EVALUATION OF THE *IN VIVO* ANTIPLASMODIAL POTENCY OF CRUDE TOAD (ANURA: BUFONIDAE) VENOM IN *PLASMODIUM BERGHEI* – INFECTED MICE

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ABSTRACT

Malaria which is still the most devastating tropical disease causing global health challenge requires an aggressive search for an alternative and non-resistant antimalarial lead compound informed this current study that elucidate the antimalarial potency of the crude venom of toad against *Plasmodium berghei* infected mice. The toad venom was extracted by manual compression of the large postorbital paratoid gland of the toad. The bioactive compounds of the venom were determined using gas chromatograph mass spectrometry procedure. The acute toxicity of the venom was established using modified Lorke procedure. Thirty mice were randomly shared into six equal groups and five groups were intraperitoneally infected with chloroquine sensitive *Plasmodium berghei* NK 65 strain. Group one, two and three were administered after establishing infection with 10, 20 and 30 mg/kg/b.wt of the toad venom, group four were not parasitized and not treated, group five were parasitized and not treated and group six were parasitized and treated with 5mg/kg chloroquine (standard control). The LD_{50} of the toad venom was computed as the geometric mean of the lowest dose that produce mortality and highest dose with no lethal effect. The result of the LD_{50} of the toad venom was 141.42 mg/kg/b.wt. Zoochemical screen revealed the presence of flavonoids, Tannins, Saponins and Carbohydrates, while GC-MS showed 25 bioactive compounds which includes; Oleic acid, Dencanoic acid and Octadencanoid acid in the toad venom. The group treated with 20 mg/kg b.wt. of the crude toad venom of an independent dose revealed a decrease of the parasitemia level (18.05± 1.01 ml/kg PBS) when compared to the standard group (21.25±0.07 ml/kg PBS). The extract at dose 20 mg/kg b.wt. was significantly ($p<0.03$) ameliorated the parasite induced decrease in the body weight (31.01 g to 37.05 g) and PCV (44.05 % to 46.28 %) of the mice. The effect of the venom on the hematological parameters was also assessed employing standard procedure. The red blood cells count (10.24±0.62 × 10^{12}/L), hemoglobin (13.08±0.95 g/dL) and total white blood cell (11.05±0.83 × 10^{12}/L) revealed a significant ($p< 0.05$) decrease in the negative group when compared to the control group (19.01±0.54 × 10^{12}/L), (26.31±0.75 g/dL) and (16.43±0.08 × 10^{12}/L) and the groups treated with 20mg/kgb.wt. of the crude extract (18.12±0.17 × 10^{12}/L), (20.108±0.63 g/dL) and (20.03±0.09 × 10^{12}/L). The survival time of the mice treated with 20 mg/kg b.wt. of the crude toad venom extract showed the highest survival time of over 40 days. Additionally the toad venom ameliorates the parasite induced liver and kidney damage when compared with the negative group. Findings from current study, thus established the antimalarial potency of toad venom and therefore it is suggested to be introduced in the development of antimalarial drugs.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Malaria continues to be the most disturbing disease of the tropics, rising infections and deaths (WHO, 2019). This remains a huge global health decry with millions of new incidence and mortality reported yearly in spite of all the control measures over the years (WHO, 2016).According to World Health Organisation world malaria report, 155 million cases of malaria occurred in 2019 with over 500,000 mortality in 11 malaria dominant countries. African regions have about 93 % with Nigeria having 25 % (WHO, 2019).

World Health Organisation (2015), modified their treatment policy in countries where malaria parasite (*Plasmodium falciparum*) is native from using of single therapy with drugs such as choloroquine, amodiquine and sulfadoxine-pyrimethamine SP to recently endorsed artemisinin- based combination therapies (ACTs). However, resistance to ACTs has risen recently in *P. falciparium* (WHO, 2015). Drug resistance by these malaria parasites is the major challenge in managing this disease, also threatening its control, treatment and elimination effort worldwide (WHO, 2015). This has necessitated an aggressive quest to develop novel drug of malaria therapy, especially in regions where resistant *Plasmodium* strains are present. This has stimulated intense drug discovery endeavour directed towards identifying novel, highly active anti-malaria drugs and the identification of quality leads from natural source would highly increase these effort (Guantani *et al*., 2011).

In the quest for finding a lasting treatment and cure to this disease the use of natural products has provided a potential technique for identifying novel anti-malarial drugs.

The bio-prospecting of secondary metabolites can be an important tool to exposing new anti-malarial drugs. The use of animal products, plants, fungi and bacteria are important sources of biologically active substance with structural diversity and novel mechanisms of action which can possibly provide patentable products (Militao *et al*., 2012). The prospective of natural products to provide the development of anti-malarial compounds is achievable (Guantani, 2011). An example is the development of captopril, this medicine was based on a research on small peptides from the venom of the South American snake (*Bothrops jararaca*) that were known to potentiate the action of bradykinin (Camargo *et al*., 2012; Harvey, 2014).

The family Bufonidae of the class of Amphibians have about 471 species (Pramuk, 2006). One of the relevant genus of this family is *Sclerophrys* (formerly *Bufo* in the new world), which has two sub-species. It is found across Western and Central Africa (Barej *et al*., 2011). The genus of the family (Bufonidae) are also in places like Nigeria, Ghana, Liberia, Sierra Leone, Cameroon, Central Africa Republic, Republic of the Congo, Democratic Republic of Congo, Ivory coast, Equatorial Guinea and Gabon respectively (Tandy, 2014).

The skin secretion and venom of amphibians are abundant source of bio-active compounds such as peptides, alkaloids, bufadienolides, biogenic amines and proteins (Gao *et al*., 2010). These molecules play an important role in the physiological functions of these animals, especially for predation and protection against microorganism (Yang *et al*., 2010).

Bufadienolides are an important group of steroid hormones that has shown vasoconstriction (Puschett *et al*., 2010), antiviral (Cui *et al*., 2011), anti-tumoral (Delebinski *et al*., 2015), anti-Leishmanial and anti-trypanosomal activities (Tempone *et al*., 2008). Venoms (toxins) from toads have long been known to include abundant

chemicals with great pharmaceutical endowment. Recent studies have noticeable therapeutic value for a plethora of diseases (Yang *et al*., 2010).

It has also been found that ancient people of Mesoamerica had used toads, *Bufo marinus* or *Bufo alvarius*, as a hallucinogen via licking toad skins directly, or smoking the prepared powder (Cui *et al*., 2011). Studies have shown that indolealkylamines (IAAs) in toad skin, primarily bufotenine are responsible for these hallucinogenic effects (Cui *et al*., 2011). Diverse strategies have been employed in the fight against malaria. Especially resistance to anti-malarial drugs, prescribing habits and therapeutic protocols, prevention of infection and the use of combination therapies, with particular weight being placed on artemisinin-based combination therapy (ACT) (Eric and Kelly, 2011).

1.2 Statement of the Research Problem

Despite the aggressive control against malaria infection, over the years, the disease remains a public health treat, especially in the developing world (Bantie *et al*., 2014).The chemotherapy that have proven successful is now facing a number of setbacks including drug resistance, side effects and not affordable by the populace.

On the other hand, efforts to develop a viable vaccine against the disease have remained elusive. This has therefore called for aggressive search for better alternatives in the management of malaria infection especially from natural source. The bioactive content identification and experimentation of toad venom against *Plasmodium* parasite has not been well exploited. Similarly, the crude toad venom may contain potent both toxic chemical substance that may have long term effect on its end users, Thus the need for toxicity assessment.

1.3 Aim and Objectives of the Study

The aim of the study is to evaluate the *in vivo* anti-plasmodial activity of toad venom on chloroquine-sensitive *Plasmodium berghei* -infected mice.

The objectives of the study are to determine;

i. the zoochemical components of toad venom.
ii. the acute toxicity test and safe dose LD_{50} of the toad venom.
iii. the antiplasmodial efficacy of crude toad venom extract on *Plasmodium berghei*-infected mice.
iv. the effects of the crude toad venom extract on the body weight, packed cell volume (PCV), haematological and biochemical parameters.
v. the bio active compounds present in the crude toad venom extract.
vi. the effect of the crude toad venom on histopathological parameters of *P. berghei*-infected mice.

1.4 Justification for the Study

Malaria control efforts are intricate by the up shoot resistance of mosquito vectors to insecticides together with challenges of not having effective anti-malaria vaccines. Thus, there is immediate need to search for effective, easily available, affordable and safe alternative anti-malaria drugs that can be composed into the existing malaria management interventions to successfully curtail the disease and for its natural elimination or eradication (Guatani, 2011).

Intensive drug discovery stimulated at developing new anti-malarial drugs or modifying the existing ones, and which aims at establishing novel compounds that exhibits high quality of experimental and clinical anti-malaria efficacy without showing any cause of resistance (Eric and Kelly, 2011).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Introduction

Malaria is one of the global killer disease (Neeta and Jobin, 2012) It affects up to 250 million and kills nearly 800,000 people per year (WHO, 2012). In every 30 seconds, a child dies as a result of malaria (WHO, 2012).

Malaria which remains a major concern in the public health sector in Nigeria, accounting for sizeable mortality and economic loss. Apart from preventive measures, early diagnosis and complete treatment are the important modalities that have been used to curtail the disease (CDC, 2015). The general management procedure should be carried out according to the clinical condition of the patient and judgment of the treating physician (CDC, 2015). Malaria is a significant public health difficulty of the country. Around 1.5 million confirmed cases are reported yearly, 40–50 % are owed to *Plasmodium falciparum* (WHO, 2015). Early and efficient malaria treatment makes it curable, while delay in treatment may lead to serious consequences including death. Quick and efficient malaria treatment is an important tool for the control and transmission of the disease (Shekins *et al*., 2014).

2.2 Malaria Parasites

Malaria parasite (*Plasmodium*) of the phylum Apicomplexa, a large group of parasitic eukaryotes (Figure 2.1). *Plasmodium* is in the order Heamosporida and the family *Plasmodiidea.* Over 200 species of *Plasmodium* have been described; of which five species are known to cause malaria in humans. They include: *P. falciparum, P. vivax*, *P. ovale*, *P. knowlesi*and *P. malariae*. Of these, *P. falciparum*, the prevalent species in Africa, is the deadliest and is responsible for approximately 90% of malaria deaths per

year (Nwabor *et al*., 2015). However, it estimates has revealed that people globally live at risk of *P. vivax* than *P. falciparum* and suffers high morbidity from *P. vivax* (WHO, 2015). Each species of the *Plasmodium* parasite differs in phenotype, immune response, geographical distribution, relapse pattern and drug response (Onasanwo *et al*., 2008).

2.3 Classification of *Plasmodium* Species

Kingdom: Protista

Infrakingdom: Alveolata

Phylum: Protozoa

Subphylum: Apicomplexa

Class: Aconoidasida

Order: Haemosporida

Families: Plasmodiidae

Genus: *Plasmodium*

Species: *P. berghei*

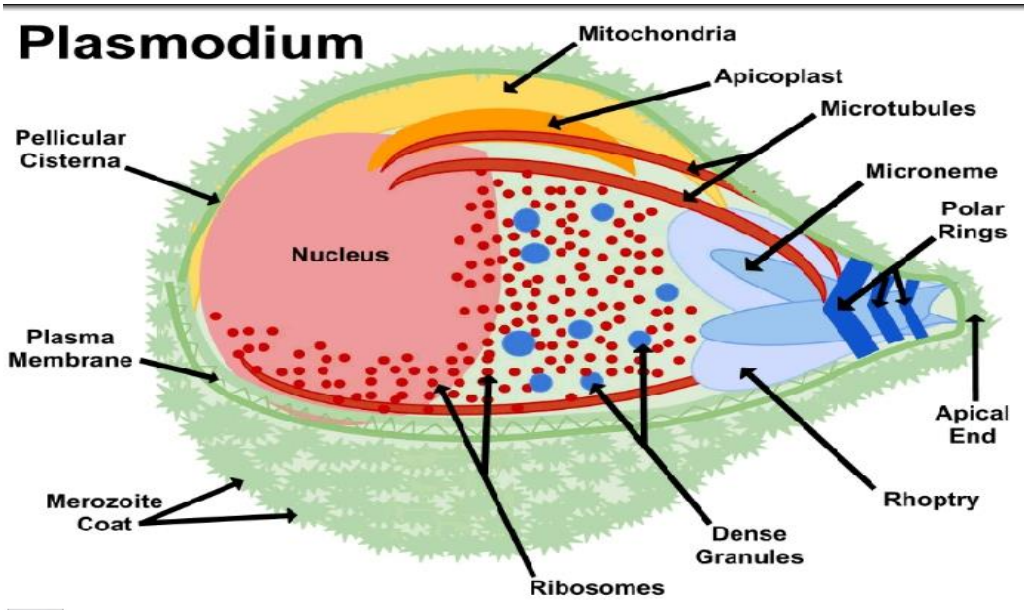


Figure 2.1 *Plasmodium* Species (Source: Nwabor *et al*., 2015)

2.4 Vectors of *Plasmodium* Species

Plasmodium species are unicellular protozoan parasite transmitted by female mosquitoes (Figure 2.2). They lay their eggs on almost any type of standing or slow-flowing water, breeding in temporary puddles such as hoof prints and tyre ruts, shallow natural ponds, marshes, irrigation ditches and water tanks (Builders *et al*., 2014). This breeding sites varies from species to species of mosquitoes 100-300 eggs are laid at once which takes 1-5 days of incubation and hatching. They hatch into tiny larvae that mainly feeds on microorganism that are found in the water developing into pupae (non-feeding) during this stage, the adults insects are shaped. The cycle takes about 7-21 days cycle which is completely dependent on the ambient temperature (Oduola *et al*., 2013).

Figure 2.2 *Anopheles gambiae* Mosquito (Source: Nwabor *et al*., 2015)

2.5 Transmission of Malaria Parasites

Malaria is an insect-borne disease spreading from one person to another by selected species of blood-sucking mosquitoes of the *Anopheles* genus which includes *A. gambiae*– the primary vector for transmission of *P. falciparum* malaria in sub-Saharan Africa (Shekins *et al*., 2014). Female mosquitoes transmit malaria during a blood meal; they need the high levels of proteins in the blood meal to develop and mature their eggs (WHO, 2012). They are attracted to humans by smell and vision. Slender, sharp, saw toothed stylets on the end of the female mosquito's proboscis pierce the skin and probe for a suitable small blood vessel. During a blood meal, the mosquito pumps saliva into the host. Chemicals in the saliva prevent the blood from clotting and act as an anesthetic to stop the host feeling the mosquito while it is feeding. The saliva leaves an allergic reaction of red, itchy swelling at the site of the bite remains characteristic to their bite. The male mosquitoes are not known to transmit or suck blood; they feed only on plants juices (CDC, 2015).

2.6 Life Cycle of *Plasmodium* Species

The alternating extracellular and intracellular forms of the *Plasmodium* parasite gives it that characteristic complex life cycle , involving sexual reproduction in mosquitoes and

asexual reproduction in the liver cells and red blood cells of humans (Neeta and Jobin, 2012). The parasite from the mosquito gains entrance into the body of its host during a blood meal by the female mosquito. The sporozoites from the mosquito's salivary glands are injected into the host's bloodstream (Exo-erthrocytic cycle). Within 30 minutes the sporozoites finds its way into the liver cells of its host, changes into a schizoint while feeding and growing on the liver content (Traore *et al*., 2006). Rapid division takes place within the next 5-8days, giving rise to merozoites in the liver cells. This cells forms balloon and burst, giving out merozoites into the bloodstream attacking the red blood cells. While the parasite is within the liver the person does not feel sick and shows no signs or symptoms of the disease. *P. vivax* and *P. ovale* can have a dormant stage in the liver called hypnozoites (WHO, 2012). These can remain in the liver for several years, causing relapses in later life. While in the liver and the red blood cells the parasite is protected from the host's antibodies (WHO, 20115). Inside the red blood cells the individual merozoites develop into trophozoites and finally into schizonts which contain up to 32 distinct merozoites (Erythrocytic cycle) which varies in species of *Plasmodium* (WHO, 2015). About 2–3 days upon invasion the red blood cells rupture, letting out the merozoites back into the bloodstream. There is a re-invasion by this new merozoite attacking uninfected red blood cells where the whole cycle is repeated (WHO, 2015). It is believed that these toxins directly stimulate the host's immune system and a highly complex immune response is initiated, resulting in bouts of chills, fever and sweats. In *P. vivax malaria* this whole cycle can occur within 48 hours, corresponding to the red blood cell cycle (WHO, 2015).

These trophozoites can develop to either male or female gametocytes, initiating the sexual stages of the parasite cycle (WHO, 2019). These gametocyte live freely in the blood stream of their host, they are picked up during a blood meal of a female mosquito,

in the body of its new host, it finds its way to the stomach where the gametocytes develops into gametes, allowing fertilization to occurs and a zygote is formed (WHO, 2015). Within 24 hours the zygotes transform into motile ookinetes that burrow into the stomach wall (Sporogonic cycle). Ookinetes encyst and become oocysts that divide to produce approximately 1,000 sporozoites each. After about 7 days the oocysts rupture, releasing their sporozoites which travels to the salivary gland of the mosquito ready for infect another human (Figure 2.3).

