Tona *et al*; 1999; Agomo *et al*., 1992; Asuzu and Chineme (1990); Makinde and Obih (1985); Koumaglo *et al*., 1992), anti-salmonella typhi activity (Akinyemi *et al*., 2005) effect on contractility of isolated uterine smooth muscle of pregnant and non-pregnant mice (Elias *et al*., 2007), toxicity and mutagenic studies (Sowemimo *et al*., 2007; Akinboro and Bakare (2007); Raji *et al*., 2005) and anti-diabetic property (Olajide *et al*., 1999) of *Morinda Lucida* extracts have all been reported.

The petroleum ether extract and fractions of the leaf samples of *Morinda lucida* have been 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.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant material

The Plant parts (leave and root) of *Morinda lucida* were collected in the month of July, 2010 at Idiche, in Okene, Kogi State, Nigeria. The plant was identified and authenticated by Mrs Jamila Ibrahim of the Department of Medicinal Plant Research and Traditional Medicine, National Institute for Pharmaceutical Research and Development, (NIPRD), Idu Abuja, Nigeria.

3.1.2 Parasites

The chloroquine sensitive *Plasmodium berghei berghei* (NK-65) was obtained from the Department of Biochemistry, Ahmadu Bello University, Zaria. The parasites were kept alive by continuous re-infestation (I.P) in mice (Carvalho *et al*., 1991) every 10 days.

3.1.3 Animals

A total of one hundred and forty seven Swiss albino mice (22-28g average body weight) were used. These were obtained from the Animal facility centre of the Department of Pharmacology and Toxicology, Ahmadu Bello University, Zaria, Nigeria and used for the study. The animals were fed ad *libitum* with standard feed and had free access to water. They were also maintained under standard conditions of humidity, temperature and 12 hrs light/darkness cycle. The animals were acclimatized for two weeks before the commencement of the study. A standard protocol was drawn up in accordance with the

Good Laboratory Practice (GLP) regulations (ENV/MC/CHEM (98) 17, 1998). The principle of laboratory animal care (NIH Publication No. 85-23, 1985) was also followed in this study.

3.1.4 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 about $1x10^7$ parasitized erythrocytes.

3.2 Methods

3.2.1 Extraction of plant materials

The root and leaf parts of the plant were air dried under shade and samples were pulverized into powder. A 70g weight of each sample was extracted successively in hexane, ethyl acetate and methanol. The extraction was done in each solvent system exhaustively for two hours, using the soxhlet apparatus, after which it was filtered hot and then allowed to evaporate. After the use of one solvent, the marc was allowed to dry before extracting in the next solvent, starting with the less polar solvent, then the medium polar, and then finishing with the polar solvent.

3.2.2 Phytochemical Screening

Phytochemical analysis to screen the crude extract for the presence of alkaloids, flavonoids, tannins, saponins, glycosides and carbohydrates was carried out according to the method described by (Odebiyi and Sofowora 1978, 1982 and 1986; Trease and Evans, 1989).

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 a test tube. The test tube was corked and shaken vigorously for 30seconds and allowed to stand for 30minutes. A honey comb forth was an indicator of the presence of saponins (Sofowora, 1993).

3.2.2.2 Test for tannin

The crude extract (0.1mL)was diluted with distilled water in the ratio of 1:4 and few drops of 10% ferric chloride solution was added. A blue to green colour indicated the presence of tannins (Evans, 1989).

3.2.2.3 Test for flavonoids

To 1.0ml of the crude extract, 1.0mL of 10% Lead acetate was added. The formation of yellow precipitate indicated a positive test for flavonoids.

Dilute NaOH (1.0mL)was added to 1.0ml of the crude extract. Precipitation showed the presence of flavonoids.

3.2.2.4 Test for Alkaloid

The crude extract (1.0mL) was shaken with 5mL of 2% HCl on a steam bath and filtrated. To 1mLof the filtrate, Wagner's reagent was added and observed for reddish brown precipitate. To another 1.0mL portion of the filtrate, Meyers' reagent was added and observed for a cream coloured precipitate

3.2.2.5 Test for glycosides

To the crude extract Fehling's reagent was added and boiled for two minutes. A brick red colouration indicates the presence of glycosides.

3.3 Preliminary Toxicology Studies

3.3.1 Acute Oral Toxicity Test (Estimation of LD_{50})

The acute toxicity of the extract was determined by evaluating its median lethal dose (LD_{50}) using the Lorkes method (Lorke, 1983). The test was carried out in two phases. Phase 1: Nine mice were divided into three groups (A, B and C) of three mice each. The three groups were administered orally with graded concentrations (10, 100 and 1000 mg kg^{-1} weight, respectively) of the crude hot methanolic extract of *Morinda lucida*. The animals were monitored for mortality, negative physical signs such as depression, loss of appetite, change in respiration, for a 24hour period. In the phase 2 experiment, nine mice were divided into three groups (D, E and F), each group consisting of three mice. These received graded concentration of 1600, 2900 and 5000 mg kg^{-1} body weight of the extract, respectively. All the Animals were monitored for pains, distress, behavioural alterations and most importantly, death for a period of 24hours.

3.3.2 Assay of Hepatic enzymes

This was done to evaluate the activities of the liver enzyme, since the liver is the site of drug metabolism, and is the major organ affected by the malaria parasite. The serum and the liver were used for this analysis. A total of twelve mice were used for the study. The mice were divided into four groups of three mice each. One group was infected on day 0, with *plasmodium berghei* parasite intraperitoneally, with the aid of needle and syringe

and after 72 hours of infection, treated with 400 mg/kg/day crude hot methanolic root extract of *Morinda lucida* for 5 days (day 2 to day 7). Another group was given 400 mg/kg/day of the crude hot methanolic root extract of *Morinda lucida* for 5 days (day 2 to day 7). The third group was infected on day 0 and not treated, while the last group was not infected, and not administered the extract, but was given 0.2mL/day. On day 8, all the Animals were sacrificed, blood collected in centrifuge tubes, dissected, and liver taken, weighed, and put in sucrose solution.

Group 1: Extract Control (Not infected, but administered 400mg/kg b.w of crude methanolic extract of *Morinda lucida* Root).

Group 2: Infected and treated with 400mg/kg b.w of crude methanolic extract of *Morinda lucida* Root).

Group 3: Infected, but not treated

Group 4: Not infected, not treated (Positive control).

3.3.2.1 Tissue collection and preparation

Collection of sample for biochemical analyses was described previously (Yakubu *et al*., 2005). Mice were anaesthetized using cotton wool soaked in chloroform and blood sample collected into a clean, dry centrifuge tubes. The blood sample were 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 the fridge until needed for analysis. The tissues (liver) were excised, weighed, and transferred into 0.25M sucrose solution. The tissues which were weighed (1g) and finely cut with clean sterile blade were homogenized in 4ml of ice cold 0.25M sucrose solution using small mortar and pestle. These were then transferred into clean centrifuge tubes

and centrifuged at 1000rpm for 10minutes. The supernatants were carefully withdrawn and also stored in the fridge, until needed for analyses.

3.3.2.2 Biochemical Analysis

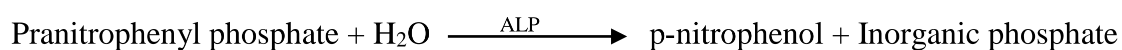
The total protein concentrations of serum and Liver were determined using biuret method (Gornall *et al*., 1949) as described by Plummer (1978). Serum and Liver collection as well as Enzyme assays were carried out using AGAPPE Diagnostic kit, Switzerland GmbH. Alkaline phosphatase (ALP) was determined based on the method of Wright *et al*., 1972, Gamma glutamyl transferase (GGT), Glutamate oxaloacetate transaminase (GOT) and Glutamate pyruvate transaminase (GPT) activities were carried out using the method described by Reitman and Frankel (1957).

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.

Determination of Alkaline phosphatase (ALP) activity

Principle; Paranitrophenyl phosphate reacts with water in the presence of alkaline phosphatase, to give paranitrophenol and inorganic phosphate.


Pranitrophenyl phosphate + H_2O $\xrightarrow{ALP}$ p-nitrophenol + Inorganic phosphate

Procedure:

The working reagent (1000µl) was mixed with 20µl of the serum and liver samples, incubated for one minute and the change in absorbance per minute during three minutes

was read at 405nm wavelength. The blank used was distilled water, and the cuvette used was 1cm light path.

Determination of Glutamate oxaloacetate transaminase (GOT) activity

Principle:

AST – Aspartate aminotransferase

MDH – Malate dehydrogenase

Procedure:

The working reagent (1000µl) was mixed with 100µl of the serum and liver samples, incubated for one minute and the change in absorbance per minute during three minutes was read at 340nm wavelength. The blank used was distilled water, and the cuvette used was 1cm light path.

Determination of Glutamate pyruvate transaminase (GPT) activity

Principle:

ALT: Alanine aminotransferase

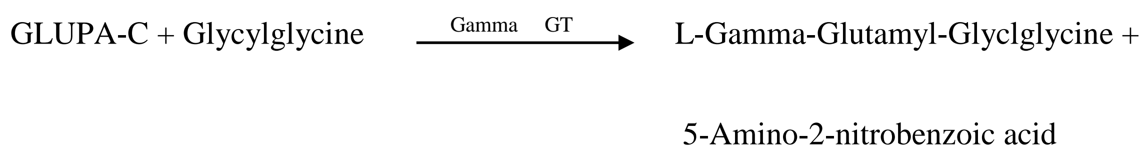
LDH: Lactate dehydrogenase

Procedure:

The working reagent was (1000µl) mixed with 100µl of the serum and liver samples, incubated at 37°C for one minute and the change in absorbance per minute during three minutes was read at 340nm wavelength. The blank used was distilled water, and the cuvette used was 1cm light path.

Determination of Gamma glutamyl transferase (GGT) activity

Principle:



GLUPA-C + Glycylglycine $\xrightarrow{\text{Gamma GT}}$ L-Gamma-Glutamyl-Glyclglycine + 5-Amino-2-nitrobenzoic acid

Procedure:

The working reagent (1000µl) was mixed with 100µl of the serum and liver samples, incubated for one minute and the change in absorbance per minute during three minutes was read at 405nm wavelength. The blank used was distilled water, and the cuvette used was 1cm light path.

3.4 In vivo Antiplasmodial Test of Extracts

3.4.1 Four-day Suppressive Test

A total of twenty five mice were used for this study. Each mouse was given standard intra-peritoneal inoculums of $1.0x10^7$ *P. berghei berghei* parasites with the aid of a 1 mL disposable syringe. The animals were divided into five groups of five mice each. Different doses of the extract (100, 200, and 400 mg/kg/day) were administered orally to three groups. Chloroquine phosphate 5mg/kg/day was given as positive control to one

group and 0.2 mL of normal saline as negative control to the last group, for four consecutive days (D_0 to D_3). On the fifth day (D_4), thin blood smears were prepared and blood films were fixed with methanol. The blood films were stained with Giemsa and then microscopically examined with 100-x magnification. The percentage suppression of parasitaemia was calculated for each dose level by comparing the parasitaemia in infected but untreated controls, with those of treated mice (Knight and Peters, 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.

3.4.2 Curative Test

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 into the mice intraperitoneally. Seventy-two hours later, the mice were divided into five groups of four mice each. Different doses of the extract (50, 100, 200, and 400 mg/kg/day) were administered orally to three groups. Chloroquine phosphate (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. 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 over a period of 28 days (D_0-D_{27}) (Ryley and Peters (1970); Chandel and Bagai (2010)).

3.4.3 Prophylactic Test

A total of twenty mice were used for the study. Five groups of four mice per group were used. Three groups were administered with 100, 200, and 400 mg/kg/day doses of the extract orally. The other two groups were administered with 5mg/kg/day of chloroquine phosphate and 0.2 mL/mouse/day of normal saline orally. The animals were dosed for four consecutive days (D_0-D_3). On the fifth day (D_4), the mice were challenged with *P. berghei berghei* infected red blood cells. Seventy-two hours later, the parasitaemia level was assessed by studying Giemsa stained thin blood smears (Peters, 1965; Okokon *et al.*, 2005). Variation in weight was monitored in the course of the study. Also, mean survival time for each group was determined arithmetically by finding the average survival time (days) of the mice (post-inoculation) in each group.

3.5 Partial purification of Crude extract

3.5.1 Column Chromatography

This was carried out in order to fractionate the crude hot methanolic extract of *Morinda lucida* using solvent partition method. Three solvent systems were used; hexane, ethyl acetate, and methanol.

The column was washed, dried, and clamped on a retort stand. A glass wool (0.1g)was inserted at the base of the column. About 30g of an already activated silica gel of 60 – 120 mesh, was mixed with 100mL of Hexane, and then packed into the column. The solvent (hexane) was gradually eluted and poured back into the column, until the column was uniformly packed.

The crude methanolic root extract of *Morinda lucida* (12g) was weighed and dissolved in the extracting solvent (methanol). An already activated silica gel of 60 – 120 mesh was then added to it, until it was observed that the silica gel had absorbed the extract. This was then dried under the sun. The final weight of the sample after drying was taken.

After the column had been uniformly packed, the dried sample (i.e. mixture of the extract and silica gel) was then poured into the column. The fractionation process was done first with the non-polar solvent, then with gradual increase in solvent polarity. The table below shows the polarity of the solvents used with gradual increase during the fractionation process.

Table 3.1: Fractionation with gradual increase in solvent polarity

S/no	Hexane	Ethylacetate	Methanol	Volume (ml)
1	100	-	-	100
2	90	10	-	100
3	80	20	-	100
4	70	30	-	100
5	60	40	-	100
6	50	50	-	100
7	40	60	-	100
8	30	70	-	100
9	20	80	-	100
10	10	90	-	100
11	-	100	-	100
12	-	90	10	100
13	-	80	20	100
14	-	70	30	100
15	-	60	40	100
16	-	50	50	100
17	-	40	60	100
18	-	20	80	100
19	-	-	100	100

3.5.2 Thin Layer Chromatography (TLC)

Each of the nineteen fractions obtained from the column chromatography were spotted in pre-coated aluminium thin layer plates, and developed in four solvent systems; 100% hexane, 100% chloroform, combination of chloroform and methanol in the ratio 9:1 and 4:1. The solvent system that gave the best result was used as a basis for pulling fractions with similar R_f values together. Four fractions were finally obtained for crude hot methanolic root extract of *morinda lucida*.

3.5.3 Curative test for partially purified *Morinda lucida* crude methanolic root extract

A total of twenty eight 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 seven groups of four mice each. Dose of 400mg/kg/day of crude hot methanolic root extract of *Morinda lucida* was administered to one group, four groups were administered 400mg/kg/day each of the four fractions obtained from the crude hot methanolic root extract of *Morinda lucida*. Chloroquine (5mg/kg/day) was given to the positive control group and 0.2 mL of normal saline to the negative control group. The extracts were given once daily for 5 days. Thin blood smears were prepared from tail of each mouse on D_2, D_5 and D_7 of the study period to monitor the parasitaemia level. Variation in weight was monitored in the course of the study. The mean survival time for each group was determined arithmetically by finding the mean survival time (days) of the mice (post-inoculation) in each group over a period of 28 days (D_0-D_{27}) (Ryley and Peters (1970); Chandel and Bagai (2010)). Thin blood smears of the mice that survived beyond 28 days were made, to investigate if actually the parasite had been cleared completely.

3.6 In vivo antiplasmodial test on combination of extracts (Curative test)

Curative test was carried out on mice to assess the antiplasmodial efficacy of combination of extracts. In this test, antiplasmodial test was carried out on the hot methanolic leaf extract of *Morinda lucida* alone, hot methanolic root extract of *Morinda lucida* alone and a combination of the hot methanolic root and leaf extracts of *Morinda lucida*, in the ratio 1:1. A total of twenty four 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 eight groups of three mice each. Two groups were administered hot methanolic leaf extract of *Morinda lucida* alone with doses 100 and 200 mg/kg /day, another two groups were administered hot methanolic root extract of *Morinda lucida* alone with doses 100 and 200 mg/kg /day, two groups were also administered a combination of hot methanolic leaf and root extract of *Morinda lucida*, in the ratio 1:1, with doses 50 and 100 mg/kg/day. Chloroquine (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 period of 28 days (D_0-D_{27}) (Ryley and Peters (1970); Chandel and Bagai (2010)). Variation in weight was also monitored in the course of the experiment.

3.7 Determination of Packed Cell Volume (PCV)

This was carried out to monitor the level of Red blood cells in the course of the experiment. The method employed was the method described by Dacie and Lewis (1991). The tips of the tails of the mice were cut, to obtain blood. The blood was

collected in heparinized capillary tubes and sealed with sealing agent (plastacin). The capillary tubes were then centrifuged, using a micro hematocrit centrifuge, at 11,000 rpm (revolution per minute), for 5minutes. The packed cell volume was then read using the micro haematocrit reader.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Phytochemical Screening

In the phytochemical screening of the crude hot methanolic root extract of *Morinda lucida* carried out (Table 4.1), it was found to contain Flavonoids, Alkaloids, Phenolic nucleus and phlobotannins.

