IN VIVO EVALUATION OF NIGERIAN TOAD (ANURA: BUFONIDAE) VENOM AS A POTENTIAL SOURCE OF ANTI-TRYPANOSOMAL AGENT IN *TRYPANOSOMA BRUCEI BRUCEI*-INFECTED WISTAR RATS

BY

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ABSTRACT

African trypanosomiasis, a vector-borne parasitic infection, remains a major parasitic disease of the developing nations, causing a significant health threat and risk to lives. The current study elucidated the *in vivo* curative efficacy of crude toad venom extract against *Trypanosoma brucei brucei*-infected wistar rats. The toad venom was extracted by pressing the paratoid glands and characterization was carried out to ascertain its bioactive components. The acute toxicity of the venom was determined using a slightly modified Lorke's method and the LD_{50} was computed. To establish the anti-trypanosomal effect of the venom, 28 male and female wistar rats were randomly split into seven groups of 4 rats each. Rats in group 1 to 6 were infected with 10^6 cells of trypanosomes per ml of blood intraperitoneally. Rats in group 1 to 4 were treated with the extract at 5, 10, 20 and 30 mg/kg body weight (b. wt.), respectively; while rats in group 5 and 6 were treated with diminazene aceturate at 5mg/kg b. wt. and normal saline at 2 ml/kg b. wt., respectively. Four rats (group 7) were neither infected nor treated and served as Normal control. The experimental animals were sacrificed and liver and kidney were obtained for histological evaluation. Zoochemical analysis showed the presence of phenols, flavonoids and terpenoids while results of acute toxicity showed the LD_{50} to be 141.42 mg/kg b. wt. The extract at 10 and 20 mg/kg b. wt. reduced parasitaemia levels significantly ($p<0.05$), prevented body weight loss and reduced the observed parasite-induced decline in packed cell volume, in comparison to Negative control. The group treated with 20 mg/kg b. wt. showed the highest anti-trypanosomal efficacy with 10 counts/ml on the 5^{th} day of treatment. This is not significantly different from the group treated with standard drug. The haemoglobin concentration (12.17±0.79 g/dL) and red blood cell count (9.88±0.68 × 10^{12}/L) showed a significant ($p<0.05$) decrease in the negative group compared to control (19.51±0.83 g/dL for haemoglobin and 18.76±0.39 × 10^{12}/L for red blood cell count) and groups treated with 20 mg/kg b. wt. of the extract (18.81±0.94 g/dL for haemoglobin and 14.65±0.40 × 10^{12}/L for red blood cell count). The survival time of rats treated with 20 mg/kg b. wt. of the extract was higher than the rats in the negative control group. GCMS revealed the presence of bioactive components including oleic acid and 9-Octadecenoic acid, methyl ester. Liver and kidney induced damage was ameliorated with the toad venom. This study, thus, established that the toad crude venom possess a promising anti-trypanosomal agent and could be developed into an alternative drug to complement treatment of trypanosomiasis.

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ABBREVIATIONS

AAT- Animal African Trypanosomiasis

ALP- Alkaline Phosphate

ALT- Alanine-amino Transferase

AMP- Antimicrobial Peptides or Proteins

AST- Aspartate-amino Transferase

b. wt. - Body Weight

BHT- Butylated Hydroxytoluene

BPP- Bradykinin Potentiating Peptides

CCAC- Canadian Council on Animal Care

CHOL- Cholesterol

CTV- Crude Toad Venom Extract

DMRT- Duncan Multiple Range Test

DMZ- Diminazene aceturate

DNA- Deoxyribonucleic Acid

DSs- Dermaseptins

EDTA- Ethylene Diamine Tetraacetic Acid

GC-MS- Gas Chromatrography-Mass Spectrometry

HAT- Human African Trypanosomiasis

HB- Haemoglobin

HBV- Hepatitis B Virus

HDL-C- High Density Lipoprotein Cholesterol

HPLC- High Performance Liquid Chromatography

LC-MS- Liquid Chromatography- Mass Spectrometry

LD- Lethal Dose

LDL-C- Low Density Lipoprotein Cholesterol

MCHC- Mean Corpuscular Haemoglobin Concentration

MCV- Mean Corpuscular Volume

MST- Mean Survival Time

NITR Nigeria Institute for Trypanosomiasis Research

NPY- Neuro Peptide Y

PBMC- Peripheral Blood Mononuclear Cells

PCV- Packed Cell Volume

PLC- Platelet Counts

Ppm-	Parts per million
PSs-	Phylloseptins
RBC-	Red Blood Cell
SEM-	Standard Error of Mean
SPSS-	Statistical Package for Social Sciences
SPYY-	Skin Polypeptide YY
STC-	Shimadzu Training Centre
T. b-	*Trypanosoma brucei*
T. b. b-	*Trypanosoma brucei brucei*
TbG-	*Trypanosoma brucei gambiense*
TbR-	*Trypanosoma brucei rhodesiense*
TRIG-	Triglyceride
TWBC-	Total White Blood Cell
VSG-	Variant Surface Glycoproteins
WBC-	White Blood Cell
WHO-	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Human African Trypanosomiasis (HAT) is a vector-borne parasitic disease instigated by the parasite species, *Trypanosoma brucei* (*T. b.*) with subspecies, *Trypanosoma brucei gambiense* (TbG) and *Trypanosoma brucei rhodesiense* (TbR). The primary route of transmission is from the bite of an infected tsetse fly, *Glossina* species. The key reservoir host of TbG are humans, while wild and domestic animals are reservoirs of TbR (Sutherland *et al.*, 2015). According to a report by World Health Organization in 2019, Trypanosomiasis is one of the endemic tropical diseases neglected in sub-Saharan African countries, risking about 70 million lives in 36 countries having numerous amount of tsetse fly as vectors. The individuals most exposed to tsetse fly, dwell in pastoral regions and largely depending on cultivation, fishing, animal farming and hunting, with inadequate access to suitable health facilities are more vulnerable to the disease, and also, movement of people, war, and poverty are vital issues that aid spread. Both TbG and TbR affect humans, with TbG responsible for more than 98 % of reported cases (WHO, 2019).

The first phase of the disease involves high temperature, headache, irritation, and joint pains, starting one to three weeks after bite. Several weeks to months later, the next phase begins with confusion, reduced coordination, shock, and inability to sleep (WHO, 2019). Discovery of parasite in a smear of blood or in the emissions of a lymph node from an infected person is the method used to diagnose the disease (Kennedy, 2013). Prevention of chronic trypanosomiasis includes screening the inhabitants at risk with blood experiments for TbG. Identifying the disease early makes it easier to treat, before it affects the nervous system. The first phase is treated with drugs like pentamidine and suramin while the next phase involves treatment with eflornithine or a mixture of nifurtimox and

eflornithine for TbG. Melarsoprol can be used to treat both phase and usually used alone for treatment of *Trypanosoma brucei rhodesiense* (TbR), due to its severe side effects. Death is the natural outcome of untreated sleeping sickness (Kennedy, 2013; WHO, 2014).

African animal trypanosomiasis (AAT) also called "nagana" is a parasitic disease in pigs triggered by *Trypanosoma brucei brucei* (*T. b. b.),* TbR, *Trypanosma congolense* and *Trypanosoma simiae* (Loker and Hofkin, 2015) and *Trypanosoma evansi* in horses, buffalo and camels also known as "surra" (Reid, 2002). Devastating economic losses in livestock from effects on their reproductive ability, anaemia and loss of condition caused by trypanosomes are particularly prominent. Other animals apart from livestock such as dogs, can also be affected. Cases that are not treated can become deadly, elevating mortality level in some epidemic (Auty *et al.,* 2015).

Transmission of *T. b.* is by vectors (arthropod) passed to mammalian hosts which is referred to as nagana when clinically diagnosed in animals and this can lead to major agricultural losses. Severe sickness with the inception of signs after a long incubation period of weeks to months can be caused by TbG. While TbR results into a chronic infection, starting a few days or weeks after bite from an infected vector; frequently, there is a remarkable inoculation chancre. Preliminary medical signs comprises of chronic headache, lack of sleep, inflamed lymph swellings, body irritations and reduced blood level. In the last phase of the disease, there is an advanced weight loss and the central nervous system also becomes affected. The disease is inevitably lethal without treatment (Sutherland *et al.,* 2015).

The fly's mid gut is inhabited by the parasite (procyclic form), from where it moves to the salivary glands for inoculation to the mammalian host through biting. The fly can be

re-infected after biting the host as the parasite is able to stay alive in the bloodstream (bloodstream form). The parasite later shifts to other parts of the host during the period of a *T. b.* infection. *T. b.* infection may be conveyed from human to human through exchange of body fluid, especially blood transfusion. Both human and animal trypanosomiasis affect the central nervous system. The last phase of development of sleeping sickness takes decades to occur in West Africa (*Gambiense*) and an infected person may only suffer from mild weakness due to the irregularity in parasitic load in blood (Sutherland *et al.,* 2015).

The common name for some frogs is toad, particularly of the family Bufonidae, categorized by their huge bumps covering the parotoid glands, short legs, dry and leathery skin. The difference between toads and frogs has not been made in scientific taxonomy although common in widespread folk culture, where toads are more terrestrial and linked with drier and bumpier skin (Feng *et al.,* 2017). The African common toad, *Sclerophrys regularis*, is a class of toad seen widely in Africa (sub-Sahara) near rivers, where it can also breed and are abundant in agricultural habitats, motane grassland, damp and arid savanna, and forest margins (Frost, 2014).

Typically, the paratoid glands are covered by the biggest bumps on the skin of a toad, normally called warts, but are totally different from pathological warts, as can be observed by their static size and only found on fit specimens and not produced because of infection. In the past few years, bioactive compounds such as bufadienolides, peptides, biogenic animes, proteins and alkaloids have been analyzed as fascinating sources from skin exudations and venom of amphibians (Ferreira *et al.,* 2013). These animals use these compounds which play an important role in their biological functions mostly for preying on other animals and defense against microorganisms. Digitalis-like aglycones and biogenic amines called bufadienolides are major molecules found mainly in toads, a

significant set of polyhydroxy C-24 steroids connected to cholesterol. Several studies on the structure–activity bond of these compounds have revealed cardiotonic, anti-viral, cytotoxic, anti-bacterial, anti-parasitic and insecticidal properties (Sciani *et al.,* 2013).

1.2 Statement of the Research Problem

African trypanosomiasis in humans and livestock has devastated the livestock industry and jeopardized the health of millions in tropical Africa where the disease is endemic leading to much affliction in societies and impoverishing the economy (Dennis *et al.,* 2017).

Although, recent advances in drug discovery has noted that current anti-trypanosomal drugs have been characterized with several adverse reactions, limited effectiveness, increasing rates of drug resistance, elevated cost, complexity in administration among others (Babokhov *et al.,* 2013; Bhattacharya *et al.,* 2020). On the other hand, the synthetic drugs discovered are not cost effective, thus, making their mass distribution less effective to the end users.

Barrett *et al.* (2011) and Abdulazeez *et al.* (2013) stated that current failure in chemotherapy of trypanosomiasis has been as a result of resistance to available drugs that have been used aggressively, its toxicity and long treatment protocols. Therefore, the need to develop an alternative and effective drug from natural products is crucial.

A major challenge is the aim to discover new drug by detecting constituents of venom with biological properties, such as analgesic, anti-inflammatory, anti-microbial, anti-viral, anti-parasitic, neurotransmission, modulators of blood coagulation and so on (Schmeda-Hirschmann *et al.,* 2016). Nevertheless, even with these glitches, researches have been done and several venom components with therapeutic potentials have been found.

1.3 Aim and Objectives of the Study

This study was aimed at evaluating the *in vivo* activities of Nigerian toad (Anura: Bufonidae) venom as a potential source of anti-trypanosomal agent in *Trypanosoma brucei brucei*-infected wistar rats.

The objectives were to determine the;

i. zoochemical components of crude extract of toad venom.
ii. bioactive metabolites in the crude extract of toad venom
iii. the acute toxicity of the crude extract of toad venom
iv. anti-trypanosomal activities of the crude extract of toad venom
v. effect of the crude extract of toad venom on some parameters in *T. b. b.*-infected wistar rats
vi. effect of the crude extract of toad venom on the histopathological parameters of *T. b. b.*-infected wistar rats.

1.4 Justification for the Study

The efforts to develop an effective, safe and easy to use chemotherapy for Animal African Trypanosomiasis (AAT) remains a demanding task. The four currently available medications have key detriments that limits wide usage (Babokhov *et al.,* 2013). Therefore, the need to develop a safer, less toxic, highly effective and available chemotherapeutic agents for the treatment of trypanosomiasis is important.

In the past few decades, researchers have diverted interest into the exploitation of animal-based therapeutic agents. Camila and Thais (2015) mentioned that researches into venom from animals, its toxins and several bioactive components gotten from natural bases are now being promoted and the focus is on the ability of these compounds to hinder parasite ecology.

About 50 % of venom represented in pharmaceuticals can be advanced (Camila and Thais, 2015), and animals contain a collection of exceptional secondary metabolites valuable for novel drug discovery (Cunha-Filho *et al.,* 2010). Findings from this study will assist stakeholders in the field of drug advancement against trypanosomiasis and provide evidence that promote traditional use of venom.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Epidemiology of African Trypanosomiasis

Major cases of African trypanosomiasis with humans as key reservoir essential for transmission, are caused by TbG, while TbR rarely affects humans and are mostly zoonotic (Franco *et al.*, 2014). The disease is dependent on the relationship between the vectors (tsetse flies) and trypanosomes (parasite), and also the host which may be TbG (humans) and/or TbR (animals). Contracting this disease primarily relies on bites from an infected tsetse fly which puts one at risk of being infected (Franco *et al.*, 2014).

The disease is mainly spread through bite from a tsetse fly of the *Glossina* species carrying the parasite. Nevertheless, people are infected via several means such as mother to child; in this transmission, the trypanosome crosses the placenta and infect the foetus, another means is by mechanical transmission which rises through vectors, majorly blood-sucking insects. There has been difficulties in evaluating the epidemiological influence and infections happening in laboratories due to accidental punctures from contaminated needles. Firstly, multiplication of trypanosomes takes place in the blood, subcutaneous tissues and lymph, which is also known as the haemo-lymphatic phase, and produces effects such as itching, bouts of fever, joint pains, headaches. Secondly, the central nervous system is infested by the trypanosomes after passing the blood-brain barrier, this is called the meningo-encephalic or neurological phase. This is when detrimental symptoms and signs of the disease starts manifesting, which includes, confusion, changes in behaviour, poor coordination and sensory disorders. Diagnosing and treating the disease is very difficult and entails an expert team (Sutherland *et al.*, 2015).

These vectors dwell in rural regions, as well as, woodlands and thickets that mark the East African savannah. They also inhabit vegetation along streams and forest in West and

Central Africa, and mainly bite during the day. Even though, both sexes of the vector can transmit the disease only a tiny percent of the flies actually carry the parasite in endemic areas. Other means of spreading the disease is possible asides the huge number transmitted by tsetse flies. Infrequently, an unborn child can be infected via the mother, and the disease can also be transferred through transfusion of blood or sexual activities although this is theoretical and has hardly been documented (WHO, 2019).

2.1.1 *Trypanosoma brucei* parasite

The two major subspecies of *T. b.* liable for trypanosomiasis in humans are, TbR having a restricted environmental range and accountable for the disease in South and East Africa, while, TbG is found in Central and West Africa. Of note is a third subspecies of *T. b.* called *Trypanosoma brucei brucei* (*T. b. b.*) that specifically affects animals (Franco *et al.,* 2014). Cattle and animals used for wild games are chief reservoirs of TbR, whereas, humans are key reservoirs for TbG, even though it can be found in other animals such as pigs, dogs and goats. The vector is mostly found in sub-Saharan Africa which makes the inhabitants the primary recipient of this disease. Variations in intensity can be observed in both forms of the disease, in the sense that, the illnesses associated with TbG can remain dormant for months or years before symptoms manifest which can last for about three years before death (Franco *et al.,* 2014).

African sleeping sickness is typically caused by TbG which is principally found in Central Africa and some parts of West Africa. This has been an important hazard in the public health sector, but has been controlled quite well over the years with 7,000 to 10,000 cases documented yearly in recent years. Angola, Central African Republic, Congo, Chad, Sudan and Northern Uganda has reported more than 95 % of human infection cases (WHO, 2019).

In TbR, which is the severe form of the disease, symptoms arises in weeks and death follows within months of infection, this form is way virulent and quicker than TbG. Additionally, parasites are enclosed by a coat made up of Variant Surface Glycoproteins (VSG) which functions to shield the trypanosomes from lysis. Numerous antibodies such as Immunoglobulin M and Immunoglobulin G, are produced by the immune system of the host after identifying the existence of glycoproteins on the coat of the trypanosomes (Franco *et al.,* 2014). The activities of these antibodies will aid the destruction of trypanosomes that flow in the blood, although, a little number found in the plasma will go through changes on their coat surface causing the development of fresh VSGs, this will lead to the inability of antibodies formed by the immune system to not spot the trypanosomes resulting in multiplication until fresh antibodies are generated to fight the new VSGs. Ultimately, the host's immunity will lack the capacity to warn off trypanosomes as a result of continuous changes in VSGs and infection will keep rising (Sutherland *et al.*, 2015).

Trypanosoma brucei is a characteristic unicellular eukaryotic cell, possessing a long tapered and streamlined body of about 8 - 50 μm (Figure 2.1A), with a cell membrane (called pellicle) surrounding the cell organelles containing the mitochondria, nucleus, ribosomes, endoplasmic reticulum, and the golgi apparatus. There is also, a rare organelle called the kinetoplast, composed of several spherical Deoxyribonucleic acid (DNA) structures which collectively form the mitochondrial DNA disc (Amodeo *et al.*, 2018) and serves as a single large mitochondrion. The basal body lying close to the kinetoplast is normally not distinguishable except when viewed under the microscope. A lone flagellum possessing a free tip that runs to the anterior end rises from the basal body and joins to the pellicle along the body surface creating an undulating membrane. A thick coat of VSGs substituted by a similar thick coat of procyclins when the trypanosomes segregates

into procyclic phase in the midgut of the tsetse fly is a structure formed on the surface of the cell in the bloodstream. A microscopic view of *T. b. spp.* is seen in figure 2.1 B, where a thin blood smear is stained with Giemsa. The trypomastigote begins to split; separating into various forms, this is seen only in African trypanosomes, and not in American trypanosomes.

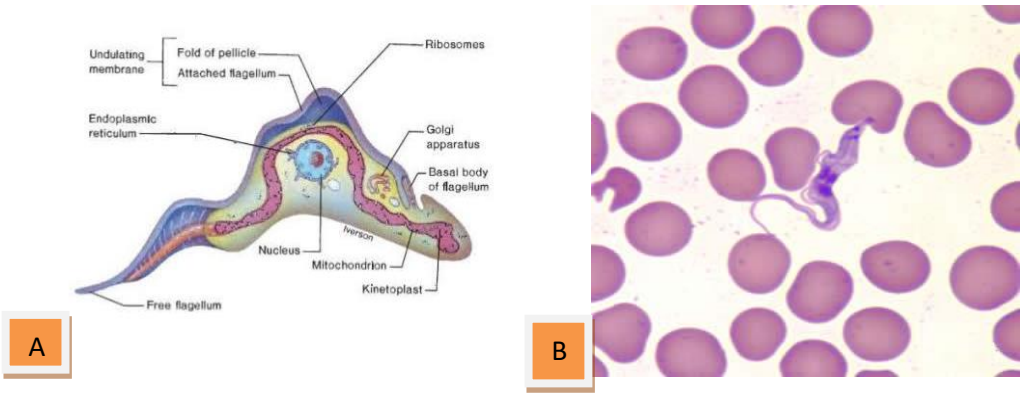

Figure 2.1: (A) *T. b.* cell (B): *T. b.* species in a Thin Blood Smear Stained with Giemsa (Source: Amodeo *et al.*, 2018)

2.1.2 Trypanosomiasis vector

The tsetse fly (genus *Glossina*) is a brown, large biting fly (Figure 2.2) commonly found in Africa of which 31 species and subspecies have been identified. Historically, the species has been grouped into three, based on its morphology, these includes the palpalis group (subgenus *Nemorhina*) originating from river banks of Central and West Africa but can spread to grassy plain areas flanked by drainage systems; *G. palpalis* and *tachinoides* are also essential AAT vectors in this group, the fusca group (subgenus *Austenina*) are usually found in the tropics of Central and West Africa; the morsitans group (subgenus *Glossina*) are found in different savannah habitats lying in the middle of the deserts and forest edges and also comprising other significant vectors of AAT such as *Glossina morsitans* species, *G. pallidipes* and *G. austeni* (Franco *et al.*, 2014)

Tsetse flies can function as vectors (biological or mechanical) and as hosts for trypanosomes thereby constituting a continuous and real menace to livestock and humans in sub-Saharan Africa, only a minority of these species are vectors of HAT but all are potential vectors of AAT. An infected fly transmits metacyclic trypomastigotes into the tissue of the skin via sucking of blood from the mammalian host. Through the bite, trypanosomes first gets into the lymphatic system and then into the circulatory system where they are converted into bloodstream trypomastigotes in the body of the host and are transferred to other body fluid sections like the lymph and spinal fluid among others and replication lingers by binary fission (Franco *et al.,* 2014).