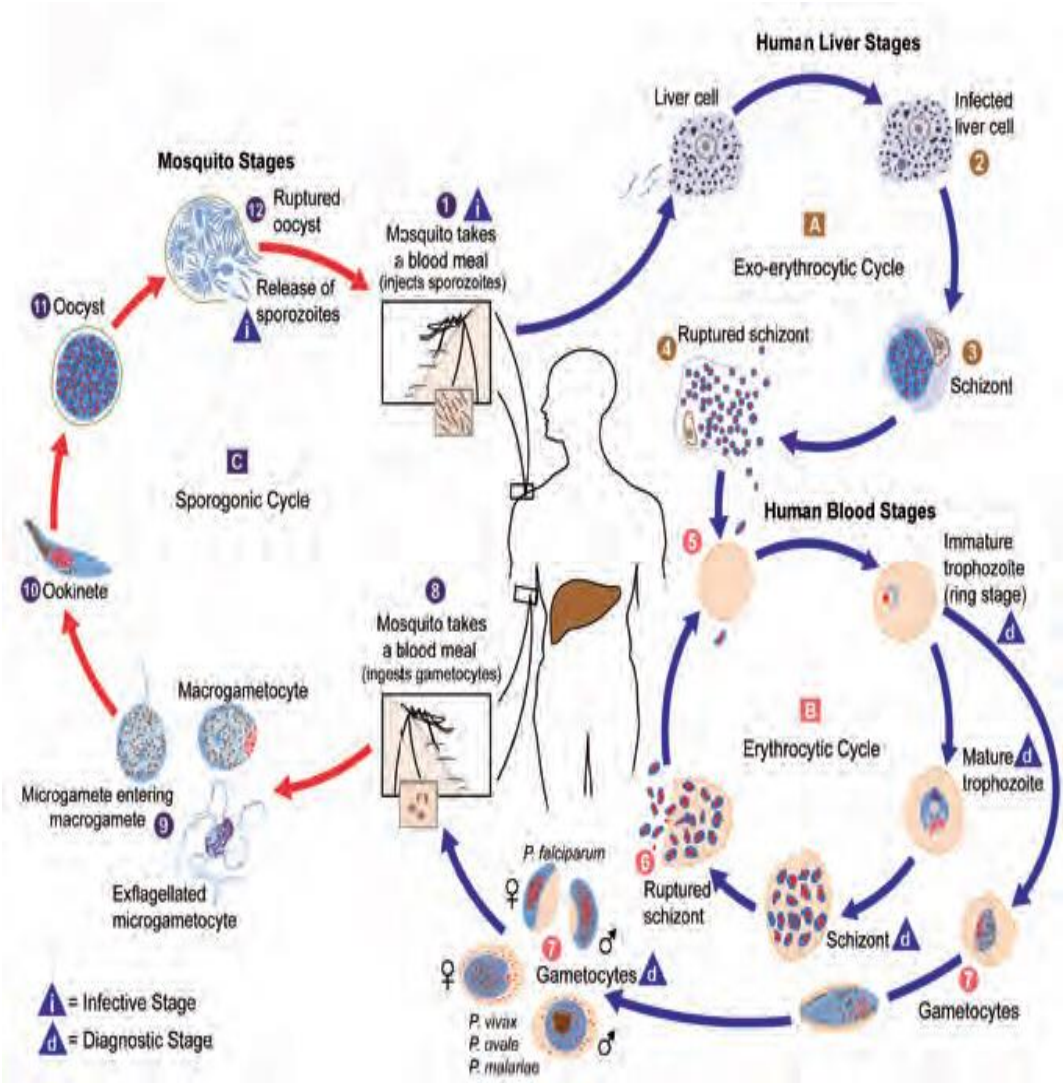


Figure 2.3: Life Cycle of Malaria parasite. (Source: Centre for Disease Control, 2015)

2.7 Clinical Features of Malaria

Fever is the well-known symptom of malaria. It can be intermittent with or without periodicity or continuous. Many cases have chills and rigors. The fever is often characterized by headache, myalgia, arthralgia, anorexia, and nausea and vomiting. The symptoms of malaria can be non-specific, sharing similar symptoms as to other diseases like viral infections, enteric fever. Persons in endemic areas are quick to suspect malaria when they have or show any of the above symptoms. It should also be suspected in those patients who have recently visited an endemic area (CDC, 2015). Although malaria is known to mimic the signs and symptoms of many common infectious diseases (CDC, 2015).

Malaria is diagnosed by clinical symptoms, microscopic examination of the blood or rapid diagnostic tests (RDTs) (WHO 2016). Rapid diagnostic tests (RDTs) use blood from a pin-prick to establish infection based on the presence of antigens. Fever, headache, chills and vomiting – the classic flu-like symptoms of malaria – appear around 9–14 days after the initial mosquito bite (WHO, 2016). This time varies according to the species of *Plasmodium*. The WHO currently recommends that all cases of suspected malaria should be confirmed by testing the presence of the parasites before treatment commences, while treatment on the basis of clinical suspicion' should only be permitted when parasitological diagnosis is not available or cannot be assessed. All clinically suspected malaria cases should be investigated immediately by microscopy and/or Rapid Diagnostic Test (RDT) (WHO, 2019).

2.7.1 Diagnosis microscopy

Microscopy of stained thick and thin blood smears remains the gold standard for confirmation of diagnosis of malaria. The advantages of microscopy are:

1. The sensitivity is high. It is possible to detect malarial parasites at low densities. Parasitic loads are better qualified using this method (Neeta and Jobin, 2012). 2. Species types are obvious and easily distinguished; also having them classified into different stage of their life cycle is achievable using this method (Neeta and Jobin, 2012).

2.7.2 Rapid diagnostic test

Rapid Diagnostic Tests are based on the detection of circulating parasite antigens. Several types of RDTs are specified to only detect *P. falciparum,* although there are others which can also identify other different species of the parasite (CDC, 2015).

RDTs vary from one product to another, resulting to different procedure that are can be used in carrying out this test. This become necessary and important that user's manual and instructions that accompany the product be thoroughly read and followed meticulously (CDC, 2015). Timing is also relevant when using RDTs, therefore result reading should be carried out and read at the specified time. It is the responsibility of the clinician or technician doing a rapid test for malaria to ensure that the kit is within its expiry date and has been transported and stored under recommended conditions (CDC, 2015).

Early diagnosis and treatment of cases of malaria aims at: Complete cure, Prevention of progression of uncomplicated malaria to severe disease, Prevention of deaths, Interruption of transmission, Minimizing risk of selection and spread of drug resistant parasites (CDC 2015).

Currently about 35 % of cases within the African region uses the RDTs techniques. Malaria may lead to anaemia and jaundice because red blood cells are rupturing occurs faster than they can be replaced; severe anaemia accounts for the high mortality rate in

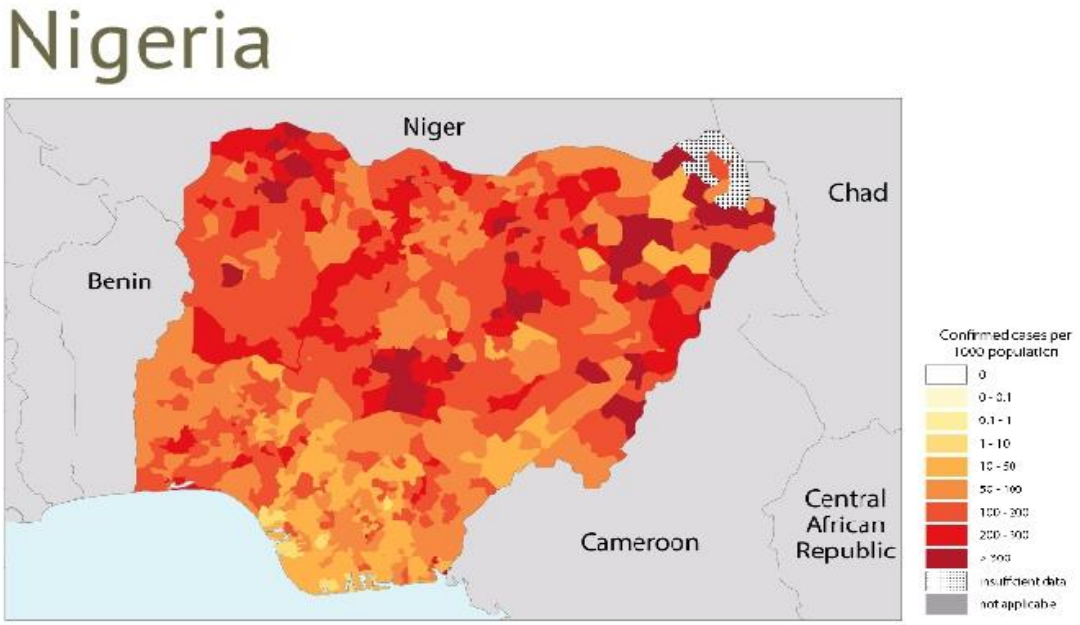
children with malaria (WHO, 2016). *P. falciparum* which is responsible for cerebral malaria is characterized with convulsion, coma and ultimately death. About 10–20 % of children with cerebral malaria die and around 7 % of those that survive suffer a permanently brain damage for the rest of their lives (WHO, 2016). There are two main theories relating to the cause of cerebral malaria.

1. Mechanical: infected red blood cells have a characteristic knob-like projections that appears at the surface their membrane, usually sticky adhering to non-infected RBCs, forming clumps, or to endothelial cells lining the surface of blood vessels. Blocking blood capillaries, thereby reducing the flow of blood resulting to tissue damage. This is extremely serious when it occurs in vessels supplying the brain when this vessels are blocked there is blood shortage to the brain consequently damaging it (Neeta and Jobin, 2012).
2. Immune response: when red blood cells are ruptured, cytokines are released in response to the toxins released from this cells producing nitric oxide gases. It is generally believed that this gases (nitric oxide) diffuses through the blood–brain barrier general anesthetic, causing lapse into unconsciousness and coma of the patient (Neeta and Jobin, 2012).

   However, scientists reported in *The Lancet* in 2002 that in African, children carrying a single genetic mutation which allowing an increased the production of nitric oxide which protects them from developing cerebral malaria. It is believed that red blood cells sticking to blood vessels can be prevented by nitric oxide, helping to regulate and keep constant blood flow to the brain (Neeta and Jobin, 2012).

2.8 Distribution of Malaria Parasites in Nigeria

Malaria is endemic throughout most of the tropics. Eighty seven countries and territories have ongoing transmission. World Health Organization (2019) reported that 229 million cases and 409 thousand deaths from malaria occurred in 2019; this is a decrease from 239 million cases in 2010 whereas 435,000 deaths were recorded in 2016. Over 94 percent of the burden occurs in the African region, with Nigeria and the Democratic Republic of Congo as high burdened countries (Figure 2.4).



I. Epidemiological profile

Population (UN Population Division)	2017	%
High transmission (>1 case per 1000 population)	145.8M	76
Low transmission (0-1 case per 1000 population)	45.1M	24
Malaria free (0 cases)	0	-
Total	190.9M	

Figure 2.4 Map of Nigeria on the Epidemiological Profile of Malaria in Nigeria. (Source: WHO, 2019)

2.9 Treatment of Malaria Parasites

Anti-malarial drugs have shown great success in treatment and curing malaria parasites. Treatment durations are all dependent on the kind of malaria (*Plasmodium* species) that is diagnosed (Builders *et al*., 2014). Travelers/ persons visiting endemic areas are advised to take anti-malarial drugs to prevent infection. Depending upon the drug prescribed, they may need to be taken up to 2 weeks before travelling, these drugs can be effective for 4 weeks or more during which the person remains protected against the parasites (Builders *et al*., 2014).

For complicated malaria cases Artemisinin-based combination therapies (ACTs) are now used as standard treatment (Eric and Kelly, 2011). A combination artemisinin or one of its derivatives with another anti-malarial or anti-malarial of a different class.

2.10 Current Recommended Drugs for ACT

1. Artemether-lumefantrine
2. Artesunate-amodiaquine
3. Artesunate-mefloquine
4. Artesunate-sulfadoxine/pyrimethamine
5. Dihydroartemisinin-piperaquine (Eric and Kelly, 2011)

Uncomplicated malaria is defined as '*a symptomatic infection with malaria parasites in the blood without signs of severity and/or evidence of organ failure or dysfunction*'. The ability to produce desired or curative measures influences the choices of ACTs among a particular populace (Neeta and Jobin, 2012). Separate tablets of antimalarial combination could be administered same time or as a co-formulation into a single tablet. ACT therapy can slow the onset of resistance as the antimalarials used in combinations have different mechanisms of action. This means that if a mutant parasite resistant to

one of the drugs arises during the course of the infection the other drug will still kill the resistant parasite (Muleye *et al*., 2019). Chloroquine is still used to cure/treat *P. vivax* malaria. However, in areas where resistance has developed an ACT is advised; a 14-day course of another drug, primaquine, is also a good alternative in addition to ACT to avoid relapse (Muleye *et al*., 2019). In cases of severe *P. falciparum* malaria, drugs are administered by intramuscular or intravenous injection of quinine followed by a complete course of an effective ACT as soon as the patient is able to take oral medication. Artemisinin suppositories are recommended where injections are infeasible (WHO, 2015).

2.11 Preventions and Control of Malaria Parasites

Malaria preventions have no sole path. Currently, control measures are a combination of mosquito management and physical barriers, which both aim to prevent mosquito bites, along with intermittent preventative treatment (IPT). These measures are geared towards a huge reduction in the transmission of the disease and host vector contact (CDC, 2015).

2.11.1 Mosquito management and physical barriers

Malaria control and prevention are mostly channeled at vector control, which has been an effective means of managing malaria transmission. The two main lines of defense are the use of indoor residual spraying (IRS) (mosquito management) and insecticide treated nets (ITNs) (physical barriers) (CDC, 2015).

These interventions may be complemented by additional strategies.

1. Indoor residual spraying (IRS): This is done by spraying insecticides inside homes, offices and dwellings in both open and dark areas, corners and edges of

buildings which are usually resting places for mosquitoes after blood meals. While this approach has been extremely fruitful, disapproval of the chemical DDT has resulted in a decline in its use. Depending upon the insecticide used, insecticide spraying is effective for 3–6 months, or 9–12 months in the case of DDT (CDC, 2015).

2. Insecticides treated nets (ITNs) are mosquito nets impregnated with pyrethroids, a class of insecticide. A once per annum insecticide treatment is required or recommended for optimum results. Long-lasting insecticidal nets are favored by the WHO; these are designed to be effective for at least 3 years, hence, cancelling insecticide treatment routines. A chemical halo that extends away from the nets is produced by the insecticide. In Africa, most endemic regions readily make this insecticide treated nets available to persons at risk of this disease (malaria) (WHO, 2019).
3. Breeding site reduction/environment management strategies is aimed at modifying the local environment preventing the larvae having access to suitable breeding conditions through improved hygiene for example, covering or removing water containers, filling in ditches or effective drainage, clearing of bushes close to human dwellings and proper refuse disposal (CDC, 2015).
4. Biological control methods introduce natural enemies to help manage mosquito populations, this natural enemies are usually stage, species and organism specific thereby other species of economic importance are not at risk of extinction. For example, parasites and pathogenic, or predatory, organisms such as insects, viruses, bacteria, protozoa, fungi, nematode worms, plants or fish (Neeta and Jobin, 2012).A key example is the larvae-eating fish and bacteria, for example, *Bacillus thuringensis* or *Bacillus sphaericus* (Figure 2.5) which

produce protein crystals that are highly toxic to the mosquito larvae, are the only methods widely employed. Most mosquitoes bite between dusk and dawn. To reduce the risk of bitten dressing modifications have been adapted at night, long-sleeved tops and trousers should be worn and insect repellent applied to the skin, also the use of fans have been used to repel them owing to the fact that they poor flyers (Neeta and Jobin, 2012).

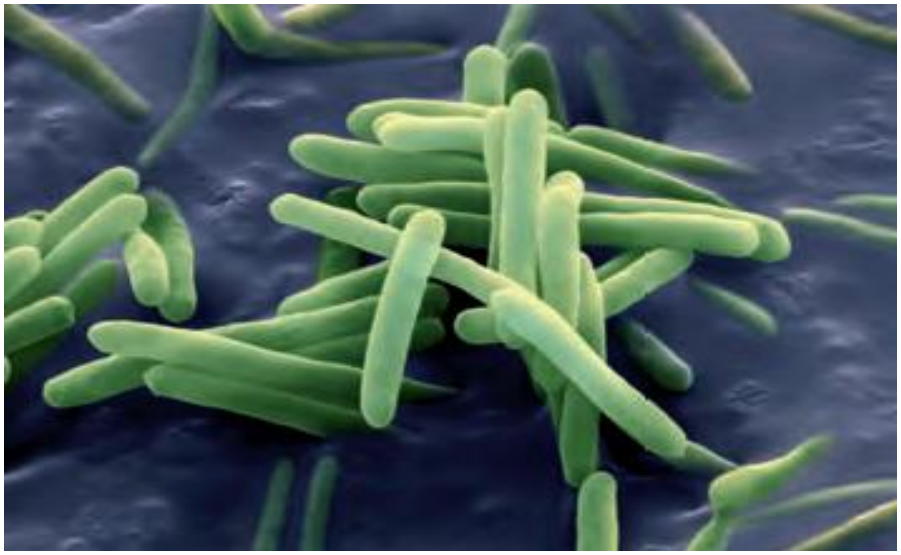


Figure 2.5 *Bacillus sphaericus*; Biological Control Agent. (Source: Neeta and Jobin, 2012).

2.12 Malaria Elimination and Eradication

Within the past 15 years great advancement has been made globally in the strive malaria, According to the World Malaria Report, incident malaria cases has reduced by 41percent between 2000 and 2015 (WHO, 2016). At the beginning of 2016, malaria was considered to be endemic in 91 countries and territories, as in 2000 with 108 endemic countries.