Table 4.1: Phytochemical constituents of methanolic root extract of *Morinda lucida*

Bioactive agents	Result
Tannins	-
Flavonoids (Phenolic nucleus)	+
Flavonoids	+
Alkaloids	+
Phlobotannins	+

Key:

+: Present

- : Absent

4.2 Preliminary Toxicological Studies

4.2.1 Acute oral toxicity (estimation of LD_{50})

The LD_{50} of the crude hot methanolic root extract of *Morinda lucida* (Table 4.2) was estimated to be 3807.89mg/kg (approximately 3800mg/kg).

Table 4.2: Acute oral toxicity test

Extract	1st part of investigation		2nd part of investigation	
	Doses (mg/kg)	Mortality	Doses (mg/kg)	Mortality
M.lucida	10	0/3	1600	0/3
(Root)	100	0/3	2900	0/3
	1000	0/3	5000	1/3

From the result compiled in the table above, the LD_{50} was estimated in the following manner:

$\sqrt{}$ minimum toxic dose x maximum tolerated dose

OR

$\sqrt{}$ maximum dose for all survival x minimum dose for all death

Minimum toxic dose = 5000mg/kg

Maximum tolerated dose = 2900mg/kg

Therefore,

$LD_{50} = \sqrt{5000 \times 2900}$

=3807.89mg/kg

$LD_{50} \approx 3800$mg/kg

4.2.2 Assay of hepatic enzymes

The specific Alkaline Phosphatase (ALP) activities for serum and liver were found to be highest in group 3, infected not treated (2681.58 ± 628.21 and 8848.89± 5387.07 for serum and liver respectively), than all other groups. The specific ALP activity for serum was lowest in group 1 (not infected, but administered extract), but the specific ALP activity for the liver was found to be lowest in group 4 animals, which were neither infected nor treated (Table 4.3)

The specific Glutamate Oxaloacetate Transaminase (GOT) activities for serum and liver for group 2 animals (infected and treated with extract) were found to be highest (6734.14 ± 1087.75 and 5643.65 ± 4814.62 for serum and liver respectively), compared to the other groups. Specific serum and liver GOT were found to be lowest (694.65 ± 156.75 and 358.12 ± 135.29 for serum and liver respectively), in group 4 animals (not infected, not treated), as shown in Table 4.4.

In Table 4.5, the specific serum and liver Glutamate Pyruvate Transaminase (GPT) activities were estimated to be highest (3490.41 ± 438.62 and 24009.63 ± 3304.22 for serum and liver respectively), in group 3 animals (infected, not treated), compared to all the other groups. The specific serum GPT was found to be lowest (323.54 ± 35.04) in group 1 animals (not infected, but treated) and specific liver GPT, was found to be lowest in group 4 animals (infected, not treated).

The specific Gamma Glutamyl Transferase (GGT) activities for serum and liver for group 3 animals (infected, not treated) were found to be highest (2495.53 ± 732.44 and 10350.79 ± 4895.68 for serum and liver respectively), compared to the other groups. Specific serum and liver GGT were found to be lowest (322.45 ± 118.84 and 52.64 ±

12.80 for serum and liver respectively), in group 4 animals (not infected, not treated), as shown in Table 4.6.

Table 4.3: Specific alkaline phosphatase (ALP) activities

GROUP	Specific enzyme activity (U/L)	
	Serum	Liver
1	1339.56 ± 319.20 [a]	147.68 ± 53.16 [a]
2	2212.16 ± 994.68 [ab]	2894.21 ± 1807.22 [ab]
3	2681.58 ± 628.21[abc]	8848.89± 5387.07 [abc]
4	2495.83 ± 189.62 [abcd]	77.41 ± 22.41[abcd]

Values are ± standard error of mean for three replicate (n).

Values with different letters (superscript) were significantly different from each other at $p< 0.05$

Group 1: Extract Control (Not infected, but administered 400mg/kg b.w of crude methanolic extract of *Morinda lucida* root).

Group 2: Infected with *Plasmodium berghei* and treated with 400mg/kg b.w of crude methanolic extract of *Morinda lucida* root).

Group 3: Infected with *Plasmodium berghei*, but not treated

Group 4: Not infected, not treated (Positive control).

Table 4.4: Specific glutamate oxaloacetate transaminase (GOT) activities

GROUP	Specific enzyme activity (U/L)	
	Serum	Liver
1	2167.35 ± 120.62[a]	412.80 ± 199.22[a]
2	6734.14 ± 1087.75[b]	5643.65 ± 4814.62[ab]
3	4361.53 ± 1723.01[abc]	43252.76 ± 27583.20[abc]
4	694.65 ± 156.75[ad]	358.12 ± 135.29[abcd]

Values are ± standard error of mean for three replicate (n).

Values with different letters (superscript) were significantly different from each other at $p < 0.05$

Group 1: Extract Control (Not infected, but administered 400mg/kg b.w of crude methanolic extract of *Morinda lucida* root).

Group 2: Infected with *Plasmodium berghei* and treated with 400mg/kg b.w of crude methanolic extract of *Morinda lucida* root).

Group 3: Infected with *Plasmodium berghei*, but not treated

Group 4: Not infected, not treated (Positive control).

Table 4.5: Specific glutamate pyruvate transaminase (GPT) activities

GROUP	Specific enzyme activity (U/L)	
	Serum	Liver
1	323.54 ± 35.04^{a}	103.57 ± 13.95^{a}
2	2454.22 ± 1097.85^{b}	1936.57 ± 962.29^{ab}
3	3490.41 ± 438.62^{bc}	24009.63 ± 3304.22^{c}
4	955.74 ± 157.25^{abd}	79.64 ± 19.62^{abd}

Values are ± standard error of mean for three replicate (n).

Values with different letters (superscript) were significantly different from each other at $p < 0.05$

Group 1: Extract Control (Not infected, but administered 400mg/kg b.w of crude methanolic extract of *Morinda lucida* root).

Group 2: Infected with *Plasmodium berghei* and treated with 400mg/kg b.w of crude methanolic extract of *Morinda lucida* root).

Group 3: Infected with *Plasmodium berghei*, but not treated

Group 4: Not infected, not treated (Positive control).

Table 4.6: Specific gamma glutamyl transferase (GGT) activities

GROUP	Specific enzyme activity (U/L)	
	Serum	Liver
1	451.48 ± 271.35^{a}	100.10 ± 28.29^{a}
2	1044.44 ± 399.01^{ab}	1787.70 ± 927.61^{ab}
3	2495.53 ± 732.44^{c}	10350.79 ± 4895.68^{c}
4	322.45 ± 118.84^{abd}	52.64 ± 12.80^{abd}

Values are ± standard error of mean for three replicate (n).

Values with different letters (superscript) were significantly different from each other at $p< 0.05$

Group 1: Extract Control (Not infected, but administered 400mg/kg b.w of crude methanolic extract of *Morinda lucida* root).

Group 2: Infected with *Plasmodium berghei* and treated with 400mg/kg b.w of crude methanolic extract of *Morinda lucida* root).

Group 3: Infected with *Plasmodium berghei*, but not treated

Group 4: Not infected, not treated (Positive control).

4.3 In vivo antiplasmodial activity of crude methanolic root extract of *Morinda lucida*

4.3.1 Four-day suppressive test

The mice in all the groups were infected with *Plasmodium berghei*. Figure 4.1 shows the mean parasite count of mice for suppressive test. The group treated with chloroquine showed significant ($p<0.05$) parasite suppression (1.84 ± 0.15), compared to all other groups. However, there was no significant difference ($p>0.05$) between the groups treated with 100 (4.44 ± 0.15), 200 (4.08 ± 0.23) and 400mg/kg (3.28 ± 0.44) crude methanolic root extract of *Morinda lucida*. The group which was not treated at all, but given 0.2ml normal saline, gave the highest parasite count (10.16 ± 1.41).

Figure 4.2 shows percentage (%) suppression of parasite for mice treated with crude methanolic root extract of *Morinda lucida*. The group treated with chloroquine had highest % suppression of parasitaemia (81.89%). The average % suppression for 100, 200 and 400mg/kg for crude methanolic root extract of *Morinda lucida* were 56.30, 59.84 and 67.72% respectively.

Figure 4.3 shows average weight of mice before infection, and after four days of treatment for suppressive test. There was no significant difference ($p>0.05$) in weight of mice before infection and after four days of treatment in all the groups.

The mean survival period in days were calculated to be 18.60 ± 0.75, $20.20.75 \pm 1.40$, 23.60 ± 1.03, 24.80 ± 1.93 and 12.80 ± 0.86, for 100, 200, 400mg/kg body weight (b.w) of crude methanolic root extract of *Morinda lucida*, 5mg/kg b.w of chloroquine and the untreated control respectively (Figure 4.4).

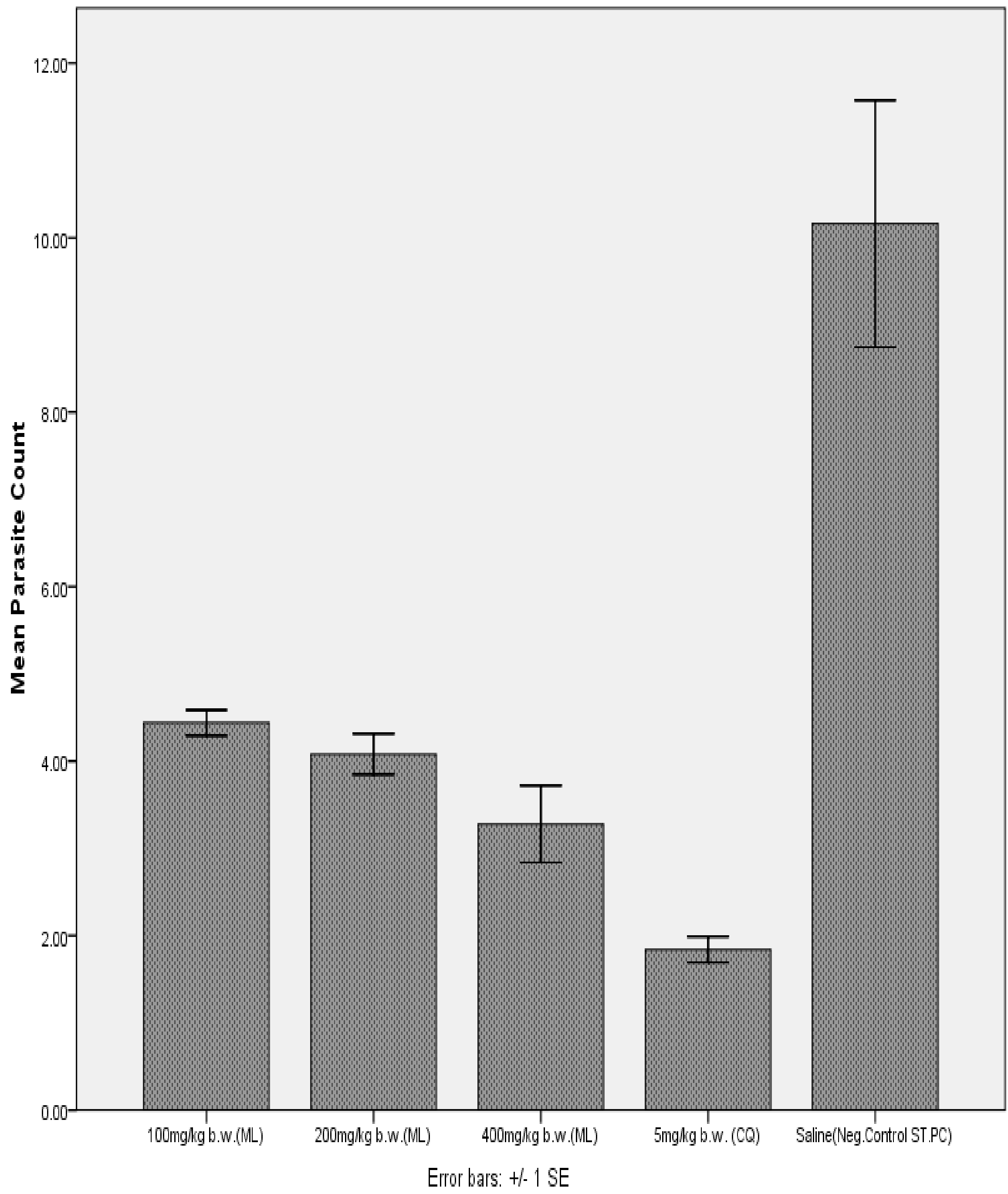


Figure 4.1: Mean parasite count of mice for suppressive test

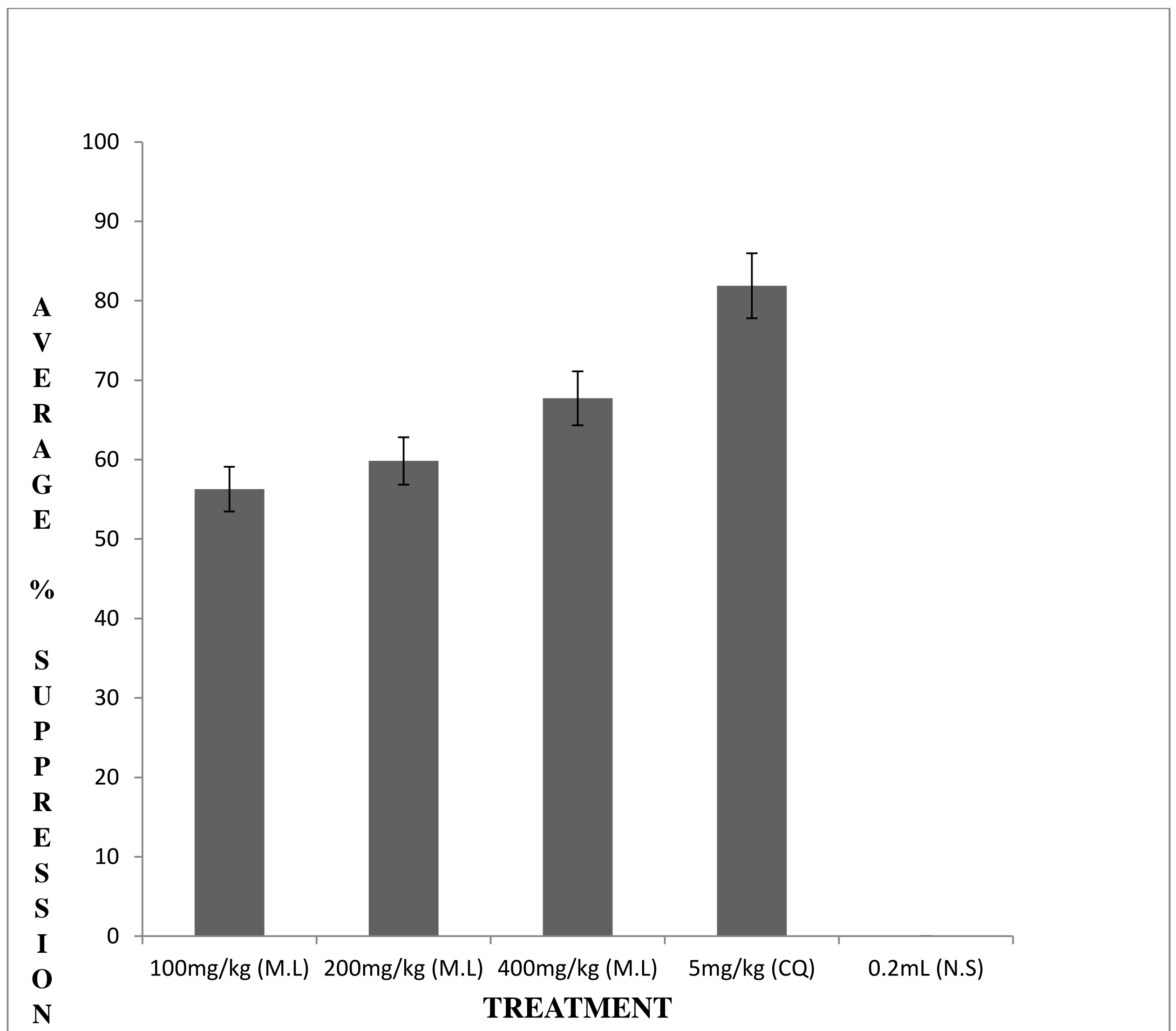


Figure 4.2: Average percentage suppression of parasite by methanolic extract of *Morinda lucida* root

Key

M.L: Methanolic root extract of *Morinda lucida*, CQ: Chloroquine, N.S: Normal saline