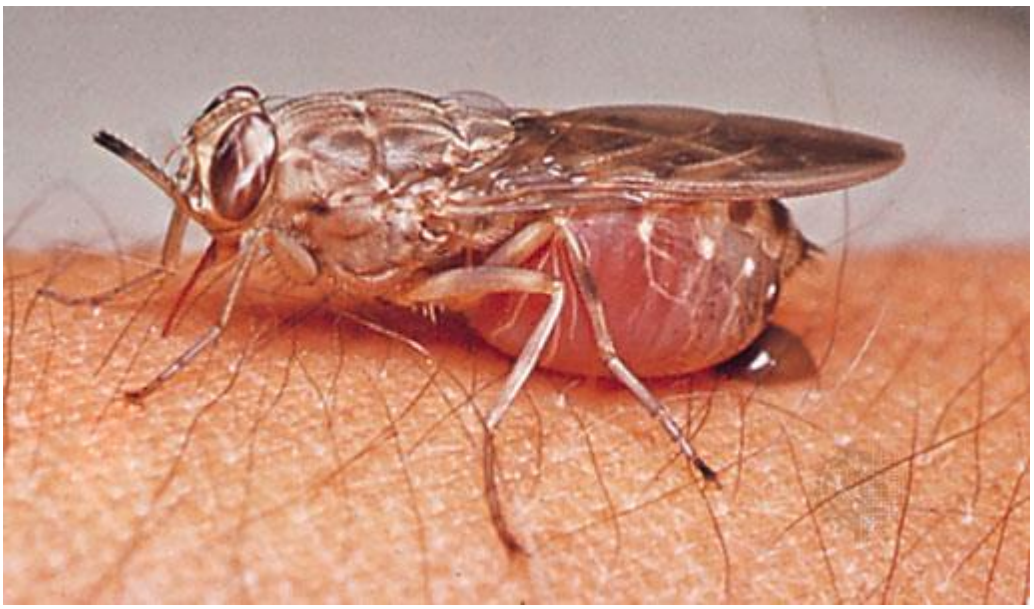

Figure 2.2: Tsetse Fly (*Glossina*) (Source: Franco *et al.,* 2014)

Tsetse flies are holometabulous insects that solely feed on blood. Specific *Trypanosoma* species determine the growth of trypanosomes in tsetse flies, such as the proboscis, which is inhabited by *T. vivax*, proboscis and midgut inhabited by *T. congolese* and *T. simiae*, TbG, TbR and *T. b. b.* inhabit the intestine. In the salivary gland of the tsetse fly, the infective forms are established. Their movement, longevity, and regular feeding make

them really effective vectors, but even a minor increase in death rate can lead to population reduction which depicts the low level of population growth. They have the ability to fly at a speed of almost 25 km/h, but they do this gradually and just for a short period of about 50 minutes and rest on trees to avoid dehydration for more than 23 hours in a day. Due to their sensitive nature to environmental settings, they are unable to endure in regions with excess heat, dryness and height (Sutherland *et al.,* 2015).

2.2 African Trypanosomiasis Distribution

Occurrence of tsetse flies relates to the dissemination of trypanosomiasis and presently take up 8,000,000 km^2. Both TbG and TbR HAT's geographical circulation is irregular and advancements in environments and migration of livestock and humans causes alterations in the level of spread and geographical location. Regions at risk of highest endemicity in Western Africa are categorized to be at average risk and mainly found in Coastal Guinea and Ivory Coast. Estimations are partially accurate for TbG than for TbR because of the presence of other infected humans instead of animal reservoirs; which is a chief factor in the former; however, various reasonable extrapolations can be made, estimating about 12.3 million humans at jeopardy of contracting TbR over a region of 0.171 million km^2 with 88 % at low to very little risk and 12 % which is about 1,500,000 humans at average to high risk (Simarro *et al*., 2012).

The Southern and Eastern areas of the continent inhabits TbR. Countries with a high prevalence rate of this HAT makes up about 50 to 1,500 cases annually, these are Uganda, Tanzania and Malawi (WHO, 2013). Both sub species of *T. brucei* are only known to affect Uganda, although their dispersal is distinct for now. The prevalence of HAT is way lower than other parasitic infections such as worm infections and malaria, though the prospects of terrible epidemic rising due to lack of surveillance makes this disease of utmost importance in endemic countries. Involvement of European countries by creating

efficient control programs led to effective maintenance in grave epidemics that took place towards the end of the 19th century to the start of the 20th, almost eradicating the disease by the 1960s. Rise in the number of cases began, following the colonial era, as a result of surveillance deficiency and lack of awareness by the local governments, even though, wars, poverty, shift in populations and unstable government also played a role. A sum of 60 million populace in 36 countries in Africa are continually opened to hazards of this disease by one of the two types of HAT, only three to four million are being scrutinized (WHO, 2013).

2.3 African Animal Trypanosomiasis Distribution

Anywhere a tsetse fly vector is found, trypanosomes definitely exists there and are prevalent in Africa. *T. vivax,* can be seen in the Caribbean, Central and South America which are parts devoid of the vector, they can also specially escalate past the 'tsetse fly belt' by spreading mechanically. The dispersal of African trypanosomes is dependent on the type of tsetse fly vector, which invades a region of 18 sub-Saharan Africa that projects from the Sahara desert's Southern edge with latitude 15 °N to Mozambique, Angola, and Zimbabwe with latitude 20 °S. Out of the three AAT, only *T. vivax* is found in the Western Hemisphere in at least 10 countries (WHO, 2013).

2.4 Signs and Symptoms of Trypanosomiasis

Symptoms of this disease takes place in two phases, in the first phase (haemolymphatic stage) causes headache, joint pain, itching, and fever. There is irregularity in fever occurrence which lasts for one to seven days with intermissions from a few days to a month or more. The trypanosomes invades the lymphatic and circulatory system connected with serious inflammation of lymph nodes, mostly of great size and this can cause the appearance of the tell-tale swollen lymph nodes behind the neck called the Winterbottom symbol. A red sore called the chancre can sometimes grow at the bite area

and if untreated, can overpower the defense of the host and cause extreme injuries, escalating the symptoms such as loss of blood, cardiac, kidney, and endocrine malfunctions. The second stage (neurological stage), starts with the invasion of trypanosomes in the central nervous system via the blood-brain wall. Interruption of sleep rotation is a major symptom of this phase from which the name "sleeping sickness" was invented. Humans infected go through an unsystematic and uneven 24-hour rhythm of the sleep-wake sequence, leading to sleep series during the day and period of staying awake at night (Lutje *et al.,* 2013).

Additional symptoms affecting the nervous system include tremor, hemiparesis, confusion, limb paralysis and general muscle weakness and damage in this phase is irreversible. Unspecified locomotion and speech disorders may arise called Parkinson-like movements. Patients may also display psychiatric disorders like hostile behaviour, irritability, apathy, and psychotic responses, which can occasionally dominate the medical diagnosis. Lack of treatment can cause permanent damage, with advanced psychological decline resulting to systemic organ collapse, coma, and ultimately death. Death can occur within months when TbR infection is left untreated, while it takes many years for death to occur when TbG infection is left untreated. (Franco *et al.,* 2014).

2.5 Life Cycle of African Trypanosomiasis

The whole life cycle of African trypanosomes is characterized by extracellular phases. Infection rises in the tsetse fly which is caused by bloodstream trypomastigotes, when it feeds on an infected host. The trypanosomes develop into procyclic trypomastigotes in the fly's midgut and replicates by binary fission, vacates and then progress into epimastigotes. In the salivary gland of the fly, the epimastigotes keep replicating still by binary fission. This cycle takes roughly three weeks. When an infected tsetse fly feed on

blood of a mammalian host, it inoculates metacyclic trypomastigotes into their skin tissue. The trypanosomes get into the bloodstream after passing through lymphatic system and then develop into bloodstream trypomastigotes in the body of the host, after, they migrate to other body sections including the lymph and spinal fluid and keeps duplicating by binary fission. TbG could be transmitted congenitally if the mother gets the disease while pregnant, although this rarely occurs (Figure 2.3) (Langousis and Hill, 2014).

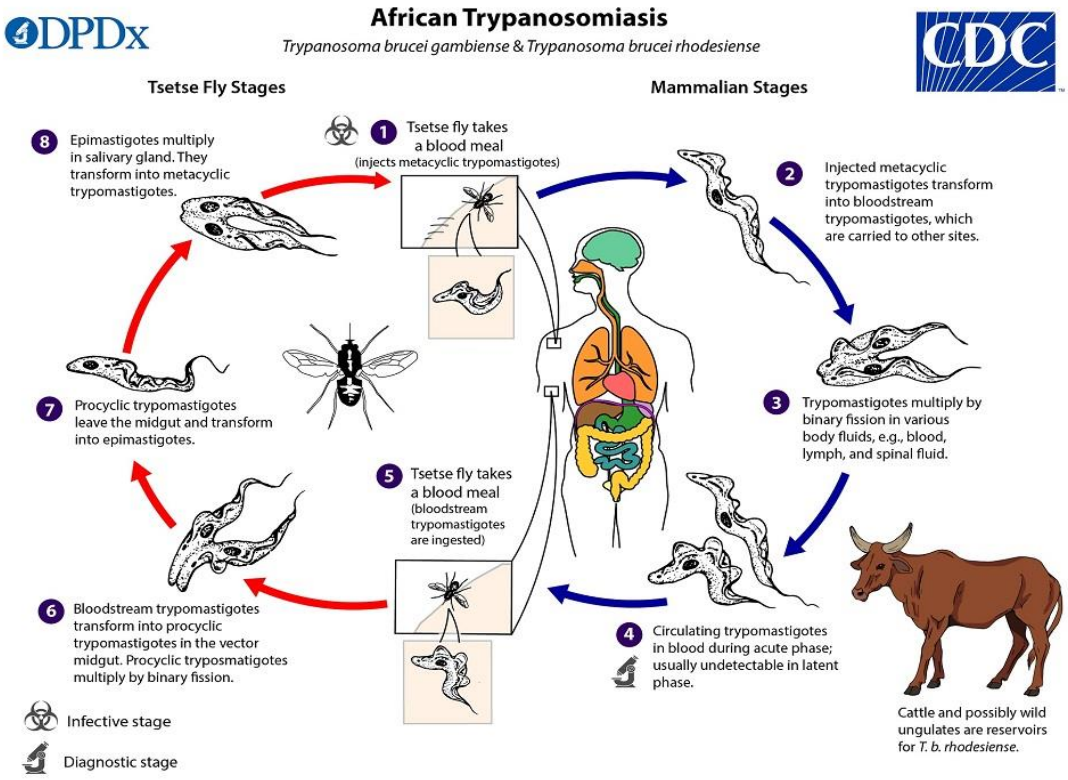

Figure 2.3: Life Cycle of African Trypanosomiasis (Source: CDC, 2017)

2.6 Impact of Trypanosomiasis in Africa

Millions of people are at risk of trypanosomiasis in 36 countries in sub-Saharan Africa. Most people affected reside in distant rural regions lacking suitable health facilities, which makes surveillance difficult and hence treatment and diagnosis of cases. Additionally, movement of people, poverty and war are significant factors that enables

transmission. About 40,000 cases were documented in 1998 but undiagnosed and untreated cases were evaluated to be 300,000 cases. Prevalence got to 50 % in various villages in Congo, South Sudan, and Angola during the most recent epidemic. Trypanosomiasis was the first and second highest cause of death ahead of HIV and AIDS in those villages. The number of cases documented reduced to 9,878 in 2009 for the first time in 50 years due to continuous efforts in controlling the disease. These reductions were constant with only 997 cases documented in 2018, the lowest level since the beginning of organized worldwide data-collection 80 years ago. 65 million people have been projected to be at risk of this disease (WHO, 2019).

2.7 Diagnosis of Trypanosomiasis

The usual means of diagnosis of trypanosomiasis is by identifying trypanosomes in a sample which can be a lymph node aspirate, bone marrow, chancre fluid, blood by microscopic analysis, and cerebrospinal fluid during the neurological phase. Diagnosis is made in three steps: using serological tests to screen for potential infection available only for TbG and inspecting for clinical signs mostly enlarged cervical lymph nodes; diagnosis is by finding out if the trypanosomes are existent in the body fluids and staging to know the level the disease has progressed to. This involves clinical analysis and in some cases examination of the cerebrospinal fluid acquired from lumbar lesion. Early diagnosis is important to avoid developing to the neurological phase in order to avoid complex and risky treatment processes. A comprehensive and vigorous screening of populations at risk is suggested to avoid long, fairly asymptomatic first phase of TbG sleeping sickness, this is necessary to detect infection early and achieve decline in transmission by their reservoir status. Investing material and human resources is vital to obtain a total and extensive screening, these resources are lacking in Africa especially in rural regions, where the

disease is prevalent, and this may result in mortality of infected people before they can be reached for diagnosis and treatment (WHO, 2019).

2.8 Treatment of Trypanosomiasis

Nature of treatment is contingent on the phase of the disease, and the sooner the disease is noticed, the better the hope of a cure. Drugs used in the first phase is more easy and safe to administer than those used in the second phase. For treatment to be absolute, the patient needs to go for regular checkups for almost two years and this involves body fluid analyses such as cerebrospinal fluid gotten from lumbar lesion, as trypanosomes may still be traceable for a long time and replicate the disease months after treatment (WHO, 2019). Achieving treatment in the second phase of the disease is solely dependent on drugs that cross the blood-barrier wall to get to the trypanosomes. These drugs are complex to administer and very toxic. Five different drugs are used in treating trypanosomiasis and are donated to WHO by producers and shared for free to endemic countries (WHO, 2019). Drugs used for treating the first phase of trypanosomiasis are: Pentamidine; this is caused by TbG. It is well endured by patients, notwithstanding its non-negligible adverse side effects. This drug was discovered in 1940. Suramin; this is also used for treating the first phase of trypanosomiasis caused by TbR. It incites some detrimental effects such as allergies and urinary tract infection. This drug was discovered in 1920.

Drugs used in treating the second phase are: Melarsoprol; TbG and TbR infections can both be treated using this arsenic derived drug, having many adverse effects, especially reactive encephalopathy which can be deadly with 3 % chances out of 10. Various regions in Central Africa has noticed rise in resistance to this drug. In present time, it is advised as a first option for treating *rhodesiense* and second option for treating *gambiense*. This drug was discovered in 1949. Eflornithine; this drug was registered in 1990 and has lower

toxicity when compared to melarsopol. It is very complicated and hard to administer and only efficient against TbG. Nifurtimox; in 2009, a treatment that combines eflornithine and nifurtimox was presented. It made the use of eflornithine simpler by decreasing the period and number of treatment and intravenous perfusions. Unfortunately, it has not been researched for TbR treatment and also not been registered for treatment of HAT but has been registered for treating American trypanosomiasis. Fexinidazole; is a novel comprehensive oral treatment for HAT used for the first phase and mild second phase of the disease and can shorten and ease the treatment of *gambiense* HAT. This drug got an encouraging scientific view from the European Medicines Agency's Committee for Medicinal Products for Human Use and has been incorporated in the WHO Essential medicines list.

2.9 Prevention and Control of Trypanosomiasis

Vaccines are not available for immunity against African sleeping sickness, lately only few medically connected options are available for prevention. However, the chances of contracting the disease from the bite of a tsetse fly is low and assessed at less than 0.1 %, using repellants, full body covering, executing bush clearing methods, keeping away from tsetse-filled regions and scrapping of wild games are best alternatives for preventing infection by inhabitants of affected regions. The pillar of the approach used to control trypanosomiasis includes vector control, consistent surveillance such as discovering and immediate treatment of fresh infections. The best method used by most countries in which this disease is endemic by screening regularly due to infeasibility in case-by-case screening, this may be in form of static or moveable centres, where medical staff journey to everyday. Efforts in screening is very necessary since initial symptoms are not obvious or severe to make persons infected with TbG find medical assistance, especially in rural regions. Furthermore, disease diagnosis is complex and medical workers may not link

these common symptoms with sleeping sickness. Regular screening allows early detection of disease and also eliminates prospective human reservoirs. Globally, only one case of trypanosomiasis transmitted through sexual activities has been documented and it's in West Africa (Paquette, 2019).

2.10 Natural Products used in the Control of Trypanosomiasis

Products naturally gotten from plants having novel potentials to produce new drugs potent against parasites has been reported. Decoctions derived from curative plants have been used since the olden days for treating sleeping sickness. Humans have used these plants and animal derived products for its medicinal and pharmaceutical properties. In regions of tropical medicine, largely used drugs for this disease are sourced from natural products (Mann and Ogbadoyi, 2012).

2.10.1 Alkaloid derivatives

Alkaloids are found in different species of animals, fungi and plants which produces various bioactive alkaloids used in treatment of a wide variety of diseases. They are categorized by natural compounds containing nitrogen. *In vitro* evaluations have been experimented using numerous alkaloids against trypanosomes. Sanguinarine and berberine are two quaternary benzylisoquinoline alkaloids observed in some plant families and quinoline alkaloids found in *Cinchona* bark have been reported to possess significant trypanocidal activity against *Trypanosoma brucei brucei*. An isoquinoline alkaloid called Emetine found in *Cephaelis ipecacuanha* (Rubiaceae), was also reported to have trypanocidal properties and used to treat amoebiasis. Marine organisms possessing some alkaloids like ascidians, tunicates, and sponges have also been assessed for its anti-trypanosomal properties (Thao *et al.*, 2014).

2.10.2 Phenolic derivatives

A prenylated phenol antibiotic called Ascofuranone, was isolated from *Ascochyta visiae* (a fungus) pathogenic to plants, tested and observed to be very effective and an inhibitor of glycerol-3-phosphate-dependent mitochondrial oxygen ingestion of *Trypanosoman brucei brucei* bloodstream forms. This inhibitory specificity is as a result of the existence of an exceptional mitochondrial electron transport system in circulatory forms of trypanosomes. This comprises of 2 enzymes (trypanosomes alternative oxidase and a glycerol-3-phosphate dehydrogenase), which work together through ubiquinone (coenzyme Q) and another enzyme called ubiquinol. Some other phenol derivatives were verified for their anti-trypanosomal efficacy, these includes gallic acid, a popular constituent of hydrolysable tannins, which is also active in procyclic and circulatory forms of *T. b. b.* (Thao *et al.*, 2014).

2.10.3 Quinone derivatives

Quinones like plumbagin, can prompt oxidative pressure in *Trypanosoma congolense* and *Trypanosoma cruzi*. Quinones reduced to semi quinone radicals by enzymes is the possible explanation given for this, like those found in trypanothione reductase, a major enzyme of trypanosomal anti-oxidant thiol metabolism and mitochondrial electron transport chain. Plumbagin, derived from *Drosera* species, is effective on *Trypanosoma brucei brucei* at the mammalian phase, whereas it's lower derivative, trans-isoshinanolone is dormant (Hoet *et al.*, 2004).

2.10.4 Terpene derivatives

Mikus *et al.* (2000) assessed the properties of some sesqui and mono-terpenes which are regularly found in essential oils, and were tested on the feasibility of *T. b. b.* circulatory forms. This experiment reported that, natural C_{13}-norisoprenoid and its 1'-epimerwere

effective on *Trypanosoma brucei brucei* bloodstream trypomastigotes (Busch *et al.*, 1998). Hoet *et al.* (2004), reported the activities of numerous sesquiterpene lactones on the action of *Trypanosoma brucei rhodesiense* blood stream trypomastigotes and the most effective molecules were mexicanin I and helenalin which had IC_{50} values of 0.32 and 0.05 μM. These sesquiterpenes were isolated from *Inula* and *Arnica* species.

2.10.5 Flavonoid derivatives

Vitex simplicifolia leaves from which artemetin was derived, showed prospective trypanocidal activity having an IC_{50} value of 4.7 μg/mL, although, this action approves usage of this plant in treating trypanosomiasis in the olden days, phytochemical findings for trypanocidal activities were not documented (Conrad *et al.*, 2018). Therefore, the plant leaves should be assessed further for compounds that have not been identified. A research on *Ageratum conyzoides* revealed the presence of many flavonoids such as eupalestin, 5' methoxynobiletine, ageconyflavone C, and pentamethoxyflavone which exhibited anti-protozoal activities. *Ageratum conyzoides* was popular in traditional medicine for its significance in treating people infected with trypanosomiasis (Ya *et al.*, 2016).