Notwithstanding, in all this advancement malaria continues to place a heavy toll on the world. More than 100 countries have eliminated malaria in the past century. While other countries have reduced their incidence of malaria by more than 50 percent (WHO, 2015).

The elimination and eradication of malaria is confronted with significant, technical, operational and financial challenges.

2.13 Emergence of Drug Resistance

Resistance to insecticides by malaria vectors has promoted disease transmission and complicated control measures (for example, those used for spraying the walls of houses or to impregnate nets) have also begun to form resistance to drugs which were formerly used to treat them. South-East Asia has the most resistant malarial parasites in the world (WHO, 2015).

In Africa drug resistance to chloroquine and sulfadoxine/pyrimethamine has increased greatly which is believed to be directly responsible for large numbers of deaths from malaria and hospital admissions for severe malaria (Builders *et al*., 2014). Implying that persons are treated with drugs that do not cure their illnesses, thereby prolong the infection and the number of red blood cells destroyed by the parasite is increased, which in turn leads to anaemia (Builders *et al*., 2014).

Due to recent current resistance of antimalarial drugs, the WHO recommends thorough monitoring of the effectiveness of any malaria treatment and prevention regime in order to follow and adapt to changes (WHO, 2015).

2.14 External Characteristics *Sclerophrys maculata*

Individuals of *Sclerophrys maculate* are among the largest toads, have adult weighing females over 1.5 kg and have snout-vent lengths greater than 22.5 cm (Gao, 2010).

Females are known to be larger than the males, and most adults range in size from about 8.5 to 15.0 cm snout-vent length. The head is broader than it is long; the snout is rounded in dorsal view and truncated in profile. Well-developed cranial crests form ridges above the eyes and join above the snout. The eyes are large and prominent and have horizontal pupils (Goa, 2010). The parotoid glands just below the eyes (Figure 2.10) are extremely large, triangular and swollen. The limbs are short. The toes are partially webbed with claws, but the fingers are not. The first finger is longer than the second. There is obvious sexual dimorphism. Irregularly scattered warts and tubercles cover both sexes. However, the skin of the female is much smoother than that of males, which has the texture of rough sandpaper, caused by the presence of numerous horny spicules on the warts and tubercles (Gao, 2010). The venter of the female and young male is creamy white; the dorsum is covered with irregular blotches of various shades of brown. The skin of adult males is more uniformly yellowish-brown in colour; the yellow is pronounced laterally and around the throat. The first finger and the inner surfaces of the second and third fingers of sexually mature males bear brown nuptial pads, and a median vocal sac opens on each side of the mouth (Gao, 2010).

Figure 2.6: ***Sclerophrys maculate*** (Source: www.google.co.uk)

2.15 Classification of *Sclerophrys maculata*

Kingdom: Animalia

Subkingdom: Bilateria

Infrakingdom: Deuterostomia

Phylum: Chordata

Infraphylum: Gnathostomata

Superclass: Tetrapoda

Class: Amphibia

Order: Anura

Family: Bufonidae

Genus: *Sclerophrys*

Species: *Sclerophrysmaculata*

Source: Amphibia Species of the World, 2019 (http://www.conabio.gob.mx/) Bufonidae

2.16 Body Wall (Skin) of *Sclerophrys maculata*

There are highly permeable to water, and absorption of water through the skin is an important rehydration mechanism, particularly in the ventral pelvic region. Which is why they habit humid environment there is an active ion transport system in the skin with a net inward movement of sodium ions. The skin is also important in gas exchange also the skin allows the secretion of whitish pasty substance (venom) which are high in bioactive compounds and are used to keep off predators (Yang *et al*., 2010).

2.17 Locomotion of *Sclerophrys maculata*

Bufo tadpoles are weak swimmers. Adults are primarily terrestrial (mostly found around wet areas or land), but are capable of swimming. On land they walk and hop, but do not leap, adult *B. bufo* move fastest at 22˚C its speed enhanced if they are acclimated at this temperature. Dehydration appears to have less impact on the locomotion of *B. marinus* than on other anurans (Pramuk, 2006).

2.18 Feeding and Digestive System *Sclerophrys maculata*

The larvae of *B. bufo* feed on the surface layers of submerged vegetation and detritus. The rasping mouth parts are used to generate fine nutrient particles that are ingested by filter feeding. During their early development, the larvae feed on the gelatinous string from which they have emerged. The adult are the insectivorous (insect eaters) this is done by well-developed tongue of the adult bears sticky, mucous secretions and is used actively to catch prey. *Bufo bufo* eat almost any prey of appropriate size, and the diet appears to reflect the food available in a particular location. Prey consists mainly of small terrestrial arthropods, including crabs, spiders, centipedes, millipedes, scorpions and a wide range of insects. Additional dietary items include earthworms, planarians, molluscs, small vertebrates including its own young, rotting fruit, dog and cat food and human faeces. Quantities of plant material and stones found often in the stomach of toads are probably ingested accidentally (Lambiris, 1973) have shown that the upper size threshold of prey selected increases, following long-term food deprivation, but the lower size threshold remains the same.

2.19 Ecology of *Sclerophrys maculata*

Though *Bufo bufo* occurs in a huge variety of habitats, it is most abundant around human habitation and, to a lesser extent, in grasslands. It occurs only rarely in forested

areas and is especially rare in rainforests, reports that it is often common along rivers and in clearings in forested areas within its natural range (Yang *et al*., 2010).

2.20 Behaviour of *Sclerophrys maculata*

Bufo bufo juveniles are active both day and night. Adults are usually nocturnal, though sometimes they feed and breed during the day. Adults take refuge in holes, crevices and burrows during the day, and may remain in these for long periods in dry conditions and during the winter months. Even when conditions are warm and wet, and thus favourable, individual toads are not active every night (Lambiris *et al*., 1973). *Bufo* species respond to prey with a behavioural pattern that has four distinct stages: orienting, approaching, fixating, and snapping although this response is instinctive, it is subject to maturation and modification (Lambiris *et al*., 1973).

2.21 Economic Significance of *Sclerophrys maculata*

Many of the introductions of *Bufo species were* made with the intention that the species should control a variety of insect pests. Nowhere was this successful. In most cases the introductions are regarded as having had a net detrimental effect. Although little is known about the species' ecological impact, *B. marinus* is not a serious economic pest. It is known to prey on domestic bees and there are numerous reports of domestic animals being poisoned and sometimes killed, as a result of eating or mouthing toads. Small enterprises have developed to supply toads for research and teaching purposes, and to produce leather goods made from toad skin (Giovanni *et al*., 1989).

In traditional Chinese medicine (TCM) processed and dried venom from parotoid glands of the toad (*Bufo bufo gargarizans*) products have been used for treating pain and inflammatory disease (Yang *et al*., 2010). Also the water extracts from the skins of *B. b. gargarizans* is known as Cinobufacini, which was developed in China about 20

years ago, and had been successfully used to treat various types of cancers with low toxicity and few sides effects (Yang *et al*., 2010). Both molecular and clinical data have revealed the chemical constituents as well as the mechanisms of action from their use (Yang *et al*., 2010). Although, different groups of constituents may have diverse functions, it is well known now that bufadienolides, such as bufalin and cinobufagin are considered as the main bio-active compounds in toads toxins. These groups of compounds are C-24 steroids with similar properties as cardiac glycosides medication such as digoxin (Pramuk, 2006).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Ethical Approval

Approval for the use of experimental animals with reference number NSVH/MLF/20/VOL1/09 (appendix A), was obtained from the school Federal Ministry of Livestock, Minna, Niger State.

3.2 Collection and Identification of Toad Species

The toad was gotten from the field (water logged areas) into a plastic perforated container in the early hours of the day at about 4 am to 6 am with temperature and humidity within 24.7 ^{o}C - 25.5 ^{o}C and 80 % - 85 %. They are starved until after the extraction process which is usually from 8am from living individuals after which they are released back into their habitat (wet lands around vegetations).

The species of toads were duly identified in the laboratory using a published proceedings of the Academy of Natural Sciences of Philadelphia (Hallowell, 1854), and an article in Zootaxa by Poynton *et al*., 2016. The species were identified to be *Sclerophrys maculata*, commonly known as Hallowell's toad, the flat backed toad and the stripped toad, of the family Bufonidae.

3.3 Extraction of Toad Venom

The toad was cleaned with cotton wool immersed in distilled water to make the body clean for venom collection. The process of extraction/secretion of the toad venom was done by having both paratoid macro glands of each specimen manually compressed allowing them to release a whitish and pasty secretion in the form of jets, which were gently collected on a clean plastic from living individuals. The secretion was then lyophilized (freeze dried) and stored at -20 ℃ after which it was scraped out into a clean

plastic container (petri dish)with cover and stored in a freezer (-20℃). (Tempone *et al*., 2008). About 0.25 g of the lyophilized venom was manually mixed with 10 ml of distilled water to obtain desired concentration for treatment.

3.4 Characterization of the Crude Toad Venom Extract

In the quest to study the distribution of secondary metabolites in the different extracts obtained, zoochemical analysis was conducted on the crude extracts to test for the presence or absence of zoochemicals.

3.4.1 Molisch's test for carbohydrates: A portion of venom was dissolved in distilled water and then a few drops of Molisch's reagent was added to the venom portion, this was then followed by addition of 1 ml of concentrated H_2SO_4 by the side of the test tube. The mixture was then allowed to stand for two minutes and 5 ml of distilled water was used to dilute it. A red or dull violet colour at the inter-phase of the two layers indicates a positive test (Sofowora, 1993).

3.4.2 Test for tannins: To 0.5 g of the venom was stirred with about 10 ml of distilled water and then filtered. Few drops of 1 % ferric chloride solution were added to 2 ml of the filtrate, the occurrence of a blue-black, green or blue-green precipitate indicates the presence of tannins (Trease and Evans, 2002).

3.4.3 Borntrager's test for anthraquinones: To 10 ml of benzene, 0.5 g of the crude toad venom extract was shaken and then filtered. Five millilitres of the 10 % ammonia solution was then added to the filtrate and thereafter shaken. Appearance of a pink, red or violet colour in the ammoniacal (lower) phase was taken as the presence of free anthraquinones and considered positive (Sofowora, 1993).

3.4.4 Liebermann-Burchard test for steroids: To 0.2 g of the venom to be tested, 2 ml of acetic acid was added, the mixture was cooled well in ice. Concentrated H_2SO_4

was carefully added. Colour development from violet to blue or bluish-green indicated the presence of a steroidal ring which means a glycone portion of cardiac glycoside (Sofowora, 1993).

3.4.5 Test for terpenoids: To 1 ml of ethanol, 0.3 g of venom was dissolved and 1 ml of acetic anhydride was added followed by the addition of concentrated H_2SO_4 carefully. Colouration changes from pink to violet showed the presence of terpenoids (Sofowora, 1993).

3.4.6 Test for saponins: One gram of the venom extract was boiled with 5 ml of distilled water and filtered. To the filtrate, 3 ml of distilled water was further added and shaken vigorously for about 5 minutes. Frothing which persisted on warming was taken as an evidence for the presence of saponins (Sofowora, 1993).

3.4.7 Shinoda's test for flavonoids: To 0.5 g of venom extract was dissolved in ethanol, warmed and then filtered. Three pieces of magnesium chips was then added to the filtrate followed by few drops of concentrated HCL. A pink, orange, or red to purple colouration indicates the presence of flavonoids (Trease and Evans, 2002).

3.4.8 Test for alkaloids: To 0.5 g of venom extract was stirred thoroughly with 5 ml of 1 % aqueous HCL on water bath and then filtered. To the filtrate, 1 ml was taken individually into 2 test tubes. Few drops of Dragendorff's reagent were added to the first portion; occurrence of orange-red precipitate was observed and taken as positive. To the second portion 1 ml, Mayer's reagent was mixed and appearance of buff-coloured precipitate indicated to be the presence of alkaloids (Sofowora, 1993).

3.4.9 Liebermann's test for cardiac glycoside: To 2.0 ml of acetic acid and 2 ml of chloroform was added to the venom. The mixture was then allowed to cool and concentrated H_2SO_4 was added. Green colour showed the entity of a glycone, steroidal part of glycosides (Sofowora, 1993).

3.4.10 Test for phenol: The presence of phenol was determined using ferric chloride test. 0.5 g of venom was boiled in 30 ml distilled water and filtered. 2 ml filtrate was mixed with 1 ml 5 % $FeCl_3$. Formation of blue, green, or violet colour indicated the presence of phenolic compounds (Trease and Evans, 2002).

3.5 Biochemical Analysis of Serum

The activity of the enzymes, aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) were determined by kinetic methods with Randox Kits (Reitman and Frankel, 1957).

3.6 Total Bilirubin Concentration

To ascertain the total bilirubin level, the method Busch (1998) were adapted using Beckman synchron bilirubin calibrator.

3.7 Gas Chromatography and Mass Spectrometry (GC-MS) Analysis

Toad Venom was sent for GC-MS analysis at Shimadzu training centre for analytical instruments (STC), Lagos. GC-MS analysis was carried out with Shimadzu Japan gas chromatography QP2010SE. The crude toad venom extract was analysed for bioactive components in GC-MS Quantitative analysis of the different compounds were performed on a GC Clarus 500 Perkin Elmer gas chromatograph equipped with column: Elite-1 (100 % Dimethyl poly siloxane), 30 m × 0.25 mm ID × 1.0 μdf at the oven temperature programme of 110 ˚C -2 minutes hold up to 280 ˚C at the rate of 5 ˚C/minute-9 minutes hold. 1μl of each sample was injected in triplicate splits and quantities represented relative area percentage as derived from the integrator. Injector temperature was 250 ˚C and the split ratio was 10:1. Helium was used as a carrier gas

with a constant flow at 1 ml/min and the detector was Mass detector-Turbo mass gold Perkin Elmer. The inlet line temperature was 200 ˚C and the source temperature was 200 ˚C. The instrument was operated at 70 eV in electron impact mode. Full-scan analyses were performed in the mass range 45-400 m/z scan. Data were evaluated by software-Turbo mass 5.1. The total GC time was 45mins and the MS time was 46 minutes. The relative amounts of individual components of the total composition were expressed as percentage peak area relative to total peak area. Qualitative different constituent were identified by performing a comparison of their relative retention times and mass spectra with those of authentic reference compounds (NIST Version 2.1) or by retention indices and mass spectra (Mondello *et al*., 1995).

3.8 Experimental Animal

The Swiss albino mice needed for the experiment were collected from Nigeria Institute for Trypanosomiasis Research (NITR), Vom Jos Plateau State. The mice of 21-45 grams were housed in plastic cages under laboratory conditions of 12 hours light / darkness cycle with standard pellet diet and water (Ukpanukpong *et al*., 2019). They were allowed to acclimatize for two weeks.

3.9 Acute Toxicity (LD_{50}) Test

The acute toxicity of the toad venom was determined using a slightly modified Lorke's method (1983) and the LD_{50} was computed as the geometric mean of lowest dose that produce mortality and the highest dose with no lethal effect. In phase one of this method, twelve (12) rats were divided into four groups (Groups I, II, III and IV) of three rats each. Rats in groups I, II, III and IV were administered 5, 10, 20 and 25 mg/kg b. wt. of the crude venom, respectively. In the absence of mortality and sign of toxicity after 24 hrs, phase two was conducted. In the phase two, twelve rats were also divided

into four groups (groups I, II, III and IV) of three rats each. Rats in groups I, II, III and IV were administered 50, 100, 200 and 500 mg/kg b. wt. of the crude venom, respectively. After 24 hrs, mortality was noted in group III and IV.

3.10 Test Parasite and Inoculation of Experimental Animals

The parasite species *Plasmodium berghei* NK-65 strain used for this study were obtained from National Veterinary Research Institute (NVRI), Vom, Plateau State. The parasites were maintained in the laboratory by continues transfer into *Plasmodium* free mice intraperitoneally throughout the period of the study. The mice were inoculated by introducing the parasite (*Plasmodium berghei*) intraperitoneally.

3.11 Determination of *In-Vivo* Antiplasmodial Activities

Thirty (30) Swiss albino mice of known weight were collected and transported to the Departmental Laboratory. They were distributed into six groups, each group comprises of four mice. The grouping and treatment given to the mice in each group is as follows: Group A, were designated as (Venom treated group one) VTG1 consist of parasitized mice administered 10 mg/kg of crude toad venom extract; Group B, were designated as (Venom treated group two) VTG2 consisting of parasitized mice administered with 20 mg/kg of crude toad venom extract. Group C, were designated as (Venom treated group three) VTG3 consisting of parasitized mice administered with 30 mg/kg of crude toad venom extract; Group D (Normal control), were designated as (Non-parasite control) NPC consisting of non-parasitized control mice administered 1 ml of distilled water; Group E (Negative group), were designated as (Parasitized non control) PNC consisting of parasitized and administered with nothing; Group F (Positive control), were designated as (Parasitized administered orthodox) PAO consisting of parasitized mice administered with 5.0 mg/kg of orthodox drug (chloroquine).

All treatment were given once daily for a succession five days. Blood samples were taken from the tail vein of the mice before treatment once daily to assess the parasitemia levels. Bioassays were carried for all the fractions as stated above (Ukpanukpong *et al*., 2019).

