# THE BIOCHEMICAL STUIDES OF THE TRYPANOCIDAL ACTIVITY OF *MITRAGYNA CILIATA AUBREV. & PELLEGR.* (RUBIACEAE)

**BY**

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**B.Sc. Biochemistry (SOKOTO), M.Sc. Biochemistry (LAGOS)**

**Matric No. 019091022**

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**DEPARTMENT OF BIOCHEMISTRY,**
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**UNIVERSITY OF LAGOS, NIGERIA**

# UNIVERSITY OF LAGOS
# SCHOOL OF POSTGRADUATE STUDIES

## CERTIFICATION

This is to certify that the thesis:

**"Biochemical Studies of Trypanocidal Activity of Mitragyna ciliata Aubrev. & Pellegr. (Rubiaceae)"**

Submitted to the School of Postgraduate Studies, University of Lagos

for the award of the degree of

## DOCTOR OF PHILOSOPHY (Ph.D)

is a record of original research work carried out

By

## OGBUNUGAFOR, HENRIETTA ARITETSOMA

**In the Department of Biochemistry**

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# DECLARATION

The Research work contained in this Thesis was undertaken entirely by me. No part of this study has been presented either in part or as a whole to any other institution or organization for the purpose of obtaining any degree or qualification.

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**OGBUNUGAFOR, HENRIETTA ARITETSOMA**

# DEDICATION

This work is dedicated to GOD ALMIGHTY who saw me through it all faithfully.

To

My late parents Mr. and Mrs. M.E. Aniretan for the big dream they had for me which has been fulfilled today.

# ACKNOWLEDGEM ENT

The acknowledgment of the wonderful people who contributed immensely to the completion of this work, actually brought tears to my eyes out of sheer joy of being so favoured.

I specially thank my indefatible committed and motivating supervisors Prof. (Mrs) V. I. Okochi and Dr (Mrs.) O. S. Odesanmi whose untiring academic and moral support saw to the successful completion of this research work. I also wish to thank Dr (Mrs.) H A. Smith who co-supervised this work at the beginning. May the good Lord bless you all abundantly!

I wish to thank my dear husband Engr. Obi Ogbunugafor (Obinakueze), for his support and forbearance throughout the duration of my academic pursuit which started early in our marital life; who gladly played the role of baby sitter in those early days of my first degree and who even had to share his house with experimental animals in transit during this study. I especially appreciate my wonderful sons, Uche,. Chidubem, Chinwike, and my lovely daughters, Unoaku and Ifeoma who faithfully stood by me, helping out in various ways - the boys with the computer work, the girls with the household chores and the caring of their father during my long hours in the laboratory. I thank them for their understanding at a time when things were difficult, when I hardly had time to prepare a decent meal for them. I thank you all very much.

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To crown it all, I am grateful to the Almighty God who endued me with strength and health for this work and for keeping His promise of making a way where there seem to be no way. All glory to Him from whom all things come. Amen!

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# DEFINITION OF TERMS

PARASITEMIA: Describes the level of parasites per ml of blood in an infected animal.

SUPRESSIVE ACTIVITY: The ability of any extract/drug to suppress parasitic infection.

THERAPEUTIC ACTIVITY: The ability of an extract/drug to clear parasites from a host after parasitic infection has been established.

OXIDATIVE STRESS: Imbalance between the productions of reactive oxygen specie and biological system's ability to readily detoxify the reactive intermediate or easily repair the resulting damage.

TRYPANOCIDE: An extract or drug that can inhibit the growth and proliferation of trypanosomes.