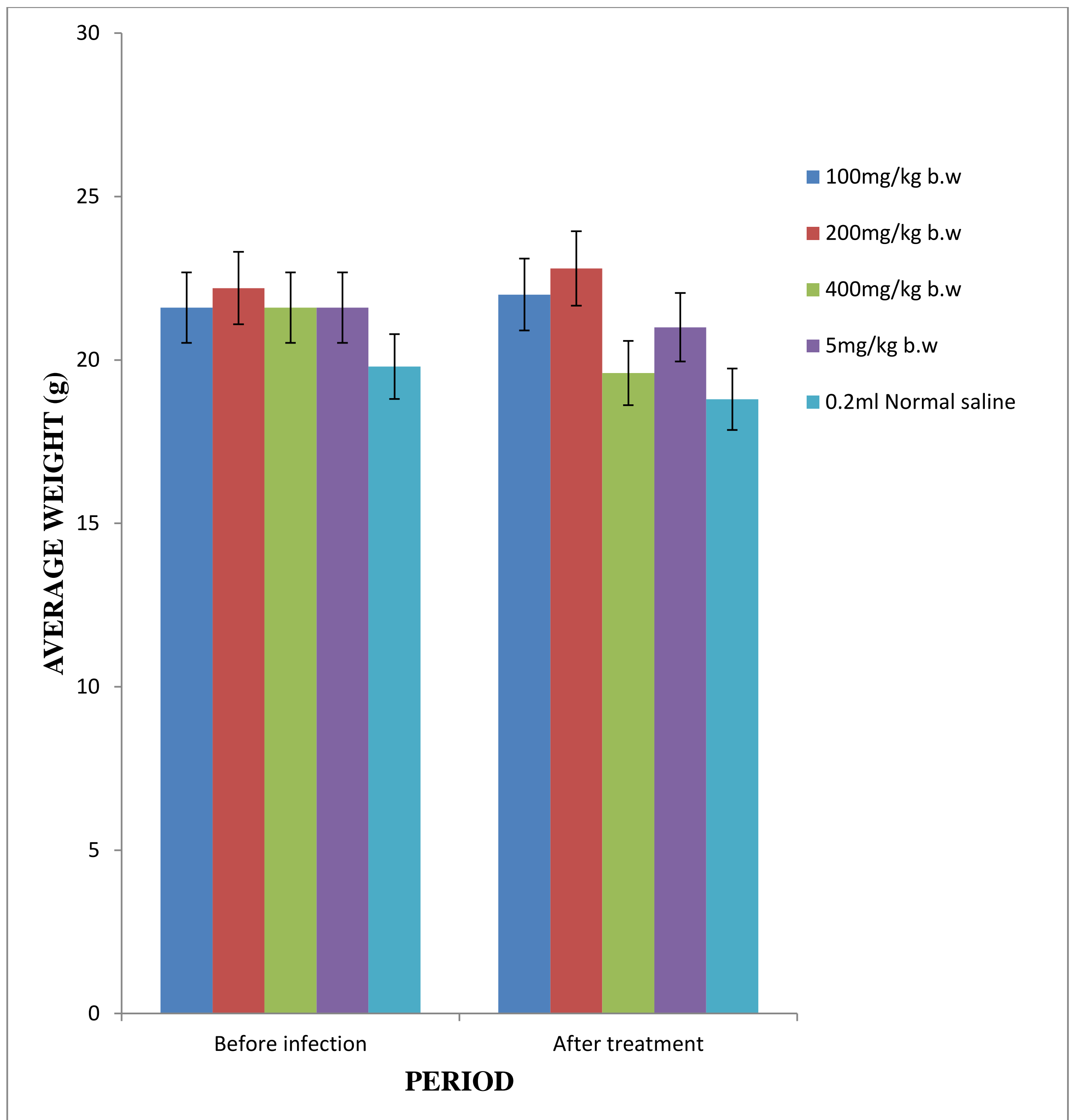


Figure 4.3: Average weight of mice, before infection and after four days of treatment (suppressive test)

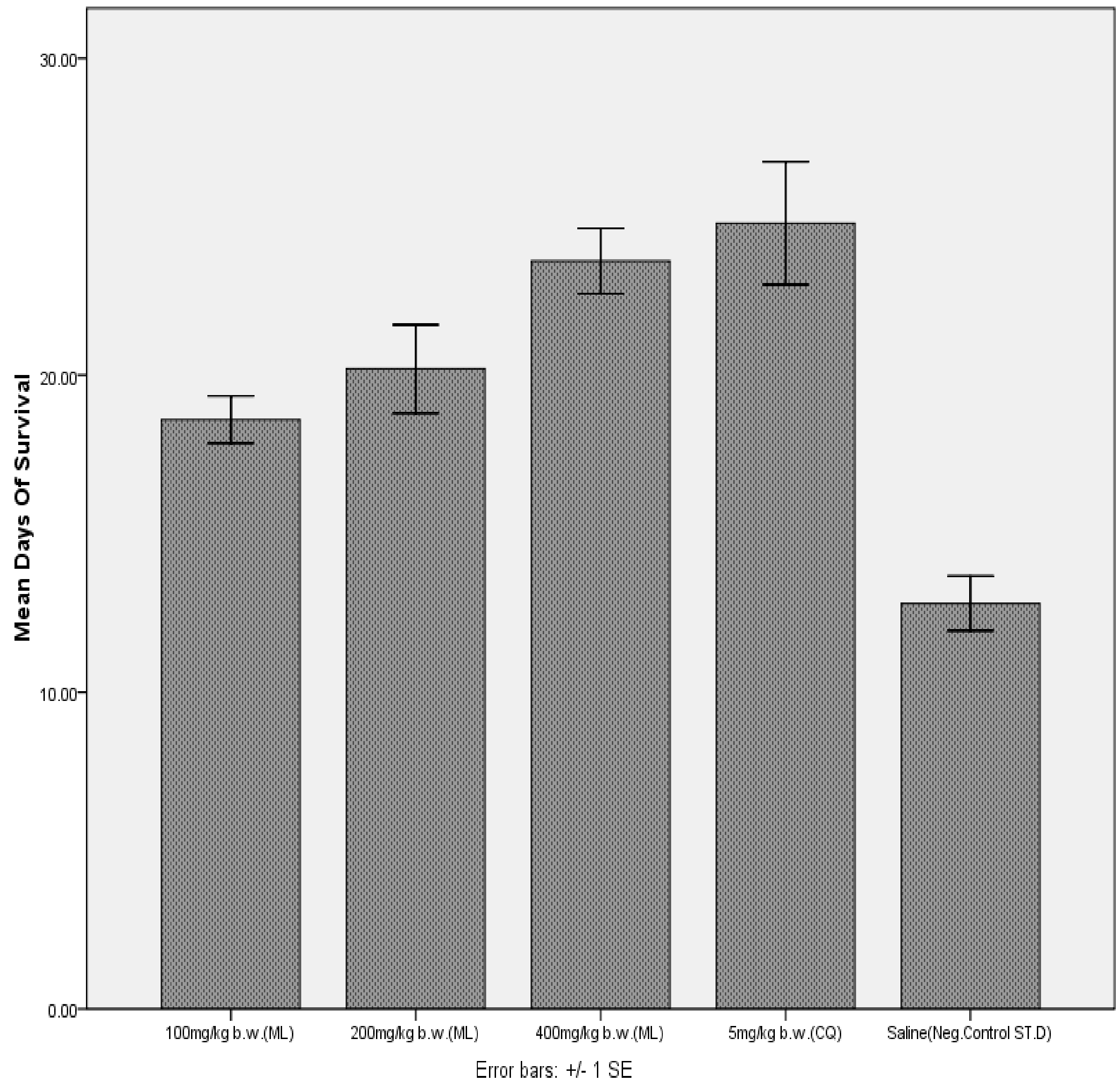


Figure 4.4: Mean Days of Survival of mice for suppressive test

ML: Crude methanolic root extract of *Morinda lucida*, CQ: Chloroquine, ST.D: Standard

4.3.2 Curative test

All the groups infected with *Plasmodium berghei* and treated with the crude methanolic root extract of *Morinda lucida* and chloroquine, showed an exponential decrease in parasite count, throughout the study period, with chloroquine showing highest parasite inhibition (8.78 ± 3.58). However, the negative control group showed exponential increase in parasite count (115 ± 5.66), throughout the study period (figure 4.5).

There was no significant weight change in all the experimental groups before infection, 72 hours after infection and after 5 days of treatment, however, those animals in the negative control group showed slight weight loss (figure 4.6).

Figure 4.7 shows the average PCV of mice before infection, 72 hours after infection and after 5days of treatment in different test groups. It can be deduced from the chart that, there was reduction in PCV of mice in all test groups, within and after the experimental period, except for the chloroquine treated group where there was an increase in the PCV level throughout the period. However, there was a slight increase in PCV between the period before infection and 72 hours after infection, for the group treated with 400mg/kg b.w of crude methanolic root extract of *Morinda lucida*.

The mean survival period in days were calculated to be 15.00 ± 0.70, 18.75 ± 0.5, 19.75 ± 1.39, 23.25 ± 1.38 and 8.75 ± 1.25, for 100, 200, 400mg/kg body weight (b.w) of crude methanolic root extract of *Morinda lucida*, 5mg/kg b.w of chloroquine and the untreated control respectively (figure 4.8).

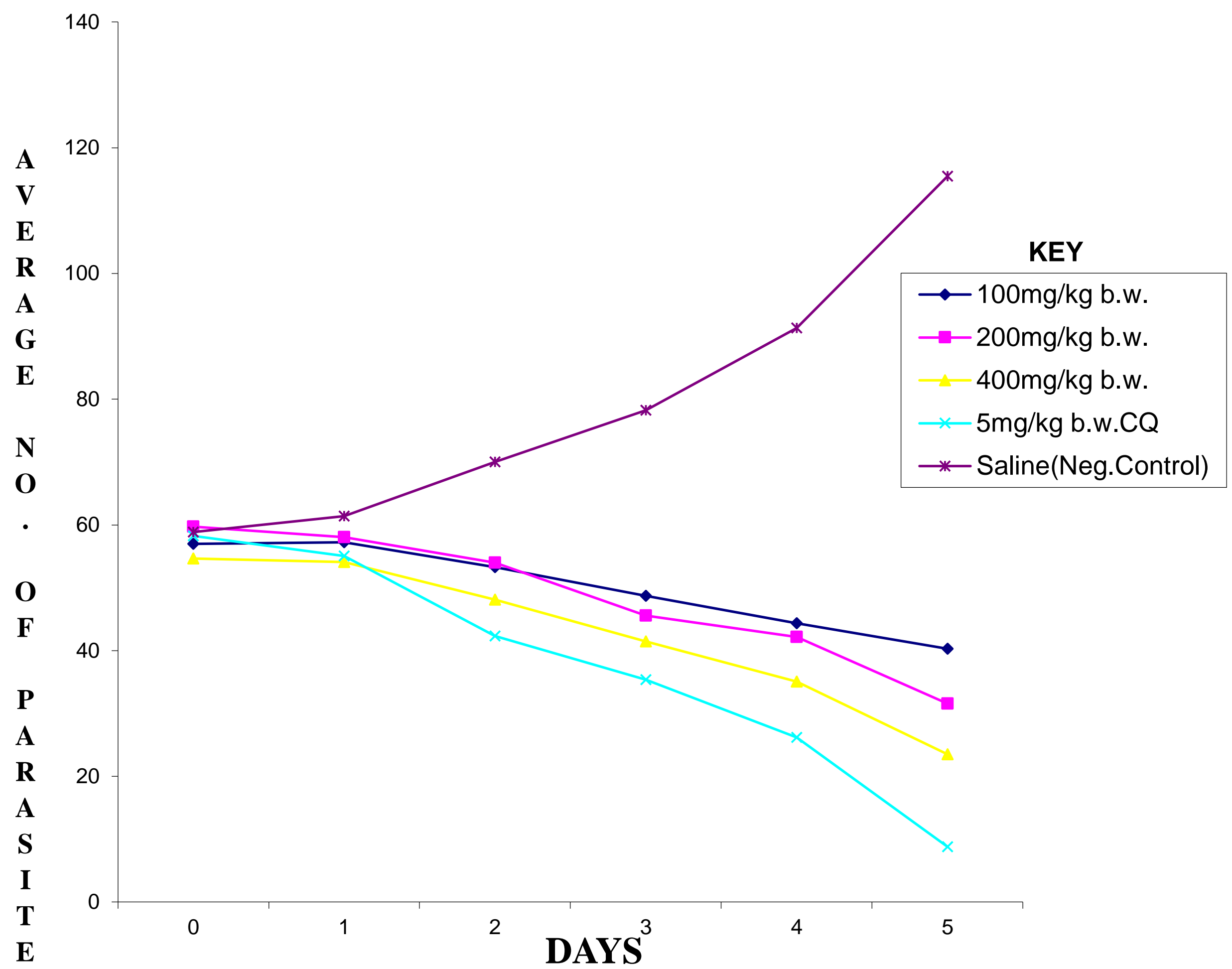


Figure 4.5: Antiplasmodial effect of methanolic root extract of *Morinda lucida* (curative test)

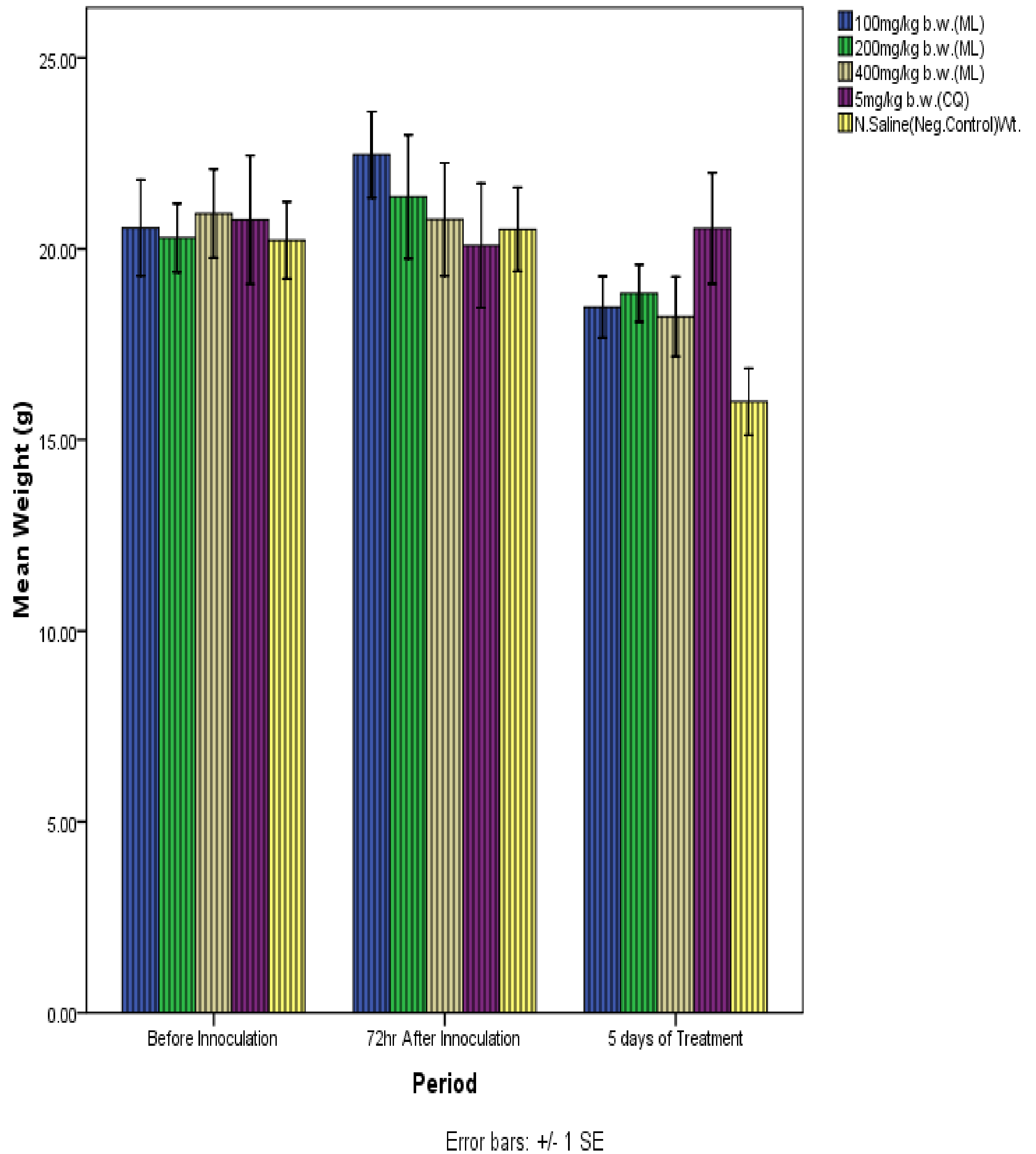


Figure 4.6: Average weight of mice, before infection, 72hours after infection, and after 5 days of treatment for curative test