3.11.1 Percentage parasitemia of red blood cell (RBC)

This is a process by which the level of the parasite (*Plasmodium berghei)* in the body of inoculated animals is determined. The method adopted were, collections of the blood sample of the experimental animals (mice) daily respectively, and a smear of thin film were made on a slide. The slides were left for a few minutes to air dry. After drying, the slides were fixed using methanol and then followed by staining with Giemzar's stain. The stain were allowed to stay on the slides for about five minutes and then washed away with water. The slides were observed under microscope using X 100 (oil immersion) for infected and uninfected RBC (equation 1). The percentage parasitemia were calculated as follows.

$$\%\ Parasitemia\ of\ RBC = \frac{number\ of\ infected\ RBC}{total\ number\ of\ RBC} \times 100\ (Equation\ 1)$$

Total Number of RBC = Number of infected RBC + Number of uninfected RBC (Ukpanukpong *et al*., 2019).

3.11.2 Packed cell volume (PCV)

Packed cell volume (PCV) were ascertained using blood collected from tail of each mouse into a heparinized micro-haematocrit capillary tubes (Globe Scientific Inc, Paramus, NJ, USA) which were filled up to ¾ th part of the tube with blood, sealed at one end with crystal seal and placed the open end of the tube to the centre and the sealed end outwards of a micro-haematocrit reader (Hawsksley & Sons LTD, England). The

blood was centrifuged at 12,000 rpm for 5 min and then the volume of the total blood and the volume of erythrocytes were measured using a ruler. Measurement will be done before infection Day zero (D0) and on Day four (D4) after infection. PCV were calculated using equation two.

$$PCV = \frac{Volume\ of\ total\ erythrocytes\ in\ a\ given\ volume\ of\ blood}{Total\ blood\ volume} \times 100 \quad \text{(Equation 2)}$$

3.11.3 Determination of body weight changes.

Body weight loss is one feature of rodent malaria infections. The effectiveness of the venom extract was determined by measuring each body weight of the mouse. The body weights of each mouse in all groups were taken before infection (D0) and after treatment (D4). The weight of each mouse was measured using sensitive electrical balance. The average body weight change of venom extract treated groups was compared with the control groups. The average body weight changes of each treated group were calculated using the following formula in equation three;

$$Average\ body\ weight\ change = Average\ D4\ weight\ of\ a\ group - Average\ D0\ weight\ of\ that\ group$$

(Equation 3).

3.11.4 Mean survival time (MST).

Mean survival time (MST) is another parameter that is commonly used to evaluate the efficacy of anti-malarial extracts. An extract that results in survival time greater than that of infected non-treated mice were considered as active. Death occurring before day 5 of infected and treated mice were regarded as toxic death. Mortality was monitored daily. The number of the days from the time of inoculation of the parasite up to death was recorded for each mouse in the treatment and control groups throughout the follow

up period. The survival time for each mouse were recorded after the treatment periods and calculated using equation four. (Girma *et al*., 2015).

$$Mean\ survival\ time\ (MST) = \frac{sum\ survival\ time\ (days) for\ all\ mice\ in\ a\ group}{total\ number\ of\ mice\ in\ the\ group}$$

(Equation 4)

3.12 Histological Techniques

The fixed organs of mice were dehydrated with ethanol. To remove the ethanol, the tissues were allowed through xylene solution facilitating molten paraffin wax infiltration at 55˚C. After that, they were embedded in a wax block, Paraffin sections of were cut with the rotary microtome and placed on cleaned glass slides. Finally, Hematoxylin and eosin were used to stain the sections. The stained slides were examined using a light microscope where the photomicrographs of the tissue samples were recorded (Kazi *et al*., 1993).

3.13 Data Analysis

The data carefully collated from the study were summarized and expressed as Standard Error of Mean (SEM), Data analysis will be performed using statistical package for social science (SPSS) version 22.0. One-way ANOVA followed by Tukey's multiple comparison tests were used to compare the results obtained from different groups and to also ascertain the level of significance. The probability value less than 0.05 were considered significant.

CHAPTER FOUR

4.0 RESULT AND DISCUSSIONS

4.1 Results

4.1.1 Zoochemical analysis of the crude toad venom extract

The crude toad venom indicated the presence of the following zoochemical components which incdude: phenol, flavonoids, tannins, carbohydrate, saponins and terpenoids and the absence of anthraquinoues, steroids, candiae glycosides and alkaloids (Table 4.1)

4.1.2 Gas chromatography and mass spectrometer analysis of crude toad venom extract

Gas chromatography and mass spectrometer analysis of crude toad venom revealed a total of 30 peaks (figure 4.1) representing 25 different bioactive compounds. The first compound identified with less retention time Dimethyl phthalate with retention time of 11.327 min followed by Dodecanoic acid and Sulfurous acid, 2-propyl tridecyl ester with retention time of 12.887 and 14.133 min respectively, while the last compound identified with longest retention time is 5, alpha. -Androstane-3.beta., 17.beta.-diol (21.881 s). However, Pentacyclo [9.1.0.0(2,4).0(5,7).0(8,10)] dode (18.81 %) and 9-Octadecenoic acid, methyl ester, (E)- (14.43 %) were the most abundant constituents in the crude toad venom (Table 4.1)

Table 4.1: Zoochemical Component of Crude Toad Venom Extract

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Zoochemical component	Inference
Phenol	+
Flavoniods	+
Alkaloids	_
Tannins	+
Steroids	_
Candiac glycoside	+
Carbohydrates	+
Saponins	+
Anthraquinoues	_
Terpenoids	+

Keys: + Present; - Absent.

Table 4.2: Chemical Composition of Crude Toad Venom (*Sclerophrys maculata*) Through Gas Chromatography and Mass Spectrometer Analysis.

Peak#	Retention Time (m)	Peak Area (%)	Height (%)	Compound Name
1	11.327	0.24	0.26	Dimethyl phthalate
2	12.887	7.11	3.60	Dodecanoic acid
3	14.133	0.06	0.12	Sulfurous acid, 2-propyl tridecyl ester
4	14.208	0.10	0.17	Tetradecanoic acid, 12-methyl-, methyl este
5	14.575	2.65	2.24	Tetradecanoic acid
6	14.952	0.17	0.15	Octadecanoic acid
7	15.787	1.15	2.33	Hexadecanoic acid, methyl ester
8	16.115	4.13	3.13	n-Hexadecanoic acid
9	16.283	1.41	1.34	Hexadecanoic acid, ethyl ester
10	16.956	1.05	1.64	9,12-Octadecadienoic acid (Z,Z)-
11	17.023	8.60	14.43	9-Octadecenoic acid, methyl ester, (E)-
12	17.190	0.98	1.47	Methyl stearate
13	17.372	10.13	6.55	Oleic Acid
14	17.405	2.35	3.56	9,12-Octadecadienoic acid (Z,Z)-
15	17.462	1.02	2.24	Ethyl Oleate
16	18.194	1.11	1.54	Hexadecanal, 2-methyl-
17	18.559	2.06	1.34	9-Octadecenamide, (Z)-
18	19.181	0.31	0.69	Hexadecanal, 2-methyl-
19	19.222	3.41	5.32	Carbamic acid, 2-(dimethylamino)ethyl este
20	19.368	1.12	1.57	Digitoxin
21	19.521	1.14	1.25	7-Hydroxyfarnesen
22	21.022	21.76	18.81	Pentacyclo[9.1.0.0(2,4).0(5,7).0(8,10)]dode
23	21.559	4.95	3.83	2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-e
24	21.748	1.08	0.87	2H-3,9a-Methano-1-benzoxepin, octahydro
25	21.881	9.07	9.36	5.alpha.-Androstane-3.beta.,17.beta.-diol, b

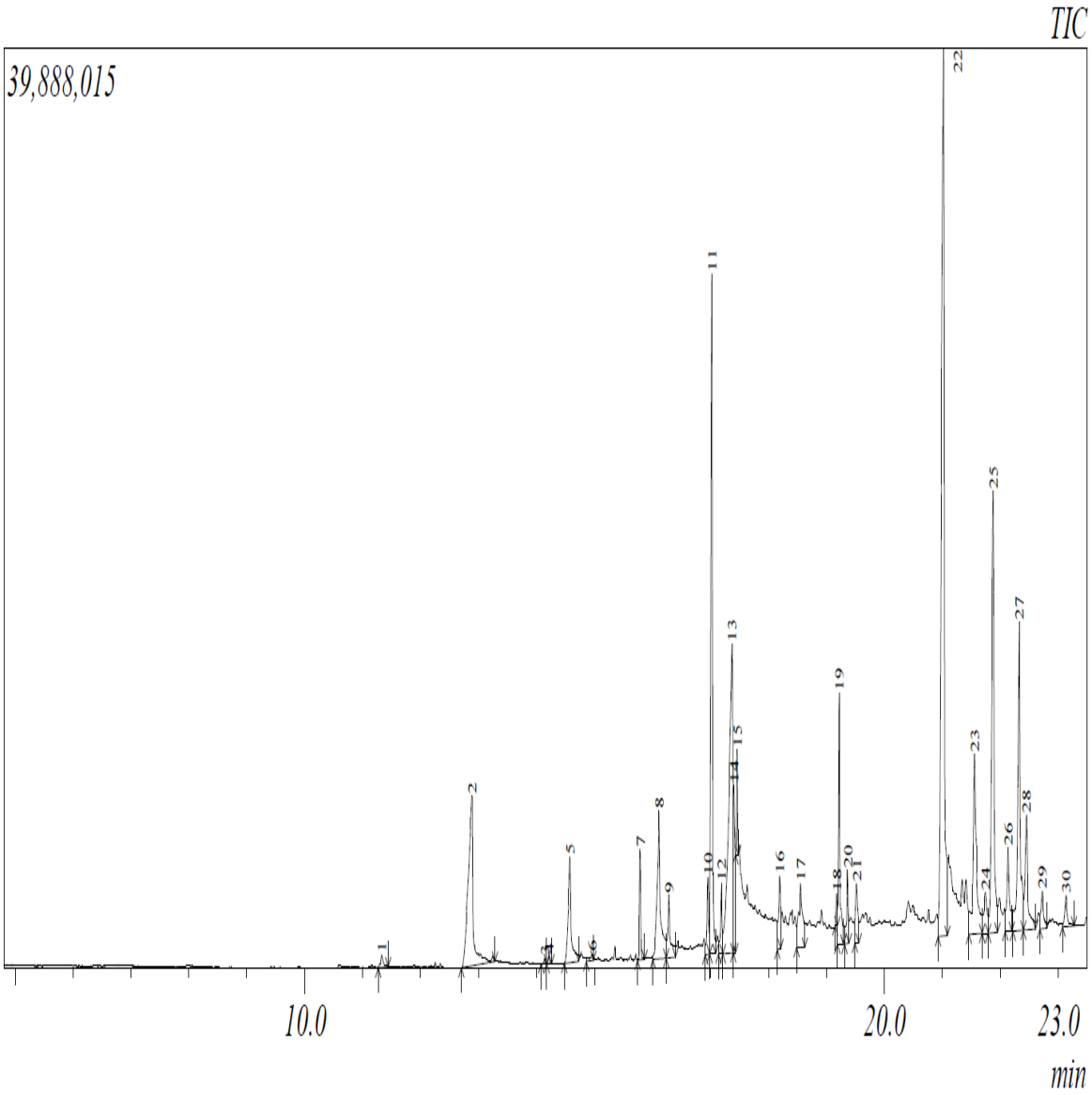

Figure 4.1: Gas chromatography and Mass Spectrometer Chromatogram of *Sclerophrys maculata* Venom

4.1.3 Acute toxicity of the crude extracts of toad (*Sclerophrys maculate*) venom

Acute toxicity study of the crude extract of toad venom revealed that the animals tolerated the extract at dose of 5, 10, 15, 20, 25 and 50 mg/kg b.wt. with no any observable adverse effect at single oral administration. However, at dose of 100 mg/kg b.wt. animals exhibited sign of adverse effect in the form of sluggishness, the adverse effect were more pronounced at higher dose of 200 and 500 mg/kg b.wt. where sluggishness, hyper cardia, erythema were observed and consequently 1 and 2 mortality were recorded at 200 and 500 mg/kg b.wt. dosing respectively. The safe dose and LD_{50} of crude toad venom are 50 mg/kg b.wt. and 141.2 mg/kg b.wt. respectively (Table 4.1).

4.1.4 Effect of the crude toad (*Sclerophrys maculata*) venom extract on parasitaemia counts of *Plasmodium berghei*-infected mice.

Treatment of *Plasmodium berghei*-infected mice with crude extract of toad venom significantly ($p<0.05$) decreases the replication of the parasite in a dose in-dependent manner. Mice treated with 20 mg/kg b.wt. of the toad venom exhibited higher anti-plasmodial activities from 17 to 3 ml/kg PBS on the 5^{th} day of treatment than those treated with 10 and 30 mg/kg b.wt. (16 to 2 ml/kg PBS and 18 to 25 ml/kg PBS). However, mice that received 5 mg/k b.wt. of chloroquine exhibited the lowest parasite counts of 0 ml/ kg PBS on the 5^{th} day of treatments. (Figure 4.2).

Table 4.3: Acute Toxicity Profile of Crude Extract of Toad (*Sclerophrys maculata*) Venom in Mice.

Dose (mg/kg b.wt.)	Number of animals	Mortality	Sign of toxicity
5	3	0	Nil
10	3	0	Nil
15	3	0	Nil
20	3	0	Nil
25	3	0	Nil
50	3	0	Nil
100	3	0	Sluggishness
200	3	1	Hyper cardia, erythema,
500	3	2	Sluggishness, Hyper cardia, erythema
control	3	0	Nil

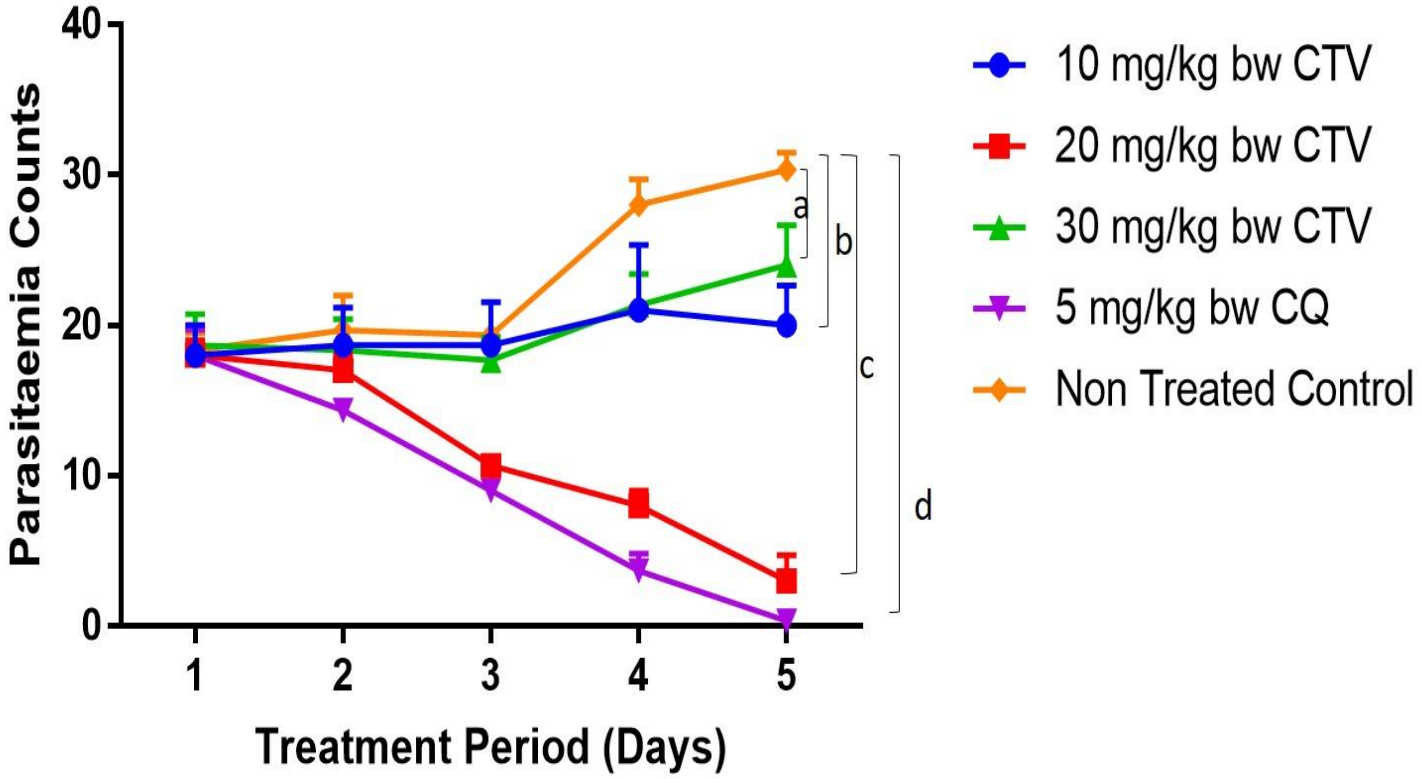

Figure 4.2: Effect of Crude Extract of Toad (*Sclerophrys maculata*) Venom on Parasitaemia Counts in *P. berghei* Infected Mice.

Key: CTV; crude extract of toad venom, CQ; Chloroquine. The superscript alphabet indicates the significant differences ($p < 0.05$) among the treatment groups. d > c > b > a

4.1.5 Effect of the crude toad (*Sclerophrys maculata*) venom extract on body weight *Plasmodium berghei*-infected mice.

There were significant ($p < 0.05$) decreases in body weight gain of *P. berghei* infected untreated mice from 32.01 g to 21.51 g when compared with the normal control (31.00 g to 36.20 g) and the chloroquine treated (30.04 g to 40.00 g) groups. However, no significant changes in body weight were observed in mice treated with the chloroquine, and those treated with toad venom at 10 mg/kgb.wt. (32.07 g to 24.61g), 20 mg/kg b.wt. (34.20 g to 32.00 g) and 30 mg/kg b.wt. (33.00 g to 28.11 g) respectively. (Figure 4.3)

4.1.6 Effect of the crude toad (*Sclerophrys maculata*) venom extract on packed Cell volume (PCV) *Plasmodium berghei*-infected mice.