2.11 Biology of Toads

Toads are characterized by their short legs, dry, leathery skin, and big bumps covering the paratoid gland, it is a known name for some frogs, particularly of the family Bufonidae. They can be found in thick vegetation, cocoa farms and by river sides in primary and secondary forests, some toad species only operate at night and the nature of their colour aids disguising (Vaughan *et al.*, 2019). A distinct difference between toads and frogs has not been made in scientific taxonomy, but it is well known in folk culture, where toads are related to rougher and drier skin, and found mostly on land. Nevertheless, in scientific taxonomy, toads are found in the families' *Bufonidae, Calyptocephalellidae,*

Bombinatoridae, Discoglossidae, Pelobatidae, Myobatrachidae, Rhinophrynidae, Microhylidae, and *Scaphiopodidae* (Vaughan *et al.*, 2019).

Typically, the biggest of the bumps on the toad's skin are those that shell the parotoid glands and are popularly called warts but totally different from pathological warts (Figure 2.4), because of their static size as seen on healthy specimens and definitely not as a result of infection. Just like most amphibians, toads, exhibit philopatry, that is, they return to a particular area to breed. American toads individually return to their place of birth to breed where they are probably going to meet siblings as prospective mates. Though innate cases within a species are likely, siblings hardly mate (Zimkus, 2014).

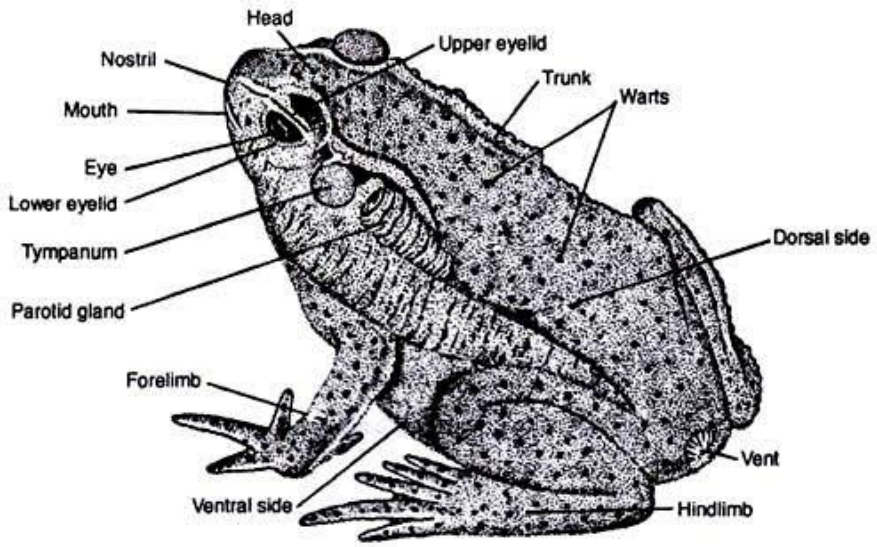

Figure 2.4: An Adult Toad in a Sitting Posture (Source: Zimkus *et al.*, 2014)

Bufotoxins are mildly toxic constituents found in the skin of toads which wards of impending predators, almost all toads and few frogs possess big glands containing poison, found at both side of their head and several small glands located on other parts of their bodies. Production of several kind of toxins and mucus by these parotoid glands makes them slippery to hold, repugnant and lethal. Immediate action of toxins causes a predator

to terminate its action, leading to the escape of the toad, but slow action of toxins may teach the predator to shun that species in future. An adaptive method (aposematism) is used by noxious toads to show their venom in bright colours, such as yellow, orange, or red sometimes with conflicting black markings on their bodies, an example is seen in poison dart frogs in the family Dendrobatidae. Although, some species such as *Allobates zaparo* is not toxic, but imitates the form of two different poisonous species with which it shares a similar range in order to mislead predators.

Table 2.1 entails known names of poisonous toads and frog found in Nigeria such as Perret's toad, Common toad and frog, Dotted reed frog, African toad and tree toad, Victoria forest tree frog (Zimkus, 2014). Some species have their warding off colours at the lower part of the body, which they blaze when confronted, by positioning themselves in a way that reveals the intense colours on their stomach, as seen in *Bombina bombina* (European fire-bellied toad) (Vaughan *et al.,* 2019). Other species such as *Rana catesbeiana*, commonly known as bull frog, squats while the eyes are closed and head pushed forward placing the poisonous glands in a perfect spot, when endangered, while, other glands on its back start to emit toxic secretions protecting the most susceptible region of its body (Vaughan *et al.*, 2019).

Table 2.1: Examples of Poisonous Toads in Nigeria (Source: Vaughan *et al.*, 2019)

S/N	Common name	Scientific name
1	Common frog	*Rana temporaria*
2	Perret's toad	*Sclerophrys perreti*
3	Dotted reed frog	*Hyperolius guttulatus*
4	Common toad	*Bufo bufo*
5	African toad	*Sclerophrys regularis*
6	Victoria forest treefrog	*Leptopelis boulengeri*
7	African tree toad	*Nectophryne afra*

2.12 Toad Venom

In toads, toxins of therapeutic efficacy can be isolated from the paratoid and skin glands, which can be used for treating varieties of diseases (Yang *et al.*, 2015). Venoms secreted from the eggs, liver, ovaries, paratoid glands and granulated glands of their skin are made up of different kind of bioactive alkaloids, majorly bufadienolides, which are cardioactive steroids such as cardenolides, cardiac glycosides from poisonous foxglove plants and also bufogenins like cinobufagin, bufotenine, cinobufotalin and bufalin (Dai *et al.*, 2016). These toxins possess a wide range of biological and defensive roles. Dried exudation from the toad's skin glands have been medically used for more than a thousand years as a local anaesthetic, anodyne cardiotonic, antineoplastic and anti-microbial agents. Various bioactive compounds observed in venoms of toad with numerous biological actions have been researched and studied for several decades, they reveal different structures with chemical properties such as peptides, alkaloids, biogenic amines, proteins and steroids (Baldo *et al.*, 2015).

All toads produce toxins but its efficiency is dependent on each species and its geographical location. These toxins used for self-protection are produced by huge glands

positioned at the dorsal part of the body and posterior to the eyes, the lesser glands also secretes toxins and are situated all over the skin. The toxins emitted are creamy white, thick and very irritating and are rapidly ejected when periglandular muscles of the skin, contracts. Toxins comprises of bufotoxins which hinder sodium networks in nerve resembling the effects of local anesthetics, serotonin, bufagenins, which possess a digitalis-like effects and catecholamines (Dai *et al.*, 2016). Toxins produced by paratoid and skin glands can be processed to serve several purposes such as ointment, as a coating agent, oral solution and injection. Cinobufacini is one of the popularly used commercial preparation, it is a sterilized hot water extract from dried toad skin. Since early 1990s, China's Food and Drug Administration Agency officially approved the use of cinobufacini as a regimen for the treatment of different kind of cancerous disease such as lung, pancreas, colon, liver and also Hepatitis B virus (HBV) (Ji *et al.*, 2014).

2.13 Therapeutic Potency of Toad Venom

A broad variety of invertebrates such as spiders, marine worms, sea anemones, hymenoptera insects, corals, scorpions, jelly fish and marine mollusks possess venomous properties including frogs, lizards, and snakes which are vertebrates. These toxins isolated from animals are complex combinations of ions, proteins, peptides, and neurotoxins and are characterized with receptor-targeted and pharmacologically active molecules that have been 'preoptimized' naturally to be used in clinical chemistry. Most of these chemical combinations are bioactive and range from minor organic molecules to increased molecular weighty proteins, including enzymes (Camila and Thaïs, 2015). Furthermore, animal toxins were used traditionally, that is, in the olden days for treating various human ailments, but the advent of biotechnology has greatly enhanced the efficiency of these toxins by purifying, identifying, and evaluating their therapeutic

functions (McCleary and Kini, 2013). The discovery of drug made of venom in this present day began in the 1970s with the development of an epic anti-hypertensive drug called captopril, whose production was grounded on the configuration of Bradykinin Potentiating Peptides (BPPs) gotten from the venom of a snake called *Bothrops jararaca* (a Brazilian viper). Chemotherapy for diseases caused by protozoa could be developed by screening of venom constituents as they are purely natural and represent above 50 % of pharmaceutical drugs in use presently (McCleary and Kini, 2013). Venoms from toad have been tested and analyzed for its potential use for treating neglected tropical diseases caused by protozoan parasites.

The skin of anurans possess cutaneous glands, which depends on sympathetic nerves to control the release of venom on the dorsal part of the animal body as a reaction to attack or provocation by predators. The substance emitted by these glands are toxic and noxious and play vital functions in regulating respiration, preventing dehydration, protection from predators, and undesirable microorganism multiplication (Azevedo *et al.,* 2011). Since the first isolation of bombinin from the skin of *Bombina variegate* (a yellow-bellied toad), and analyzed to possess anti-microbial properties, and various anti-microbial peptides (AMP), several researches and studies have been done on protozoan targets, which focus specially on dermaseptins (DSs) and to a smaller level phylloseptins, temporines, bombinins and skin peptide YY (Azevedo *et al.,* 2011).

2.13.1 Actions of dermaseptins on parasitic diseases

Dermaseptins (DSs) are a family of peptides isolated from the skin secretion of frogs from the *Phyllomedusa* genus (Azevedo *et al.,* 2011). The structure contains 24 to 34 amino acid elongated cationic molecules that bend into amphiphilic helices when in hydrophobic

media. They apply a cytolytic influence on a wide range of fungi, viruses, and bacteria with little or no toxicity on blood cells of mammals (Pinto *et al.,* 2013). Feder *et al.* (2000) researched on the use of synthesized analogs and dermaseptin to determine the probability that combination is essential for dermaseptin cytotoxicity. The combinations exhibited leishmanicidal activities in the micromolar range, while single compounds had no intense effect on anti-leishmanial activities, whereas, certain dermaseptin derived peptides exhibited improved anti-bacterial effectiveness and decreased haemolytic action. These outcomes back the concept of peptide combination in solution.

Products of dermaseptins also showed anti-malarial activities by preventing the development of *Plasmodium falciparum* by lysis of infected red blood cells, although still maintained a fragile cytotoxic result, with lysis of uninfected red blood cells (Krugliak *et al.,* 2000). These peptides were more active against trophozoite progress and less against the parasite ring phase. However, no compound was selective of phase and revealed same inhibitory action against young, ring-phase, and more grown trophozoites (Dagan *et al*., 2002; Efron *et al.,* 2002).

Some dermaseptins have been isolated and purified for their cytolytic action against protozoa and bacteria, these dermaseptins were from *Pithecopus oreades* and *Pithecopus hypocondrialis* (Brand *et al.,* 2006; Savoia *et al.,* 2008). From *Pithecopus oreades*, a 29 residue lengthy peptide was first isolated and had a mass molecule of 2.8 kDa, later it was seen in *Pithecopus hypocondrialis*, which showed trypanocidal activities against *Trypanosoma cruzi* trypomastigote and epimastigote forms and also leishmanicidal activities against *Leishmania amazonensis, Leishmania major*, and *Leishmania. chagasi* promastigotes (Brand *et al.,* 2006) with little cytotoxic effects on red and white blood cells (Savoia *et al.,* 2008; Zampa *et al.,* 2009).

Pinto *et al*. (2013) investigated the efficacy of dermaseptins, which were isolated from the emission of *Pithecopus nordestina*. The two peptides exhibited positive activities against *Trypanosoma cruzi* trypomastigotes by totally clearing them at 0.25 to 0.68 mM concentrations, but showed no leishmanicidal action against *Leishmania infantum*. Pérez-Cordero *et al*. (2011) researched on the action of a dermaseptin peptide and some AMPs isolated from some venoms, they revealed the inhibition of *Leishmania panamensis* and *Leishmania major* by stopping the growth of promastigotes and amastigotes.

2.13.2 Phylloseptin activities on parasitic diseases

These were first isolated from the skin emissions of *Pithecopus oreades* and *Pithecopus hypochondrialis* (Leite *et al.,* 2005). *Pithecopus burmeinsteri, Pithecopus bicolor, Pithecopus azurea, Pithecopus tomopterna, Pithecopus rohdei, Pithecopus nordestina,* and *Pithecopus tarsius,* also possess phylloseptins (PSs) in their skin (Azevedo *et al*., 2011; Pinto *et al.,* 2013). Only their anti-microbial action has been noted against protozoa, bacteria, and fungi, with substantial toxicity at elevated concentrations in mammalian cells (Leite *et al*., 2005). Till date, only, phylloseptins isolated from *Pithecopus oreades* (Leite *et al.,* 2005), and phylloseptins from *Pithecopus nordestina* have been confirmed against trypomastigotes of *Trypanosoma cruzi*. *Leishmania infantum* promastigotes were also tested on by phylloseptins, and exhibited lesihmanial activities with an IC_{50} of 10.6 mM (Pinto *et al.,* 2013), making leishmania promastigotes almost 30 times more resistant to than *Trypanosoma cruzi* trypomastigotes.

2.13.3 Effect of temporins on parasitic diseases

Temporins comprises a big family of a-helical AMPs secreted by the skin of New World ranid frogs and Eurasian (Abbassi *et al.,* 2013). Temporins have been tested against the activities of gram-positive bacteria, viruses, and methicillin-resistant *Staphylococcus*

aureus. Only a few temporins are active against gram-negative bacteria, yeasts and fungi and this is due to a net charge of +3 (Abbassi *et al.*, 2013). A major importance of temporins is the lack or almost devoid of toxicity to mammalian cells.

Up till date, just three studies assessed the anti-protozoal action of temporins. *Rana temporaria* (European red frog) skin emissions revealed anti-leishmanial activities against promastigotes of *Leishmania donovani* and amastigotes of *Leishmania pifanoi*, at micromolar range and had no cytolytic activity against red blood cells in humans and also protected biological function in serum. The isolated compounds were temporins A and B, 13-amino-acid AMPs which activated deaths of parasite cell by puncturing the plasma membrane. The skin of *Pelophylax saharica* (Saharan frog) was isolated to produce temporins which exhibited an extensive range of activities against yeasts, bacteria, and filamentous fungi (Abbassi *et al.*, 2013).

2.13.4 Action of bombinins and bombinins H

Bombina species secretes anti-microbial peptides such as Bombinins and bombinins H from their skin (Simmaco *et al.*, 2009), they are efficient against Gram-positive and negative bacteria, and *Candida albicans* with no significant lysis, although bombinins H generally have low anti-bacterial action and high lysis activity (Simmaco *et al.*, 2009). Bombinins H were grouped into several groups of different peptides, where bombinins H2 and H4 were effective against amastigotes of *Leishmania pifanoi* and promastigotes of *Leishmania donovani* at micromolar ranges, though, the LC_{50} values of H4 were very much lower than those of H2 (Mangoni *et al*., 2006).

2.13.5 Skin polypeptide YY (SPYY)

Genus *Phyllomedusa* produces Skin Polypeptide YY (SPYY), which is the only peptide pharmacologically and architecturally associated to the Neuro Peptide Y (NPY) family

(Azevedo *et al.,* 2011). A comprehensive variety of essential regulatory purposes including, circadian rhythm regulation, sympathetic vascular control, automatic function, and central regulation of endocrine are facilitated by these peptides and are assumed to prompt numerous biological actions, by triggering specific membrane-bound receptors (Azevedo *et al.,* 2011). These peptide exhibits anti-microbial activities against gram-negative and positive bacteria and fungi. SPYY also stopped development of amastigotes and promastigotes of *Leishmania major* and had no toxic effect on red blood cells.

2.13.6 Effect of bufadienolides on parasitic diseases

Bufadienolides have been reported to contain anti-tumor and anti-microbial properties, they are cardioactive C-24 steroids obtained from both animals and plants (Gao *et al.,* 2011). The compounds isolated from paratoid glands of a toad (*Rana jimi*) included bufadienolide, hellebrigenin, and telecinobufagin and they all exhibited anti-parasitic efficacy (Tempone *et al.,* 2008). Telocinobufagin had the ability to halt the development of amastigotes and promastigotes of *Leishmania chagasi* but was ineffective on trypomastigotes of *Trypanosoma cruzi*. Whereas, hellebrigenin had both leishmanicidal and trypanocidal properties. They also had to no cytotoxic effect on the red blood cells and peritoneal macrophages of mice, for both steroid.

A bio-prospective anti-proliferative action in extracts of toads (*Rhinella marina* and *Rhaebo guttatus*) was conducted by analyzing the venom extracts through Liquid Chromatography- Mass Spectrometry (LC–MS) and High Performance Liquid Chromatography (HPLC). Four bufadienolides comprising of telocinobufagin, marinobufagin, bufalin, and resibufogenin were revealed from *Rhinella marina*, while *Rhaebo guttatus*, comprised of marinobufagin only. Each extract displayed cytotoxicity, where *Rhinella marina* extracts were similar to doxorubicin, only *Rhaebo guttatus* extract

caused permeability in the membrane of human red blood cells. The extracts were examined for their selective action using Alamar Blue assay to determine their influence on human peripheral blood mononuclear cells (PBMC). They exhibited up to 80-fold extra selectivity against leukemia cells when related to segregating white blood cells. In order to confirm these anti-proliferative activities, bromodeoxyuridine (BrdU) was integrated into Deoxyribonucleic acid (DNA) and was measured in HL-60 (human leukemia) cells treated with *Rhinella marina* extracts. These extracts reduced the absorption of BrdU at each concentrations. Conclusively, nine extracts of *Rhaebo guttatus* and *Rhinella marina* venom indicated prominent fatal and selective effects on tumor lines, particularly extracts of *Rhinella marina,* emphasizing the importance of exudations from toad parotoid gland as a potential basis for novel lead anti-cancer compounds (Ferreira *et al.,* 2013).

Past *in vitro* analyses proved the multiplication of bufadienolides such as rhamnoside, gamabufotalin, bufotalin, epoxy-marinobufagin, hellebrin, bufalin, hellebregenin, resibufogenin 3- acetate, marinobufagin, cinofagin, and telocinobufagin that might be toxic to living cells (Sciani *et al.,* 2013). They were isolated from skin emissions of *Rhinella Bufo* and *Rhaebo* species, extracted from plants namely *Urginea maritima, Urginea aphylla, Urginea maritima* and *Urginea hesperia,* and a Chinese drug (Ch'an Su) that was used traditionally, revealing activities against tumor lines like, colon, leukemia, melanoma, breast, prostate, nervous system and primary liver carcinoma (Cunha-Filho *et al*., 2010; Banuls *et al.*, 2013).

Various toad species found in the genus *Bufo*, possess a chemical component called bufotenin, present in their eggs and venom but more common in *Incilius alvarius* previously known as *Bufo alvarius* (Colorado River toad) which is the only toad species, having bufotenin in adequate quantities for a psychoactive effect (Dai *et al.,* 2016).

Digoxin-like cardiac glycosides in tally with bufotenin are also found in *Bufo* species and consumption of the venom can be lethal. Intake of eggs and venoms by humans gave rise to numerous documented cases of poisoning, some of which lead to death (Baldo *et al.*, 2015).

Present-day reports showed that toad venoms comprises of bufotenin that was used as a street drug, that is, a hypothetical aphrodisiac, meaning, it is not an aphrodisiac but absolutely a deadly poison, consumed orally in the form of chan' su as a psychedelic (Dai *et al.*, 2016), and was also smoked when formed from dried *Bufo* skin. Usage of chan'su and love stone; an associated toad venom preparation used as an aphrodisiac in some sub regions of North America, has ensued in numerous cases of poisoning and has a resulted in a minimum of one death (Baldo *et al.*, 2015).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Ethical Clearance

Ethical clearance for the use of experimental animal with reference number, NSVH/MLF/20/V0L1/07 (appendix A), was obtained from the Ministry of Livestock and Fisheries, Minna, Niger State.

3.2 Collection of Toads

Forty (40) toads were collected from several locations in Federal University of Technology, Bosso campus, Minna, Niger State (Figure 3.1), with temperature and humidity within 24.7 ^{o}C - 25.5 ^{o}C and 80 % - 85 %. They were collected at dawn around 4:00 am to 6:00 am from swampy water logged areas into a plastic container with adequate aeration and returned to their various habitat after extraction. Plate I shows a specimen sample which was preserved as a voucher and deposited in the Laboratory.