M.L: Crude methanolic root extract of *Morinda lucida*, CQ: Chloroquine

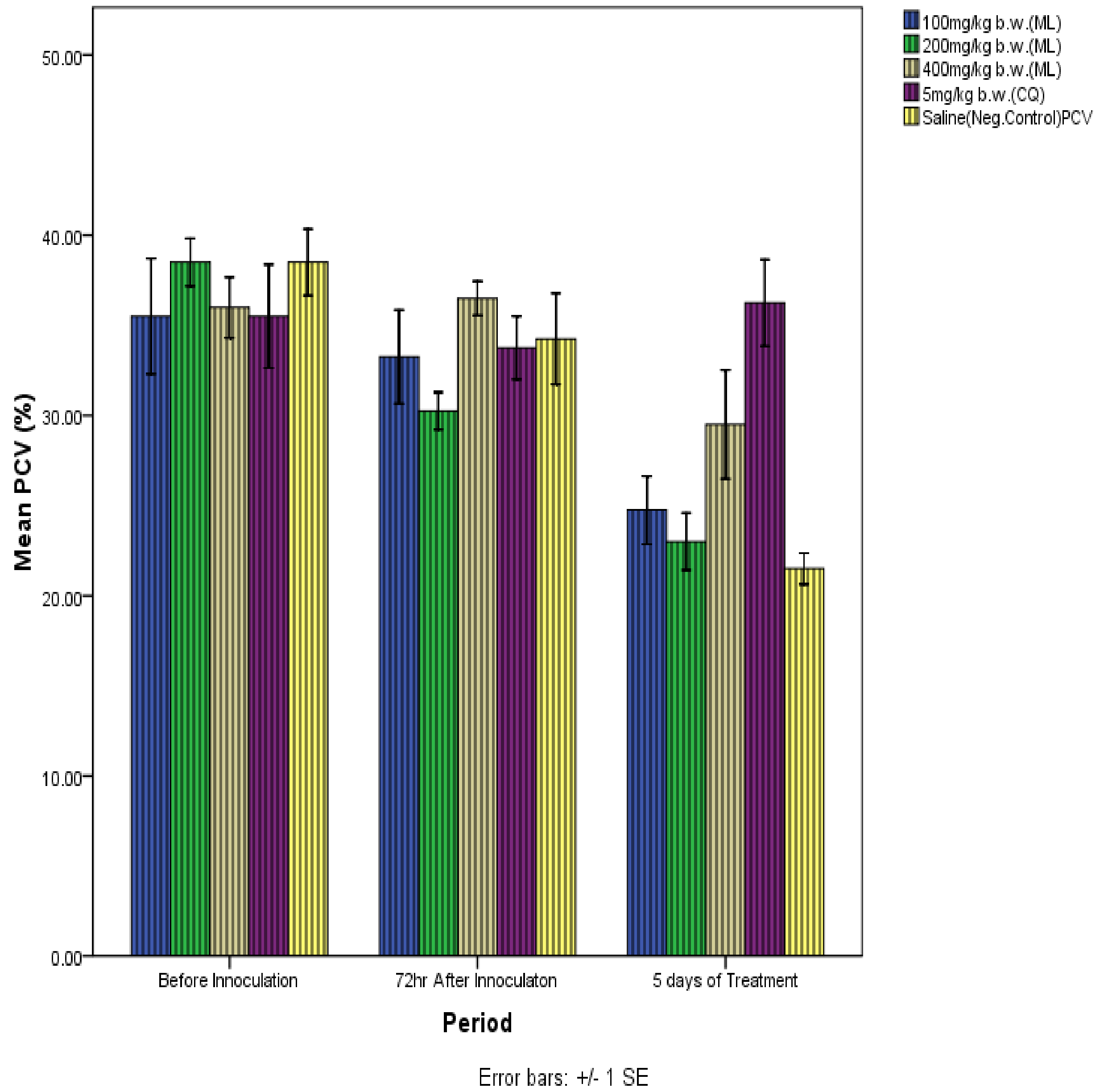


Figure 4.7: Average Pack Cell Volume (PCV) of Mice, before infection, 72hours after infection, and after 5 days of Treatment

M.L: Crude methanolic root extract of *Morinda lucida*, CQ: Chloroquine

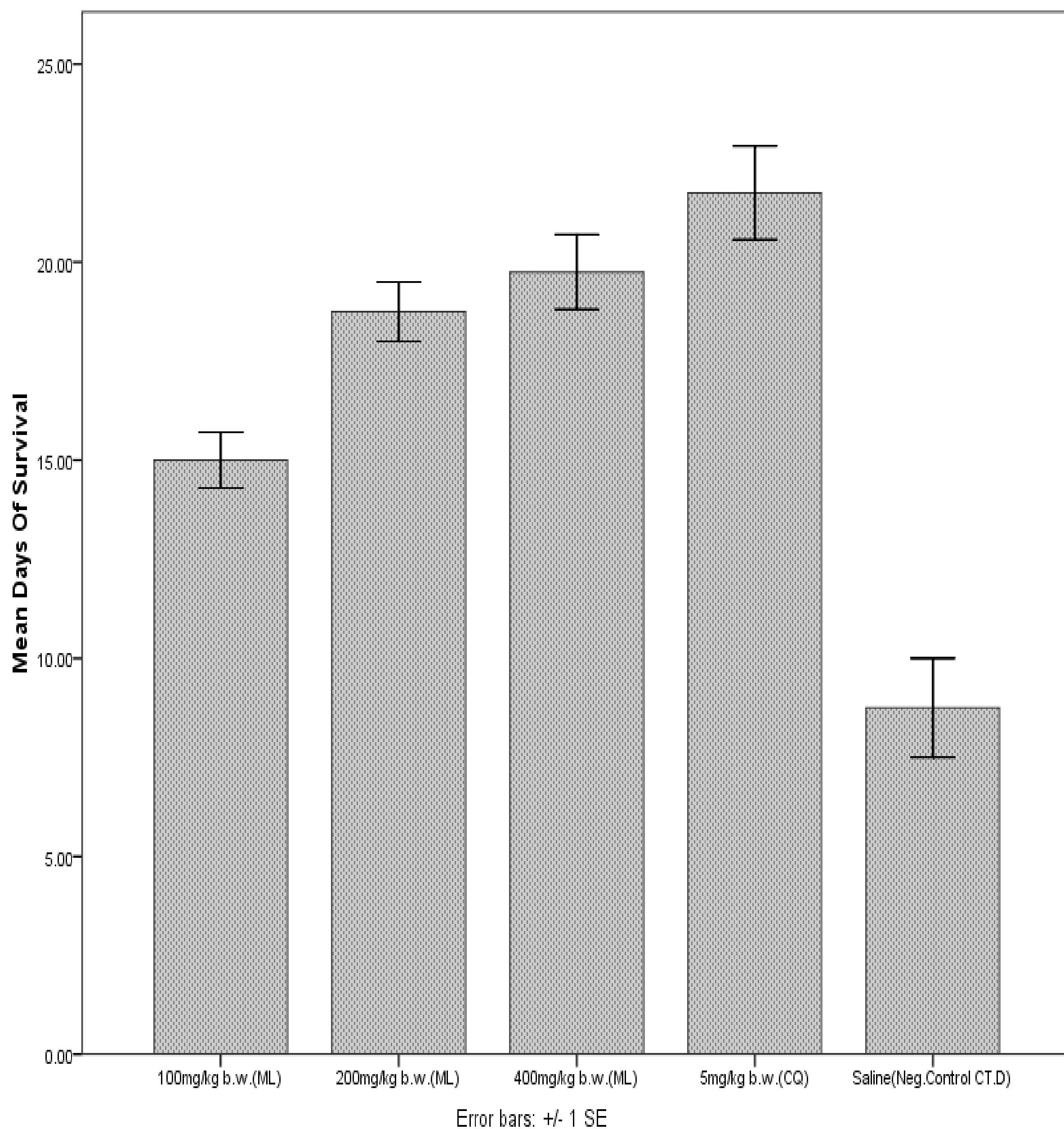


Figure 4.8: Mean days of survival of *Plasmodium berghei* infected mice treated with methanolic root extract of *Morinda lucida*

M.L: Crude methanolic root extract of *Morinda lucida*, CQ: Chloroquine

4.3.3 Prophylactic Test

Table 4.7 shows the mean parasite count of mice 72 hours after infection with *Plasmodium berghei*, upon administration of crude methanolic root extract of *Morinda lucida* and chloroquine. The mean parasite counts were 32.00 ± 2.20, 25.60 ± 1.06, 23.80 ± 0.74, 6.38 ± 0.31 and 42.25 ± 0.95 for 100, 200, 400mg/kg of crude methanolic root extract of *Morinda lucida*, 5mg/kg chloroquine and 0.2ml normal saline groups respectively.

The percentage (%) prophylaxis of mice administered with different doses of crude methanolic root extract of *Morinda lucida* and chloroquine, and subsequently infected with *Plasmodium berghei* is shown in figure 4.9. Percentage prophylaxis was calculated to be 24.26, 39.41, 43.67 and 84.91% for doses of 100, 200, 400mg/kg b.w of the methanolic root extract, and 5mg/kg chloroquine respectively.

In all the extract treated groups and the negative control group, there was slight increase in weight after extract administration, but after 72 hours of infection with *Plasmodium berghei*, there was drop in weight. However for the chloroquine treated group, there was slight increase in weight after extract administration and after 72hours of infection (figure 4.10).

In the negative control group, chloroquine treated group and the groups treated with 200 and 400mg/kg b.w of methanolic root extract of *Morinda lucida*, there was increase in PCV after extract administration and after 72 hours of infection, but for the group treated with 100mg/kg b.w of methanolic root extract of *Morinda lucida*, there was decrease in PCV after extract administration and 72hours after infection with *Plasmodium berghei* (figure 4.11).

Table 4.7: Mean parasite count of mice for prophylactic test

Drug/ Extract	Dose (mg/kg/day)	Mean Parasite Count
M. lucida	100	32.00 ± 2.20
M. lucida	200	25.60 ± 1.06
M. lucida	400	23.80 ± 0.74
Chloroquine	5	6.38 ± 0.31
Normal saline	0.2ml/mouse/day	42.25 ± 0.95

Values are ± standard error of mean for four replicate (n).

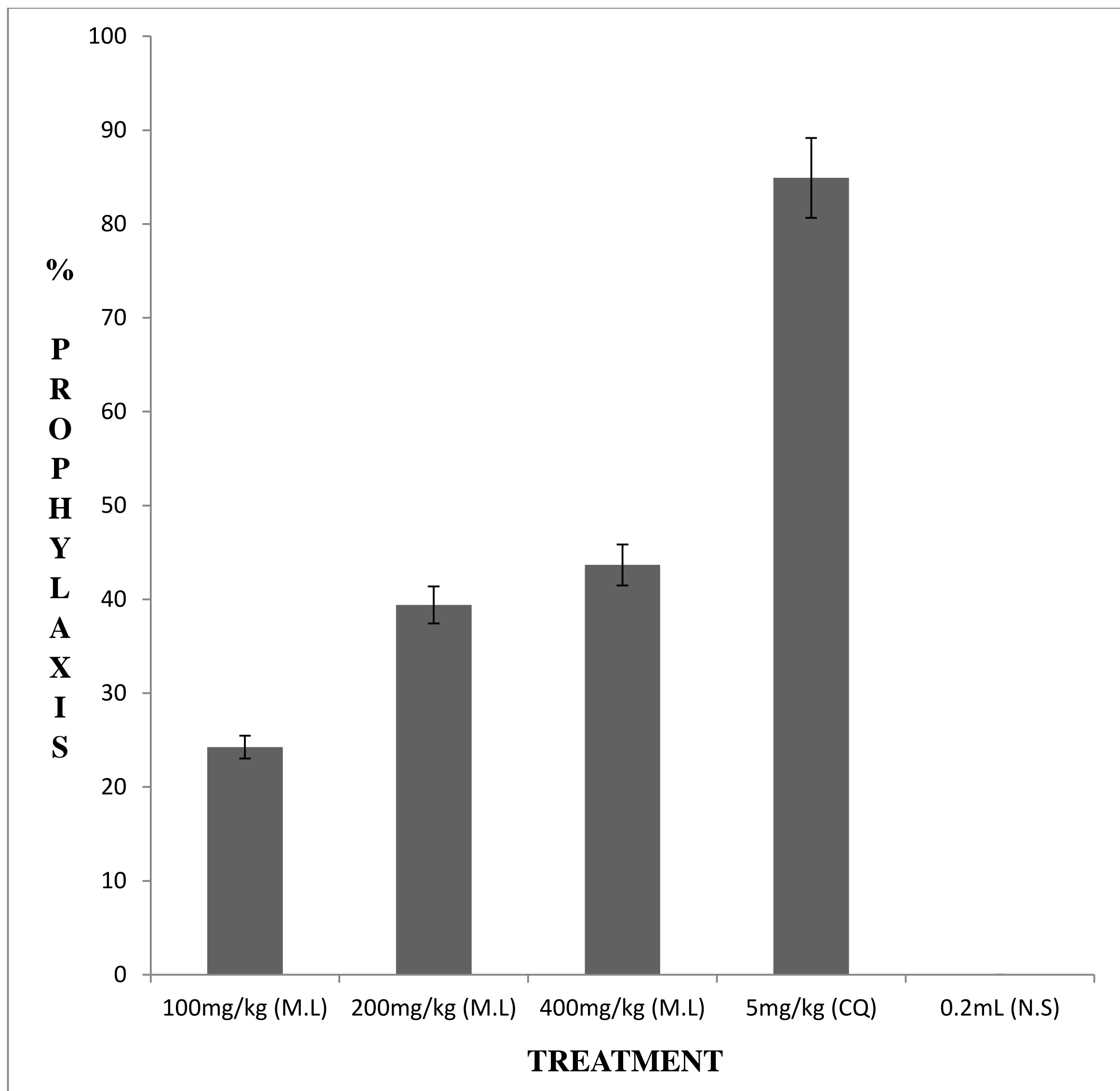


Figure 4.9: Average percentage prophylaxis of mice administered with methanolic root extract of *Morinda lucida*, and infected with *Plasmodium berghei*

Key:

M.L: Methanolic root extract of *Morinda lucida*, *CQ*: Chloroquine, N.S: Normal saline

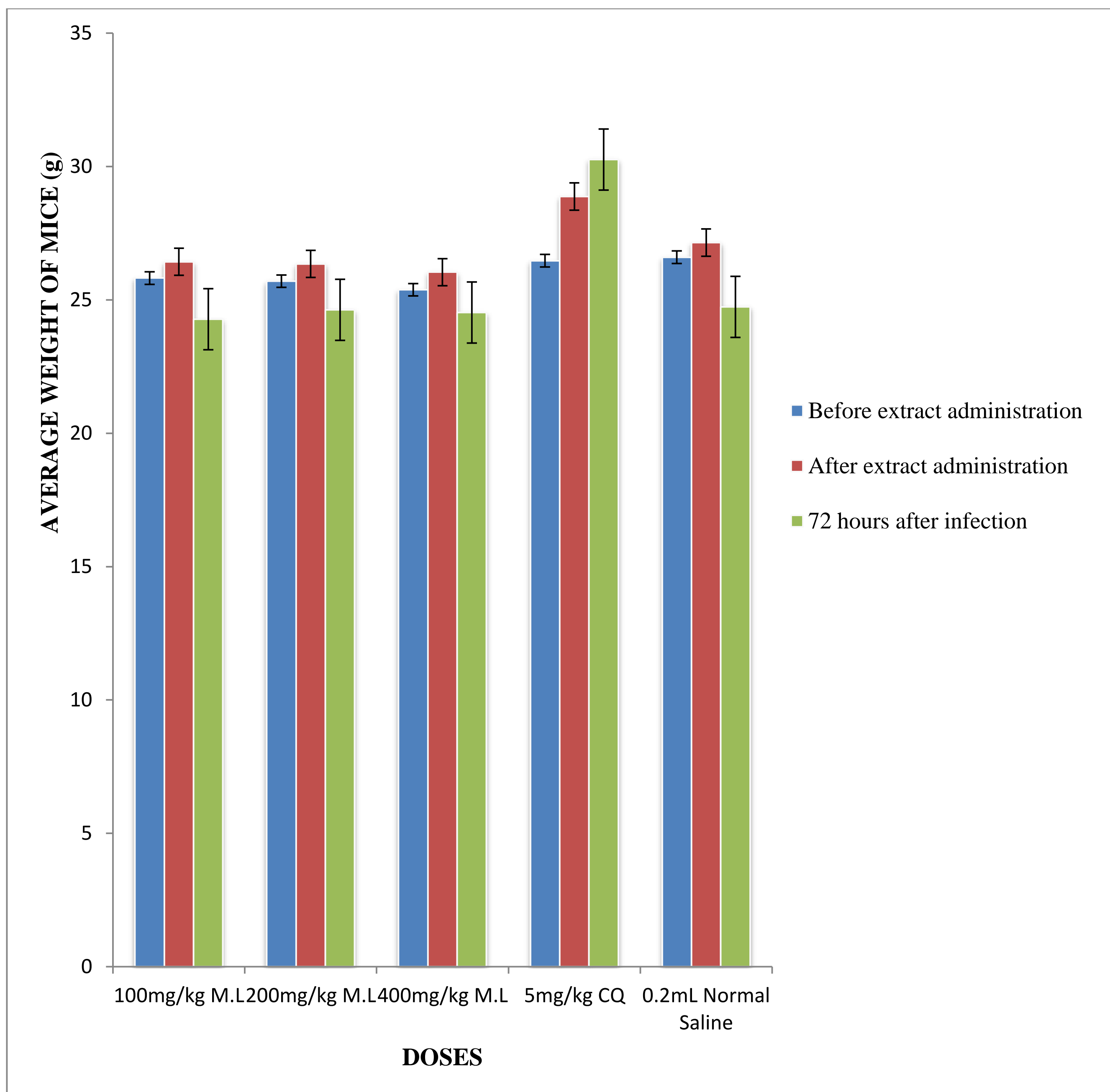


Figure 4.10: Average Weight of Mice for Prophylactic test

Key:

M.L: Methanolic root extract of *Morinda lucida*, *CQ*: Chloroquine, N.S: Normal saline