There were significant ($p < 0.05$) decreases in packed cell volume (PCV) gain of *P. berghei* infected untreated mice from 40 % to 33 % when compared with the normal control (42 % to 43 %) and the chloroquine treated group which had 41 % to 46 %. However, no significant changes in PCV were observed in mice treated with the chloroquine which had a PCV before inoculation to be 45 % and after treatment 44 % and those treated with toad venom at 10, 20 and 30 mg/kg b.wt. had their PCV to be 40 % to 41 %, 45 % to 46 % and 38 % to 39 % before the inoculation and after treatment respectively (Figure 4.4).

4.1.7 Effect of the crude toad (*Sclerophrys maculata*) venom extract on survival time (MST) of *Plasmodium berghei*-infected mice.

There were significant ($p < 0.05$) increase in the survival time of the *P. berghei* infected and treated mice when compared to the infected and untreated mice. It was observed that those treated with toad venom of 20 mg/kg b.wt. and the group treated with chloroquine had the highest longevity of over 27 days to 30 days compared to those of 10 mg/kg b.wt. of 21 days and 30 mg/kg b.wt. of 9 days respectively (Figure 4.5).

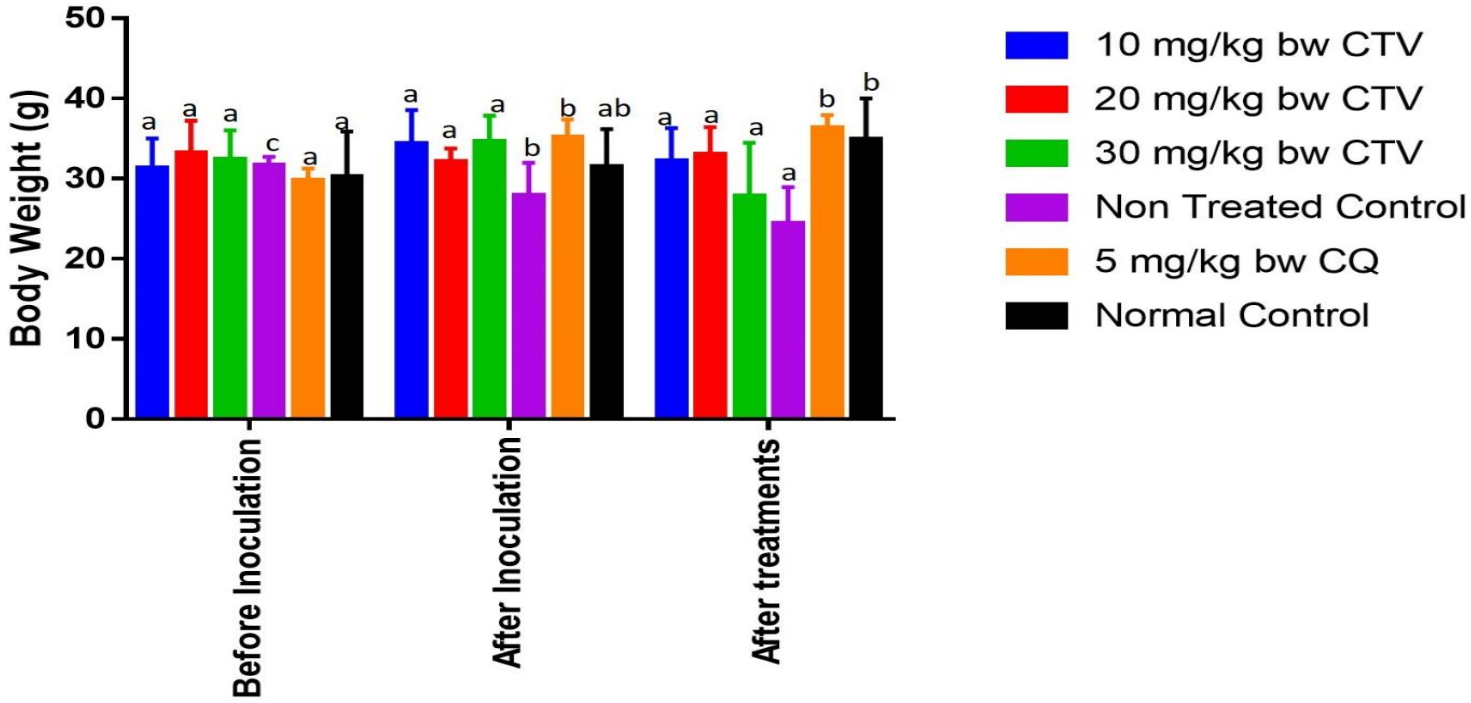

Figure 4.3: Effect of Crude Extract of Toad (*Sclerophrys maculata*) Venom on Body Weight Changes in *P. Berghei*- Infected Mice.

Key: CTV; crude extract of toad venom, CQ; Chloroquine. The superscript alphabet indicates the significant differences ($p < 0.05$) among the treatment groups. d > c > b > a

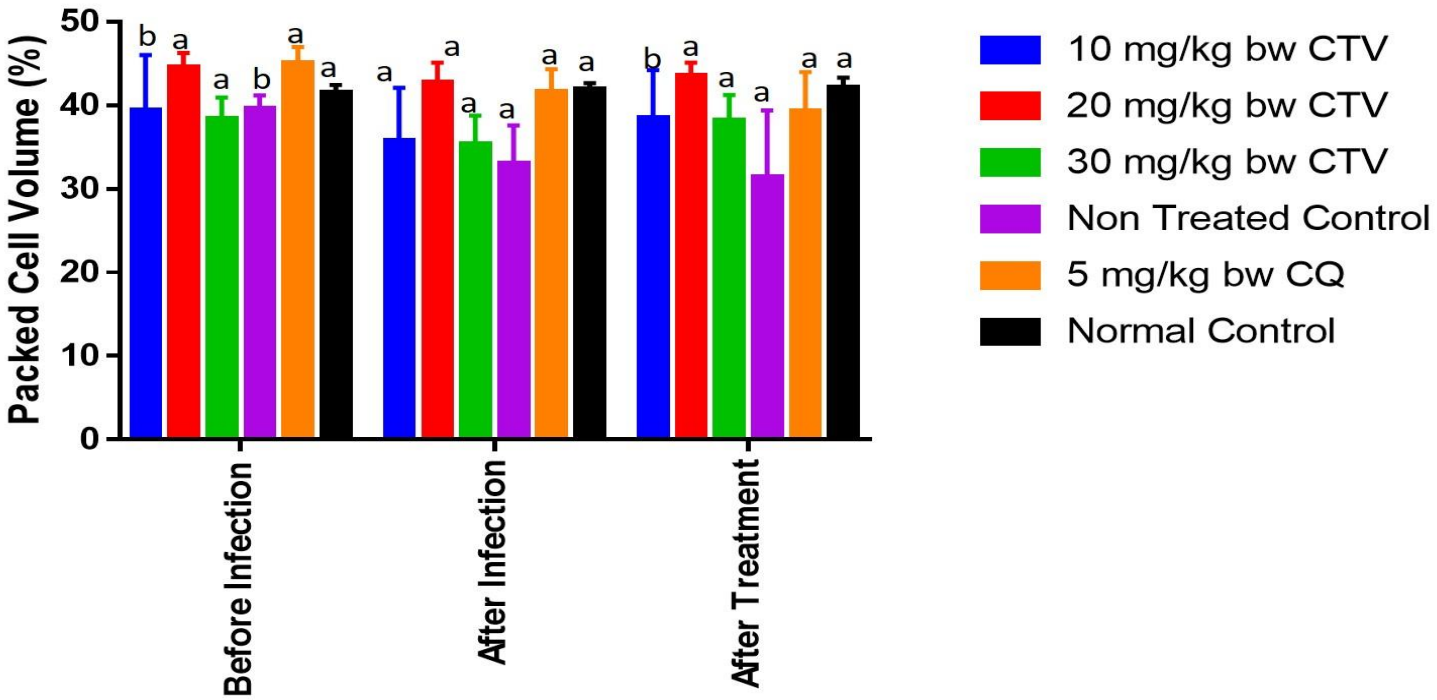

Figure 4.4: Effect of Crude Extract of Toad (*Sclerophrys maculata*) Venom on Packed Cell Volume (PCV) in *P. berghei* Infected Mice.

Key: CTV; crude extract of toad venom, CQ; Chloroquine. The superscript alphabet indicates the significant differences ($p < 0.05$) among the treatment groups. d > c > b > a

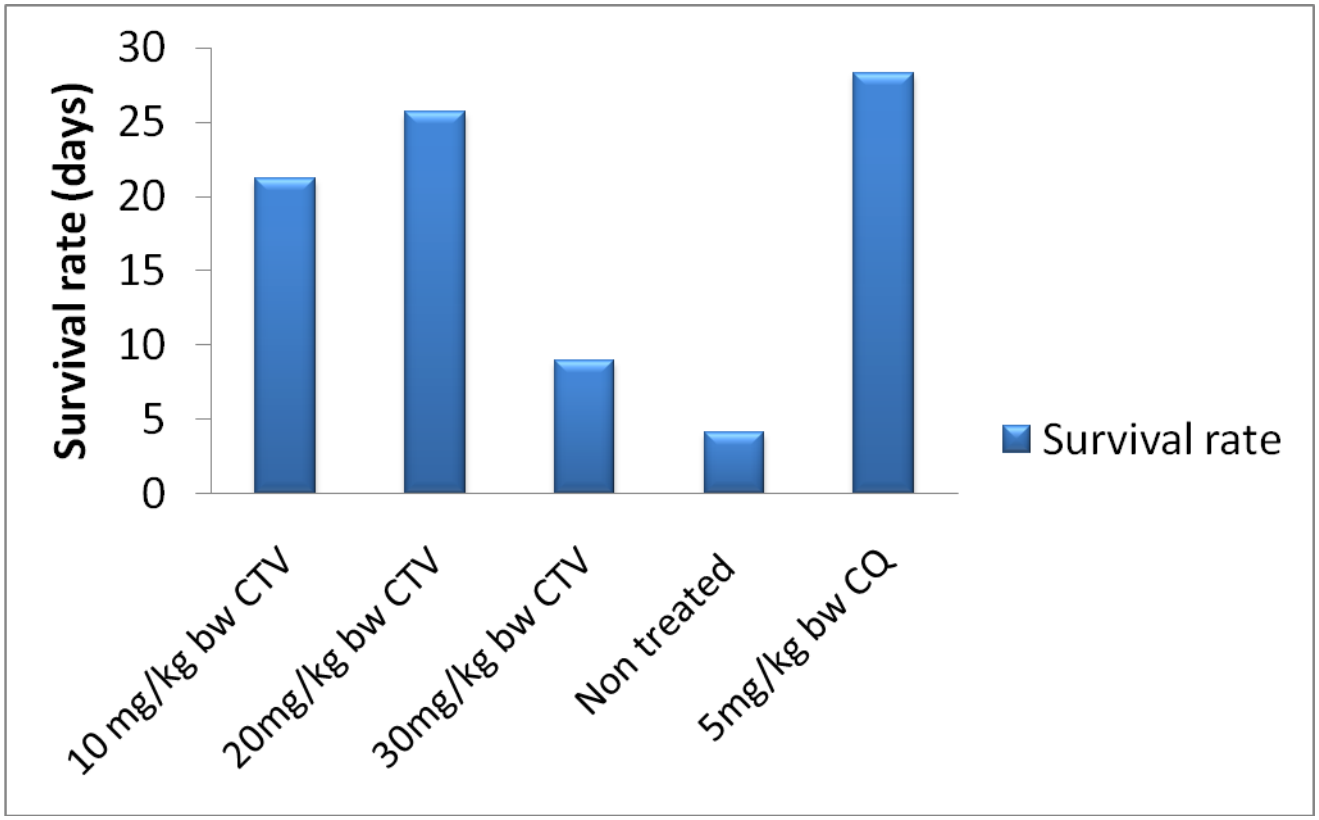

Figure 4.5: Effect of the Crude Extract on Survival Rate of *Plasmodium berghei*-Infected Mice.

Key: CTV; crude toad venom, CQ; Chloroquine

4.1.8 Effect of crude toad (*Sclerophrys maculata*) venom extracts on biochemical parameters; (liver function parameters).

Serum concentrations of transaminases (AST and ALT) and ALP activities were significantly higher while the levels of albumin and total proteins were significantly lower in the *P. berghei* infected untreated mice when compared with the concentrations found in the normal control and chloroquine treated mice. Treatments of *P. berghei* infected mice with the crude extract of toad venom at concentrations of 10 and 20 mg/kg b.wt. resulted in the decrease concentrations of the ALT, AST and ALP while increasing the levels of albumins and proteins compared to the levels detected in the untreated control and those treated with 30 mg/kg b.wt. toad venom. However, no significant differences exist ($p>0.05$) in the AST, ALT ALP, total proteins and albumins concentrations among the mice treated with 10 and 20 mg/kg b.wt. (Figure 4.5). Total bilirubin concentrations were higher in untreated mice and mice treated with 30 mg/kg b.wt. when compared with the normal control and chloroquine treated groups. However, 10 and 20 mg/kg b.wt. of toad venom decreases the levels of both total and direct bilirubin when compared with the untreated control (Figure 4.6).

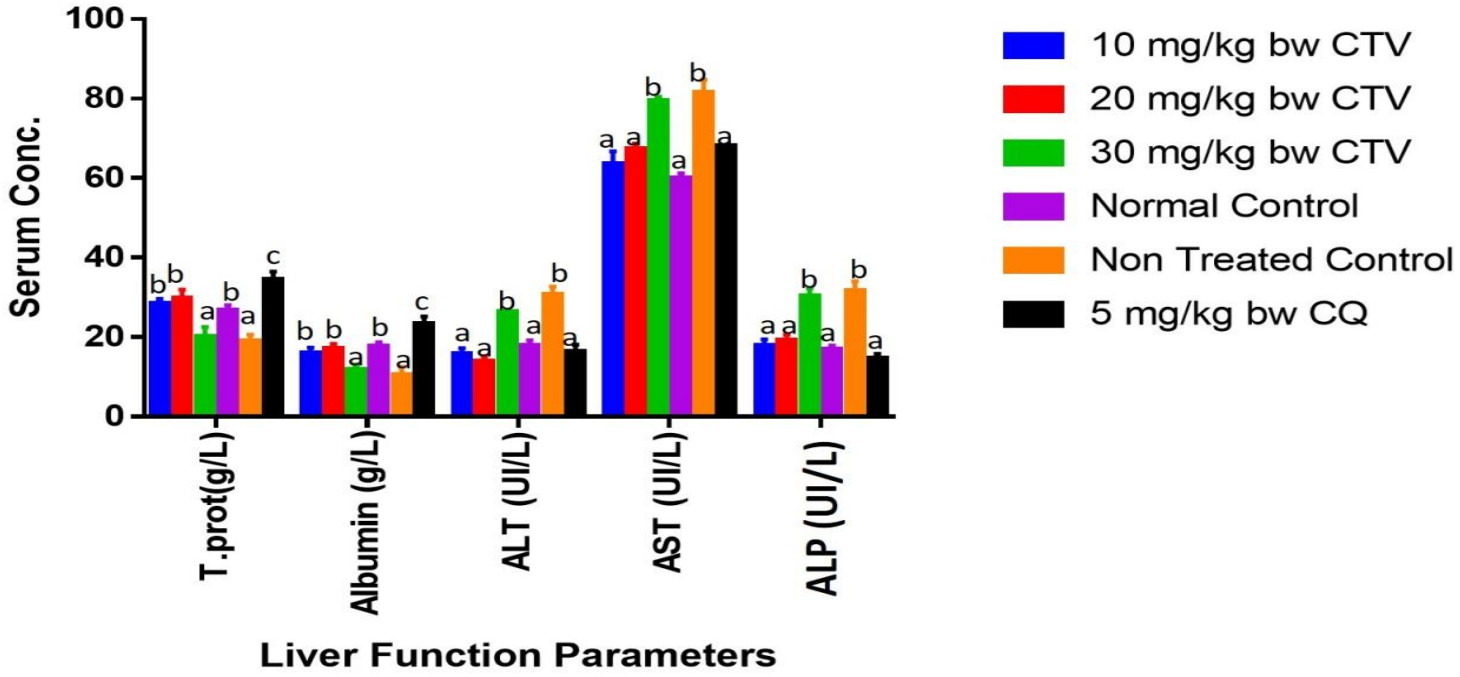

Figure 4.6: Effect of Crude Extract of Toad (*Sclerophrys maculata*) Venom on Serum Biomarkers of Liver Function in *P. berghei*-Infected Mice.