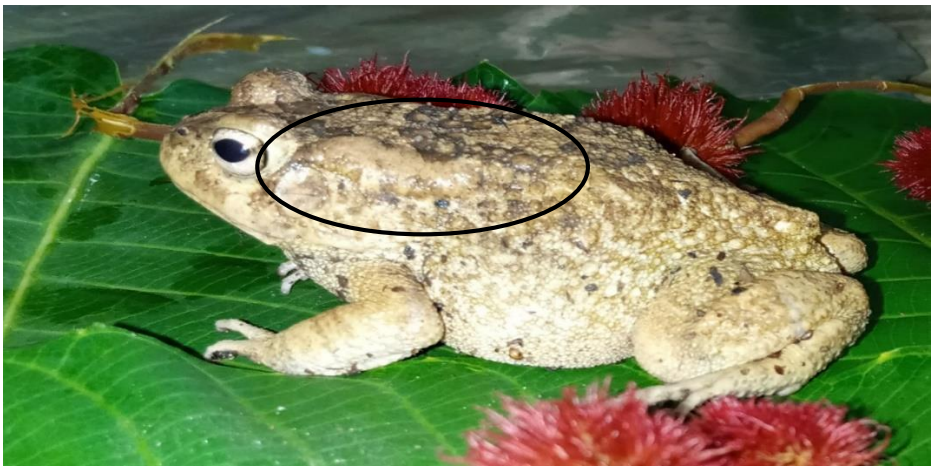

Plate I: A Toad (*Sclerophrys maculata*) with Visible Paratoid Gland (Source: Field Photograph)

3.3 Species Identification

The toads collected were 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 striped toad, of the family Bufonidae.

3.4 Collection of Experimental Animals

Sixty (4-6 weeks old) wistar rats weighing between 120 to 160 g (Plate II) were acquired from Nigeria Institute for Trypanosomiasis Research (NITR), Vom, Plateau State. They were kept under standard laboratory settings, fed pelleted diet and given clean water (Abdulazeez *et al*., 2013). The animals were allowed to acclimatize for a period of 2 weeks before commencing the experiment. The principle guiding laboratory animals' use and care was strictly followed during experiment as described by the Canadian Council on Animal Care (CCAC, 2020).

Plate II: Experimental Wistar Rats

3.5 Collection and Maintenance of *Trypanosoma brucei brucei* (*T. b. b.*)

Trypanosoma brucei brucei parasite was acquired from NITR, Vom, Plateau State and preserved in the Biochemistry department's animal house, Federal University of Technology Minna, and maintained by continuous passage of infected blood from donor rats into the normal rats (Abdulazeez *et al.*, 2013).

3.6 Toad Venom Extraction

Toads were cleaned with cotton wool immersed in distilled water to avoid contamination during venom collection. Plate III shows the process of extraction achieved by massaging and pressing of paratoid macroglands to release a whitish and sticky substance known as the venom, collected into a clean petri dish (Plate IV) and lyophilized (freeze dried) after which it was dispatched into eppendorf tubes (Plate V) and stored in a freezer (-20 ^{o}C) (De Medeiros *et al.*, 2019). 0.25 gram of the lyophilized venom was mixed with 10 ml of distilled water to obtain the desired stock solution.

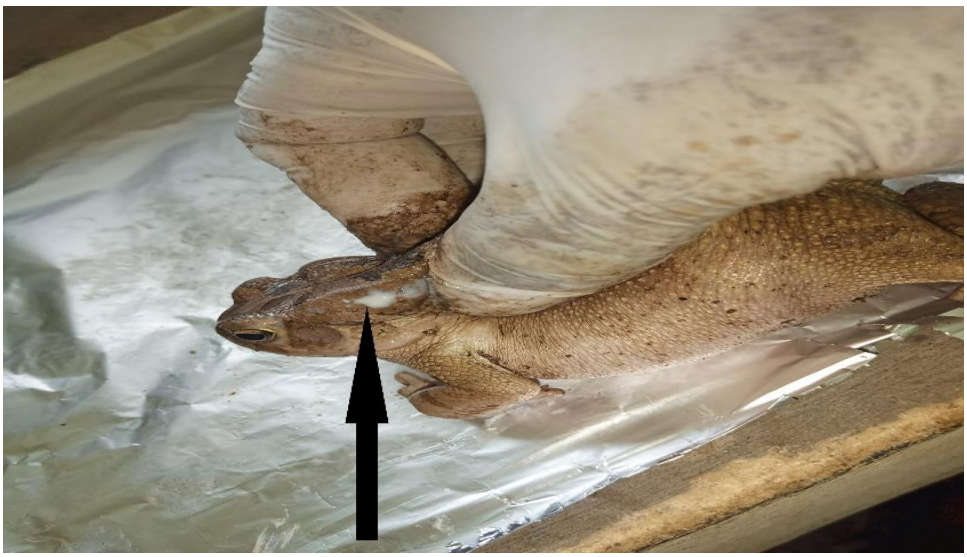

Plate III: Venom Emission from the Paratoid Gland (Source: Field Photograph)

Plate IV: Collection of Venom into a Clean Petri Dish

Plate V: Freeze Dried Venom in Eppendorf Tubes

3.7 Zoochemical Analysis of Crude Toad Venom Extract (CTV)

Zoochemical analysis of CTV was conducted to assess the occurrence or lack of zoochemicals as described below:

3.7.1 Carbohydrates (molisch's test): To each venom ration, droplets of molisch's reagent was added and disbanded in distilled water, after which, 1 ml of concentrated H_2SO_4 was added by the test tube's side. After which it was allowed to stand for 2 mins before being diluted with 5 ml of distilled water. A red or dull violet colour at the interphase of the two layers showed a positive result (Sofowora, 1993).

3.7.2 Tannin test: 10 ml of distilled water was mixed with approximately 0.5 g of and then sieved, to 2 ml of the filtrate, drops of 1 % ferric chloride solution was added. The formation of a blue-green, green or blue-black precipitate shows the occurrence of tannins (Trease and Evans, 2002).

3.7.3 Anthraquinones (borntrager's test): 10 ml of benzene was thoroughly mixed with 0.2 g of venom and sieved, 5 ml was taken from 10 % ammonia solution and then added to the sieved solution and subsequently shaken. The presence of a violet, red or pink colour in the ammoniacal (lower) phase depicts the existence of free anthraquinones (Sofowora, 1993).

3.7.4 Steroids (liebermann-buchard test): 2 ml of acetic acid was added to 0.2 g of the venom, the mixture was then cooled in ice for a sufficient time, after which concentrated H_2SO_4 was carefully added. The change in colour from violet to bluish-green or blue showed the occurence of a steroidal ring, that is, a glycone ration of cardiac glycoside (Sofowora, 1993).

3.7.5 Terpenoid test: A tiny portion of the venom was liquefied in ethanol, followed by the addition of 1 ml of acetic anhydride and concentrated H_2SO_4. A colour change from pink to violet displayed the occurrence of terpenoids (Sofowora, 1993).

3.7.6 Saponin test: 1 g of the venom and 5 ml of distilled water was boiled and sieved. 3 ml of distilled water was further added to the sieved solution and shaken actively for roughly 5 mins. Continued frothing while warming served as indication for the presence of saponins (Sofowora, 1993).

3.7.7 Flavonoids (shinoda's test): Approximately 0.5 g of venom was liquefied in ethanol, warmed and then sieved. To the sieved solution, three pieces of magnesium chips was added followed by droplets of concentrated HCl. The exhibition of red to purple, pink and orange colouration showed the presence of flavonoids (Trease and Evans, 2002).

3.7.8 Alkaloid test: Little amount of venom was mixed with 5 ml of 1 % aqueous HCl on water bath which was then sieved and then 1 ml was collected separately into two test tubes. Droplets of dragendorff's reagent was added to the first test tube; indication of orange-red precipitate was taken as positive. To the second test tube, 1 ml of Mayer's reagent was added and the formation of buff-coloured precipitate will be a sign for the occurrence of alkaloids (Sofowora, 1993).

3.7.9 Cardiac glycoside (liebermann's test): 2 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. The presence of a green color showed a unit of a glycone, steroidal part of glycosides (Sofowora, 1993).

3.7.10 Phenol test: Ferric chloride test was used to determine the presence of phenol. 0.5 g of venom was boiled in 30 ml distilled water and sieved. 2 ml of the sieved solution was mixed with 5 % $FeCl_3$. Formation of blue, green, or violet color showed the presence of phenolic compounds (Trease and Evans, 2002).

3.8 Gas Chromatography and Mass Spectrometry (GC-MS)

Toad Venom was sent for GC-MS analysis at Shimadzu Training Centre for analytical instruments (STC), Lagos. The analysis was carried out with GCMS-QP2010SE, Shimadzu Japan. The GC-MS device includes an injector and GC interfaced to mass spectrophotometer. The GC was conditioned in this format: Helium as carrier gas flow rate (1.56 ml/min), Column oven temperature (60 ^oC), injection temperature (200 ^oC), injection mode split ratio (1:1), the system was temperature programmed from 60 ^oC (at 10 ^oC/min) to 160 ^oC (held for 2 mins) then at (10 ^oC/min) to 250 ^oC and the injection volume was 0.5 µL while the mass spectrophotometer was conditioned as follows: Interface temperature 250 ^oC solvent cut time 4.5 mins and ion source temperature 200 ^oC and acquisition was in the scan mode.

3.9 Acute Toxicity (LD_{50}) of the Crude Toad Venom Extract

The acute toxicity of the CTV 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 shared into 4 groups (Groups I, II, III and IV) of three (3) rats each. Rats in groups I, II, III and IV were administered 5, 10, 20 and 25 mg/kg b. w. of the CTV, respectively. In the absence of mortality and sign of toxicity after 24hrs, phase two was conducted. In the phase two, twelve (12) rats were also distributed into 4 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. w. of the CTV, respectively.

3.10 Anti-trypanosomal Bioassay of the Crude Toad Venom Extract

3.10.1 Experimental design

Therapeutic potency of the CTV was carried out according to a modified method of Debela *et al.* (2020) in a complete randomized experimental design. For this purpose, twenty-eight (28) rats were randomly shared into seven groups of four rats each. Blood from a highly parasitized rats was obtained from the tail into a clean glass container, and then few drops of phosphate buffered saline glucose was added and mixed. Healthy rats in Groups I, II, III, IV, V, and VI were infected intraperitoneally with 0.2 ml of the mixed solution (Bulus *et al.*, 2012) while Group VII (normal control) were not infected. Treatment commenced 72 hours after inoculation when parasites were detected in the blood stream of inoculated rats and lasted for five (5) days as follows: Group I**;** infected with *T. b. b.* and treated with 5 mg/kg b. wt. of CTV, Group II; infected with *T. b. b.* and treated with 10 mg/kg b. wt. of CTV, Group III; infected with *T. b. b.* and treated with 20 mg/kg b. wt. of CTV, Group IV; infected with *T. b. b*. and treated with 30 mg/kg b. wt. of CTV, Group V; infected with *T. b. b.* and treated with (5 mg/kg b. wt.) diminazene aceturate (DMZ) (Berenil) (Standard/Positive control), Group VI; infected with *T. b. b.* and not treated (Negative control) administered normal saline, Group VII; not infected with *T. b. b.* and not treated (Normal control). Each rat was monitored after treatment till its death and survival time was also recorded. The *in vivo* efficacy was determined based on the variations in the intensities of parasitaemia, weights and packed cell volume of the animals during the trial period.

3.10.2 Effect of crude toad venom extract on parasitaemia level in *Trypanosoma brucei brucei*-infected wistar rats

The parasitaemia levels were examined using a direct method. The infected rats were tested via the rapid matching method (Debela *et al.,* 2020). Briefly, the tail of an infected animal was punctured using lancet and a clean slide was used to drop the blood on, then a cover slip was placed on it and viewed under microscope at X40 magnification. The quantity of parasites was then counted at four separate fields of the microscope (Bulus *et al.,* 2012).

3.10.3 Effect of crude toad venom extract on packed cell volume in *Trypanosoma brucei brucei*-infected wistar rats

The PCV of each rat was determined before and after infection, and after treatment. Blood was obtained by bleeding tail vein of rat in heparinized microhaematocrit capillary tubes. These tubes were positioned in a microhaematocrit centrifuge with closed ends outwards which were sealed by a crystal seal. The blood from each rat was centrifuged at 12, 000 rpm for 5 mins (Mzena *et al.,* 2018). The quantity of the total blood and erythrocytes was measured and PCV calculated according to Mengistie formula in equation 1 below:

$$PCV = \frac{\text{Volume of erythrocytes in a given volume of blood}}{\text{Total Blood Volume}} \times 100 \quad \text{..... (Equation 1)}$$

Where PCV = Packed cell volume

3.10.4 Effect of crude toad venom extract on body weight of *Trypanosoma brucei brucei*-infected wistar rats

The body weight (g) of each rat in all the groups was measured before infection (day 1) after infection (day 3) and after treatment (day 7), the different body weights were measured using a sensitive digital weighing balance (Mengistie *et al.,* 2012).

3.10.5 Effect of crude toad venom extract on mean survival time of *Trypanosoma brucei brucei*-infected wistar rats

The mean survival time (MST) for each rat was documented by day-to-day checking of the cages for cases of any mortality, this was done for six (6) weeks after treatment. The figure of deceased rats was noted all through the experimental period. The mean survival time was then calculated (Equation 2) (Mengistie *et al.,* 2012).

$$MST = \frac{\text{Sum survival time for all rats in a group (days)}}{\text{Total number of rats in that group}} \times 100 \quad \text{..... (Equation 2)}$$

Where MST= Mean survival time

3.10.6 Effect of crude toad venom extract on relative organ body weight of *Trypanosoma brucei brucei*-infected wistar rats

Rats were sedated and sacrificed, internal organs comprising the heart, kidneys, liver, spleen, and lungs, were cautiously removed and weighed. These organs were then preserved immediately in 10 % buffered formaldehyde solution for histopathological studies (Mayur *et al.,* 2017). Each rat's relative organ weight was calculated using the formula in equation 3 below:

$$ROW = \frac{organ\ weight\ (g)}{body\ weight\ of\ animal\ on\ sacrifice\ day\ (g)} \times 100 \quad \text{..... (Equation 3)}$$

Where ROW= Relative Organ Weight

3.10.7 Effect of crude toad venom extract on biochemical parameters in *Trypanosoma brucei brucei*-infected wistar rats

Two rats from each group were sedated by ether suffocation and pooled blood was collected by cardiac puncture on day 8 into plain tubes to obtain sera for biochemical examination. Levels of creatinine, Alanine-amino Transferase (ALT), Aspartate-amino Transferase (AST), and Alkaline Phosphatase (ALP), were evaluated in the control and treated groups respectively (Kazi *et al.,* 2018). The action of all serum enzymes was determined using agappe kits according to the manufacturer's instruction.

3.10.8 Effect of crude toad venom extract on haematological parameters in *Trypanosoma brucei brucei*-infected wistar rats

A sufficient amount of blood was obtained from rats in each group into Ethylene Diamine Tetraacetic Acid (EDTA) bottles for haematological studies. Parameters such as Red Blood Cell (RBC) counts, Haemoglobin (HB) concentration, Total White Blood Cell (TWBC), Platelets Counts (PLC), Mean Corpuscular Volume (MCV) and Mean Corpuscular Haemaoglobin Concentration (MCHC) were estimated by a fully automated Abacus Junior (Amatya *et al.*, 2017).

3.11 Histopathological Studies

Liver and kidneys were washed with normal saline, and dehydrated with ethanol. Ethanol was removed to ease molten paraffin wax penetration at 55 ^{o}C by passing the tissues through a xylene solution, and afterwards, fixed in wax block. 6 µ thickness of paraffin units were cut out with rotary microtone and dropped on clean glass slides. Conclusively, haematoxylin and eosin were used to stain the units (Kazi *et al.,* 2018). The stained slides were examined using light microscope at ×40 magnification where the tissues samples were captured.

3.12 Data Analysis

The data gathered from parasitaemia count, PCV, body weight change, mean survival time, relative organ body weight, biochemical and haematological parameters were subjected to One-Way Analysis of Variance (ANOVA) using Statistical Package for Social Sciences (SPSS Version 20.0). Datas were presented as mean ± standard error of Mean (SEM). Significant differences between means were determined using Duncan Multiple Range Test (DMRT) at 5 % level of significance

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Results

4.1.1 Zoochemical compositions of crude toad venom extract (CTV)

Zoochemical analysis of crude extract of toad venom showed the presence of phenols, tannins, flavonoids, cardiac glycoside, carbohydrate, terpenoids and absence of saponins, alkaloids, steroids and anthraquinones (Table 4.1)

4.1.2 Gas chromatography and mass spectrometer analysis of crude toad venom

The GC-MS analysis of the toad venom is represented in table 4.2 and figure 4.1. 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 was Dimethyl phthalate with retention time of 11.327 mins followed by Dodecanoic acid with 12.887 mins retention time, Sulfurous acid and 2-propyl tridecyl ester had a retention time of 14.133 mins, while the last compound identified with longest retention time is 5, alpha. -Androstane-3.beta., 17.beta.-diol (21.881 mins). 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: Zoochemical Composition of Crude Toad Venom Extract

Zoochemicals	Inferences
Phenols	+
Flavonoids	+
Alkaloids	-
Tannins	+
Steroids	-
Cardiac glycoside	+
Carbohydrate	+
Saponins	-
Anthraquinones	-
Terpenoids	+

KEYS: +; presence, -; absence

Table 4.2: Chemical Composition of Toad Venom 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 ester
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

Peak#	Retention Time (m)	Peak Area (%)	Height (%)	Compound Name
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

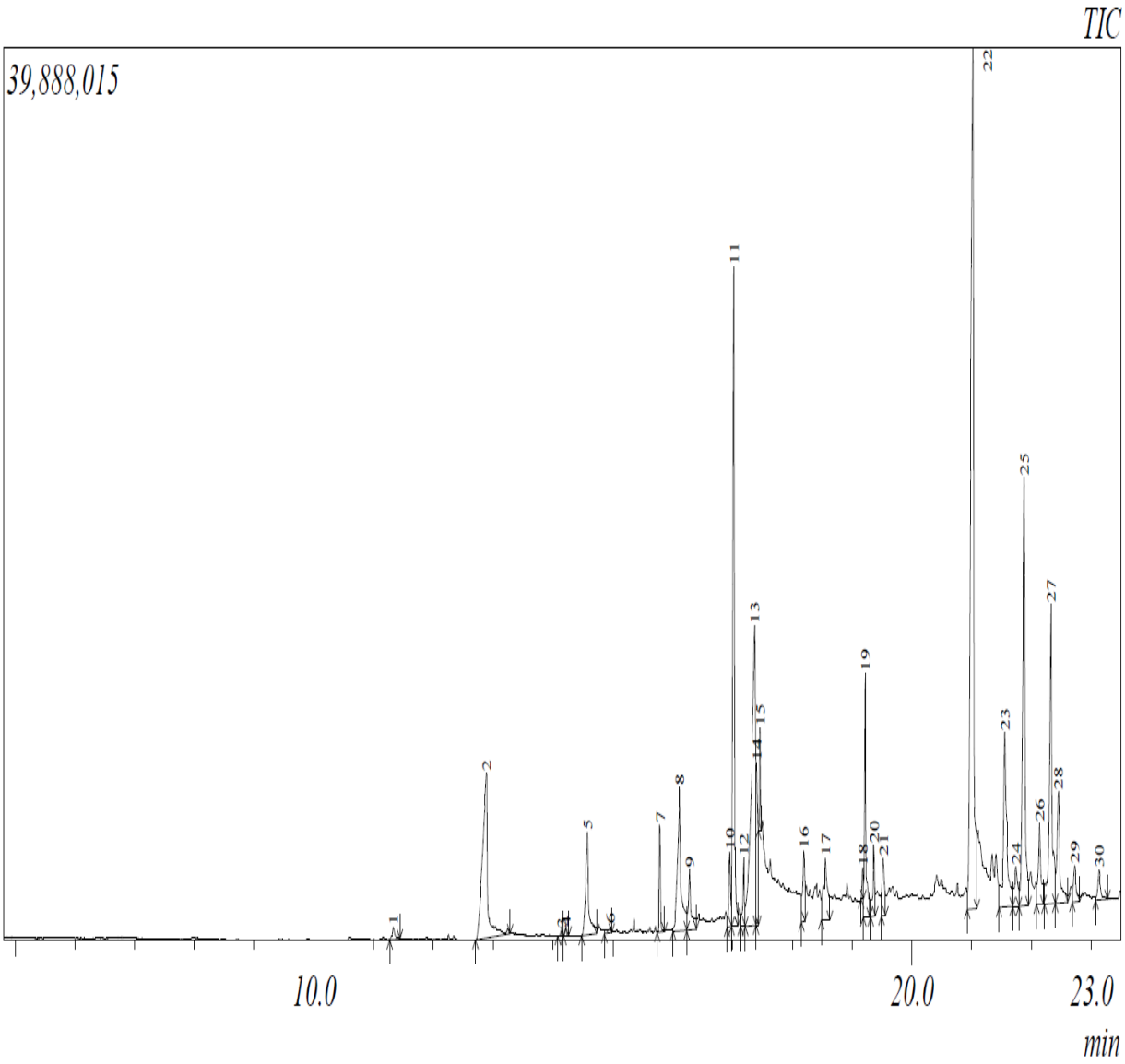

Figure 4.1: Gas Chromatography and Mass Spectrometer Chromatogram of Toad Venom

4.1.3 Acute toxicity of crude toad venom extract

The CTV revealed that the animals tolerated the extract at doses 5, 10, 15, 20, 25 and 50 mg/kg b. wt. with no observable adverse effect at single oral administration. However, at a 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 a higher dose of 200 mg/kg b. wt. and 500 mg/kg b. wt. where sluggishness, hyper cardia, erythema were observed and consequently 1 and 2 animal mortality were recorded at 200 and 500 mg/kg b. wt. dosing respectively. The LD_{50} of crude extract of toad venom was extrapolated to be 141.42 mg/kg b. wt. (Table 4.3).