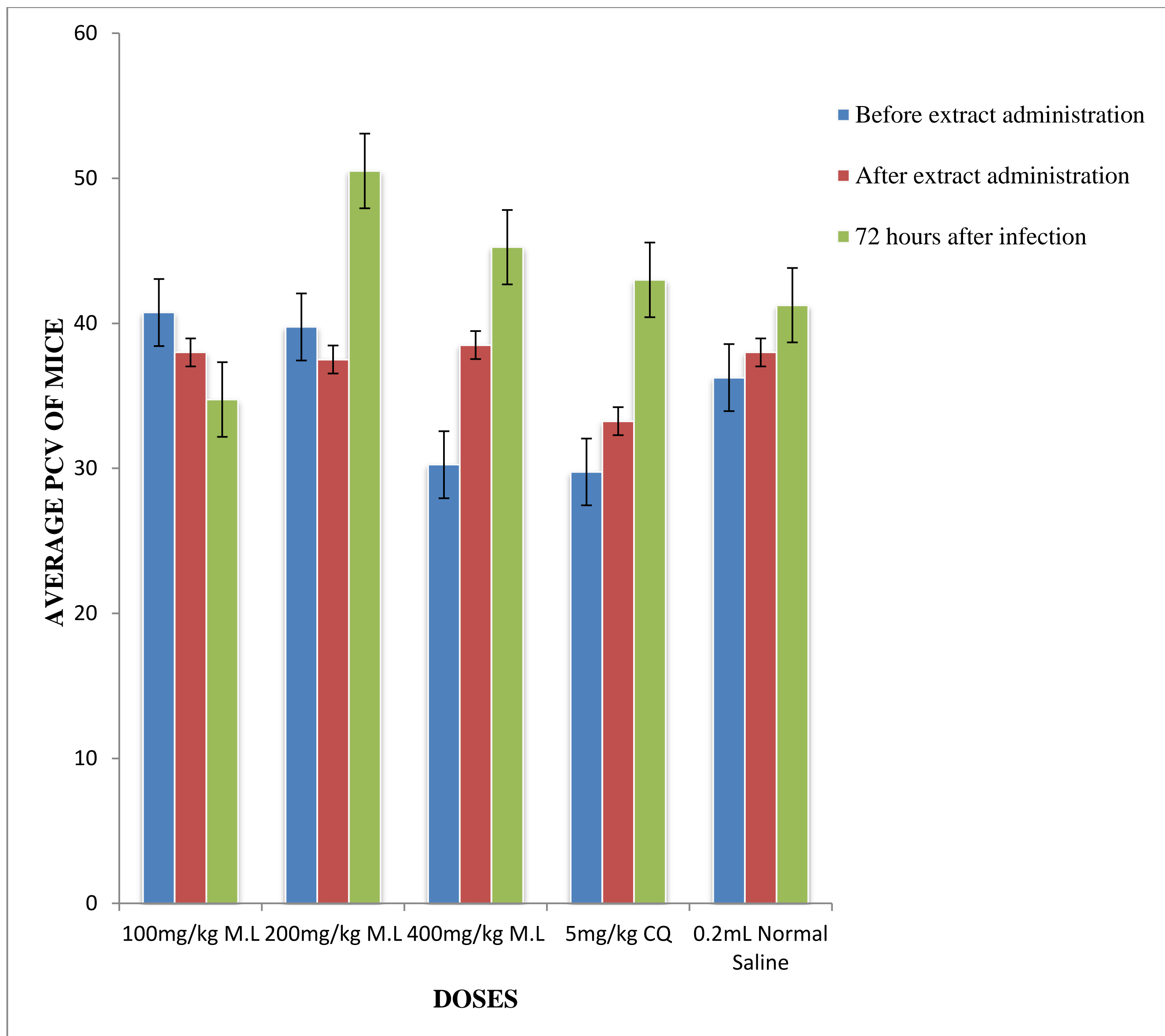


Figure 4.11: Average PCV of mice for prophylactic test

Key:

M.L: Methanolic root extract of *Morinda lucida*, CQ: Chloroquine

4.4 Partial purification of crude methanolic root extract of *Morinda lucida*

4.4.1 Column chromatography

Fractionation was carried out on the crude methanolic root extract of *Morinda lucida*, by column chromatography. This was done by gradually increasing the polarity of solvent used at each step. At the end, nineteen fractions were obtained, which were then subjected to TLC, in order to pull together those fractions with similar R_f values.

4.4.2 Thin Layer Chromatography (TLC)

TLC was carried out on the nineteen fractions obtained from the Column Chromatography, which gave a total of four fractions (fractions 2-6 were pulled together, fractions 7-11, 12 & 13, and 14-19 were pulled together). The plates below shows pictures of the Thin Layer plates used. Ultra-Violet (UV) lamp and spraying with 20% sulphuric acid were used as means of identification of spots.

In the thin layer plates below, same samples (fractions 1-7) were spotted on two different silica gel pre coated aluminium foils, but developed in different solvent systems (100% petroleum ether and 100% chloroform).

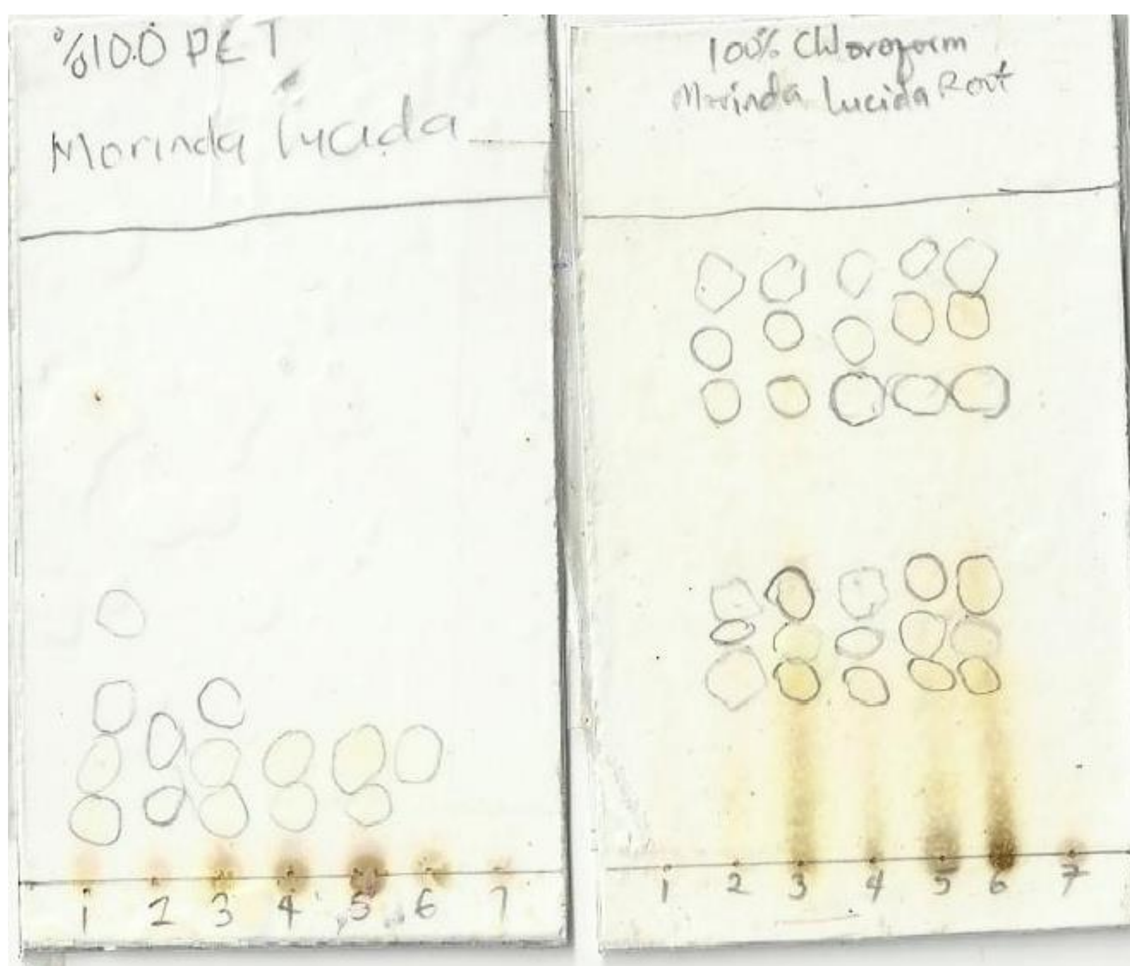


Plate I: Thin layer chromatography aluminium foil plates showing the various spots identified for fractions 1-7

Key:

PET: Petroleum ether

In the thin layer plates below, same samples (fractions 7-12) were spotted on two different silica gel pre coated aluminium foils, but developed in two different solvent systems (mixture of chloroform and methanol in the ratio 9:1 and 4:1).

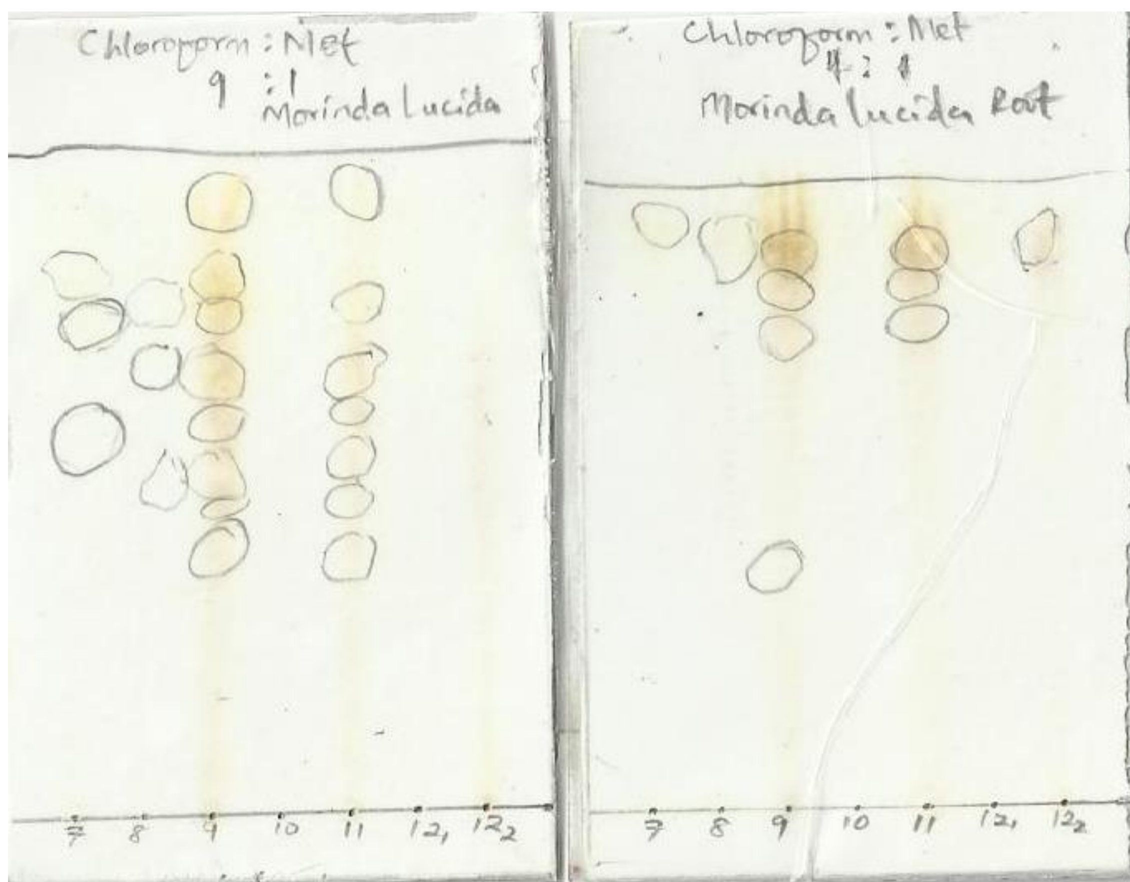


Plate II: Thin layer chromatography aluminium foil plates showing the various spots identified for fractions 7-12

Key:

Met: Methanol

In the thin layer plates below, same samples (fractions 12-17) were spotted on two different silica gel pre coated aluminium foils, but developed in different solvent systems(mixture of chloroform and methanol in the ratio 9:1 and 4:1).

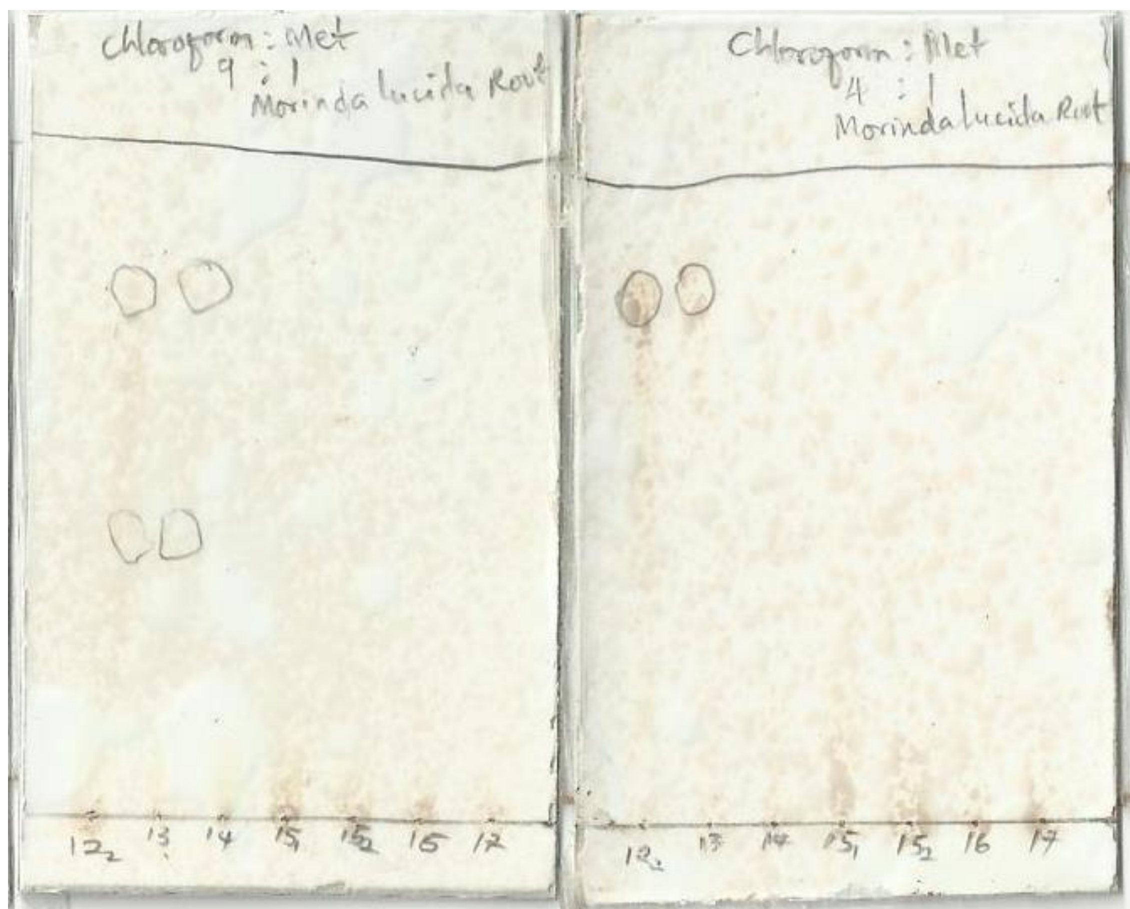


Plate III: Thin layer chromatography aluminium foil plates showing the various spots identified for fractions 12-17

Key:

Met: Methanol

In the thin layer plates below, same samples (16-19) were spotted on two different silica gel pre coated aluminium foils, but developed in different solvent systems (mixture of chloroform and methanol in the ratio 9:1 and 4:1).