Key: CTV; crude extract of toad venom, CQ; Chloroquine. T. proteins; total proteins, AST; aspartate transaminases, ALT; alanine transaminase; ALP; alkaline phosphatase. The superscript alphabet indicates the significant differences ($p < 0.05$) among the treatment groups. d > c > b > a

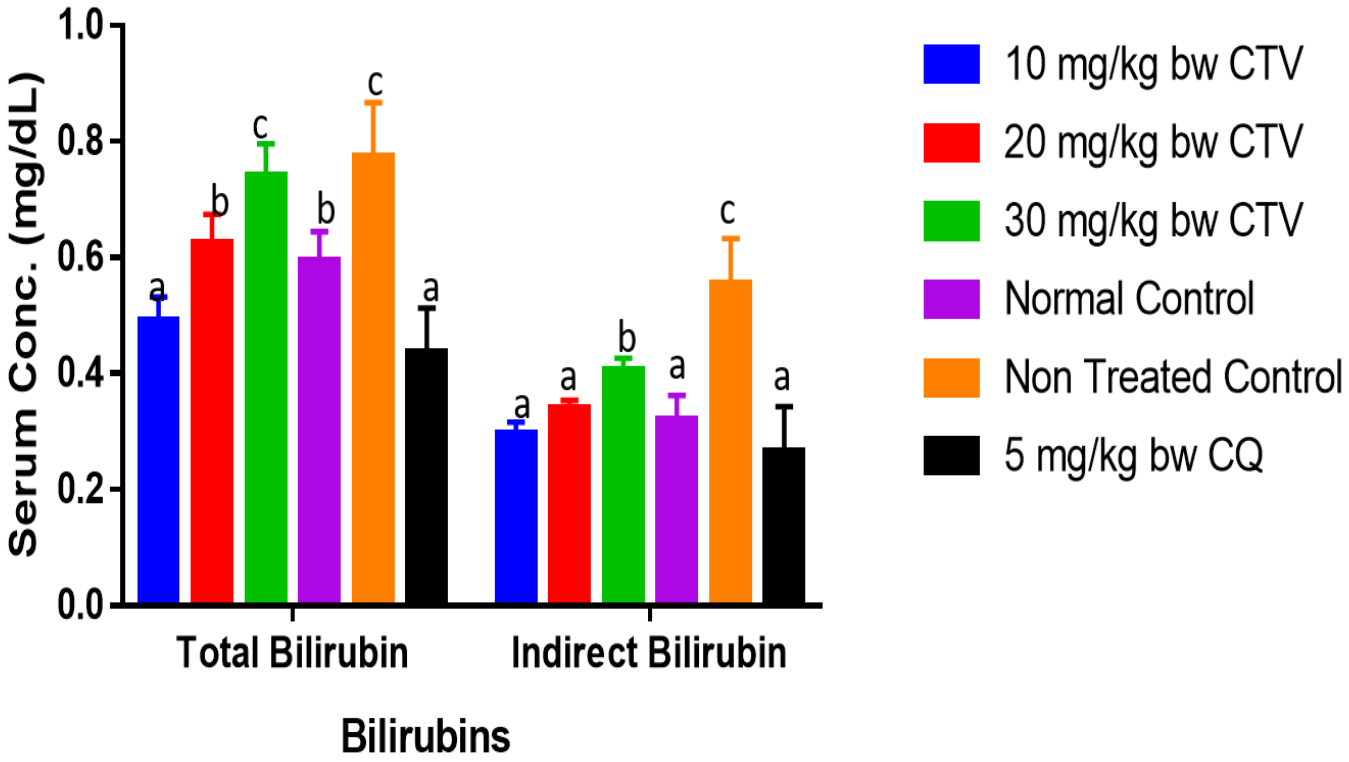

Figure 4.7: Effect of Crude Extract of Toad (*Sclerophrys maculata*) Venom on Serum Bilirubin Concentrations in *P. berghei*-Infected Mice.

Key: CTV; crude extract of toad venom, CQ; Chloroquine. The superscript alphabet indicates the significant differences ($p < 0.05$) among the treatment groups. d > c > b > a

4.1.9 Effect of crude toad (*Sclerophrys maculata*) venom extraction biochemical parameters; (kidney function parameters)

Serum urea and creatinine concentrations in *P. berghei* infected untreated mice were significantly higher than the concentrations found in the normal control and chloroquine treated groups. Treatments of *P. berghei* infected mice with the crude extract of toad venom at concentrations of 10 and 20 mg/kg b.wt. resulted in the decrease concentrations of the urea and creatinine compared to the untreated control and those treated with 30 mg/kg b.wt. toad venom. Treatment of the infected mice with 30 mg/kg b.wt. did not decrease the elevated urea and creatinine when compared with the untreated groups. However, no significant differences exist ($p>0.05$) in the uric acid concentrations among all the experimental groups (Figure 4.7).

4.1.10 Effect of crude extract of toad (*Sclerophrys maculata*) venom on haematological parameters

Haematological parameters including the hemoglobin, packed cell volume, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentrations, red blood cells, total white blood cells and platelet counts were significantly ($p < 0.05$) higher in *P. berghei* infected mice and mice treated with 30 mg/kg b.wt. of toad venom when compared with the normal control and those treated with 5 mg/kg b.wt. of chloroquine. However, mice treated with 10 mg/kg b.wt. and those treated with 20 mg/kg b.wt. of toad venom had higher counts of hemoglobin, packed cell volume, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentrations, red blood cells, total white blood cells and platelets when compared with the untreated controls (Figure 4.8).

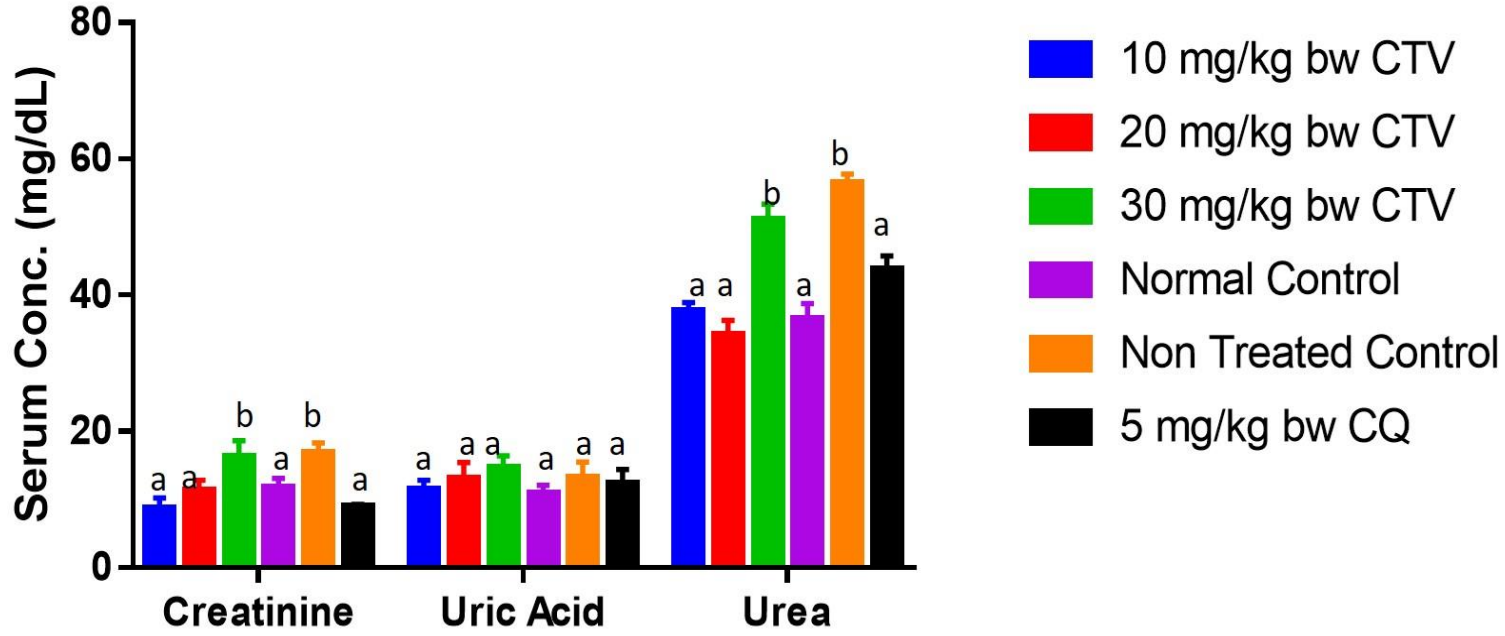

Figure 4.8: Effect of Crude Extract of Toad (*Sclerophrys maculata*) Venom on Serum Urea, Uric Acid and Creatinine Concentrations in *P. berghei*-Infected Mice.

Key: CTV; crude extract of toad venom, CQ; Chloroquine. The superscript alphabet indicates the significant differences ($p < 0.05$) among the treatment groups. d > c > b > a

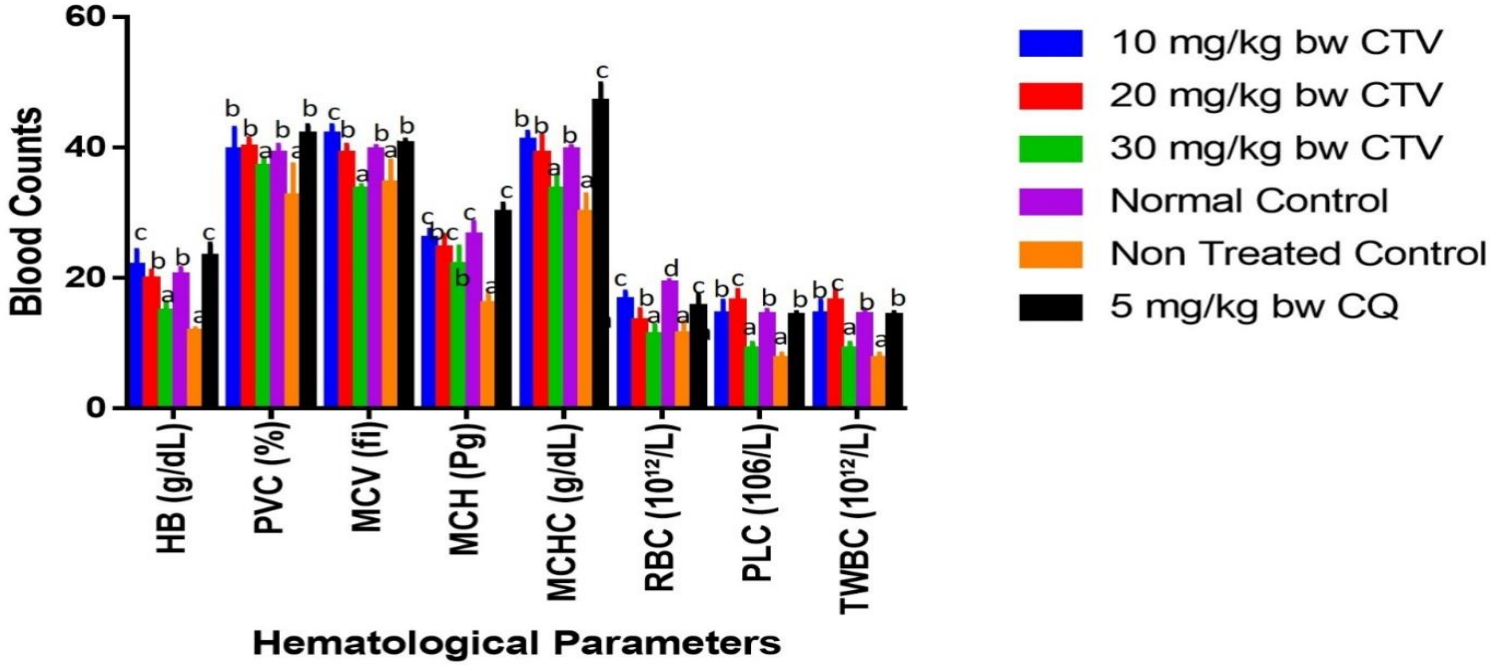

Figure 4.9: Effect of Crude Extract of Toad Venom on Haematological Parameters of *P. berghei*-Infected Mice.

Key: CTV; crude extract of toad venom, CQ; Chloroquine. HGB; Hemoglobin, PCV; packed cell volume, MCV; mean cell volume, MCH; mean cell hemoglobin, MCHC; mean cell hemoglobin concentrations, RBC; red blood cells, TWBC; total white blood cells, PLC; platelet**.** The superscript alphabet indicates the significant differences ($p < 0.05$) among the treatment groups. d > c > b > a (using Duncan Multiple Range Test)

4.1.11 Effect of crude extract of toad (*S. maculata*) venom on organs body weight

The liver body weight ratio and spleen body weight ratio were significantly higher in *P. berghei* infected untreated mice when compared with the normal control and the chloroquine treated group. Similarly, mice treated with 30 mg/kg b.wt. of crude extract of toad venom had higher liver body weight ratio and spleen body weight ratio when compared with the normal control and other experimental groups. However, no significant differences ($p > 0.05$) exist in heart, kidney and lung body weight ratio among the normal control, untreated group and treatment groups (Table 4.4).

4.1.12 Histopathological findings of the kidneys and livers of *Plasmodium berghei*-infected mice

The organs (liver and kidney) of the mice treated with the crude toad venom extract of 10, 20 and 30 mg/kg b.wt., 5 mg/kg of chloroquine and control group where subjected to tissue microscopic analysis. The findings revealed that the normal architecture of their tissue was preserved with no feature of acute or chronic damage. The liver of the mice infected and treated with the crude toad venom extract, positive group (5 mg/kg CQ) and the normal control group showed that their hepatic tissue with a preserved architecture composed of cords of normal hepatocytes, portal tract and central vein. While in the untreated group, it was observed to have a degenerated hepatocyte. In the kidney, the mice infected and treated with the crude toad venom extract, positive group (5 mg/kg CQ) and the normal control group revealed that the renal tissue had preserved architecture composed of normal glomerili tubules and unmarkable interstitium. While in the untreated group a degenerated glomerulli and a collapsed of the capsular space was observed in Plate I to VI below.

Table 4.4 Effect of Crude Extract of Toad Venom on Organs Body Weight of *P. Berghei*-Infected Mice

Organ Weight	Liver (g)	Heart (g)	Kidney (g)	Lung (g)	Spleen (g)
10 mg/kg b.wt. CTV	1.49±0.03[a]	0.19±0.01 [a]	0.49±0.04 [a]	0.35±0.01 [a]	0.27±0.01 [a]
20 mg/kg b.wt. CTV	1.53±0.01 [a]	0.19±0.01 [a]	0.45±0.02 [a]	0.36±0.00 [a]	0.26±0.02 [a]
30 mg/kg b.wt. CTV	1.95±0.02[b]	0.21±0.00 [a]	0.42±0.03 [a]	0.36±0.01 [a]	0.41±0.03 [b]
Normal Control	1.59±0.01 [a]	0.18±0.01 [a]	0.43±0.02 [a]	0.35±0.02 [a]	0.25±0.00 [a]
Non treated Control	1.57±0.01 [a]	0.19±0.00 [a]	0.41±0.01 [a]	0.33±0.01 [a]	0.21±0.02 [a]
5 mg/kg b.wt. CQ	2.19±0.00 [b]	0.20±0.01 [a]	0.44±0.01 [a]	0.37±0.03 [a]	0.55±0.01 [b]

Key: CTV; crude extract of toad venom, CQ; Chloroquine, g; gram. The superscript alphabet indicates the significant differences ($p < 0.05$) among the treatment groups. d > c > b > a

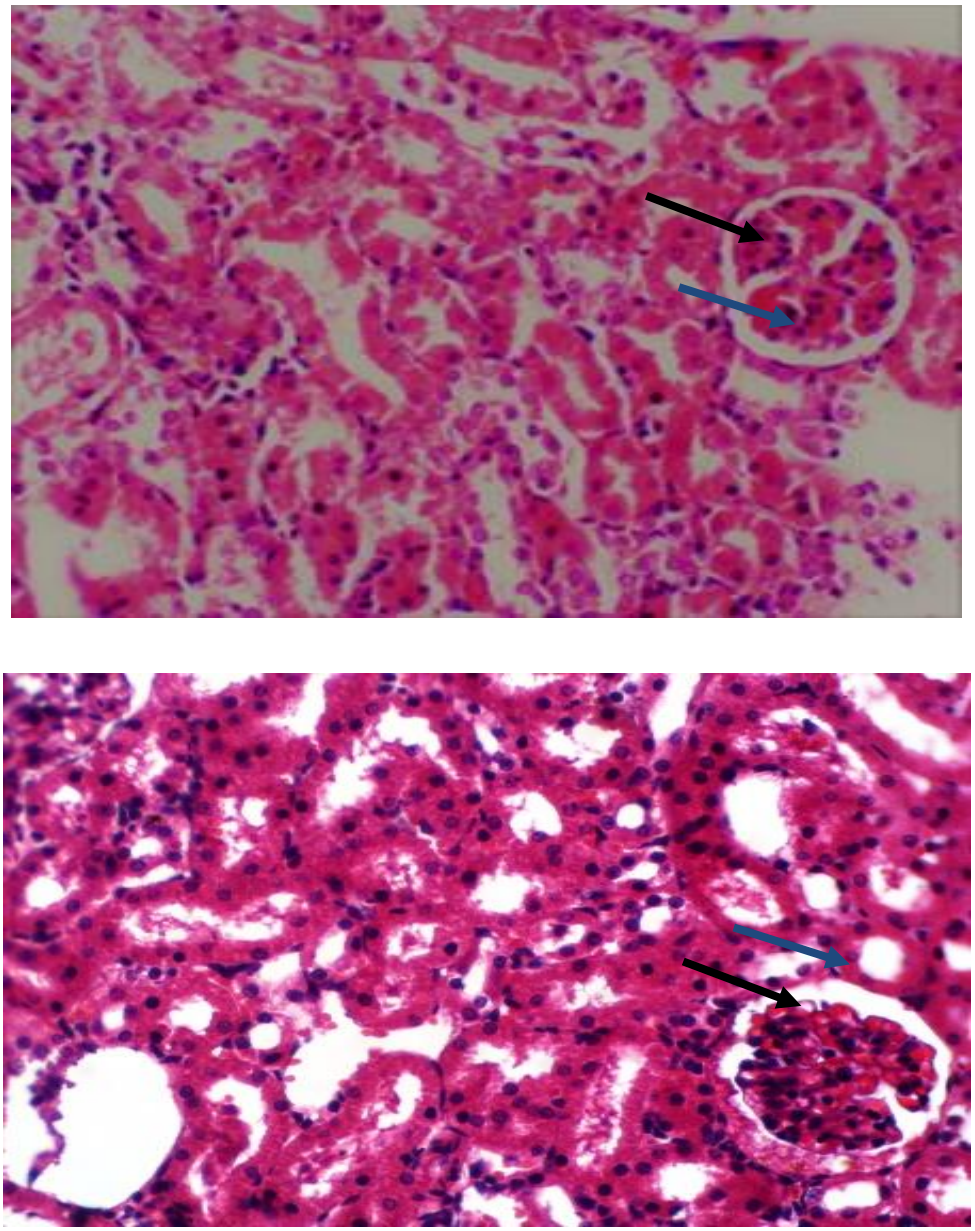

Plate I: Photomicrographs of the Kidney (×40; eosin and haematoxylin) 10 & 20 mg/kg b.wt. of the Crude Extract