4.1.4 Effect of crude toad venom extract on parasitaemia counts in *Trypanosoma brucei brucei*-infected wistar rats

Infection of rats with *T. b. b.* parasite caused progressive increase in *T. b. b.* replication. However, treatment of the infected rats with crude extract of toad venom at concentrations of 10 mg/kg b. wt. and 20 mg/kg b. wt., significantly ($p < 0.05$) reduced the parasite replication to 15 and 10 counts/ml on the 5^{th} day of treatment respectively, when compared to the untreated control and those treated with 5 mg/kg b. wt. and 30 mg/kg b. wt., where the parasites multiplied to 50 counts/ml on the 5^{th} day in both groups. Infected rats treated with diminazene aceturate almost totally eliminated the parasite from blood circulation, having 4 counts/ml on the 5^{th} day (Figure 4.2).

Table 4.3: Acute Toxicity Outline of Crude Toad Venom Extract in Wistar Rats

Dose (mg/kg b. wt.)	No 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

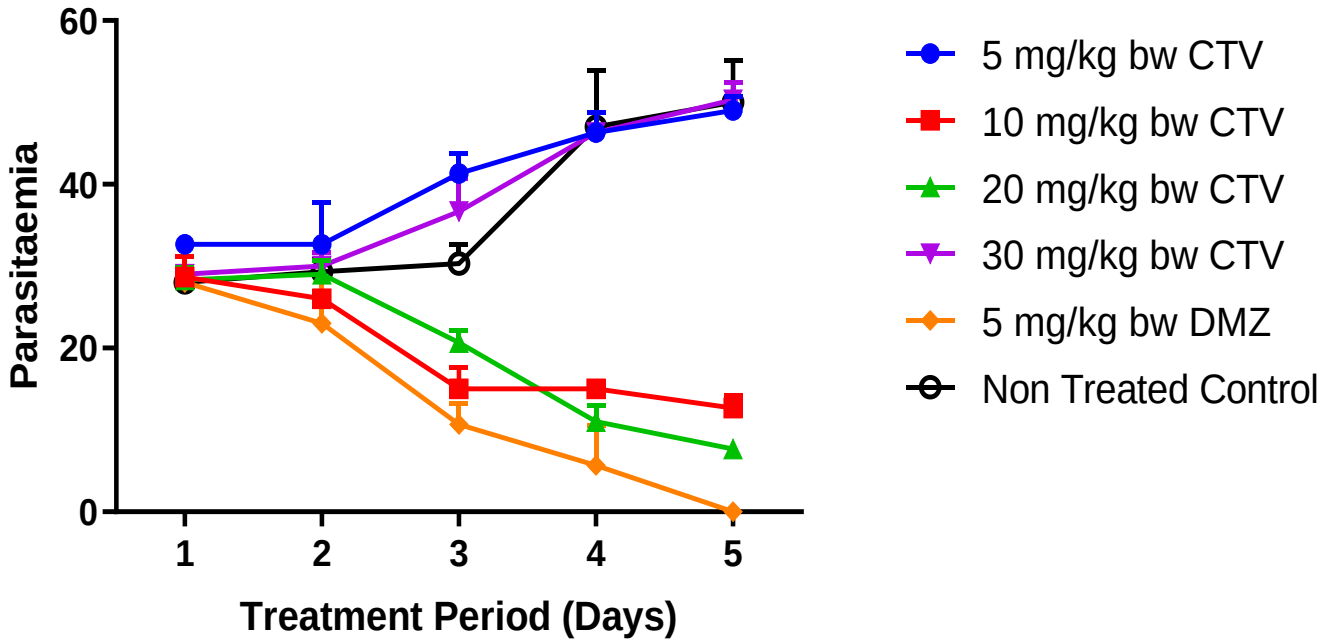

Figure 4.2: Effect of CTV on Parasitaemia Counts in *Trypanosoma brucei brucei*-Infected Wistar Rats

Key: CTV; crude toad venom extract, DMZ; diminazene aceturate

4.1.5 Effect of crude toad venom extract on packed cell volume in *Trypanosoma brucei brucei*-infected wistar rats

There was significant ($p < 0.05$) decrease in packed cell volume (PCV) in *T. b. b.*-infected non-treated rats when compared with the normal control, the rats had 30 % of blood before inoculation, 25 % after inoculation and 22 % after treatment. Similarly, rats treated with 5 mg/kg b. wt. and 30 mg/kg b. wt. also exhibited loss of PCV when compared with other treatment group. Although rats treated with 10 mg/kg b. wt. of CTV showed significant increase in PCV with 33 % of blood before inoculation; 32 % after inoculation; and 35 % after treatment, while rats treated with 20 mg/kg b. wt. of CTV also had significant effect in rise of PCV with 31 % of blood before inoculation; 35 % after inoculation; and 34 % after treatment. The group treated with 5 mg/kg b. wt. of diminazene aceturate had the highest rise in PCV when compared to other treated groups with 30 % before inoculation, 31 % after inoculation, and 36 % after treatment (Figure 4.3).

4.1.6 Effect of crude toad venom extract on body weight of *Trypanosoma brucei brucei*-infected wistar rats

Rats infected with *T. b. b.* parasite caused significant loss of body weight from 125 g to 110 g, when compared with the normal control. However, treatment with the crude extract of toad venom at all concentrations did not cause any significant ($p > 0.05$) loss of body weight when compared with the normal control. However, rats treated with 5 mg/kg b. wt. of diminazene aceturate increased the body weight gain of the animals after treatments from 125 g to 150 g (Figure 4.4).

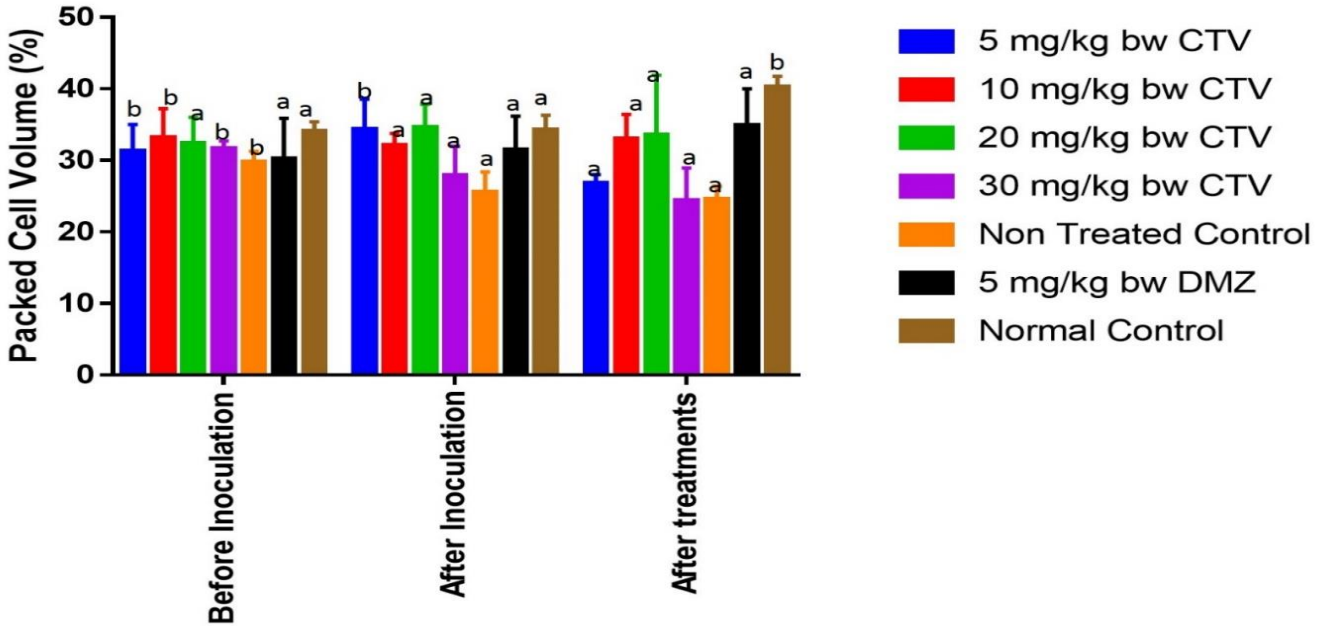

Figure 4.3: Effect of CTV on Packed Cell Volume in *Trypanosoma brucei brucei*-Infected Wistar Rats

Key: CTV; crude toad venom extract, DMZ; diminazene aceturate. The superscript alphabet indicates the significant differences ($p < 0.05$) among the treated groups. d > c > b > a

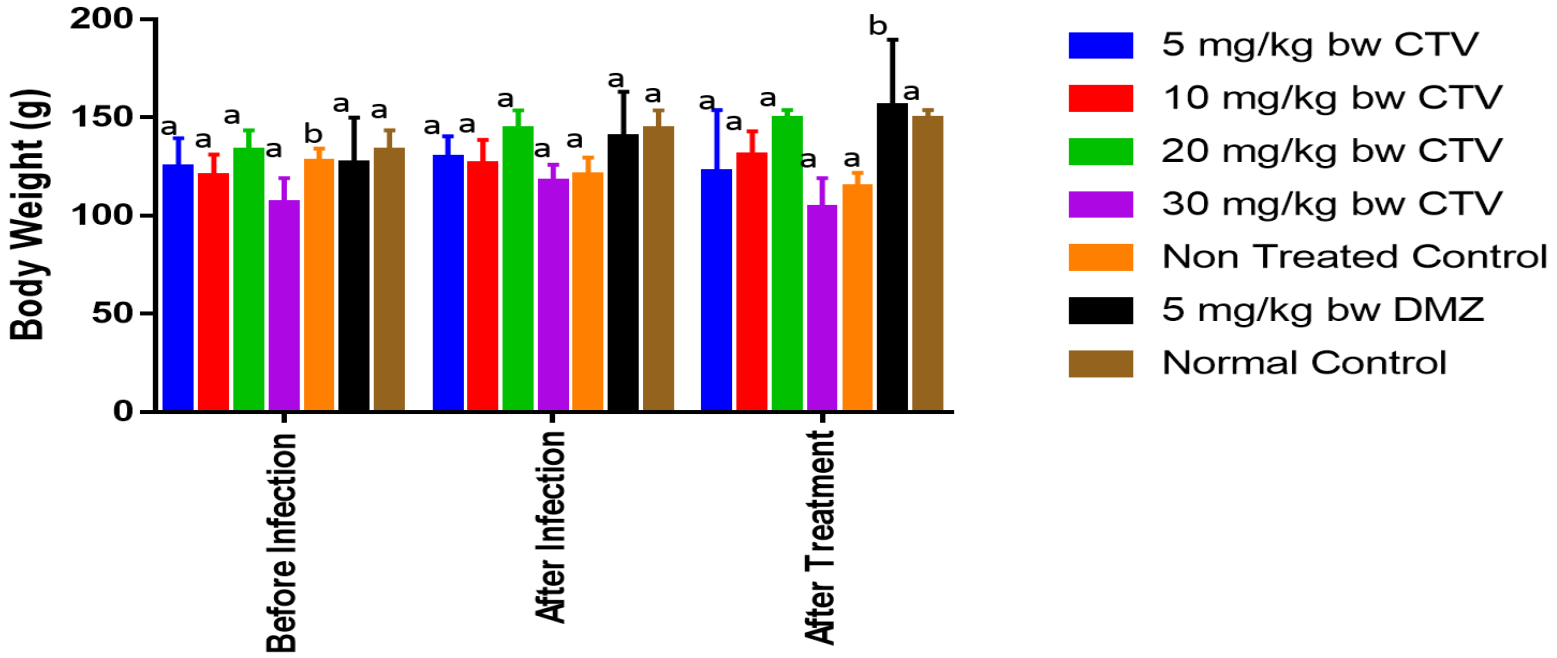

Figure 4.4: Effect of CTV on Body Weight in *Trypanosoma brucei brucei*-Infected Wistar Rats.

Key: CTV; crude toad venom extract, DMZ; diminazene aceturate. The superscript alphabet indicates the significant differences ($p < 0.05$) among the treated groups. d > c > b > a

4.1.7 Effect of crude toad venom extract on survival rate of *Trypanosoma brucei brucei.*-infected wistar rats

T. b. b.-infected non-treated rats had the least survival rate of 6 days comparable ($p > 0.05$) with rats treated with 5 mg/kg b. wt. and 30 mg/kg b. wt. of toad venom extract with a rate of 7 and 8 days respectively. However, rats treated with 10 and 20 mg/kg b. wt. of crude toad venom extract had 19 and 24 days of survival respectively, which was significantly ($p < 0.001$) higher when compared with the non-treated control. Rats treated with diminazene aceturate had the longest survival time of 32 days (Figure 4.5).

4.1.8 Effect of crude toad venom extract on relative organ body weight of *Trypanosoma brucei brucei*-infected wistar rats

There were no significant differences ($p > 0.05$) in the relative liver, lung, kidney, heart and spleen body weight ratio of *Trypanosoma brucei brucei*-infected non-treated rats, infected treated with diminazene aceturate and infected treated with toad venom of 5 mg/kg b. wt., 10 mg/kg b. wt., 20 mg/kg b. wt., and 30 mg/kg b. wt. when compared with the normal control (Table 4.4).

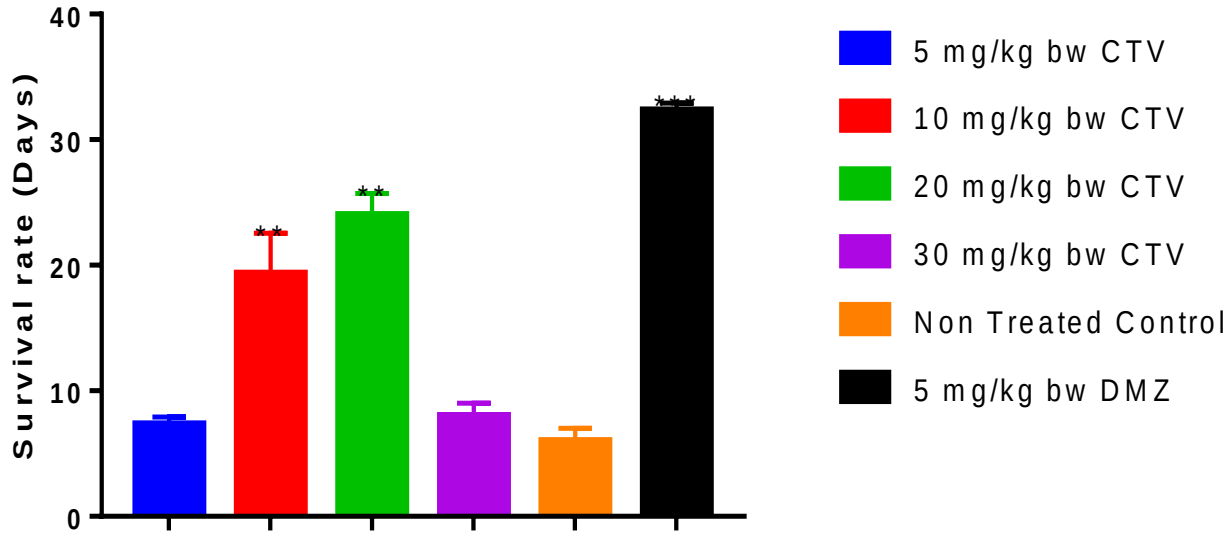

Figure 4.5: Effect of CTV on Survival Rate of *Trypanosoma brucei brucei*-Infected Wistar Rats

Key: CTV; crude toad venom extract, DMZ; diminazene aceturate. The superscript alphabet indicates the significant differences ($p < 0.05$) among the treated groups. ** $p < 0.05$, *** $p > 0.0001$

Table 4.4: Effect of Crude Toad Venom Extract on Relative Organ Body Weight of *Trypanosoma brucei brucei*-Infected Wistar Rats

Organ Weight	Liver (g)	Heart (g)	Kidney (g)	Lung (g)	Spleen (g)
5 mg/kg b. wt. CTV	2.35±0.00[a]	0.23±0.01 [a]	0.032±0.00 [a]	0.26±0.00 [a]	0.31±0.01 [a]
10 mg/kg b. wt. CTV	2.83±0.00[a]	0.25±0.02 [a]	0.032±0.00 [a]	0.25±0.01 [a]	0.35±0.01 [a]
20 mg/kg b. wt. CTV	2.64±0.00[a]	0.22±0.01 [a]	0.031±0.00 [a]	0.26±0.00 [a]	0.32±0.01 [a]
30 mg/kg b. wt. CTV	2.64±0.01[b]	0.24±0.02 [a]	0.035±0.00 [a]	0.25±0.01 [a]	0.36±0.00 [b]
Normal Control	2.87±0.02[a]	0.25±0.01 [a]	0.035±0.0 [a]	0.29±0.01 [a]	0.34±0.00 [a]
Non treated Control	2.27±0.01[a]	0.23±0.01 [a]	0.036±0.00 [a]	0.24±0.01 [a]	0.33±0.01 [a]
5 mg/kg b. wt. DMZ	2.70±0.00[a]	0.23±0.01 [a]	0.035±0.01 [a]	0.25±0.02 [a]	0.35±0.01 [b]

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

4.1.9 Effect of crude toad venom extract on biochemical parameters in *Trypanosoma brucei brucei*-infected wistar rats

Serum lipid profile concentrations in *T. b. b*-infected non-treated rats such as, triglyceride (160 mmol/L); cholesterol (240 mmol/L), and Low Density Lipoprotein Cholesterol (110 mmol/L) were significantly higher ($p < 0.05$), while the rate of High Density Lipoprotein Cholesterol (90 mmol/L) was significantly lower ($p < 0.05$) when compared with rats treated with 5 mg/kg b. wt. diminazene aceturate, where triglyceride was 120 mmol/L; cholesterol, 150 mmol/L, LDL-C, 70 mmol/L; and HDL-C, 170 mmol/L. The group treated with 30 mg/kg b. wt. also exhibited higher LDL-C, triglyceride and cholesterol rates when compared with the control group. However, treatments of rats with 10 mg/kg b. wt. of crude toad venom extract where LDL-C was 60 mmol/L; triglyceride, 110 mmol/L, and cholesterol, 160 mmol/L and those treated with 20 mg/kg b. wt. had LDL-C of 75 mmol/L, triglyceride, 100 mmol/L and cholesterol, 190 mmol/L, significantly reduced the elevated levels of LDL-C, triglyceride and cholesterol while increasing the level of HDL-C when compared with the control group (Figure 4.6).

The effect of crude toad venom extract on liver function parameters in *T. b. b.*-infected rats are presented in figure 4.7. *T. b. b.*-infected non-treated rats exhibited higher serum activities of ALP, 140 mg/dL; AST, 75 mg/dL; and ALT, 90 mg/dL, as well as decreased serum concentrations of total proteins, 20 mg/dL; and albumins, 5 mg/dL, when compared with the normal control rats where ALP was 100 mg/dL, AST, 50 mg/dL; ALT, 40 mg/dL; total proteins, 48 mg/dL; and albumin, 23 mg/dL. Rats treated with 5 and 30 mg/kg b. wt. of toad venom also exhibited higher serum AST, ALT and ALP while decreasing total proteins and albumin concentrations when compared with the control. However, rats treated with 10 mg/kg b. wt. had ALP, 108 mg/dL; AST, 55 mg/dL; ALT, 40 mg/dL; total proteins, 45 mg/dL, and albumin, 20 mg/dL and those treated with 20 mg/kg b. wt. of

CTV had ALP, 100 mg/dL; AST, 50 mg/dL; ALT, 35 mg/dL; total proteins, 47 mg/dL; and albumins, 25 mg/dL, and also the group treated with 5 mg/kg b. wt. of DMZ significantly ($p < 0.05$) lowered the actions of ALT, ALP and AST, while increasing the concentrations of total proteins and albumins in the serum of the infected animals (Figure 4.7).