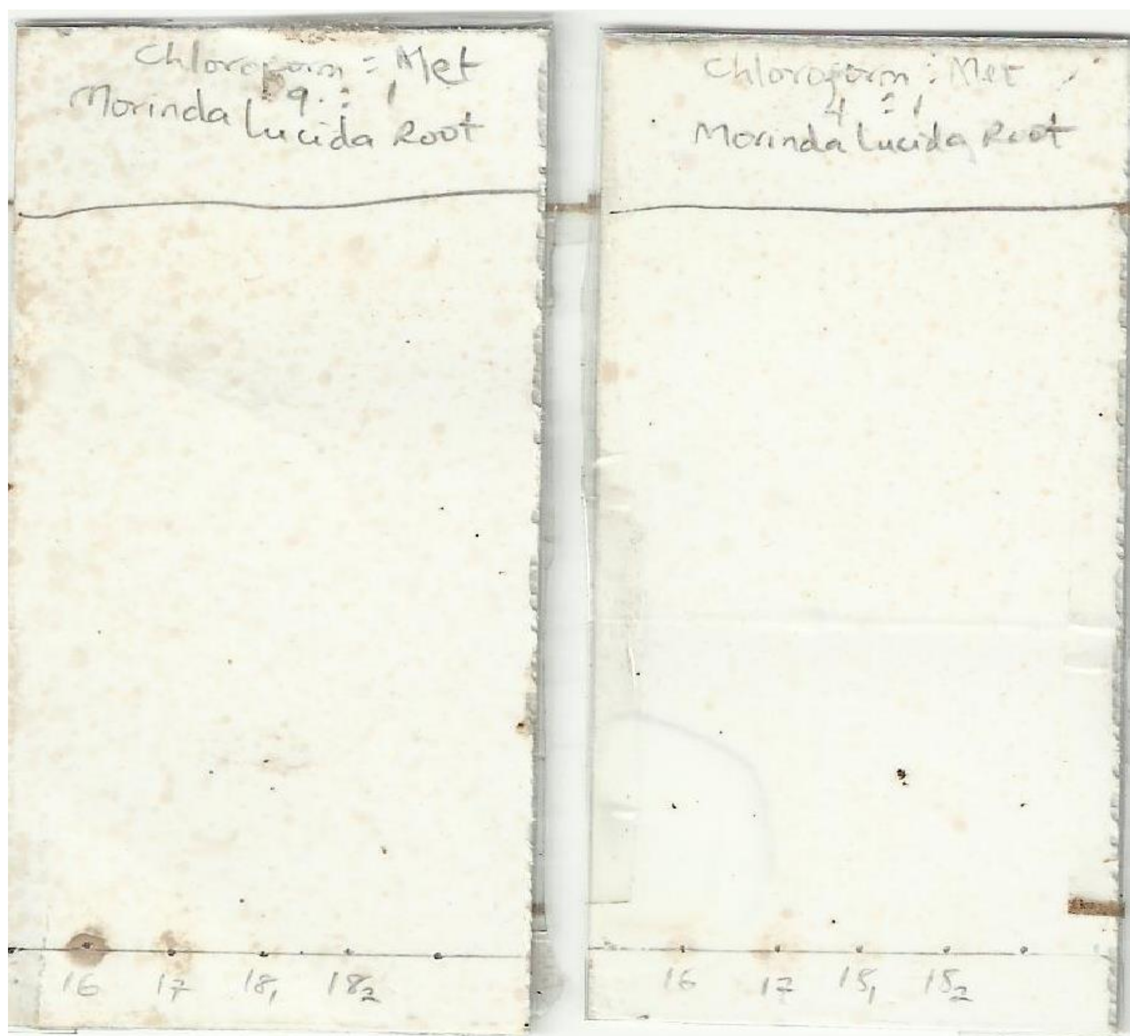


Plate IV: Thin layer chromatography aluminium foil plates showing the various spots identified for fractions 16-19

Key:

Met: Methanol

4.4.3: Antiplasmodial efficacy of the various fractions of the crude methanolic root extract of *Morinda lucida*

There was an exponential decrease in parasite count in all the test groups (crude, fractions 1,2,3,4 and chloroquine), but of course, there was an increase in parasite count for the negative control group, throughout the test period (Figure 4.12). There was no significant difference in parasite inhibition after the experiment, between the crude methanolic root extract (11.50 ± 1.27) of *Morinda lucida* and fraction 4 (12.05 ± 0.29) (Figure 4.12).

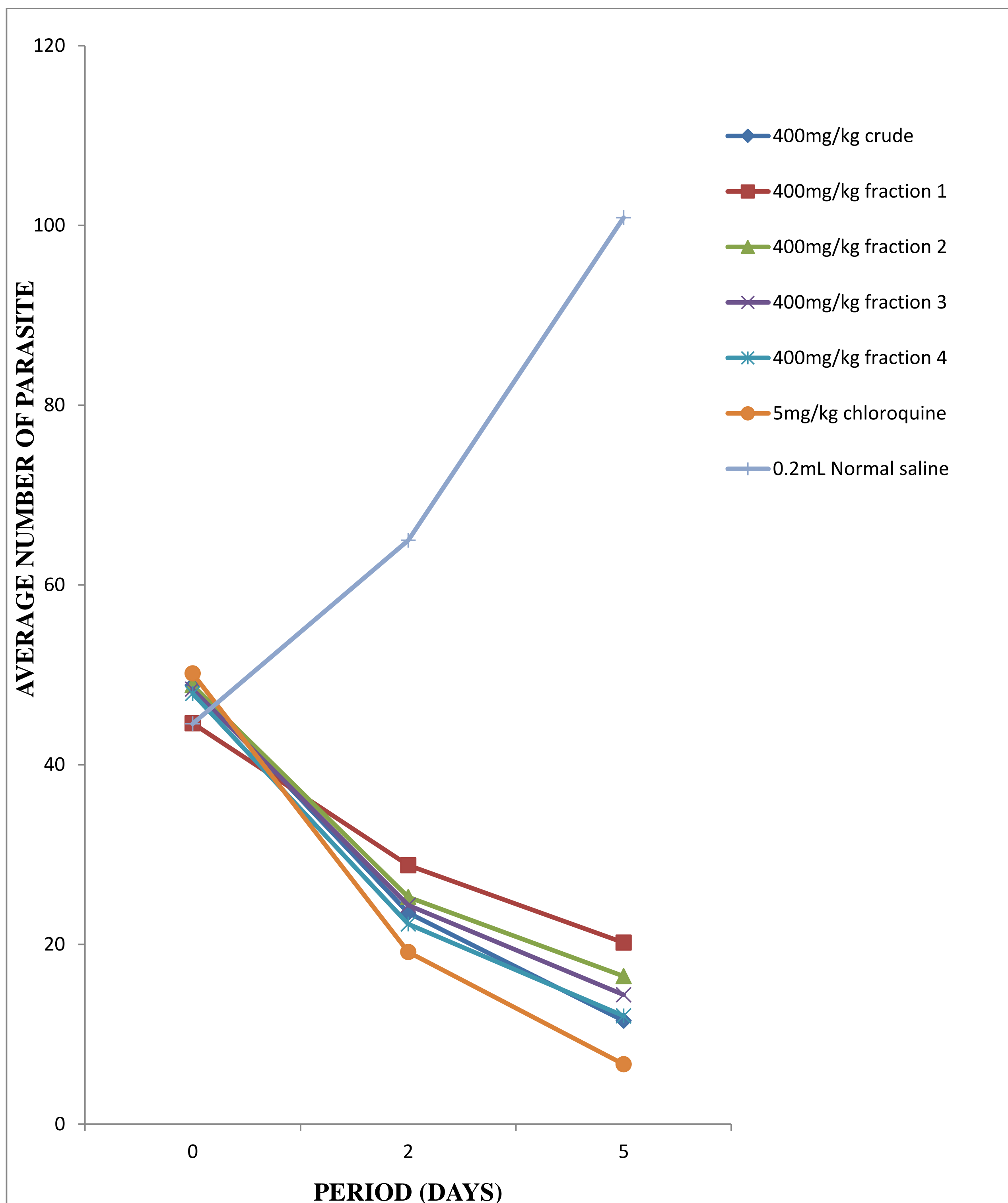


Figure 4.12: Antiplasmodial activity of the various fractions of crude methanolic root extract of *Morinda lucida*.

4.5 In vivo Antiplasmodial activity of combination of methanolic root and leaf extracts of *Morinda lucida*

The chloroquine treated group showed remarkable parasite inhibition at the end of the experiment (8.50 ± 1.26) compared to all other test groups. There was no significant difference in parasite inhibition between the groups treated with 200mg/kg b.w leaf alone (20.00 ± 1.29), 200mg/kg b.w root alone (21.35 ± 1.85) and 100mg/kg b.w combination of methanolic root and leaf extracts of *Morinda lucida* (22.50 ± 1.29) (Figure 4.13).

There was no significant difference in weight of mice in all the test groups between the periods before and after the experiment (Figure 4.14).

In all the experimental groups, there was significant decrease in PCV levels after infection with *Plasmodium berghei* and after treatment, except for chloroquine treated group, where there was a decrease in PCV after infection, but subsequently increased after 5 days of treatment (Figure 4.15).

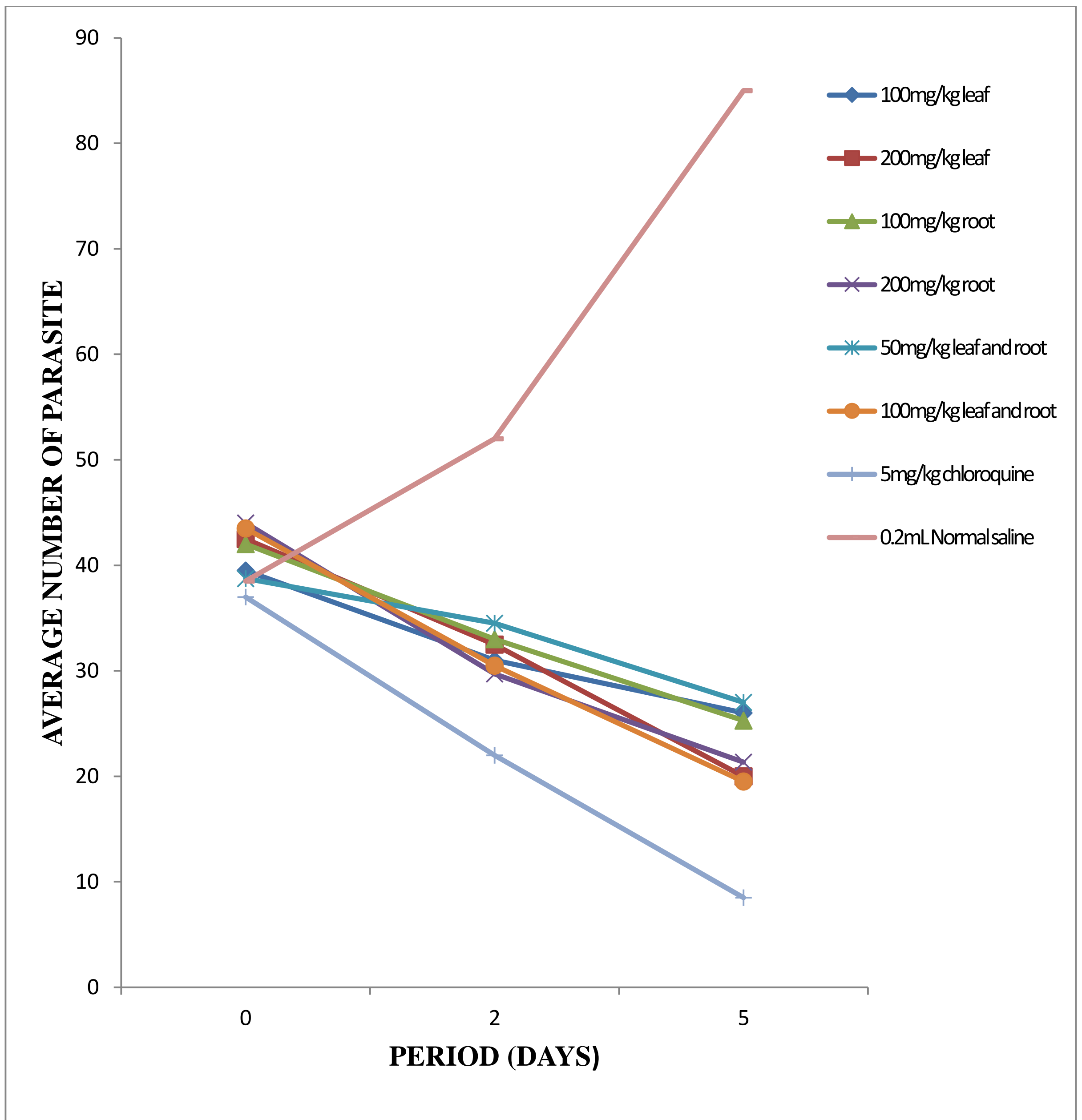


Figure 4.13: Average number of parasite of mice treated with a combination of the methanolic root and leaf extracts of *Morinda lucida*

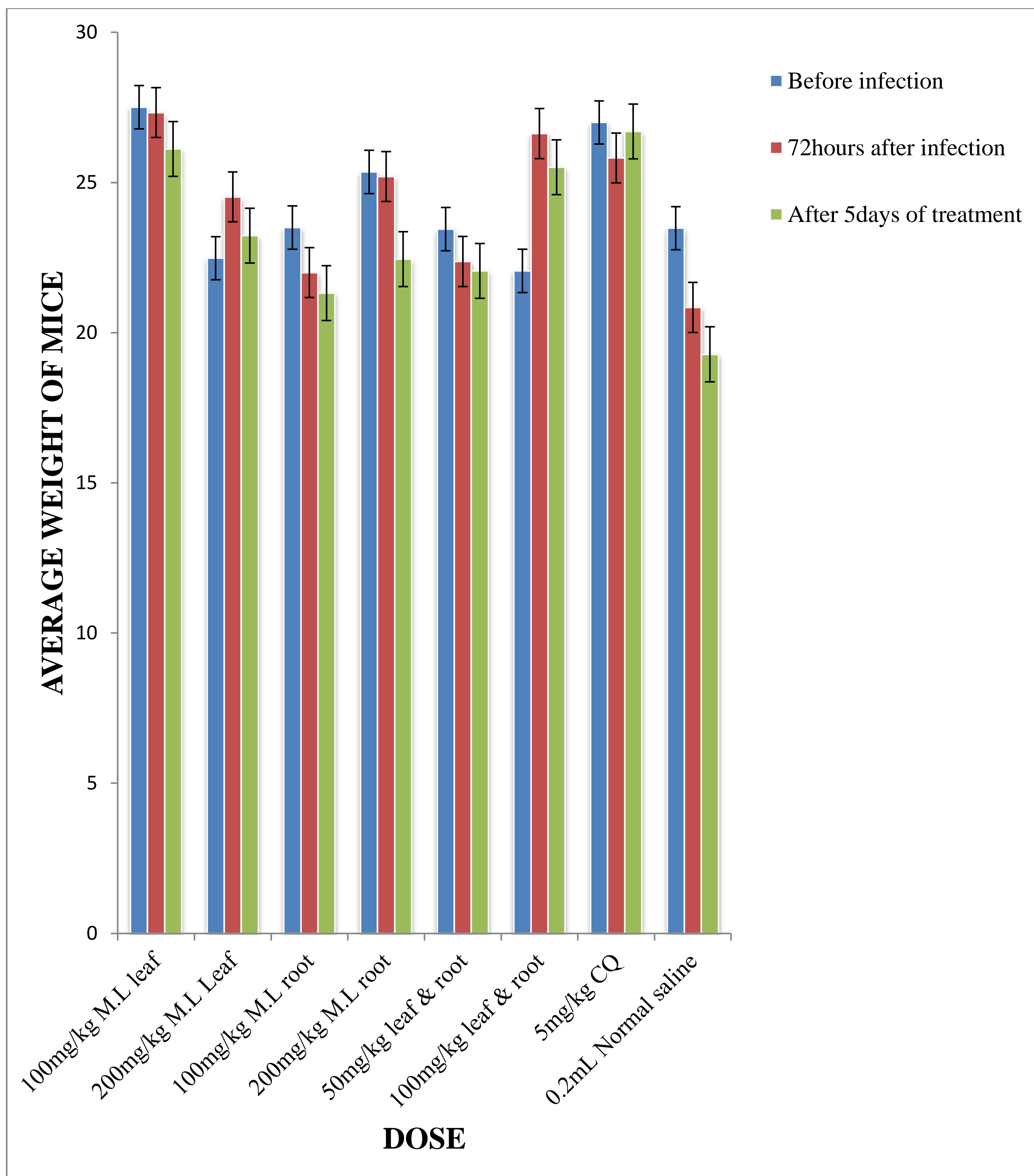
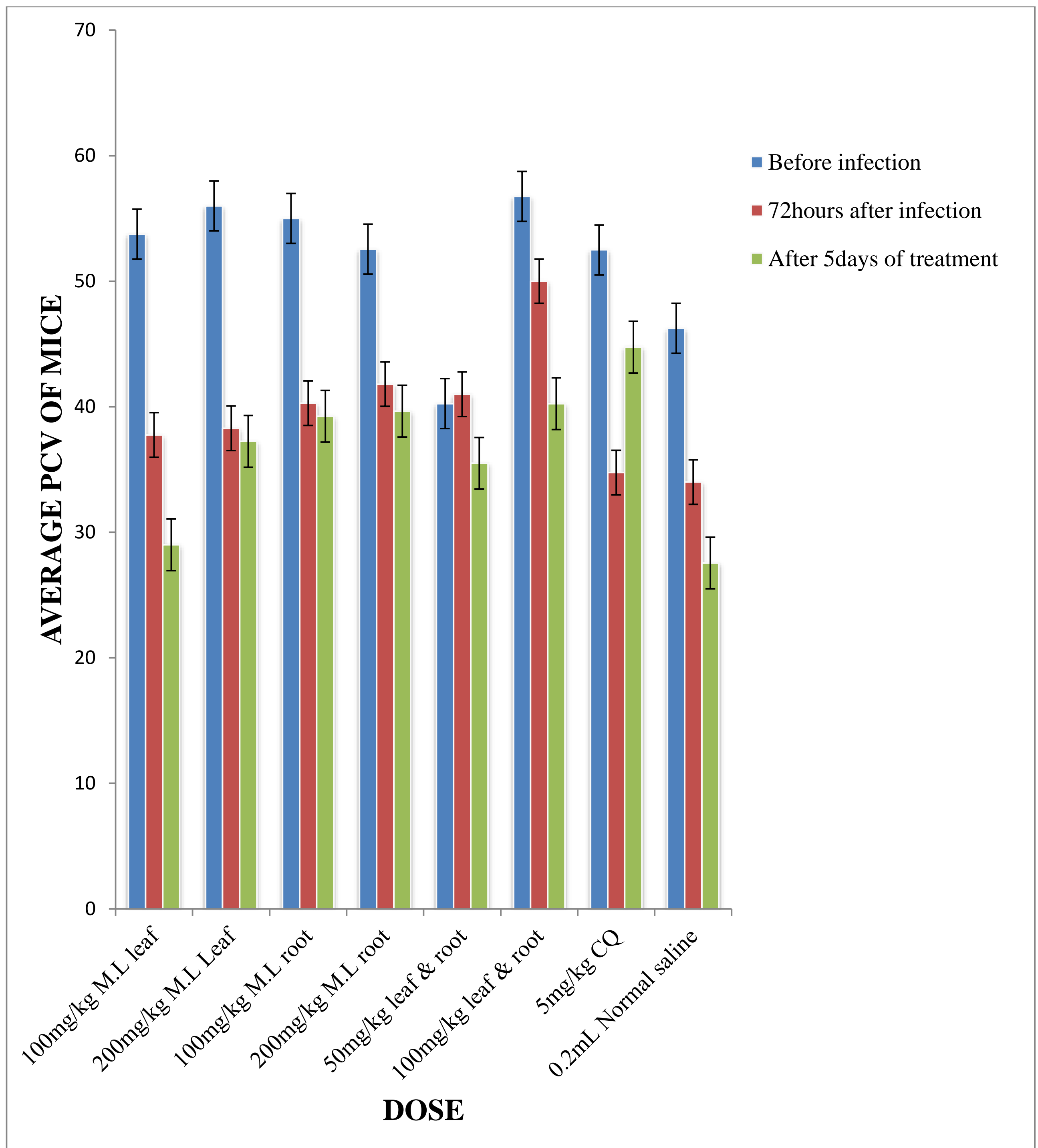


Figure 4.14: Average weight of mice treated with a combination of the root and leaf methanolic extracts of *Morinda lucida*



pFigure 4.15: Average PCV treated with a combination of the root and leaf methanolic extract of *Morinda lucida*

4.6 Discussion

Morinda lucida, commonly known as "Ugigo" by the Ebira people in Kogi State North-Central Nigeria, is used in the treatment of malaria, fever, amongst other ailments. The phytochemical screening result showed the presence of alkaloids, flavonoids, phenolic nucleus, and phlobotannins, which confirms the works of others on phytochemical constituents of *Morinda lucida.* This is done to know the secondary metabolites present in the plant sample before its use in antimalarial studies to ascertain what could be responsible for its antimalarial properties, even though it has been established by others (Tona *et al*., 1999; Agomo *et al*., 1992; Makiinde and Obih, 1985; Awe and Makinde, 1998).