# ABBREVIATIONS

DALY..... Daily Adjusted Life Years

DNDI..... Drugs of Neglected Disease Initiative

FAO..... Food and Agricultural Organization

ILRAD..... International Laboratory for Research on Animal Diseases

$LD_{50}$..... Minimum Lethal Dose

WOAH.....World Organization for Animal Health

$R_f$.....Retention Factor

WHO/TDR.....WHO Research and Training in Tropical Disease

WHO..... World Health Organization

# ABSTRACT

The extracts of many plants used in traditional medicine in Africa have been shown to contain therapeutic agents supporting their use in the treatment and management of many disease endemic to the region. Trypanosomiasis (sleeping sickness) is one of such diseases that still are a major health challenge in sub-Saharan Africa, in the quest to combat this problem, the search for trypanocidal agents from medicinal plants has arisen. The aim of this study was to investigate and corroborate the claim in traditional medicine of the safety and efficacy of the extract of *Mitragyna ciliata Aubrev and Pellegr* (Rubiaceae) in the treatment of trypanosomiasis. The study started with initial screening hydroethanolic extracts of the roots of two plants- *Mitragyna ciliata* Aubrev. & Pellegr. (Rubiaceae) and *Ritchea longipedicellata Gilg.* (Capparidaceae), for toxicity and antitrypanosomal activity. Toxicity test result after single dose administration of extracts to rats for 21 days, show that both plant extracts exhibited a marked decrease in ALP, ALT, AST and creatinine levels ($p<0.05$). These results indicated that the plant extracts was well tolerated and that they have a positive hepatoceflular effect. Investigation of the trypanocidal activity of *Mitragyna ciliata* root extract indicates that it had 76 % suppressive and 54.24 % therapeutic activities at 50mg/kg dose, while *R. longipedicellata* showed no trypanocidal activity. The combination (1:1 v/v) of both plant extracts exhibited complete suppressive activity at the tested doses (200 and 400 mg/kg) and 100 % therapeutic activity at 400 mg/kg. The potency of *M. ciliata* hydroethanolic root extract resulted in its selection for further investigation. Evaluation of *M. ciliata* extract showed that the extract had low in vitro antioxidative activity (25.25 %) and thin layer chromatography indicated that eluted fractions were mainly alkaloids. Activity-guided fractionation reveal n-butanol fraction as the active fraction producing 66.61 % inhibition of parasites growth The effects of the active traction

on oxidative stress enzymes in infected rats which might explain its mode of action, showed elevated superoxide dismutase activity (SOD) ($p < 0.05$), while catalase activity was depressed ($p < 0.05$). Data suggest alteration in the oxidative status of rats creating oxidative stress in the animals which caused parasite clearance. Hematological profile of infected and fraction treated rats was consistent with the major characteristics of trypanosomiasis anaemia leucocytopaenia and thrombocytopaenia. *In vivo* evaluation of serum $Ca^{2+}$ levels in active fraction indicated depressed ($p < 0.05$) levels in the treated rats ($2.53 \pm 0.036$ mmol/L) in comparison to untreated/infected ($17.79 \pm 0.034$ mmo/L). Results might indicate that the bioactive agent (butanol fraction) had an effect on the $Ca^{2+}$ metabolism in the animals which underlines the important role $Ca^{2+}$ plays in control of trypanosomal infection. Data show that *M. ciliata* root extract is a potential trypanocide and substantiates the use of the plant in the treatment of the disease in ethno medicine. It also provides some biochemical basis for the trypanocidal activity of the plant.

# CHAPTER ONE

# INTRODUCTION

## 1.1 BACKGROUND OF STUDY

Trypanosomiasis (sleeping sickness), caused by flagellated protozoan parasites - trypanosomes, continues to pose grave economic and social problems in sub-Saharan Africa. Combating this disease, which is transmitted by the insect vector, tsetse fly is still a major health challenge in Africa. This disease is restricted to Africa between latitude $15^0$ N and $20^0$ S from the southern edge of the Sahara desert to Zimbabwe, Angola, and Mozambique (WOAH, 2005). Trypanosomiasis which infects both humans and livestock is considered one of the most neglected diseases in terms of drug development in Africa (Truc, 2003).

Human African Trypanosomiasis (HAT) has recently become resurgent in Africa (WHO, 2000; Scovill *et al.*, 2001; Mishina *et al.*, 2007); with World Health Organization (WHO) reporting that about 60 million people are at risk and 50,000 people are infected annually (WHO, 1998; Mishina *et al.*, 2007). Animal trypanosomiasis is invariably the greater epidemic across Africa than the human trypanosomiasis; with dire economic consequences - 4.5 billion US dollar losses each year (WHO/TDR, 2005). The disease reduces growth rate, milk productivity and strength of farm animals leading to death of infected animals (WOAH, 2005). *T. brucei brucei,* the animal species used for this study, infects cattle, sheep, goats, pigs, camels, dogs, and cats (WOAH, 2005). The infection contributes to poverty more through its effect on livestock than through its effects on humans.

The search for vaccines remains elusive and the prospects of prophylactic immunization are poor since the parasites change their surface coat to evade the host immune system in a process known as antigenic variation (Rudenko and Taylor, 2006). This results in cyclic parasiteamia and aneamia; major characteristic features of trypanosomiasis (Naessens *et al.*, 2005). Existing trypanocides some of which have been on the market for over forty years (Olila *et al.*, 2002) are faced with drug resistance, toxicity and a lack of guaranteed supply problems (Burchmore *et al.*, 2002). Chemotherapy remains the principal means of intervention (Khan and Omar, 2007). Consequently, this scourge has become a pressing challenge to African medical scientists for possible home-grown action based on the rich bio-resources of their various communities.