Trypanosoma brucei brucei-infected non-treated rats exhibited higher serum concentrations of electrolytes including potassium (15 mEq/L), sodium (130 mEq/L), chloride (80 mEq/L), bicarbonate (52 mEq/L) (Figure 4.8), urea (42 mg/dL), uric acid (22 mg/dL) and creatinine (20 mg/dL) (Figure 4.9) when compared with the normal control rats with values of potassium (8 mEq/L), sodium (100 mEq/L), chloride (55 mEq/L), bicarbonate (25 mEq/L), urea (12 mg/dL), uric acid (14 mg/dL) and creatinine (10 mg/dL). Rats treated with 5 and 30 mg/kg b. wt. of CTV also exhibited higher serum concentrations of the electrolytes including potassium, sodium, chloride, bicarbonate, urea, uric acid and creatinine when compared with the control. However, rats treated with 20 mg/kg b. wt. of CTV with values of potassium (2 mEq/L), sodium (95 mEq/L), chloride (58 mEq/L), bicarbonate (25 mEq/L), urea (22 mg/dL), uric acid (15 mg/dL), and creatinine (18 mg/dL) and those treated with 5 mg/kg b. wt. of DMZ significantly ($p < 0.05$) lowered the serum concentrations of these electrolytes when compared with non-treated control.

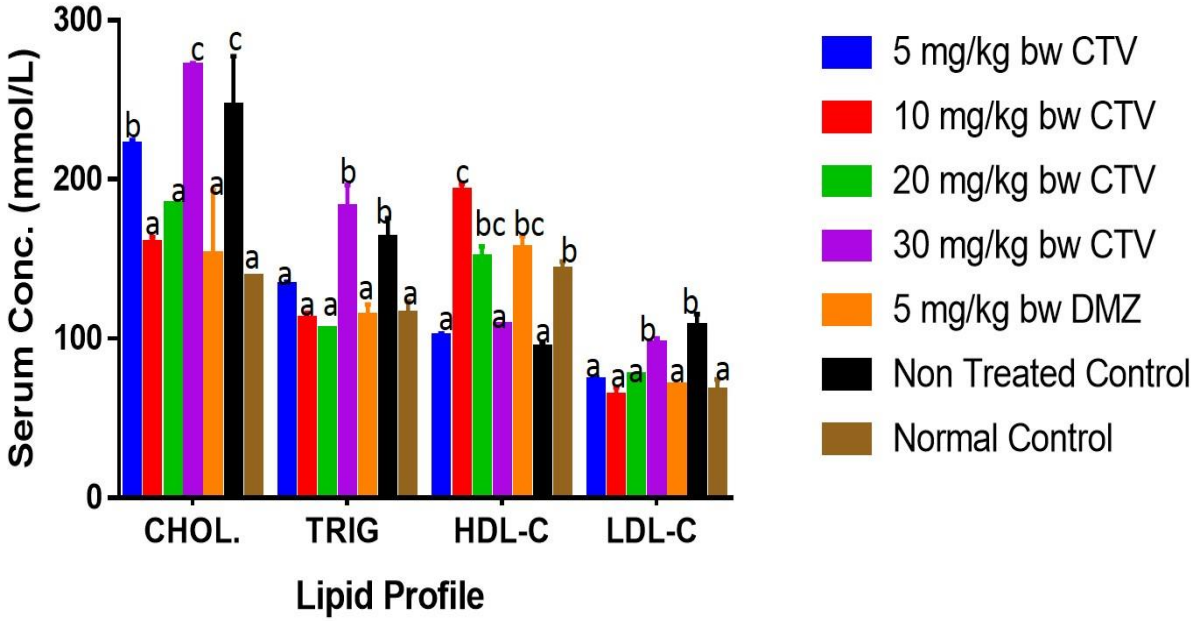

Figure 4.6: Effect of CTV on Lipid Profile in *Trypanosoma brucei brucei*-Infected Wistar Rats.

Key: crude toad venom extract, (CTV); diminazene aceturate, (DMZ); cholesterol (CHOL); triglyceride, (TRIG); high density lipoprotein cholesterol, (HDL-C); low density lipoprotein cholesterol, (LDL-C). The superscript alphabet indicates the significant differences ($p < 0.05$) among the treated groups. d > c > b > a

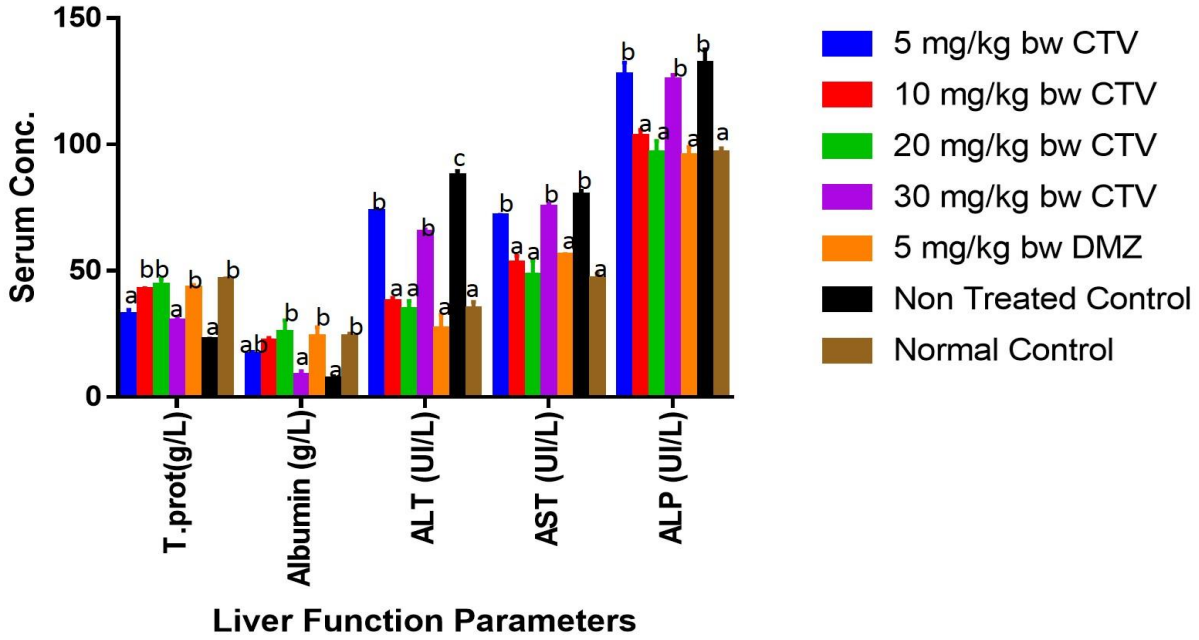

Figure 4.7: Effect of CTV on Liver Function Parameters in *Trypanosoma brucei brucei*-Infected Wistar Rats

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

4.1.10 Effect of crude toad venom extract on haematological parameters in *Trypanosoma brucei brucei*-infected wistar rats

Haematological parameters such as MCV, HB, RBC, MCHC, PLC and TWBC were significantly ($p < 0.05$) lesser in *T. b. b.*-infected non-treated rats, where MCV was 32.83 ± 0.84^{a}; HB, 12.17 ± 0.79^{a}; RBC, 9.88 ± 0.68^{a}; MCHC, 28.83 ± 0.69^{a}, PLC, 6.94 ± 0.87^{a}; and TWBC, 6.94 ± 0.76^{a} when compared with the normal control group where MCV was 40.91 ± 0.83^{b}; HB, 19.51 ± 0.83^{b}, RBC, 18.76 ± 0.39^{c}; MCHC, 39.13 ± 0.87^{b}, PLC, 13.65 ± 0.98^{b}; and TWBC, 13.65 ± 0.76^{c}. However, rats treated with 10 mg/kg b. wt. of CTV had higher counts when compared with infected non-treated group, where MCV was 41.03 ± 0.83^{b}; HB, 23.56 ± 0.45^{b}; RBC, 17.56 ± 0.59^{c}; MCHC, 42.37 ± 0.78^{bc}; PLC, 12.86 ± 0.59^{b}; and TWBC, 12.86 ± 0.58^{c}. The group treated with 20 mg/kg b. wt. of CTV also had higher counts, where MVC, 38.24 ± 0.79^{ab}; HB, 18.81 ± 0.94^{ab}; RBC, 14.65 ± 0.40^{b}; MCHC, 37.08 ± 0.95^{b}, PLC, 15.12 ± 0.79^{c}; and TWBC, 15.12 ± 0.73^{d}, when compared with the non-treated control. (Table 4.5).

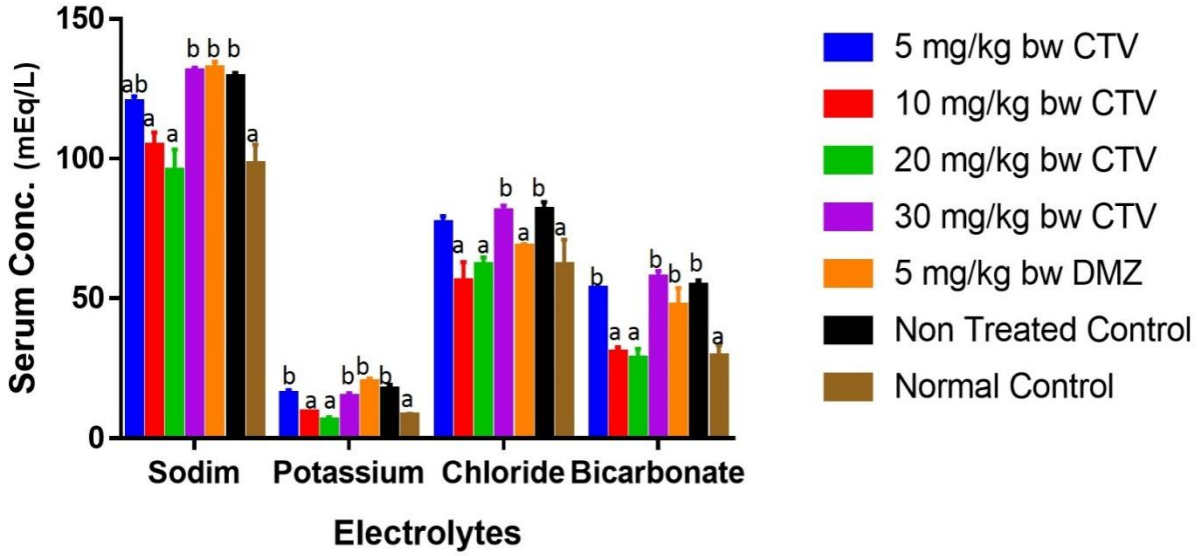

Figure 4.8: Effect of CTV on Serum Electrolyte Concentrations in *Trypanosoma brucei brucei*-Infected Wistar Rats.

Key: crude toad venom extract, (CTV); diminazene aceturate, (DMZ). The superscript alphabet indicates the significant differences ($p < 0.05$) among the treated groups. d > c > b > a

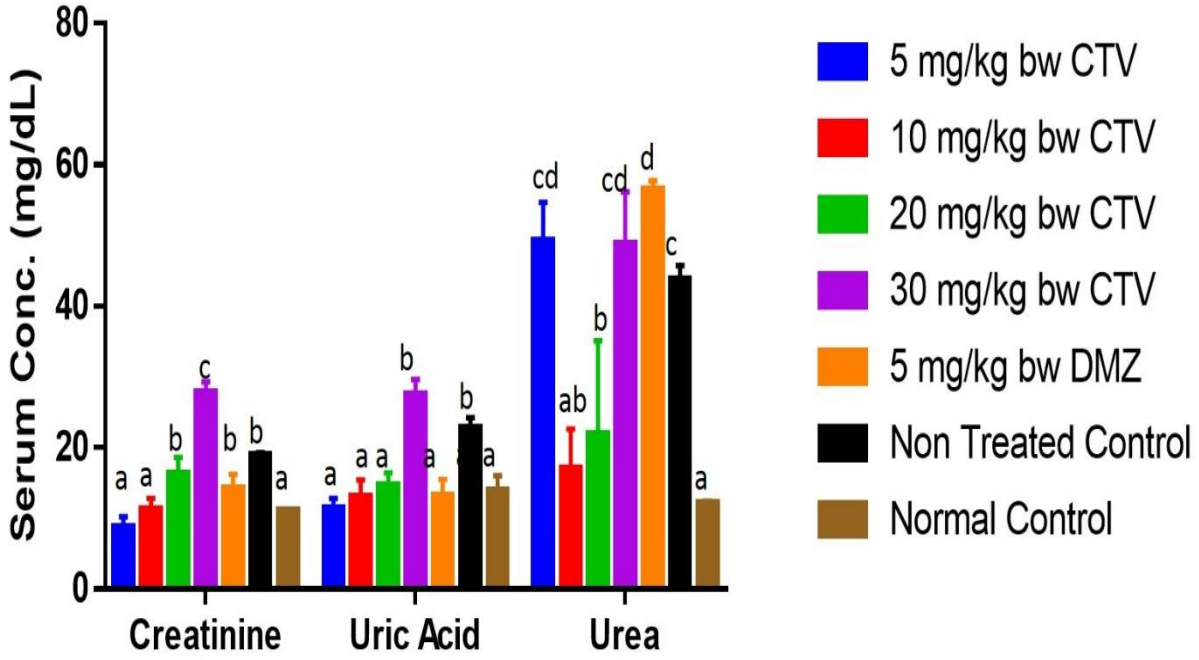

Figure 4.9: Effect of CTV on Serum Urea, Uric acid and Creatinine Concentrations in *Trypanosoma brucei brucei*-infected Wistar Rats.

Key: crude toad venom extract, (CTV); diminazene aceturate, (DMZ). The superscript alphabet indicates the significant differences ($p < 0.05$) among the treated groups. d > c > b > a.

Table 4.5: Effect of Crude Toad Venom Extract on Haematological Parameters in *Trypanosoma brucei brucei*-Infected Wistar Rats

	5 mg/kg b. wt. CTV	10 mg/kg b. wt. CTV	20 mg/kg b. wt. CTV	30 mg/kg b. wt. CTV	Normal Control	Non-Treated Control	5 mg/kg b. wt. DMZ
HB (g/dL)	13.21±0.45^{a}	23.56±0.45^{b}	18.81±0.94^{ab}	15.89±0.38^{a}	19.51±0.83^{b}	12.17±0.79^{a}	21.89±0.56^{b}
PCV (%)	32.43±0.35^{a}	42.45±0.35^{b}	41.53±0.30^{b}	36.23±0.84^{a}	40.24±1.54^{b}	36.72±0.58^{a}	41.32±0.35^{b}
MCV (fi)	32.43±0.83^{a}	41.03±0.83^{b}	38.24±0.79^{ab}	34.13±0.97^{a}	40.91±0.83^{b}	32.83±0.84^{a}	40.13±0.87^{b}
MCH (Pg)	19.43±0.97^{a}	25.76±0.97^{b}	23.89±0.87^{b}	20.09±0.73^{ab}	28.02±0.87^{c}	17.78±0.75^{a}	29.14±0.90^{c}
MCHC (g/dL)	32.44±0.78^{b}	42.37±0.78^{bc}	37.08±0.95^{b}	35.12±0.48^{b}	39.13±0.87^{b}	28.83±0.69^{a}	45.13±0.68^{c}
RBC (10^{12}/L)	11.67±0.59^{a}	17.56±0.59^{c}	14.65±0.40^{b}	10.13±0.78^{a}	18.76±0.39^{c}	9.88±0.68^{a}	14.14±0.76^{b}
PLC (10^{6}/L)	7.45±0.59^{a}	12.86±0.59^{b}	15.12±0.79^{c}	9.76±0.83^{a}	13.65±0.98^{b}	6.94±0.87^{a}	14.62±0.37^{b}
TWBC (10^{12}/L)	9.34±0.58^{b}	12.86±0.58^{c}	15.12±0.73^{d}	9.76±0.89^{b}	13.65±0.76^{c}	6.94±0.76^{a}	14.62±0.90^{c}

Key: crude toad venom extract, (CTV); diminazene aceturate, (DMZ), haemoglobin, (HB); packed cell volume, (PCV); mean cell volume, (MCV); mean cell hemoglobin, (MCH); mean cell hemoglobin concentrations, (MCHC); red blood cells, (RBC); total white blood cells, (TWBC); platelet counts, (PLC)**.** The superscript alphabet indicates the significant differences ($p < 0.05$) among the treated groups. d > c > b > a

4.1.11 Histopathological evaluation of liver and kidney of *Trypanosoma brucei brucei*-infected wistar rats

Microscopic analysis of the tissue's units (liver and kidney) of rats that were treated with 10, 20 and 30 mg/kg b. wt. of CTV and 5 mg/kg b. wt. of DMZ exhibited standard architectures with unremarkable alterations in the histological and cellular structures of all organs. In kidney, the cellular structures of glomeruli, capsules and interstitium were preserved with no features of acute or chronic damage while the liver section showed hepatic tissues with preserved architecture made up of cords of standard hepatocytes, typical portal tracts and central vein, there were also no feature of acute or chronic damage. On the other hand, the results indicated disruption in the normal architecture of infected non-treated rats and rats treated with 5 mg/kg b. wt. of CTV; this includes degeneration of glomeruli and collapse of corpuscular capsule of the kidney while degeneration of hepatocytes was observed in the liver. Below are photomicrographs of the liver with the black arrow depicting the hepatocytes (Plate VI to XII) and photomicrographs of the kidney with the black and blue arrow pointing to the glomeruli and capsular space respectively (Plate XIII to XIX).

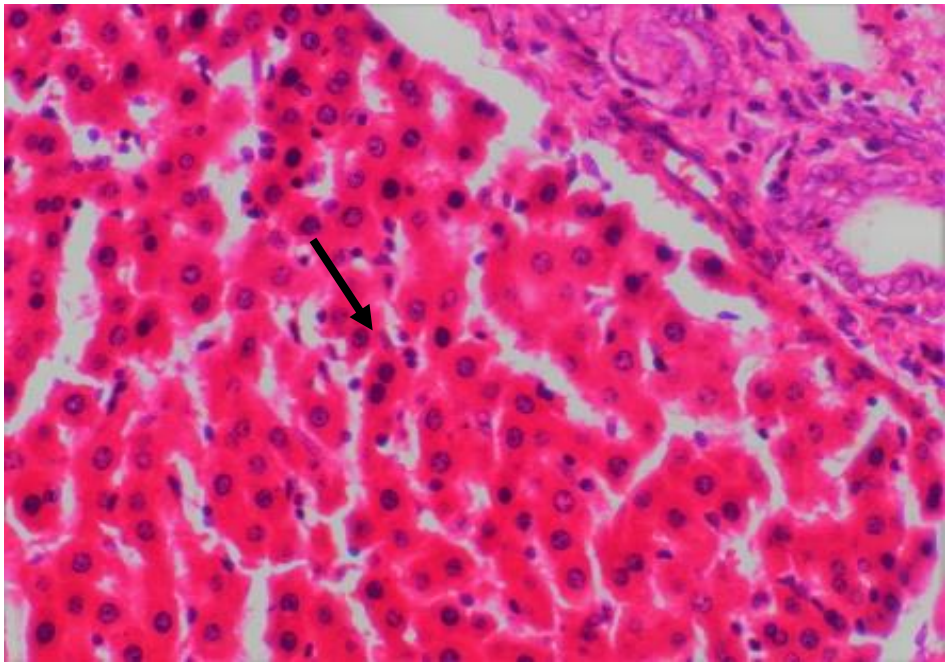

Plate VI: Photomicrograph of Liver (Normal Control)
KEY: Black arrow; Hepatocytes

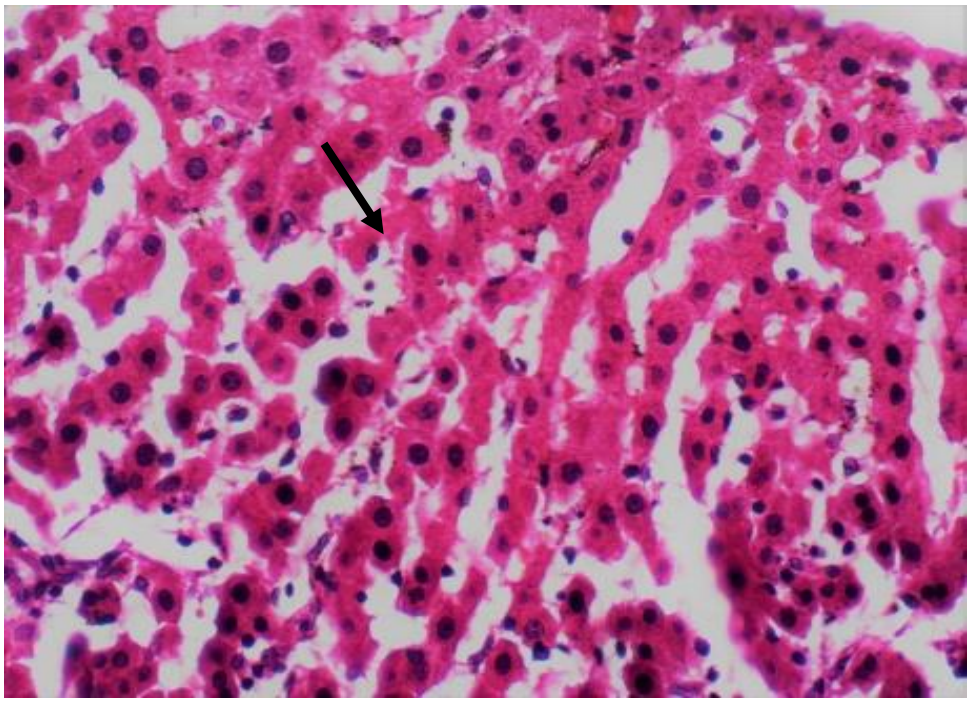

Plate VII: Photomicrograph of Liver (5 kg/mg b. wt. of DMZ)
KEY: Black arrow; hepatocytes, DMZ; diminazene aceturate.