During the acute toxicity studies, it was observed on the first day of administration of the crude methanolic root extract of *morinda lucida*, that especially those given the highest dose became calm, depressed, lost appetite, after about 40 minutes of administration and went to sleep 2hours later. One out of the three mice that were given 5000mg/kg body weight died after 48 hours of extract administration. It was observed before it died that, it was panting and stooling differently from the normal faeces of mice. From the result of the LD_{50} (Table 4.2), it can be concluded that the methanol extract of the root of *Morinda lucida* is only slightly toxic, suggesting that extract is within the safety level (Klaasen *et al*., 1995).

Elevation or depression of the levels of activity of specific enzymes may indicate the presence of a disease or damage to a specific tissue (Nelson and Cox, 2005). Analysis of serum enzymes have been reported to be of value and are early warning signs for certain diseased conditions. It has been reported that changes in enzyme levels are a good marker of soft tissue damage (Wilkinson, 1962). He also noted that damage to body

cells result in the alteration of membrane permeability and consequent release of enzymes into the extracellular fluid (ECF). Elevated enzyme levels may also result from effect of plasmodium lyses resulting from effect of the host defence mechanism.

There was no significant difference ($p>0.05$) in serum and liver Alkaline phosphatase (ALP) concentration in all the group of animals (Table 4.3).

The concentration of serum Glutamate Oxaloacetate Transaminase (GOT) in group 2 animals (6734.14 ± 1087.75) was significantly ($p<0.05$) higher than groups 1 (2167.35 ± 120.62) and 4 (694.65 ± 156.75), but showed no significant difference ($p>0.05$) with group 3 animals (4361.53 ± 1723.01). The concentration of liver GOT showed no significant difference ($p>0.05$) in all the groups (Table 4.4).

The concentration of serum Glutamate Pyruvate Transaminase (GPT) in group 3 animals (3490.42 ± 438.62) was significantly ($p<0.05$) higher than groups 1 (323.54 ± 35.04) and 4 (955.74 ± 157.25), but showed no significant difference ($p>0.05$) with group 2 (2454.22 ± 1097.85). However, liver GPT was significantly ($p<0.05$) higher in group 3 animals (24009.63 ± 3304.22) than the rest of the groups (103.57 ± 13.95, 1936.57 ± 962.29 and 955.74 ± 79.64 for group 1, 2 and 4, respectively) (Table 4.5).

The concentration of gamma glutamyl transferase (GGT) in U/L for animals in group 3 (2495.53 ± 7324.44 and 10350.79 ± 4895.68 for serum and liver respectively) were significantly ($p<0.05$) higher than all the other groups (Table 4.6). However, the concentration of gamma glutamyl transferase (GGT) in U/L for animals in group 4 (322.45 ± 118.84 and 52.64 ± 12.80 for serum and liver respectively) were significantly ($p<0.05$) lower than the group 3 animals, but there was no significant difference ($p>0.05$) between it and groups 1 (451.48 ± 271.35 and 100.10 ± 28.29 for serum and

liver respectively) and 2 animals (1044.44 ± 399.01 and 1787.70 ± 927.61 for serum and liver respectively) (Table 4.6).

It can be deduced from the enzyme assay studies that, the specific enzyme activities in serum and liver for ALP, GGT, GPT and liver GOT were highest in the group 3 animals (those infected, not treated) compared to other groups, except for serum GOT specific activity, which was found out to be highest in the group 2 animals (infected, but treated). This implies that, the more elevated levels in serum and liver ALP, GGT, GPT and liver GOT in group 3 animals could be attributed to cell damage caused by the plasmodium parasite, which causes the enzymes to leak into the blood and also causes the uncontrolled proliferation of the liver cells (hepatocytes). However, the more elevated GOT levels in group 2 animals (infected and treated) than the rest groups, could be attributed to cell damage caused by the plasmodium parasite, and a slight damage caused by the methanolic root extract of *Morinda lucida*. However in general terms, the serum and liver enzyme activities in group 1 animals (extract control) compared to group 4 (not infected, not treated) were not significantly different ($p>0.05$), except for GOT, where there was significant difference ($p<0.05$) between the two groups.

The in vivo Antiplasmodial studies carried out on the methanolic root and leaf extracts, have confirmed the works of others on the Antimalarial activity of extracts of the plant parts (leaf, stem bark and root) (Asuzu and Chineme, 1990; Makinde and Obih, 1985; Koumaglo *et al.*, 1992). It has been reported that the stem bark infusion is used as an antimalarial (Burkill, 1997).

It has been reported that the petroleum ether extract and fractions of the leaf samples 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, the most active being damnacanthal.

Despite the level of work that has been carried out on *Morinda lucida* plant, more areas of research on the plant still needs to be explored. This research work aimed at studying the antimalarial activity of crude hot methanol extract of the root parts, fractions of the crude extract of the root part, and a combination of the hot methanol root and leaf of *Morinda lucida*.

The four-day suppressive test carried out on hot methanol root extract, showed appreciable suppressive effect (figure 4.1), as the mice in two groups out of the three groups treated with the crude methanolic root extract survived beyond twenty days (figure 4.4). There was no significant difference in weight for all the groups, before and after treatment ($p>0.05$).

The curative test showed appreciable curative effect (figure 4.5), though none of the groups treated with the extract survived up to 28 days, when compared with the experimental animals in the Cholroquine treated group, where one of the animals survived beyond 28 days. There was no significant difference ($p>0.05$) in the body weight of all the test animals, before infection, after 72 hours of infection, and after 5 days of treatment, when compared with the negative control group, where there was significant ($p<0.05$) drop in body weight, during the test period. There was significant ($p<0.05$) difference in the pack cell volume (PCV) in all the groups, throughout the test period. There was increase in the PCV of the chloroquine treated group after 5days of

treatment, compared to all the extract treated groups and the negative control group, where there was drop in PCV, though the negative control group being drastic (figure 4.7). The drop in the PCV that is responsible for malarial anaemia occurs both through an increase in the rate at which old Red blood cells are broken and a decrease in the rate at which new ones are produced. Plasmodium not only causes the rupture of parasitized red blood cells, but stimulates the activity of macrophages in the spleen, which then destroys both parasitized and unparasitized red blood cells.

The crude methanolic root extract showed minimal prophylactic activity, when compared with the chloroquine group (figure 4.9).

The result from the antimalarial study of the crude hot methanol extract of the root of *Morinda lucida* suggests that, the extract has more suppressive and curative effect, than prophylactic effect, as evident from the percentage prophylaxis.

Column chromatography was carried out on the crude hot methanol root extract of *Morinda lucida*, where about nineteen fractions were obtained. Thin layer chromatography was carried out on these fractions, and those fractions with similar R_f (Retardation factor) values were pulled together. The thin layer aluminium foil plates used for the thin layer chromatography are shown in plate I. After pulling together fractions with similar R_f, 4 fractions were obtained finally, and curative test was carried out on these fractions.

The curative test carried out on the fractions of the crude hot methanol extract of the root showed that, two of the fractions (fractions 3 and 4) showed effective antimalarial activity as the crude (figure 4.12). This implies that, the crude extract and fractions 3 and 4 exhibited a significant curative effect during established infection comparable to that of the standard drug, chloroquine as demonstrated in the mean survival time of

mice in the extracts and chloroquine treated groups (29.25±1.43, 11.25±0.75, 11.75±1.60, 24.25±1.11, 28.50±1.32), for crude extract, fraction 1, fraction 2, fraction 3, fraction 4 respectively, while in the chlloroquine treated group, two of the mice survived beyond 30 days of the study.

Curative test carried out on the combination of the methanolic extract of the root and leaf showed that parasite reduction in mice was more when treated with the combination of the two extracts, than when treated single extract (figure 4.13).

The presence of flavonoids and other bioactive constituents, is likely to have contributed to the observed antiplasmodial activity of *Morinda lucida* root and leaf extracts.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

From the studies carried out, it has been observed that the crude methanolic root extract of *Morinda lucida* is only slightly toxic (as evident from the acute oral toxicity study and enzyme assay), and has some (67.72%) antimalarial activity, as well as its fractions. When also combined with crude methanolic leaf extract, it showed more effective parasite inhibition. It can be concluded that *Morinda lucida* root and leaf extracts possess antimalarial activity and the results of this study justifies and confirms its usage traditionally, as antimalarial remedy.

5.2 Recommendations

It is evident that great attention has been paid to *Morinda lucida*, however, several compounds should be waiting to be discovered, since it has not been systematically studied for its biochemical composition. It is therefore, recommended that, more research be carried out, in order to isolate active constituents that are yet to be discovered. Also, more work should be done on the fraction that gave the best antimalarial activity, to know the active ingredients contained in it, and possible mechanism of action. The fraction should equally be tested for its toxic potentials to the system.

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APPENDICES

APPENDIX A

PREPARATION OF WORKING REAGENTS

a) 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

b) Determination of Total Protein

Biuret reagent

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

Standard protein (Bovine Serum Albumin)

The standard was prepared by dissolving 1g of Albumin standard in 100mL of water. It was serial diluted and then absorbance was taken at 580nm 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.

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

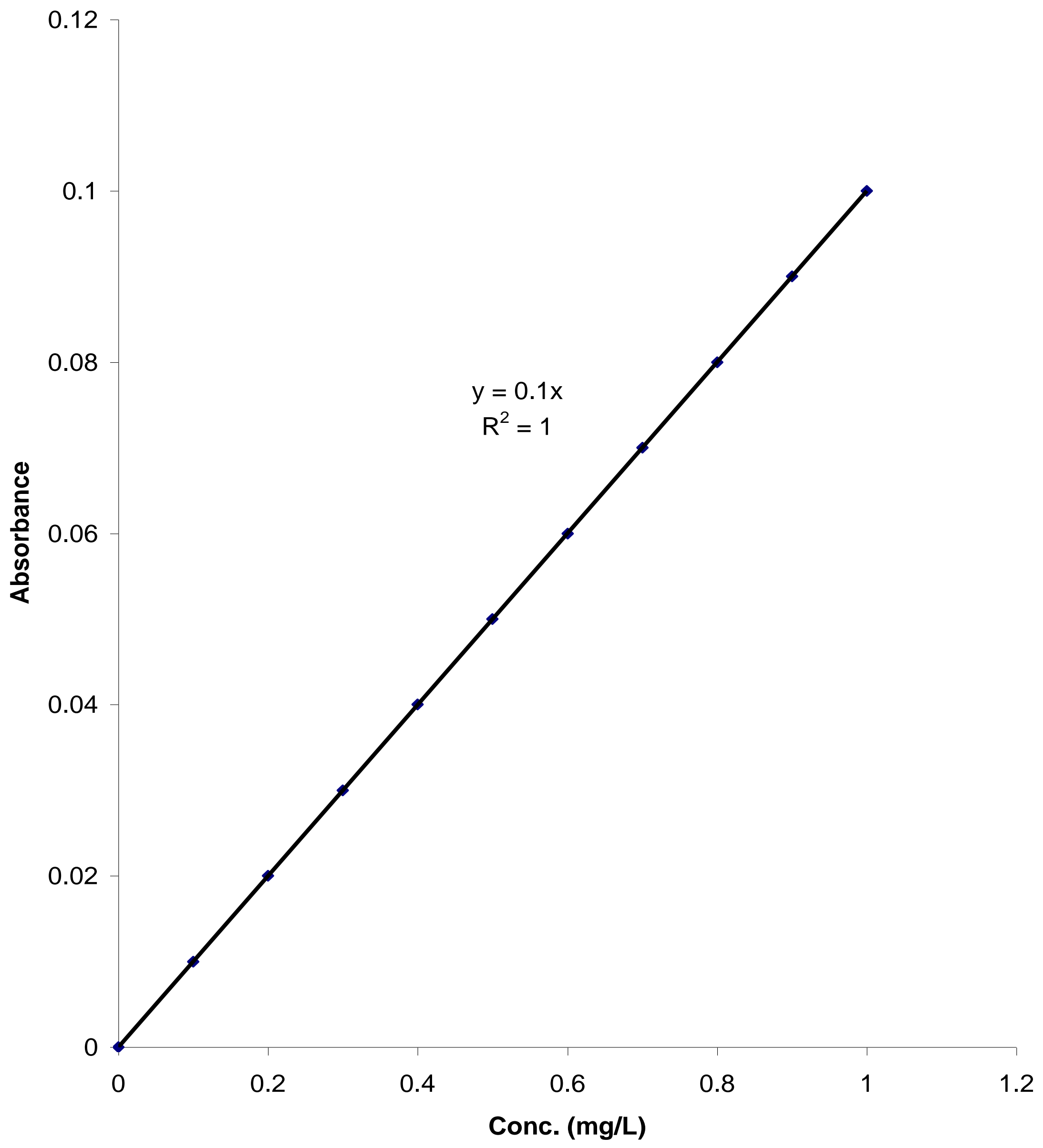
Key:

BSA: Bovine Serum Albumin

BR: Biuret reagent

ABS: Absorbance

Wavelength used – 580nm



Calibration curve of Absorbance against Wavelenght

c) Determination of Alkaline phosphatase (ALP)

Preparation and Stability of working reagent;

4 volume of reagent 1 (R1) was mixed with 1 volume of reagent 2 (R2). The working reagent is stable for 30 days, at 2 – 8°C.

Reagent composition:

Reagent 1: Alkaline Phosphatase (S.L) R1	2 x 24ml / 2 x 40mL
• Diethanolamine buffer (pH 10.2)	125mmol/L
• Magnesium Chloride	0.65mmol/L
Reagent 2 : Alkaline Phosphatase (S.L) R2	2 x 6mL /2 x 10mL
• P-Nitrophenyl phosphatase	50mmol/L

d) Determination of Glutamate oxaloacetate transaminase (GOT)

Preparation and Stability of working reagent;

Reagent 2 (R2) was reconstituted with the volume of reagent 1 (R1). The reconstituted reagent is stable for 30 days at 2 – 8°C.

Reagent composition:

SGOT R1	**2 x 63mL / 4 x 50mL / 2 x 205mL**
• Tris Buffer (pH 7.8)	88mmol/L
• L-Aspartate	260mmol/L
SGOT R2	**6 x 20mL / 4 x 50mL / 8 x 50mL**
• Malate dehydrogenase	≥ 600 U/L
• Lactate dehydrogenase	≥ 900 U/L
• NADH	0.20 mmol/L
• α - ketoglutarate	12 mmol/L

e) Determination of Glutamate pyruvate transaminase (GPT)

Preparation and Stability of working reagent;

Reagent 2 (R2) was reconstituted with the volume of reagent 1 (R1). The reconstituted reagent is stable for 50 days at 2 – 8°C.