Research into African medicinal plants has been intensified in recent times with the real hope of finding an alternative to synthetic drugs for the management of the disease. This is predicated on the fact that many of these aqueous, ethanolic, distilled, condensed or dried extracts used in ethno medicine for the treatment of the diseases have been shown to be efficacious (Asuzu and Chineme 1990; Freiburghaus *et al.*, 1997; Atawodi *et al.*, 2003; Wurochekke and Nok, 2004; Atidehou *et al.*, 2004; Atawodi, 2005; Ogbadoyi *et al.*, 2007). Furthermore, studies have shown a high correlation between pharmacological activity/clinical use of plant isolates and their established use as herbal medicine (Agbaje and Onabanjo, 1991; Iwu, 1993; Schuster *et al.*, 1999).

Herbal medicine is experiencing a renaissance in recent times. Several factors including their organic nature; the drop in the efficacy of conventional medicine especially antibiotics; the non-existence of an effective conventional medicine treatment for many chronic diseases such as asthma, arthiritis, hypertension, diabetes and the side effects of conventional medicine is encouraging people to look for other forms of treatment.

Traditional medicine practitioners in Nigerian use many plants for the treatment of sleeping sickness. *Mitragyna ciliata Aubrev* and *Pellegr (Rubiaceae)* is an example of such plants used by traditional healers in south-eastern Nigeria for the treatment of trypanosomiasis (Iwu, 1993). *Mitragyna ciliata (*known) as *'abura'* in Yoruba, *'uburu'* in Igbo, '*guleya*' in Hausa), is an erect woody plant found in abundance in Nigeria and it is also used by traditional medicine practitioners in the treatment of bacteria infections especially gonorrhoea, hypertension, arthritis, mental disorder, epilepsy, and bronco-pulmonary disease (Aji *et al.*, 2001). As a consequence of its ethno medical uses the pharmacological, biochemical and molecular studies of this plant is important in order to scientifically establish its various pharmacological effects. It is also of vital importance that plants used in traditional medicin e to treat African trypanosomiasis be validated and their active principles identified in order to design inexpensive standardized medicines for developing countries.

The overall objective of this work therefore, was to evaluate the trypanocidal activity of *Mitragyna ciliata,* using animal models with *T. brucei brucei* infection. Since reports on plant extracts active against trypanosomes are not as abundant as those on other protozoal diseases such as malaria, establishing the toxicological effect, trypanocidal effect and the biochemical basis for the activity of this plant will be a useful contribution to veterinary medicine in the management and control of animal trypanosomiasis (FAO, 1998).

## 1.2 STATEMENT OF PROBLEM

Vaccine development against trypanosomiasis has been difficult due to the capacity for antigenic variation of the parasites (Rudenko and Taylor, 2006). This means that chemotherapy is the principal means of intervention for the management of the disease. Earlier drugs for the treatment of the disease are old, faced with toxicity, unavailability, long treatment regimen and irregular supply problems (Buchmore *et al.*, 2002).

Coupled with these, is the recent development of resistance to these drugs by the parasite (WHO, 2000). Later drugs were effective but are expensive, eliminating the target groups who are the poor people of Africa to which the disease is restricted.

Also, the administration of these drugs need specialized health staff in specific centers further removing these services from the reach of the people. Furthermore, developing new formulations by pharmaceutical companies and licensing of new trypanocides have been slow, due to non-profitability.

This has led to scientists resorting to the search for trypanocides from medicinal plants used by traditional healers for the treatment of trypanosomiasis. This approach is central to finding a home-grown solution to the treatment of the disease which is endemic to Africa.

## 1.3 SIGNIFICANCE OF STUDY

The significance of this study is the investigation of *Mitragyna ciliata*, a medicinal plant used in traditional medical system, in order to provide the biochemical basis for the anti-trypanosomal activity of the plant.