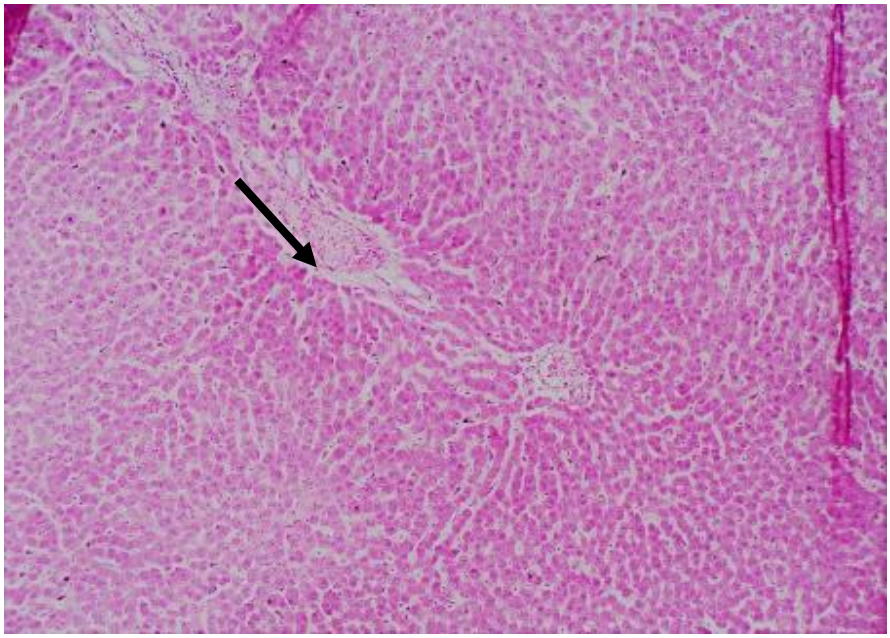

Plate VIII: Photomicrograph of Liver (5 mg/kg b. wt. of CTV)
KEY: Black arrow; hepatocytes, CTV; crude extract of toad venom

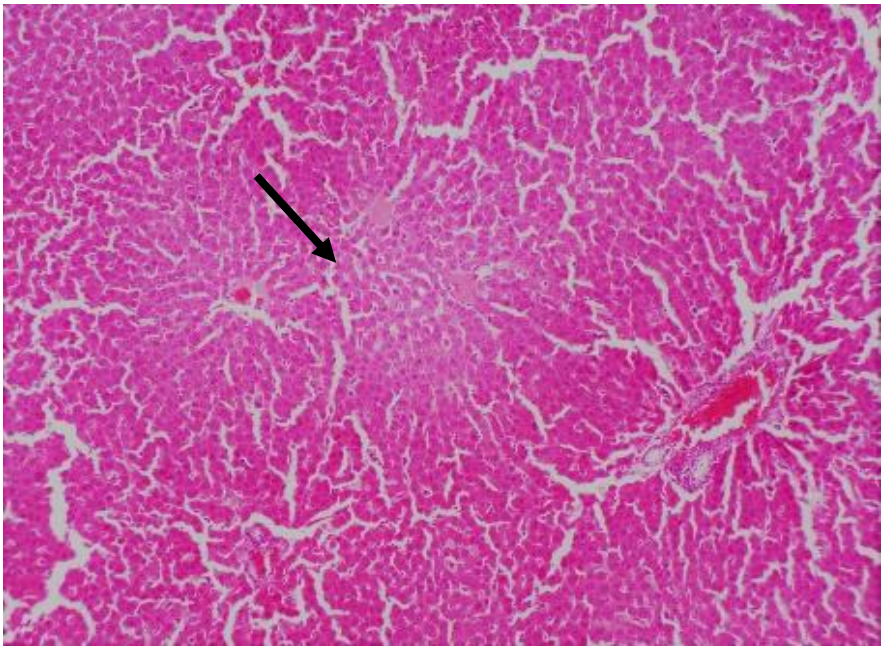

Plate IX: Photomicrograph of Liver (Negative control)
KEY: Black arrow; hepatocytes

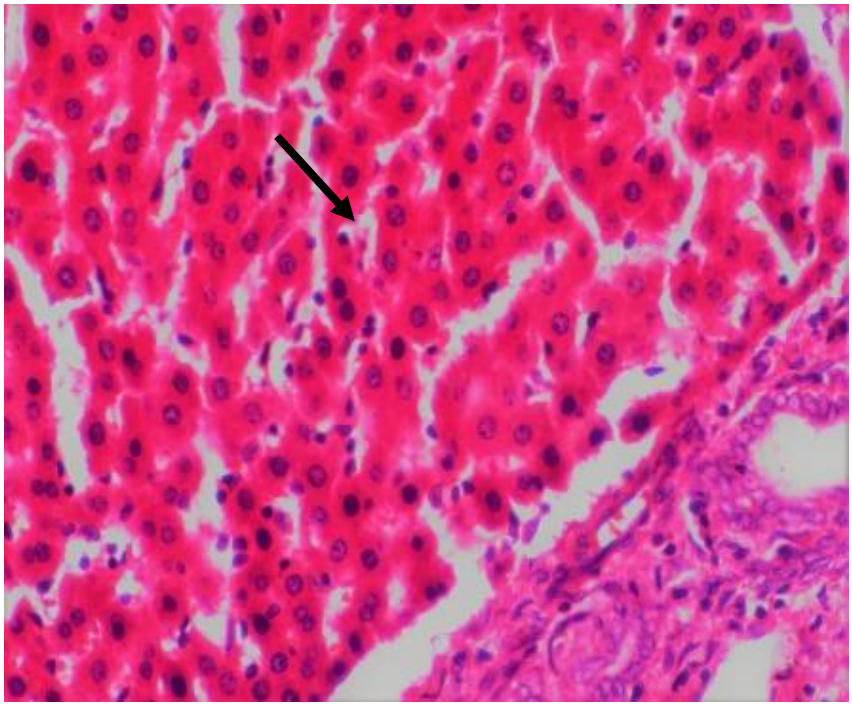

Plate X: Photomicrograph of Liver (10 mg/kg b. wt. of CTV)
KEY: Black arrow; hepatocytes, CTV; crude extract of toad venom

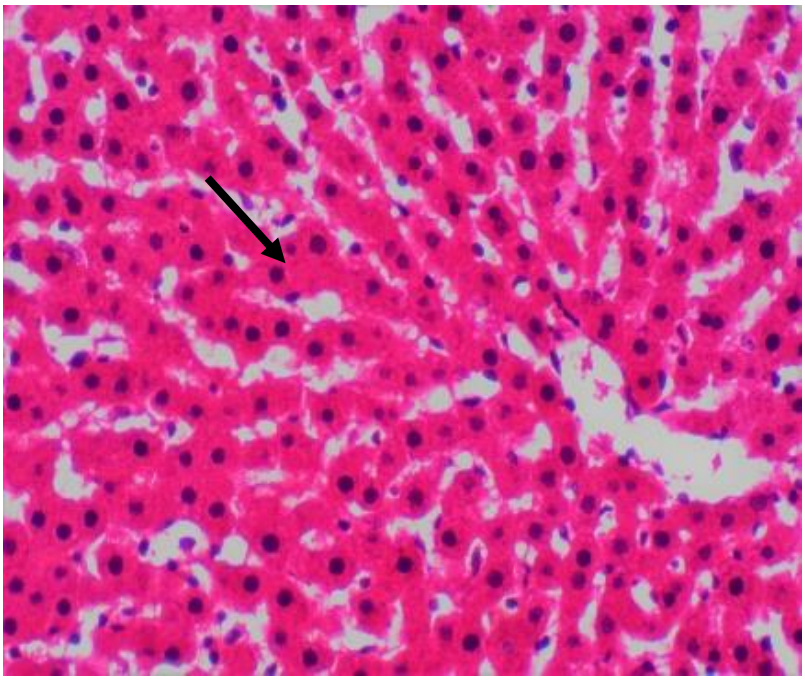

Plate XI: Photomicrograph of Liver (20 mg/kg b. wt. of CTV)
KEY: Black arrow; hepatocytes, CTV; crude extract of toad venom

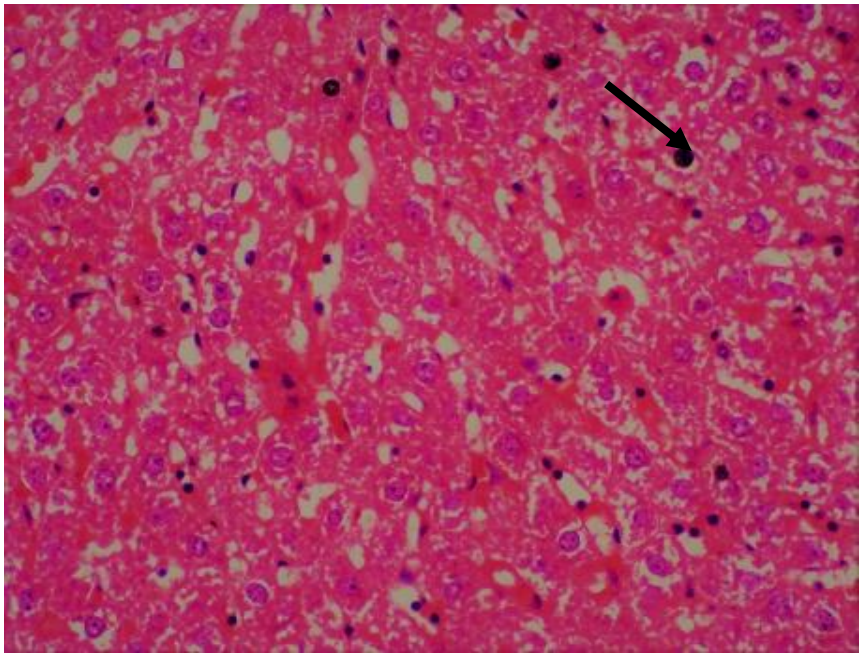

Plate XII: Photomicrograph of Liver (30 mg/kg b. wt. of CTV)
KEY: Black arrow; hepatocytes, CTV; crude extract of toad venom

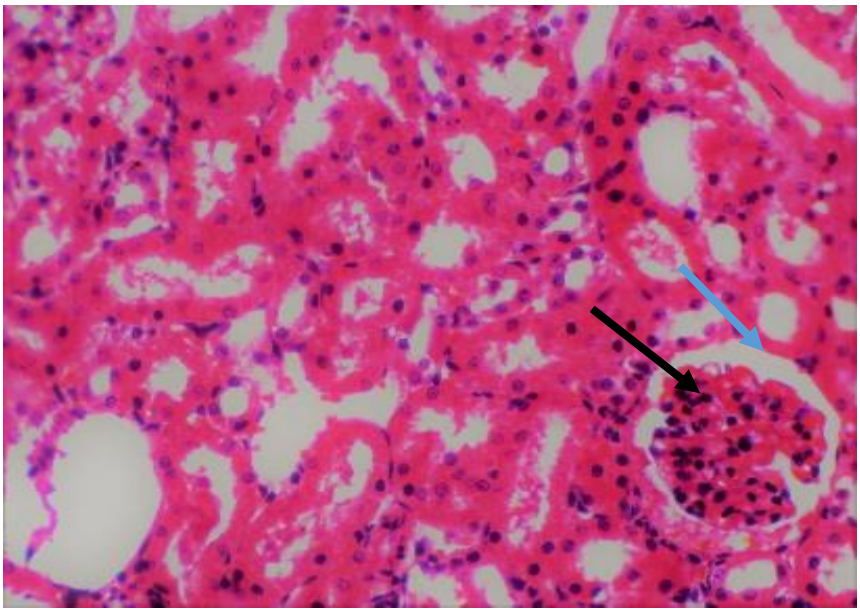

Plate XIII: Photomicrograph of Kidney (Normal control)
KEY: Black arrow; glomeruli, Blue arrow; capsular space

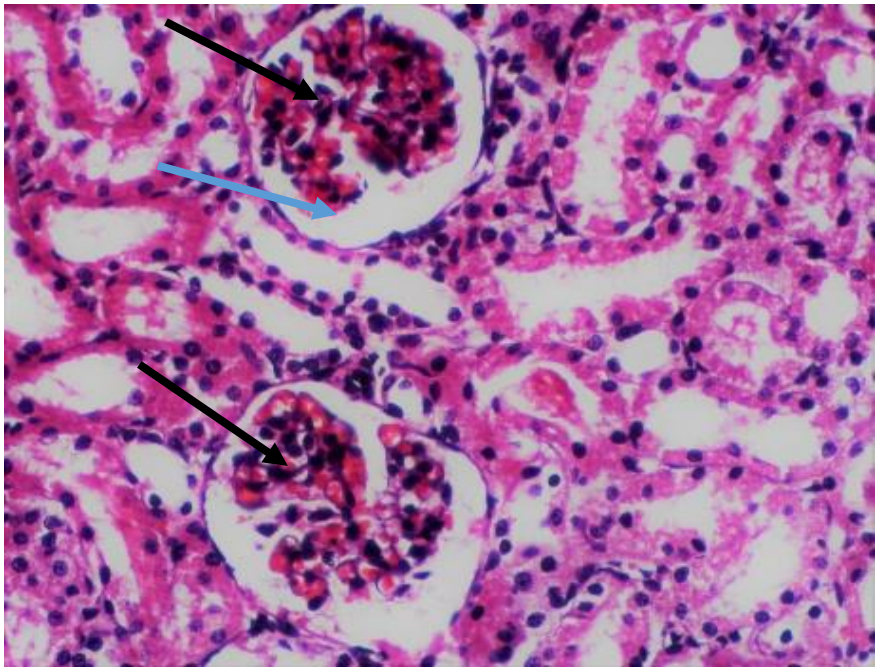

Plate XIV: Photomicrograph of Kidney (5 mg/ kg b. wt. of DMZ)
KEY: Black arrow; glomeruli, Blue arrow; capsular space, DMZ; diminazene aceturate

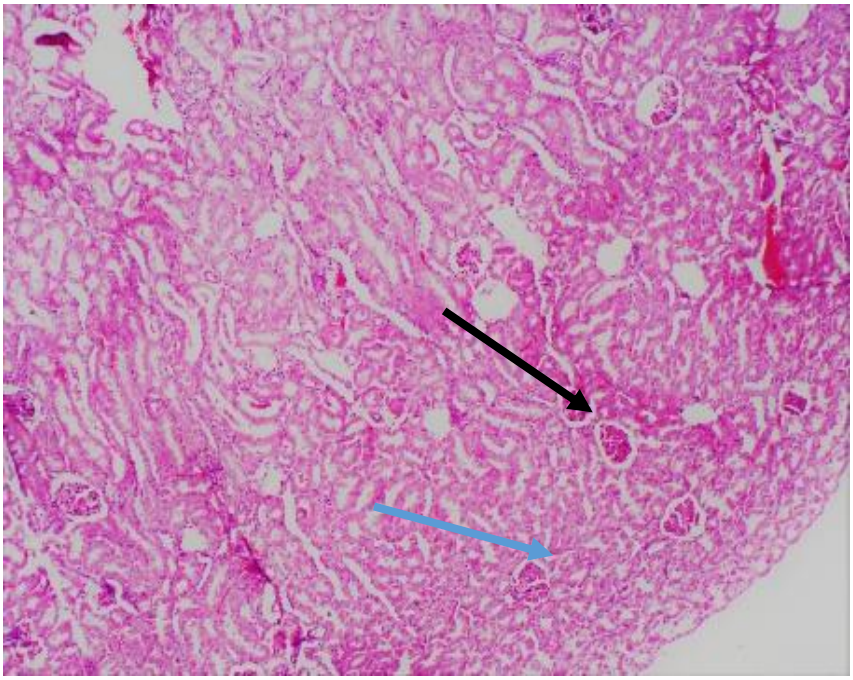

Plate XV: Photomicrograph of Kidney (5 mg/kg b. wt. of CTV)
KEY: Black arrow; glomeruli, Blue arrow; capsular space, CTV; crude extract of toad venom

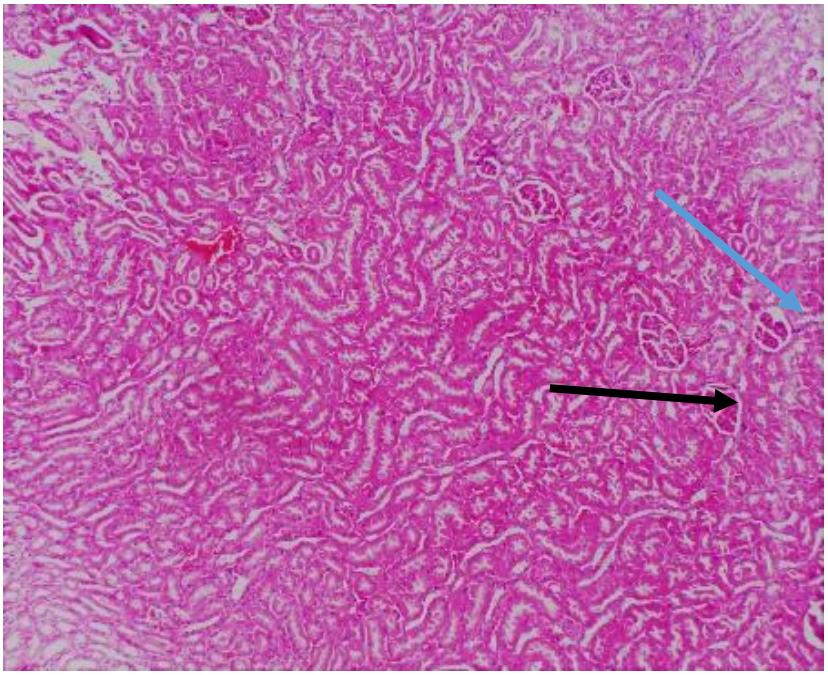

Plate XVI: Photomicrograph of Kidney (Negative control)
KEY: Black arrow; glomeruli, Blue arrow; capsular space

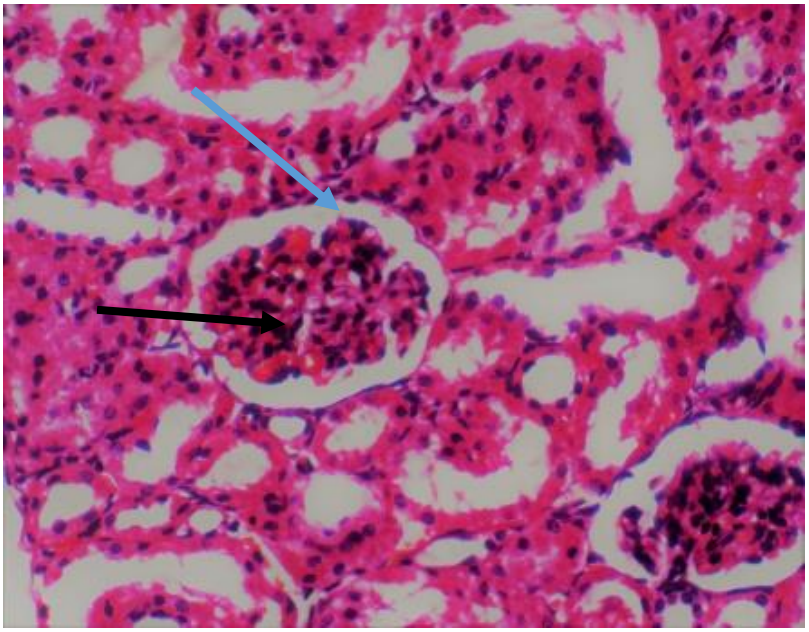

Plate XVII: Photomicrograph of Kidney (10 mg/kg b. wt. of CTV)
KEY: Black arrow; glomeruli, Blue arrow; capsular space, CTV; crude extract of toad venom