KEY: Black arrow; Glomeruli, Blue arrow; Capsular space

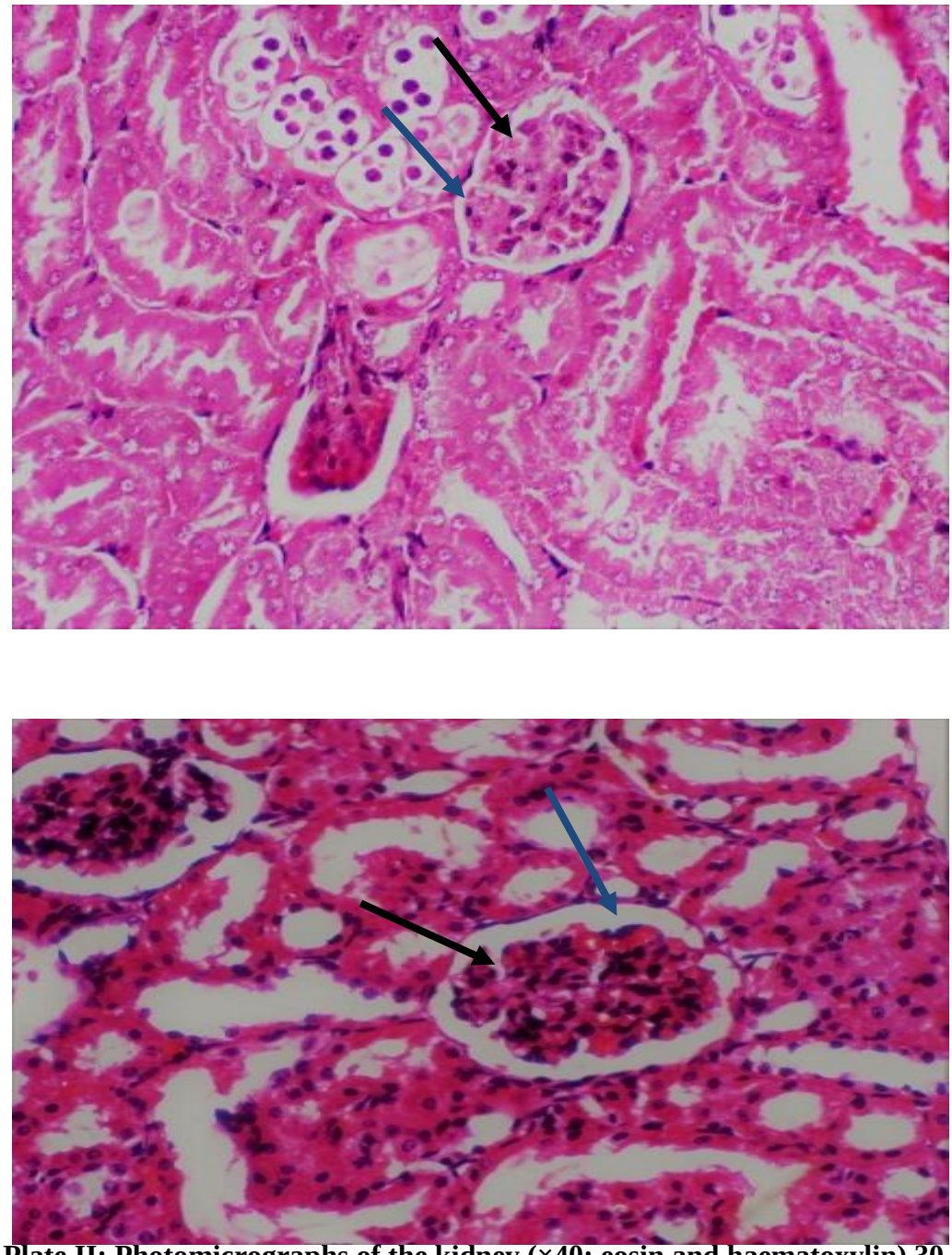

Plate II: Photomicrographs of the kidney (×40; eosin and haematoxylin) 30 mg/kg b.wt. of the Crude Extract and 5 mg/kg of CQ

KEY: Black arrow; Glomeruli, Blue arrow; Capsular space, CQ; Chloroquine

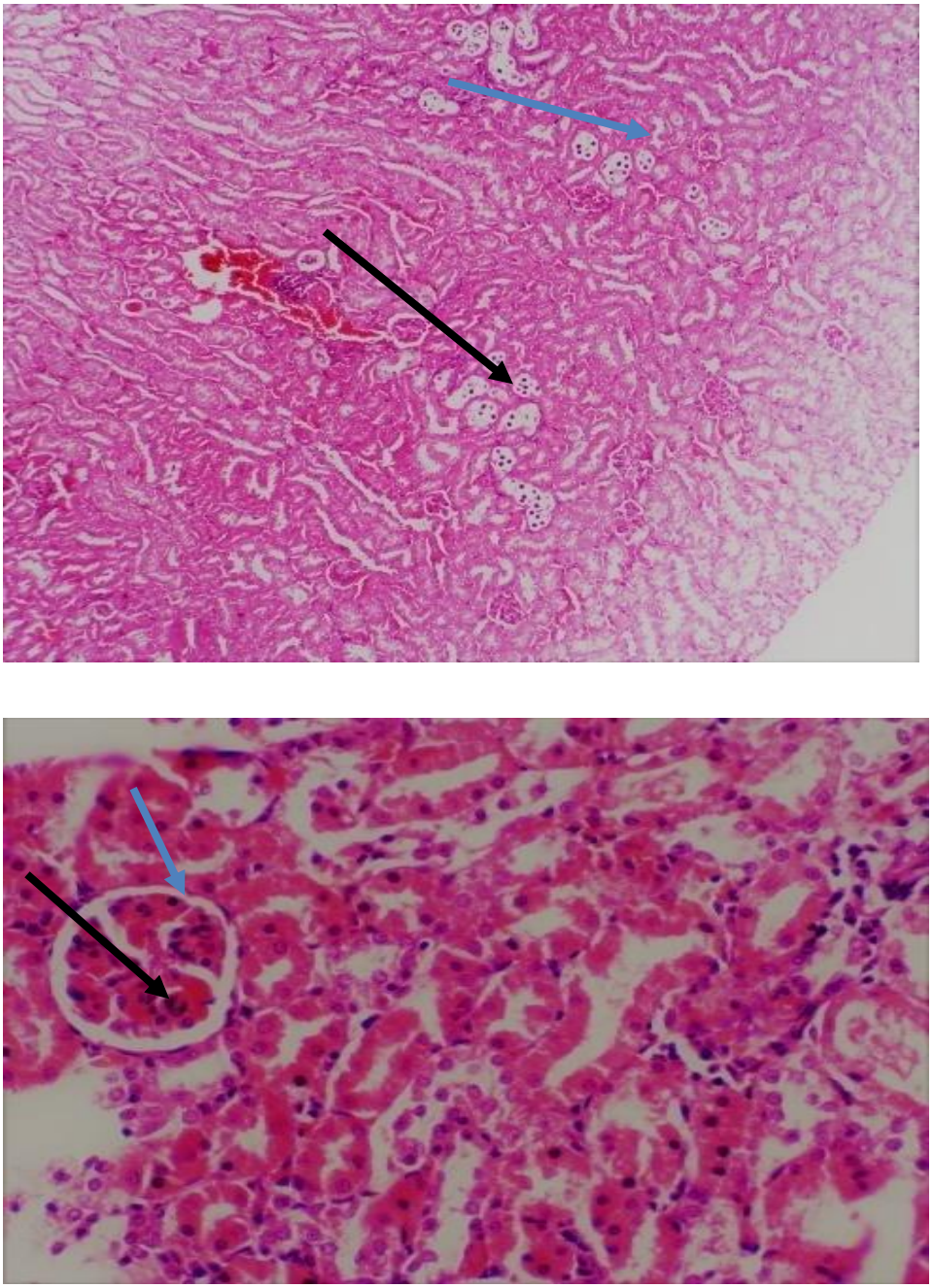

Plate III: Photomicrographs of the Kidney (×40; eosin and haematoxylin) Negative and Normal Control

KEY: Black arrow; Glomeruli, Blue arrow; Capsular space

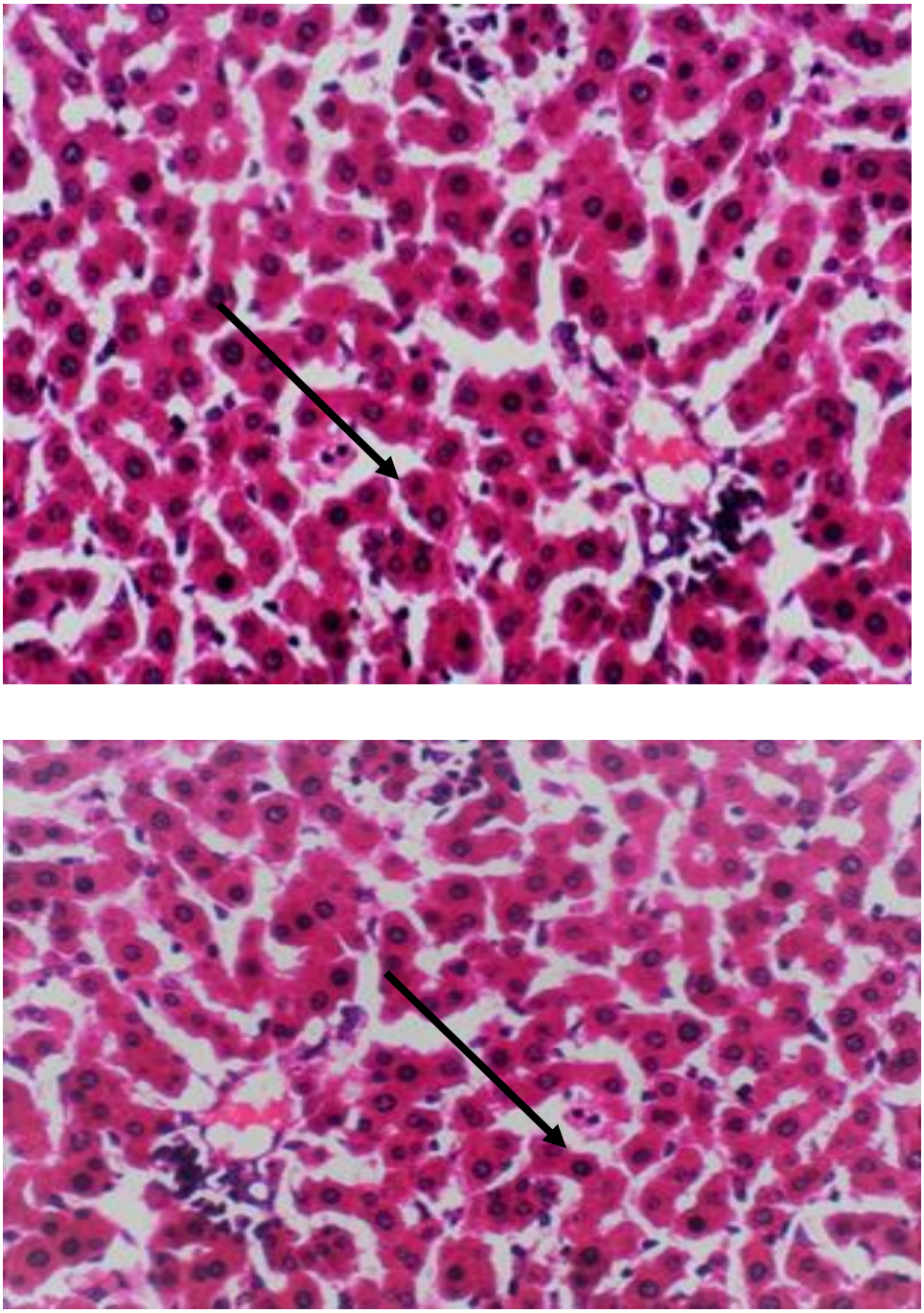

Plate IV: Photomicrographs of the Liver (×40; eosin and haematoxylin) 10 and 20 mg/kg b.wt. of the Crude Extract

KEY: Black arrow; hepatocyes

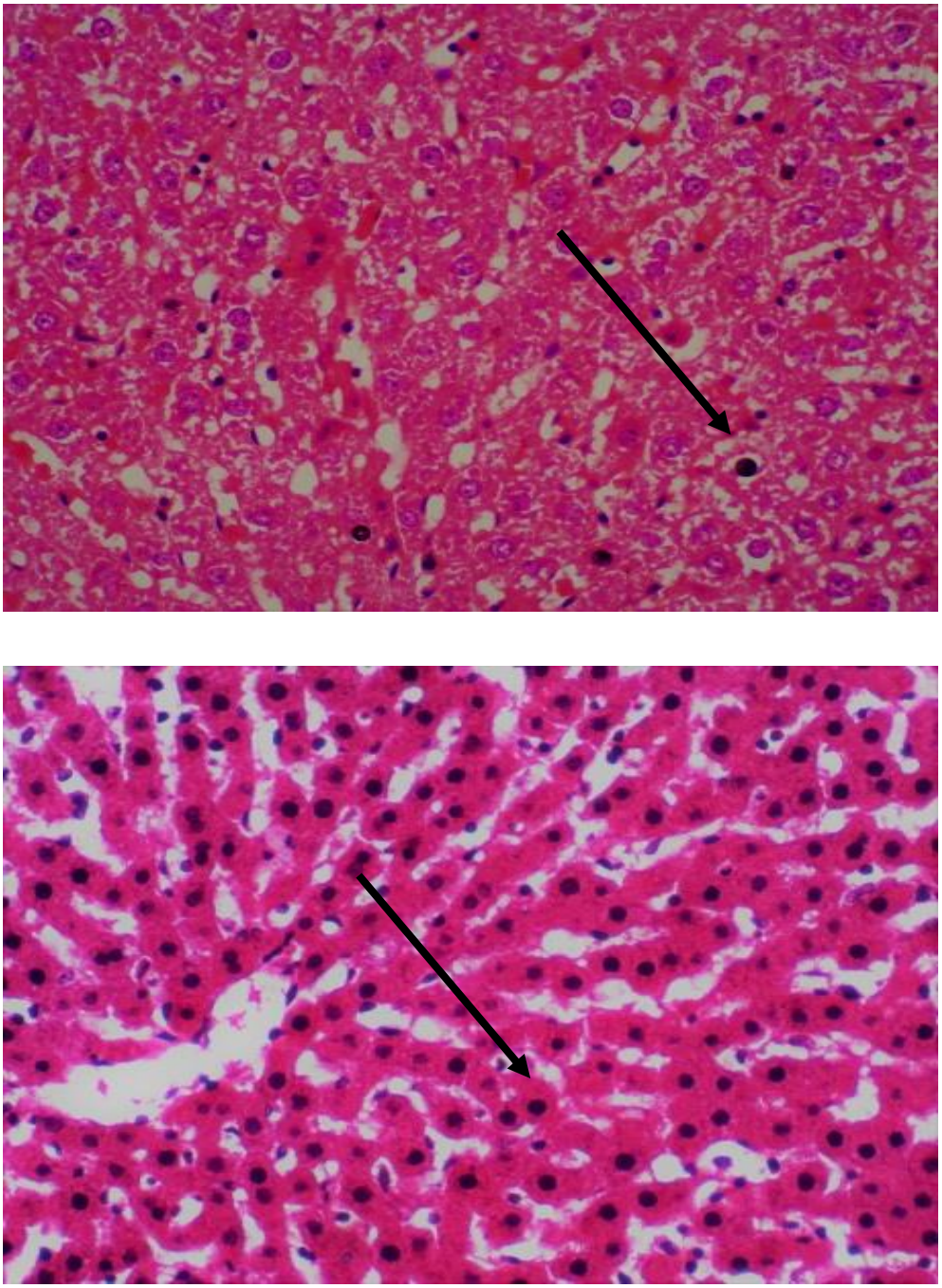

Plate V: Photomicrophages of the Liver (×40; eosin and haematoxylin) 30 mg/kg b.wt. of the Crude Extract and 5 mg/kg (CQ)