Reagent composition:

SGPT R1	**2 x 63mL / 4 x 50mL / 2 x 205mL**
Tri Buffer (pH 7.5)	110mmol/L
L-Alanine	550mmol/L
SGPT R2	**6 x 20mL / 4 x 50mL / 8 x 50mL**
Lactate dehydrogenase (LDH)	≥ 200 U/L
NADH	0.20mmol/L
α-Ketoglutatrate	16 mmol/L

f) Determination of Gamma glutamyl transferase (GGT)

Preparation and Stability of working reagent;

Four volume of reagent 1 (R1) was mixed with 1 volume of reagent 2 (R2). The working reagent is stable for 21 days, at 2 – 8°C.

Reagent composition:

Reagent 1: Gamma GT (S.L) R1 2 x 24mL

- Tris buffer pH (8.25) 133mmol/L
- Glycylglycine 138mmol/L

Reagent 2: Gamma GT (S.L) R2 2 x 24mL

- Glupa-C 23mmol/L

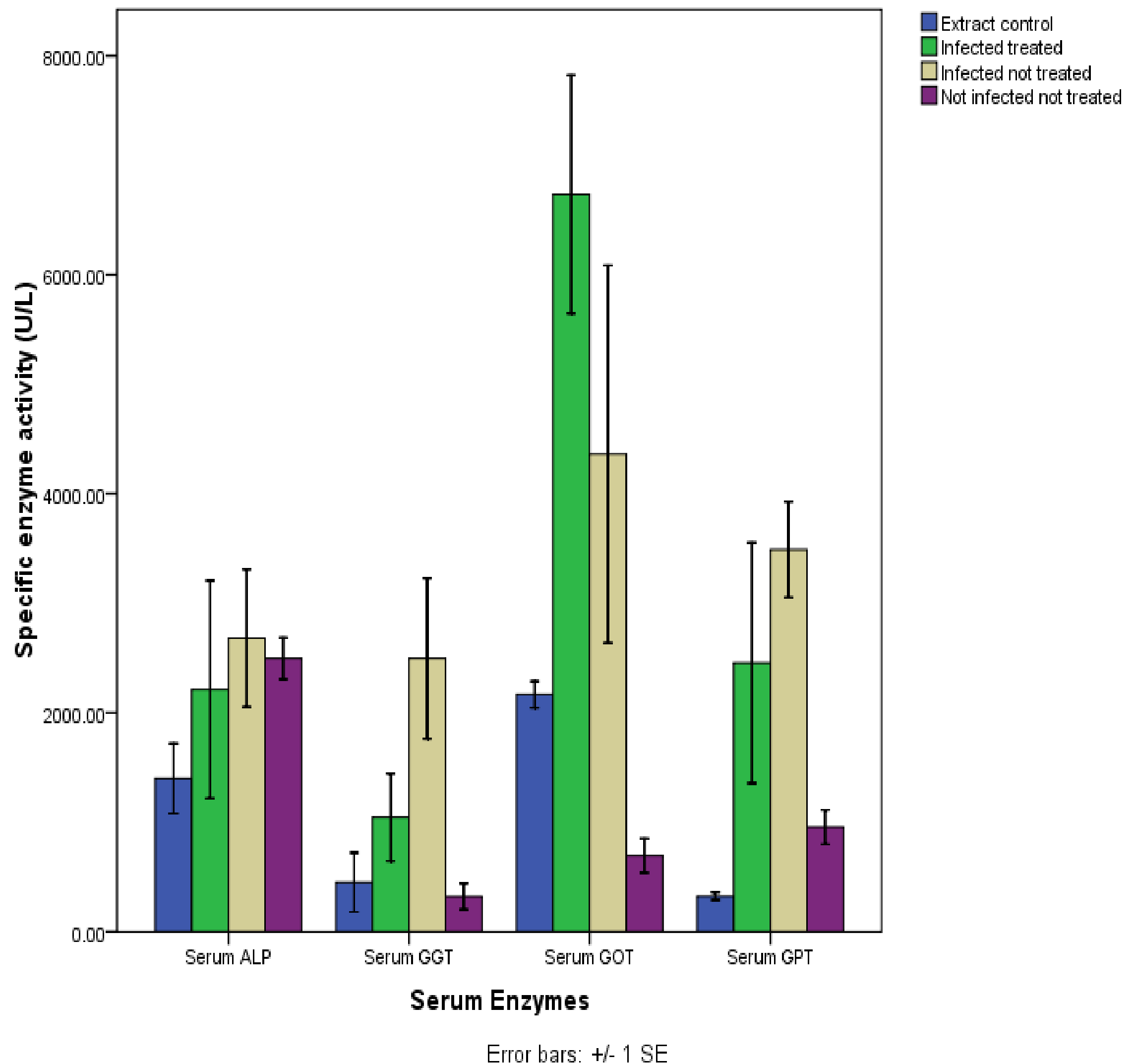
g) **Calculation**

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

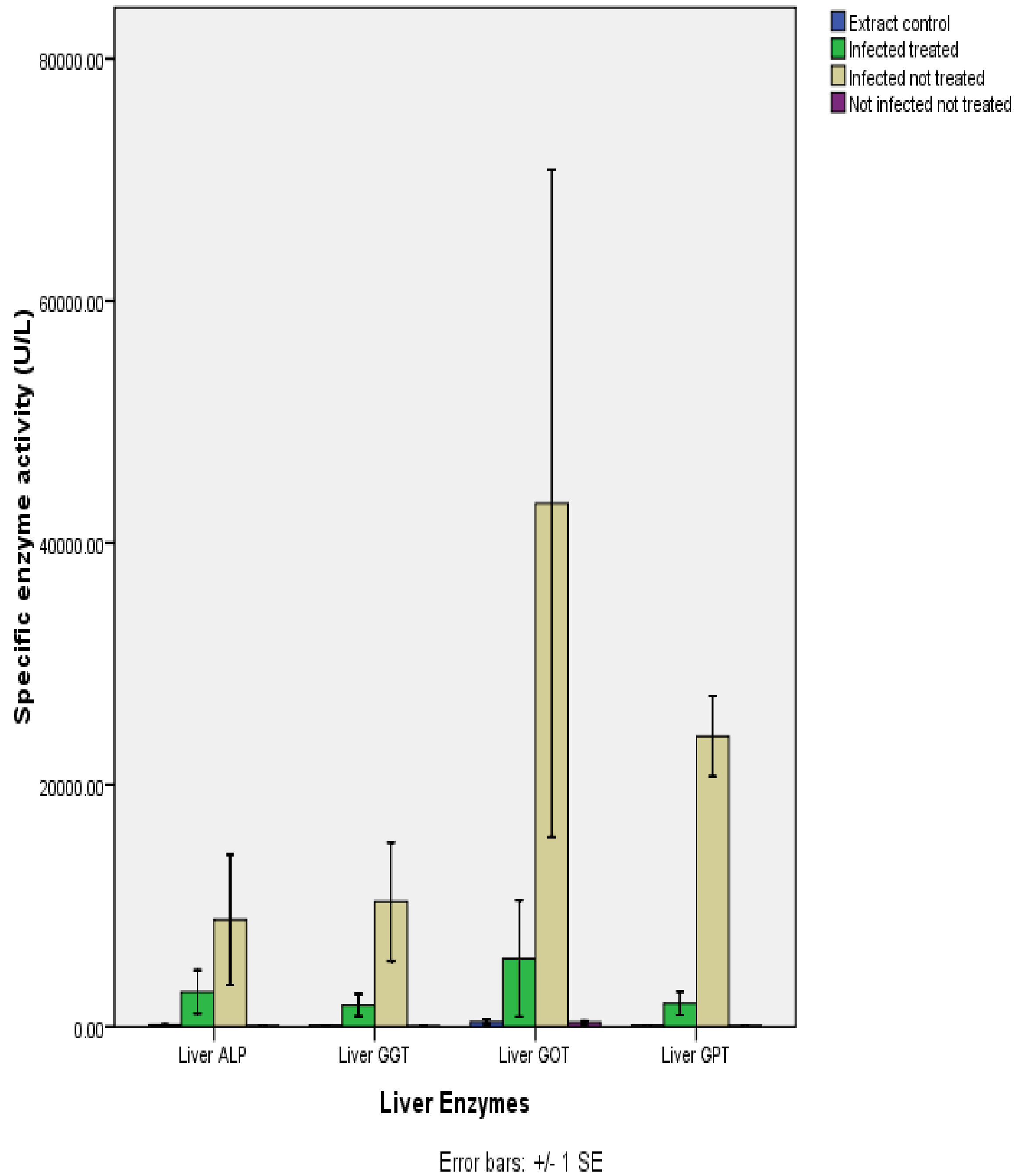
Specific enzyme activity = $\frac{\text{Enzyme activity} \times \text{dilution factor}}{\text{Total protein}}$

APPENDIX B

Specific Enzyme activities of Alkaline phosphatase (ALP), Gamma glutamyl transferase (GGT), Glutamate oxaloacetate transaminase (GOT) and Glutamate pyruvate transaminase (GPT)



Specific Alkaline phosphatase, Gamma glutamyl transferase, Glutamate oxaloacetate transaminase and Glutamate pyruvate transaminase activities in the Serum



Specific Alkaline phosphatase, Gamma glutamyl transferase, Glutamate oxaloacetate transaminase and Glutamate pyruvate transaminase activities in the Liver

APPENDIX C

Four-day suppressive test with crude methanolic root extract

a) Mean Parasite Count

Drug/ Extract	Dose (mg/kg/day)	Mean Parasite Count
M. lucida	100	4.44 ± 0.15
M. lucida	200	4.08 ± 0.23
M. lucida	400	3.28 ± 0.44
Chloroquine	5	1.84 ± 0.15
Normal saline	0.2ml/mouse/day	10.16 ± 1.41

b) Average % parasitaemia and average % suppression

Drug/Extract	Dose (mg/kg/day)	Average % parasitaemia	Average % suppression
M. lucida	100	2.22± 0.07	56.30
M. lucida	200	2.04± 0.12	59.84
M. lucida	400	1.64±0.22	67.72
Chloroquine	5	0.92± 0.73	81.89
Normal Saline	0.2ml/mouse/day	5.08± 0.71	0.00

c) Average weight of mice, before infection and after treatment for suppressive Test

Drug/Extract	Dose (mg/kg/day)	Weight before infection (g)	Weight after treatment (g)
M. lucida	100	21.60 ± 1.69	22.00 ±1.00
	200	22.20 ± 1.24	22.80 ±1.93
	400	21.60 ± 1.29	19.60 ±1.54
Chloroquine	5	21.60 ± 1.03	21.00 ±1.58
Normal saline	0.2ml/day	19.80 ± 0.80	18.80 ±0.74

d) Mean days of survival of mice for suppressive test

Drug / extract	Dose (mg/kg/day)	Mean days of survival
M. lucida	100	18.60 ± 0.75
M. lucida	200	20.20 ± 1.40
M. lucida	400	23.60 ± 1.03
Chloroquine	5	24.80 ± 1.93
Normal Saline	0.2ml/mouse/day	12.80 ± 0.86

APPENDIX D

Curative test on Crude methanolic root extract of *Morinda lucida*

Dose	Days of Treatment					
(mg/kg/day)	D_0	D_1	D_2	D_3	D_4	D_5
100 M.L	57.00±1.82	57.25±1.60	53.30±2.04	48.73±2.06	44.35±1.77	40.27±1.21
200 M.L	59.73±2.07	58.03±2.20	54.00±1.59	45.60±1.00	42.20±2.38	31.57±1.21
400 M.L	54.65±2.17	54.10±2.35	48.15±1.18	41.45±0.92	35.10±1.04	23.50±1.45
5mg CQ	58.25±1.81	55.05±1.58	42.33±0.96	35.38±2.36	26.18±1.69	8.78±3.58
0.2ml N.S	58.85±3.00	61.37±2.77	70.00±3.17	78.23±2.75	91.35±1.92	115±5.66

Key:

M.L: *Morinda lucida,* CQ: Chloroquine, N.S: Normal saline

APPENDIX E

Prophylactic test with crude methanolic root extract

a) Average weight of mice before and after administration of extract, and after 72 hours of infection

Drug/ Extract	Dose (mg/kg/day)	Average weight of Mice		
		D_0	D_4	D_7
M. lucida	100	25.81± 1.05	26.43 ± 1.10	24.28±0.91
M. lucida	200	25.70 ± 0.93	26.35 ± 0.89	24.63 ± 1.15
M. lucida	400	25.38 ± 0.51	26.04 ± 0.58	24.53±1.41
Chloroquine	5	26.47 ± 1.64	28.88 ± 1.36	30.26±1.50
Normal saline	0.2ml/mouse	26.60 ± 0.17	27.15 ± 1.07	24.74±0.64

Key:

D_0: Day 1 of extract administration, D_4: after 4 days of administration (before infection), D_7: 72 hours after infection.

b) Average packed cell volume (PCV) of mice before and after administration of Extract, and After 72 hours of Infection

Drug/ Extract	Dose (mg/kg/day)	Average weight of Mice		
		D_0	D_4	D_7
M. lucida	100	40.75 ± 0.48	38.00 ± 2.38	34.75±1.38
M. lucida	200	39.75 ± 2.66	37.50 ± 2.33	50.50 ± 1.56
M. lucida	400	30.25 ± 8.26	38.50 ± 1.71	45.25 ± 2.02
Chloroquine	5	29.75 ± 1.93	33.25 ± 1.79	43.00 ±4.10
Normal saline	0.2ml/mouse/day	36.25 ± 1.03	38.00 ± 1.47	41.25 ± 3.90

Key:

D_0: Day 1 of extract administration, D_4: after 4 days of administration (before infection), D_7: 72 hours after infection.

APPENDIX F

Antiplasmodial efficacy of fractions of crude methanolic root extract of *Morinda lucida*

a) Mean parasite count of mice treated with crude and fractions of root hot methanolic extract of *Morinda lucida*

DRUG/ EXTRACT	DOSE (mg/kg/day)	DAYS OF TREATMENT		
		DO	D2	D5
Crude	400	48.85±2.06	23.50±1.45	11.50±1.27
Fraction 1	400	44.60±2.21	28.80±1.37	20.20±1.96
Fraction 2	400	48.85±1.30	25.25±1.53	16.45±1.07
Fraction 3	400	48.40±0.99	24.35±0.61	14.40±1.49
Fraction 4	400	47.90±1.28	22.25±0.67	12.05±0.29
Chloroquine	5	50.15±0.60	19.15±0.91	6.65±0.21
Normal Saline	0.2ml/day	44.55±0.86	64.95±1.02	100.87±2.66

b) Mean days of survival of mice treated with fractions of the crude methanolic root extract

Extract (400mg/kg)	Mean survival time (days)
Crude methanolic root extract	29.25±1.43
Fraction 1	11.25±0.75
Fraction 2	11.75±1.60
Fraction 3	24.25±1.11
Fraction 4	28.50±1.32
Chloroquine	28.00±0.00
Normal saline	5.75.00±2.14

Appendix G: Antiplasmodial efficacy of Combination of methanolic extracts of leaf and root

a) Mean Parasite Count

DRUG/ EXTRACT	DOSE (mg/kg/day)	DAYS OF TREATMENT		
		D_0	D_2	D_5
M.L leaf	100	39.50±2.22	31.00±1.29	26.00±1.29
	200	42.25±1.25	32.50±2.21	20.00±1.29
M.L Root	100	42.00±1.75	33.00±2.15	25.28±1.65
	200	44.00±1.25	29.70±1.26	21.35±1.85
M.L leaf and root	50	38.75±0.75	34.50±2.50	29.00±1.83
	100	43.50±1.26	30.50±1.71	22.50±1.29
Chloroquine	5	37.00±1.92	22.00±1.83	8.50±1.26
Normal Saline	0.2ml/day	38.80±2.50	52.00±2.16	85.00±5.50

Key:

M.L: *Morinda lucida*

b) Average weight of infected mice treated with combination of leaf and root of *Morinda lucida*

DRUG/ EXTRACT	DOSE (mg/kg/day)	DAYS OF TREATMENT		
		Before infection	D_0	D_5
M.L leaf	100	27.51±0.80	27.33±1.0	26.12±1.19
	200	22.48±0.66	24.52±0.28	23.23±0.72
M.L Root	100	23.50±0.85	22.00±1.06	21.32±1.18
	200	25.35±1.25	25.20±1.22	22.45±2.44
M.L leaf & root	50	23.45±1.94	22.37±2.17	22.06±2.51
	100	22.06±2.51	26.63±1.47	25.21±0.97
Chloroquine	5	27.00±1.45	25.82±1.44	26.70±1.19
Normal Saline	0.2mL	23.48±0.98	20.84±0.50	19.28±1.02

Key:

M.L: *Morinda lucida*

c) Average packed cell volume (PCV) of infected mice treated with combination of leaf and root of *Morinda lucida*

DRUG/ EXTRACT	DOSE (mg/kg/day)	DAYS OF TREATMENT		
		Before infection	D_0	D_5
M.L leaf	100	53.75±3.19	37.75±4.80	29.00±4.02
	200	56.00±3.49	38.28±4.09	37.25±4.48
M.L Root	100	55.00±4.80	40.28±1.19	39.25±2.36
	200	52.55±3.35	41.80±2.08	39.65±3.48
M.L leaf & root	50	40.25±4.51	41.00±1.23	35.50±2.47
	100	56.75±4.75	50.00±4.02	40.25±1.89
Chloroquine	5	52.50±3.07	34.75±3.09	44.75±8.25
Normal Saline	0.2mL/day	46.25±2.63	34.00±4.19	27.55±5.10

Key:

M.L: *Morinda lucida*