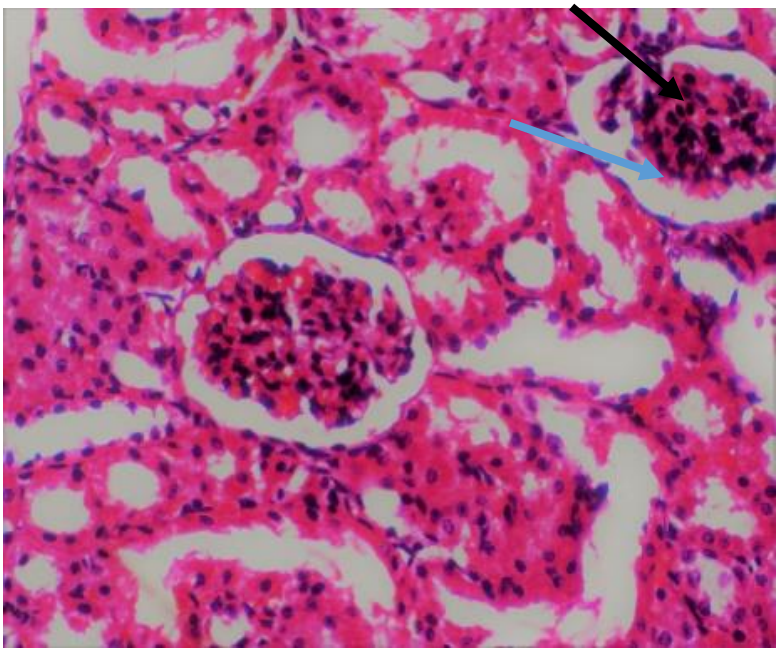

Plate XVIII: Photomicrograph of Kidney (20 mg/kg b. wt. of CTV)
KEY: Black arrow; glomeruli, Blue arrow; capsular space, CTV; crude extract of toad venom

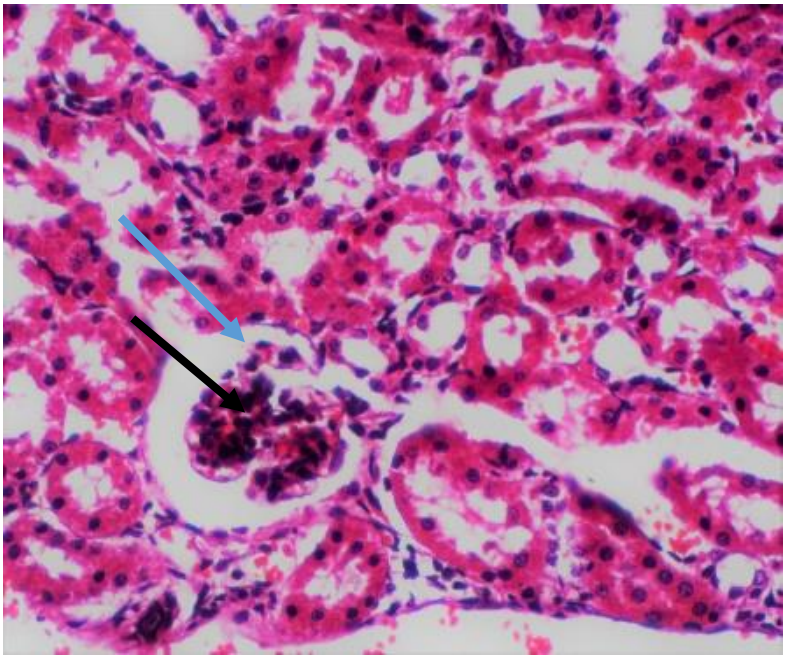

Plate XIX: Photomicrograph of Kidney (30 mg/kg b. wt. of CTV)
KEY: Black arrow; glomeruli, Blue arrow; capsular space, CTV; crude extract of toad venom

4.2 Discussion

4.2.1 Zoochemical composition of crude toad venom extract (CTV)

The use and quest for natural product derived drugs have enhanced in recent years. Microbiologists, natural-products chemists, biochemists, botanists, pharmacologists, zoologists globally, are continuously in search of natural-products for bioactive zooconstituents that could function as a drug lead for treatment of numerous human ailments including trypanosomiasis. Zoochemicals are animal equivalent of phytochemicals in plants which are secondary metabolites of plants well-known for its diverse biochemical effects on living organisms (Sultana *et al.,* 2009). The presence of important zoochemicals including phenols, flavonoids, tannins, cardiac glycoside, carbohydrate and terpenoids in the crude toad venom extract is an indication of its potential therapeutic effect, which is similar to the discoveries of Shittu *et al*. (2015), who detected the existence of cardiac glycoside, tannins and flavonoids in Nigeria bee propolis (*Apis mellifera*). In agreement with the zoochemical findings from this study, the use of animals' venom in treating several illness is growing round the world. Prior studies have reported the anti-parasitic activities of flavonoid and phenols in experimental animals (Adesina *et al.,* 2020). However, the present study pioneered the report of anti-trypanosomal activities of toad venom against *T. brucei brucei*-infected rats.

4.2.2 Gas chromatography and mass spectrometry of toad venom

The toad venom was analyzed and observed to possess 25 bioactive metabolites; these includes oleic acid and its derivatives, methyl ester, dodecanoic acid, hexadecanoic acid, ethyl ester and 9-Octadecenoic acid (Z), methyl ester, as revealed by the GC-MS analysis. Octadenoic acid, methyl ester and hexadenoic acid has also been found in *Argenteohyla siemersi* (frog) skin secretions which contributes to their defense mechanisms (Fusco *et al.,* 2020). These compounds have been earlier reported with several bioactivities including anti-microbial (Wagh *et al.,* 2007), anti-oxidant (Wei and Shibamoto, 2007),

anti-inflammatory (Yunfeng *et al.*, 2007). By implication, the recorded anti-trypanosomal properties of the crude toad venom could be connected to the existence of the aforementioned bioactive compounds.

4.2.3 Acute toxicity of crude toad venom extract

Acute toxicity is mainly conducted to observe the short-term adverse effects of a drug when given in a multiple or single dose during a 24 hours period (Colerangle, 2017). The outcome of this current study revealed that intraperitoneal administration of the crude toad venom extract exhibited a LD_{50} of 141.42 mg/kg b. wt. which is an indication that the venom is safe at all tested doses.

4.2.4 Effect of crude toad venom extract on parasitaemia counts in *Trypanosoma brucei brucei*-infected wistar rats

Trypanosoma brucei infection is a serious parasitic infection that is associated with severe complications and multiple damage if left untreated. In this study, the continuous multiplication of *T. b. b.* in the blood of the infected untreated rats is in line with the report of previous studies (Kennedy, 2013). *Trypanosoma brucei* is a well-known parasite that multiply in thousands in less than 24 hours. Their hyper proliferation ability is the major reason behind their infiltration and damage to multiple organs. However, it was observed that the infected animals treated with CTV at concentrations of 10 and 20 mg/kg b. wt. significantly decreased the multiplication ability of the parasite, hence demonstrating the anti-trypanosomal effect in experimental animals. This observation agrees with the experiment of Camila and Thais (2015), which reported the action of *Apis mellifera* against pathogenic protozoan parasites. Previous study has also reported that, during trypanosome infections the parasite are sequestered in several organs including the liver (Akpa *et al.*, 2008), leading to gradual tissue damage (Ekanem and Yusuf, 2005). This

effect has been described to be directly related to the levels and severity of parasitaemia and anaemia respectively (Anosa, 1988). Thus, elimination of parasite from the body system and concurrently increasing the immune system of the host may possibly be very important in the regulation of African sleeping sickness (Hoet *et al.,* 2004). Interestingly, it was observed that, the toad venom, in addition to its anti-parasitic properties also significantly improved the immune capacity of the animals and prevented the parasite induced alterations in haematological and biochemical parameters in *T. b. b.*-infected rats.

4.2.5 Effect of crude toad venom extract on packed cell volume in *Trypanosoma brucei brucei*-infected wistar rats

The significant decrease in the level of PCV of the *T. b. b.*-infected non-treated rats is a sign of anaemic condition caused by the parasite infection. However, the infected rats treated with CTV at concentration of 10 and 20 mg/kg b. wt. showed increase in the packed cell volume level, which is similar to the report of Cunha- Filho *et al.* (2010), who attributed the venom extract of *Rhinella marina* (a toad species) to be a positive anti-proliferative agent by not causing haemolysis of human erythrocytes at high concentrations. Thereby, indicating the ameliorative potentials of toad venom on the effect caused by the parasite on haematological parameters. Also, in line with this observation, Shittu *et al.* (2015) observed that venom from insect significantly improved the PCV of the infected animals.

4.2.6 Effect of crude toad venom extract on body weight of *Trypanosoma brucei brucei*-infected wistar rats

Loss of body weight of infected *T. brucei brucei* rats could be attributed to the pathological state of diseased rats which caused loss of appetite, thereby affecting overall feed consumption (Lawal *et al.,* 2016). However, treatment with the CTV at all

concentrations did not cause any significant weight loss when compared to normal control.

4.2.7 Effect of crude toad venom extract on survival rate of *Trypanosoma brucei brucei*-infected wistar rats

Bioactive components that can extend survival time of infected rats in comparison with negative controls are considered as active agents against the disease (Abdu *et al.,* 2008). The infected non-treated rats had the lowest survival days when compared to the groups treated with 5 and 30 mg/kg b. wt. of CTV, however rats treated with 10 and 20 mg/kg b. wt. of CTV had higher survival days, while the group treated with DMZ had the highest. These correlates with the review of Wink (2012), stating that secondary metabolites such as terpernoids, phenols, and cardiac glycosides serve as anti-trypanosomal agents, so these might be the reason for the prolonged survival rates.

4.2.8 Effect of crude toad venom extract on relative organ body weight of *Trypanosoma brucei brucei*-infected wistar rats

The weight of the spleen, lungs, kidneys, heart and liver of the treated and control group showed no significant change suggesting that the administration of the crude toad venom extract intraperitoneally had no influence on their organs. The procedure of weighing of relative organs in toxicity studies is due to their sensitivity to envisage toxicity and this associates well with histopathological changes (Mayur *et al.,* 2017).

4.2.9 Effect of crude toad venom extract on biochemical parameters in *Trypanosoma brucei brucei*-infected wistar rats

Several studies have conveyed that trypanosomiasis infection could compromise the integrity of the hepatocyte with the consequent release of enzymes into the serum (Ashok *et al.,* 2002). Therefore, estimation of biochemical indices has been widely accepted as a bio-indicator of liver damage (Yusuf *et al.,* 2018). The significant increase in serum ALT and AST of *T. brucei brucei*-infected rats is obviously as a result of the leakages of this enzyme from the liver into the serum (Yusuf *et al.,* 2012). However, the fact that ALP, AST and ALT activities in serum of rats treated with the CTV compared well ($P > 0.05$) with the control value suggests that the crude extract of toad venom was able to reverse the injurious effects of trypanosome infection to the liver, or prevented the parasite infection from infiltrating the hepatocyte. This effect suggests that the liver's integrity has been preserved by the toad venom despite the *T. brucei brucei* infection. Generally accepted biomarkers of endoplasmic reticulum and plasma membrane such as activities of ALP in organs and body fluid are often used to evaluate the reliability of the endoplasmic reticulum and plasma membrane (Akanji *et al.,* 1993). The *T. brucei brucei* parasite mediated compromised liver function was also evident by the alteration in serum ALP activities in the infected non treated rats. The surge in ALP activities in serum can be ascribed to the activation of the enzyme molecules by the crude toad venom extract or activation of the enzyme molecules in situ (Hassan *et al.,* 2011). A rise in the activities of ALP could place one's lively cells at risk, and these cells are usually reliant on diverse phosphate esters for their necessary development as they might be lacking vital energy due to indiscriminate hydrolysis of phosphate ester (Oyewo *et al.,* 2012). The usual activity of enzymes can be affected by these alterations in the animals. However, the fact that the serum ALP of rats treated with toad venom compared well with the control value suggests that the toad venom have some functions in preserving structural integrity of hepatocellular membrane (Umar *et al.,* 2019).

The concentrations of total proteins, is also an important indicator of normal or impaired functions of liver. The observed decrease in the total proteins in serum of infected untreated rats when compared with the control rats signifies a concession of the synthetic capacity of the liver rising from the *T. brucei brucei* infection. Reduction in total protein could lead to dehydration which is injurious to cellular homeostasis and could adversely upset the metabolic activities of the liver and subsequently the well-being of the animals (Adesina *et al.,* 2020). Nevertheless, the CTV significantly protected the animals from the parasite mediated alterations in total protein. Urea, uric acid and creatinine are vital excretory metabolites and widely accepted clinical markers of kidney integrity (Lawal *et al.,* 2015). Increased levels of these metabolites in infected untreated rats when compared to treated and control groups suggest a condition of *T. b. b.* mediated compromised kidney function. Interestingly, groups treated with 10 and 20 mg/kg b. wt. of CTV significantly decreased the level of these metabolites, suggesting kidney function restoration.

4.2.10 Effect of crude toad venom extract on haematological parameters in *Trypanosoma brucei brucei*-infected wistar rats

Extrapolations from this present study can be related to the previous study of Shittu and Eyihuri (2015) which reported that bee venom improved the PCV, HB, and RBC of *Plasmodium berghei* parasitisized mice, when compared to infected untreated control, this is comparable as both diseases are caused by protozoans. White blood cell (WBC) play an active role in the immune system of hosts (Lawal *et al.,* 2016), the results of the effect this parasite had on WBCs may be because of the utilization of the WBC to fight off foreign substance or the stress induced by the parasite in the animals. However, the significant intensification in the WBC counts in the CTV treated groups when compared with non-treated animals suggests that the CTV stimulated immune responses. The crude extract was probably able to offer some defense mechanisms and released usage of

endogenous immune responses, to combat the stress prompted by the trypanosomes (Yusuf *et al.,* 2012).

4.2.11 Histopathological evaluation of liver and kidney of *Trypanosoma brucei brucei*-infected wistar rats

Parasites invasion of tissues can result in hepatomegaly in internal organs of infected rats showing that *T. brucei brucei* is pathogenic in rats (Ukpai and Nwabuko, 2014) which correlates with the observation seen in the infected untreated rats showing degeneration of glomeruli and collapse of capsular space in the kidney and degeneration of hepatocytes in the liver. However, the infected rats treated with CTV was able to reduce damage to the liver and kidney, which might be related to the fact that anti-protozoal drugs directly reduce the degree of liver invasion by trypanosomes (Ogunbawo *et al.,* 2001). In conclusion, the crude extract of toad venom has ameliorated the adverse effects of *T. brucei brucei* induced liver and kidney damage and could therefore, be recommended as an effective natural product for the management of *T. brucei brucei* infection and associated complications.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The findings from this study revealed that Crude Toad Venom contains bioactive compounds of therapeutic significance such as Oleic acid, methyl and ethyl ester, 9-Octadecenoic acid (Z), Hexadecanoic acid among others, and zoochemical components made up of tannins, phenols, flavonoids, cardiac glycoside, and terpenoids. Acute toxicity study of the crude toad venom extract indicates that the toad venom is safe for oral administration at tested doses. The venom also demonstrates a dose dependent anti-trypanosomal activities with a considerable antioxidant activities. The toad venom ameliorated parasite induced liver and kidney damage, haematological and biochemical alterations that were associated with *T. b. brucei* infection as well as promoted their survival time.

5.2 Recommendations

Further studies to establish the sub chronic toxicity of the toad extract is suggested. The determination and purification of bioactive compounds in the venom is recommended. The Toad Venom can also be tested on other parasites of public health importance.

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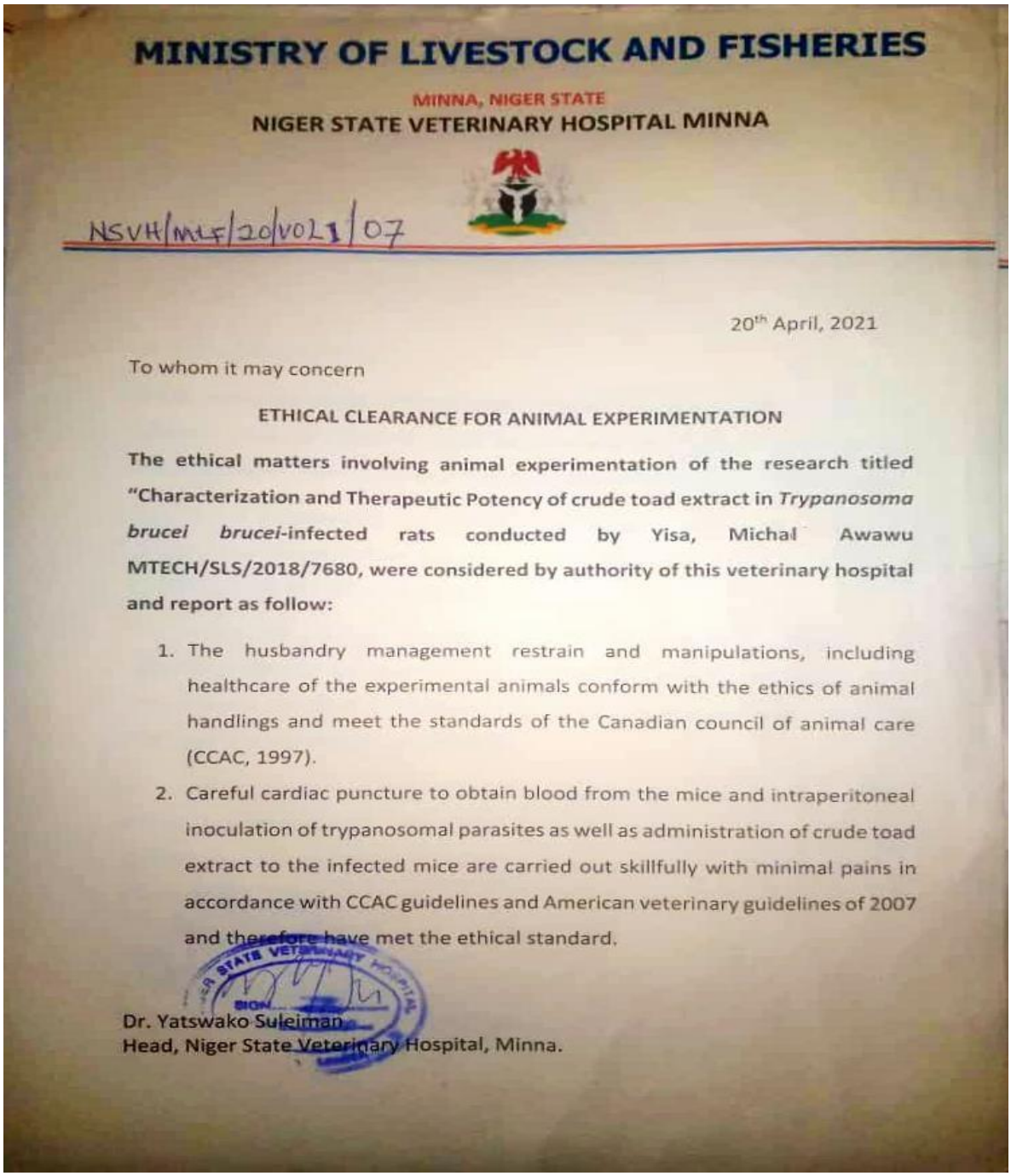
APPENDIX

APPENDIX A



MINISTRY OF LIVESTOCK AND FISHERIES

MINNA, NIGER STATE

NIGER STATE VETERINARY HOSPITAL MINNA

NSVH/MLF/20/2021/07

20th April, 2021

To whom it may concern

ETHICAL CLEARANCE FOR ANIMAL EXPERIMENTATION

The ethical matters involving animal experimentation of the research titled "Characterization and Therapeutic Potency of crude toad extract in *Trypanosoma brucei brucei*-infected rats conducted by Yisa, Michal Awawu MTECH/SLS/2018/7680, were considered by authority of this veterinary hospital and report as follow:

1. The husbandry management restrain and manipulations, including healthcare of the experimental animals conform with the ethics of animal handlings and meet the standards of the Canadian council of animal care (CCAC, 1997).
2. Careful cardiac puncture to obtain blood from the mice and intraperitoneal inoculation of trypanosomal parasites as well as administration of crude toad extract to the infected mice are carried out skillfully with minimal pains in accordance with CCAC guidelines and American veterinary guidelines of 2007 and therefore have met the ethical standard.

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