KEY: Black arrow; hepatocytes, CQ; Chloroquine

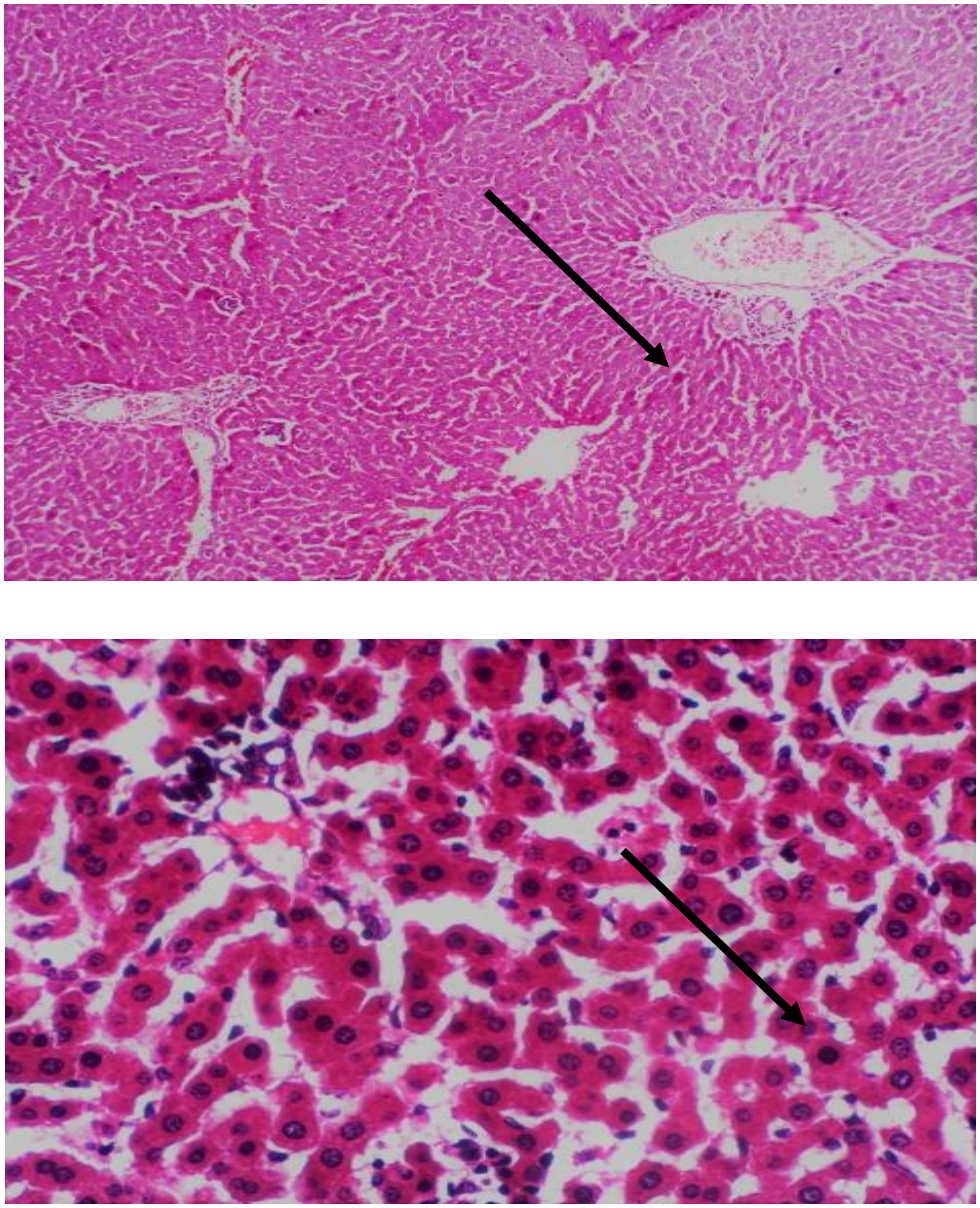

Plate VI: Photomicrographs of the Liver (×40; eosin and haematoxylin) Negative and Normal Control

KEY: Black arrow; hepatocytes

4.2 Discussion

4.2.1 Zoochemical component of crude toad venom extract

In the recent past, much concern has been channelled on the use of natural products as an optional therapy for treatment of different ailments especially the oxidative and parasitic diseases (Lawal *et al*., 2015; Lawal *et al*., 2016). Zoochemicals are secondary metabolites of plants known to exhibit diverse pharmacological and biochemical effects on living organisms (Sultana *et al*., 2009). Therefore, the presence of phenols, flavonoids, tannins, cardiac glycoside, carbohydrate and terpenoids in crude toad venom is an indication that the crude extract of toad venom would exhibit some therapeutic effect against *Plasmodium* parasite. This is similar to the finding of Adeshina *et al*., (2020) who reported antiplasmodial activities of phenol and flavonoids in *Plasmodium berghei*- infected mice. The zoochemical components are indicator that the crude toad venom extract has anti-parasitic potentials.

4.2.2 Gas chromatography and mass spectrometry of the crude toad venom extract

Extract of the crude toad venom was subjected to gas chromatography and mass spectrometry which revealed 25 (twenty five) different bioactive compounds; these compounds include Dodecanoic acid, octadecanoic acid and oleic acid, which have also been reported by Anand, (2015) in *Pseudoglochindion anamalayanum*(gamble)plant to have anti-microbial and anti-parasitic potency. The observed anti-plasmodial activities of the crude toad venom extract could be attributed to the presence of these compounds (Adams, 2007). Therefore, toad venom could serve as a new template for the developments of safe and effective anti-plasmodial drug.

4.2.3 Acute toxicity of the crude toad venom extract in mice

Though medicinal derived natural products are widely acceptable and employed in the treatment of many diseases, their toxicity must not be neglected. Acute toxicity is the adverse effects that occur within 24 hours of ingesting single dose of substance (Amos *et al*., 2015). Results of the present study suggested that the toad venom exhibited low safe dose and LD_{50} of 141.42 mg/kg b.wt. Thus toad venom should be used for therapeutic purpose only at doses below the established safe dose.

4.2.4 Effect of the crude toad extract on the parasitemia count in *Plasmodium berghei*-infected mice

Analysis of parasitaemia counts indicated significant parasitaemia suppression by toad venom at concentration of 10 and 20 mg/kg b.wt. compared to the negative control after the 4-day suppression test (Fentahun *et al*., 2017). However, the higher suppression of *Plasmodium* parasite observed in mice treated with 10 and 20 mg/kg b.wt. compared to those treated with 30 mg/kg b.wt. showed that the toad venom would be better antimalarial at lower dose than at higher dose. The lower activities observed at higher dose of toad venom could be attributed to antagonistic effect of the bioactive components at higher dose. The higher anti-*Plasmodial* effect demonstrated by 5 mg kg b.wt. chloroquine is not surprising, because, chloroquine has recently shown disruption of malarial parasite's cell membrane and induction of auto-parasite digestion via the formation of FP-chloroquine complex which impaired the haeme polymerization (Noedl *et al*., 2003). This is similar to the study of Builder (Builders *et al*., 2014) who reported higher parasitaemia inhibition for chloroquine.

4.2.5 Effect of the crude toad extract on the pack cell volume on *Plasmodium berghei*-infected mice

Anaemia is a feature of malaria infected mice, this occurs due to the destruction of the red blood cells. Therefore, effective anti-malarial agents are expected to reverse or prevent the parasite induced anaemic condition (Fentahun *et al*., 2017). The significant decrease in the pack cell volume (PCV) of the untreated mice is a sigh of anaemic condition caused by this parasite. However, mice treated with the crude toad venom extract of 20 mg/kg b.wt. showed the highest pack cell volume followed by 10 and 30 mg/kg b.wt. This agrees with the findings of Cunha-filho *et al*., (2010), who revealed the presence of anti-haemolysis properties in the venom extract of a toad species (*Rhinella marina*). Therefore, the indication of enhanced resistance to erythrocyte haemolysis by the crude toad venom prevented the parasitic induced anaemic condition in the mice.

4.2.6 Effect of the crude toad extract on the body weight of *Plasmodium berghei*-infected mice

Body weight loss is a general feature of *Plasmodium*- infected mice (Fentahun *et al.,* 2017). The high weight loss is attributed to the depressing effect of the plasmodium infection on feed uptake appetite (Chindull *et al.,* 1998). In this study there was no significant change in the body weight observed in the mice treated with chloroquine and those treated with toad venom of 10, 20 and 30 mg/kg b.wt. This may be due to the ability of the crude toad venom to reverse the loss of appetite and increase the feed uptake of the mice.

4.2.7 Effect of crude toad venom extract on the mean survival time of *Plasmodium berghei* -infected mice

Treatments with the toad venom elongated the mean survival time of the experimental mice indicating that the toad venom suppressed *P. berghei* and reduced the overall pathologic effect of the parasite on the mice. The longest survival time of mice as a result of the administration of the 20 mg/kg b.wt. of toad venom could be linked to the presence synergetic anti-*Plasmodial* active secondary metabolites at that dose. Similar result on mean survival time of mice was reported by Bantie *et al*., (2014). However, neither the extracts nor the standard drug cured the infection. This could be due to recrudescence of *P. berghei* parasites after apparent cure. Although the activities demonstrated by crude toad venom was lower than those of a standard drug (chloroquine), it is possible that a combination of toad venom with chloroquine would act synergistically to exhibit higher antimalarial activities than the chloroquine alone.

4.2.8 Effect of crude toad venom extract on the organ body weight of *Plasmodium berghei* -infected mice

The kidney, liver, heart lung and spleen body weight of the untreated mice showed a significantly low weight when compared with the weight of the other groups. This may be due the degenerates tissue found in the organs of the untreated groups according to the histopatholgical findings. While the weight of the heart, liver, lung, spleen and kidney of the groups treated with the crude toad venom extract, 5 mg/kg CQ and the normal group had no significant change in their organ body weight suggesting that the crude toad venom that was administered intraperitoneally produced no effect on the normal growth, this is similar to Mayur *et al*. (2017) who used extract of *Mardenia tenacissima* leaves on experimental rats in relative organs toxicity studies and histopathological changes.

4.2.9 Effect of crude toad venom extraction the biochemical parameters in *Plasmodium berghei*-infected mice

Evaluation of biochemical parameters holds a pivotal position in assessing the integrity of liver and kidney following parasitic infection as well as during administration of plant extract for oral remedy (Ibrahim *et al*., 2020). The most relevant and widely used biochemical parameters in assessing liver integrity includes AST, ALT, ALP, total proteins, albumins and bilirubins (Lawal *et al*., 2015). These enzymes and proteins were primarily found in the liver and are release in substantial amount to the serum when the liver's integrity has been compromised (Umar *et al*., 2019; Yusuf *et al*., 2018). Consequently, *P. berghei* infected untreated mice exhibited significant up regulated activities of AST, ALT, ALP, total proteins and bilirubins when compared with the control mice and chloroquine treated mice. This is similar to the report of Umar *et al.*, (2019) who admitted that venom of *Heterometrus xanthopus* exhibited significant increase of biochemical indices monitored in the serum of experimental rats.

As mentioned earlier, these up regulations of biochemical indices of liver integrity are secondary events that follows liver assault. The *Plasmodium* parasite must have mediated hyper expression of ALT, ALP and AST genes and consequently resulted in higher enzyme activities and release into the serum. However, it is clear from these findings that toad venom at 10 and 20 mg/kg b.wt. exhibited significant ameliorative effect by decreasing the levels of these biochemical parameters to a level comparable to the control group.

Uric acid, urea and creatinine are important excretory metabolites and are widely accepted clinical markers of kidney integrity (Bashir *et al*., 2015). Infected untreated mice exhibited high levels of serum urea and creatinine when compared to treatment

and control groups, suggesting a condition of *Plasmodium berghei* mediated compromised kidney function. Interestingly treatments with toad venom at concentration of 10 and 20 mg/kg b.wt. significantly decreases the urea and creatinine levels, thus suggesting the restoration of kidney function.

It's noteworthy, that the aberrant biochemical and haematological parameters that were observed in *P. berghei* infected untreated mice were significantly ameliorated in mice treated with 10 and 20 mg/kg b.wt. of crude toad venom. Thus indicating the complete anti-plasmodial effect as well as its ability to prevent *Plasmodium* induce alterations in haematological and biochemical parameters in mice.

4.2.10 Effect of crude toad venom extract on haematological parameters in *Plasmodium berghei*-infected mice

As a carrier of genetic materials, nutrients, metabolites across the body, haematopoietic system is one of the most important system in human and are most susceptible to assaults by parasitic infection as well as by administration of natural product during treatment regimens (Berinyuy *et al*., 2015). Hematological parameters are therefore, widely used in assessing the extent of parasitic infection as well as assessing the health status of an animal (Berinyuy *et al*., 2015). The increases in PCV of mice treated with the crude toad venom is an indication of enhanced resistance to erythrocyte haemolysis. This agrees with Shittu and Eyihuri (2015) who reported that bee venom were able to reverse the anaemic condition induced by *Plasmodium berghei* -infected mice The toad venom, therefore, prevented the parasite-induced anaemic condition in mice.

The significant decrease in white blood cell (WBC) counts in the *P. berghei* infected untreated mice suggested an exhaustion of immune cells as a results of it engagement to ameliorate the stress induced by the plasmodium parasite (Lawal *et al*., 2015). This will

affect the animals' ability to fight further infection and thus compromise the overall health status of the animal. Similarly, the significant ($p < 0.05$) decrease in erythrocytic indices, including HGB, PCV, MCH, MCHC, in *P. berghei* infected untreated mice when compared with the control and the chloroquine treated mice is an indication of erythrocytic destructive effect of *Plasmodium* parasite. Furthermore, the *Plasmodium* parasite must have inhibited the release of erythropoietin in the kidney, a humoral mediator of RBC production (Mishra and Tandon, 2012). Interestingly, treatments of the *P. berghei* infected mice with toad venom at 10 and 20 mg/kg b.wt. significantly restored the levels of these hematological component to their respective normal levels, thus, suggesting that crude extract of toad venom at doses of 10 and 20 mg/kg b.wt mediated erythropoietic properties of the mice during parasitaemia infection.

4.2.11 Histopathological evaluation of liver and kidney of *Plasmodium berghei*-infected mice

Plasmodium parasite are known to have complex life cycle, where one of this cycle occurs in the liver, therefore the need for histopathological evaluations of tissues of the liver and kidney to examine the pathogenic level of crude toad venom extract on them. The presence of parasite in the tissue can cause hepatomegaly of the internal organs. (Ukpai and Nwabuko, 2014). The histopathological findings of the liver revealed a degenerated hepatocyte in the liver of the untreated group, and preserved architecture of normal hepatocyte, portal tracts and central veins of the groups treated with the crude toad venom extract, normal and standard group.While the kidney of the untreated group also revealed a degenerated glomeruli, with a collapse of the capsular space, and preserved architecture of normal glomeruli, tubules and unremarkable interstitium were observed in other groups. This is similar to Ogunbawo *et al*. (2001) who reported the effect of anti-protozoal drugs and the histopathology on try pa bosom all infected rats.

This indicated that the crude toad venom extracts had no adverse effect on the normal growth of the body organs of the mice.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The present study indicated promising in vivo anti-plasmodial effect of the crude toad venom. The zoochemical constituent of the crude toad venom indicated the presence of phenol, flavonoids, tannis, carbohydrates, saponins and terpenoids. The crude toad venom was also found to be safe at the maximum dose of 141.42 mg/kg b.wt. The antimalarial effect of the crude toad venom was revealed to be less at higher dose (30 mg/kg b.wt.) as compared to that by the lower doses (10 and 20 mg/kg b.wt.).

The crude toad venom had protected against loss of packed cell volume and body weight, and displayed ameliorative effect on *P. berghei* mediated alterations in biochemical and haematological parameters in mice.

Gas chromatography and mass spectrometry (GS-MS) analysis indicated twenty five (25) bioactive compounds some of which include Dodencanoic acid, Octadencanoic acid, oleic acid. The effect of the crude toad venom on histopathological parameters of *P. berghei* infected mice revealed that there was no remarkable feature of acute or chronic damages on the organs of the mice.

Therefore, the crude toad venom could potentially be used as a new source for the development of new natural-based antimalarial agent. Moreover, the data could be used as additional evidence to uphold claims by the Nigerian traditional medicine practitioners on the effect of traditionally used venoms to manage malaria.

5.2 Recommendations

Based on the results obtained from this study, knowledge about the activity of the molecules of this venom is needed through intensive research, such as the isolation and characterization of the active principle and in vivo tests with the venom and/or the isolated substance, aimed at the identification of an efficacious novel molecule to be used in antimalarial therapy.

Further toxicological are required to move forward in the exploitation of toad venom as a new antimalarial agent.

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APPENDIX

APPENDIX A

MINISTRY OF LIVESTOCK AND FISHERIES

MINNA, NIGER STATE

NIGER STATE VETERINARY HOSPITAL MINNA

NSVH/MLF/20/VOL1/09.

Date: 22nd April, 2021

TO WHOM IT MAY CONCERN

Ethical approval for the use of laboratory mice to conduct a research titled "Preliminary Evaluation of the in-vivo Anti plasmodium potency of crude toad venom in *plasmodium berghei* infected mice by Abuh, Ojomona Oboro M.Tech/SLS/2018/8159.

The ethical committee of Veterinary Hospital considers this research worthwhile due to its potential benefits. *Plasmodium berghei* has been found to cause malaria in certain rodents. Realistically any research involving mice to understand the role of *plasmodium berghei* in the cycle of malaria has a benefit that outweigh any risk that might occur to the mice. The use of mice is therefore ethical and justified.

2. Population of the mice involved in the experimentation have also met the ethical standard and morally justified.

3. The laboratory procedures with respect to handling, housing, inoculation and subsequent administration of the extracts are humane, well safeguarded and non-brutal.

NIGER STATE VETERINARY HOSPITAL SIGN DATE

Dr. Yatswako Suleiman
Head, Niger State Veterinary Hospital,
Minna.