## 1.4 OBJECTIVES OF STUDY

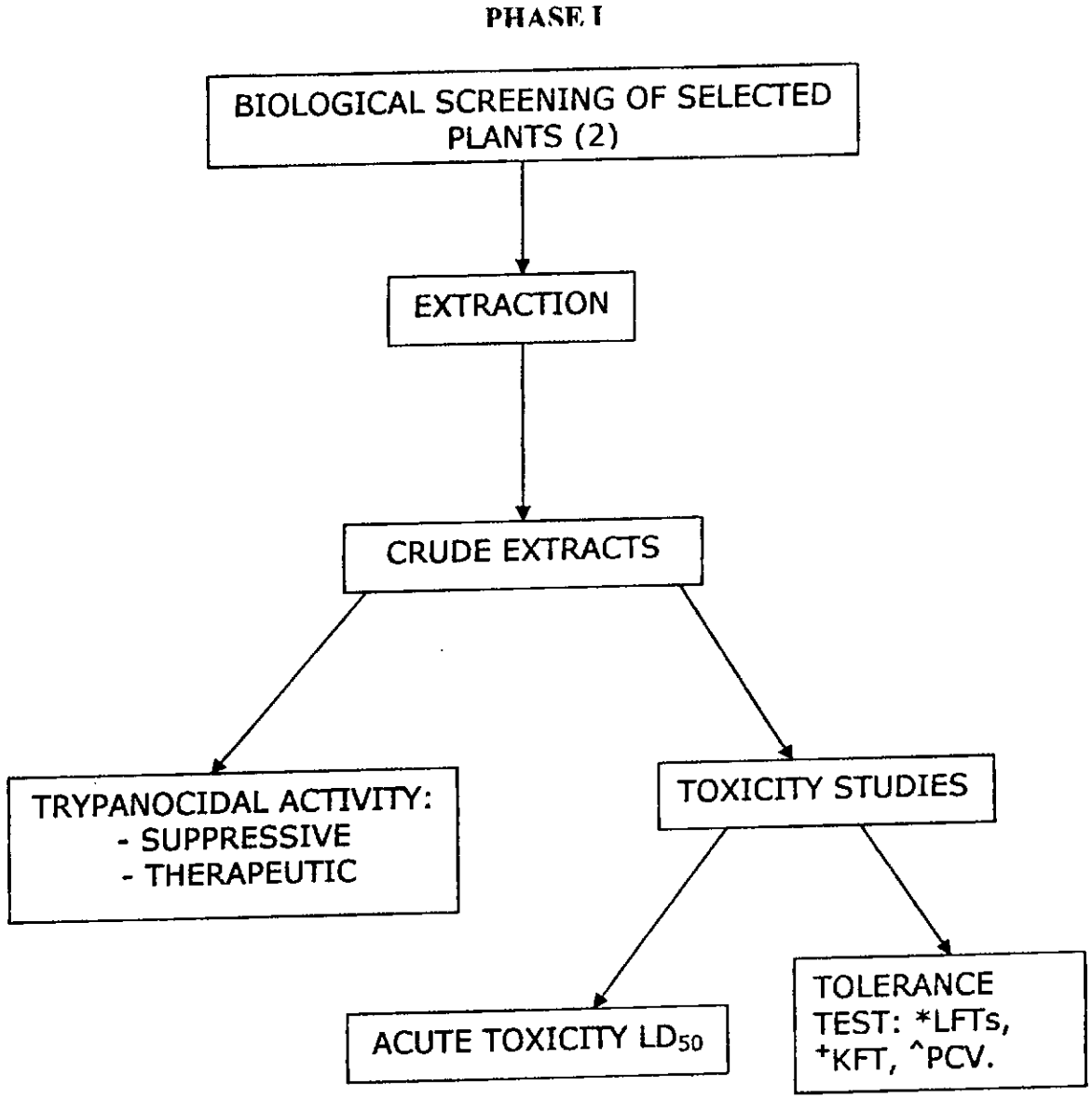
- To biologically screen selected plants *Mitragyna ciliata Aubev & Pellegr (Rubiaceae)* and *Ritchea capparoides Gilg (Capparidaceae)* used in traditional medicine in Nigeria for the treatment of trypanosomiasis using *T. brucei - brucei* infected animals.

- To investigate toxicity studies of the ethanolic root extract of *M. ciliata* on rats in order to establish its toxic level.

- To undertake the *in vitro* studies and activity-guided fractionation of the extract in *T. brucei - brucei* infection.

- To evaluate the trypanocidal activity of the extract by carrying out hematological and biochemical studies in *T. brucei - brucei* infected rats.

## 1.5 STUDY DESIGN




*Liver Function Tests
+Kidney Function Test
^Packed Cell Volume

**Fig. 1a: The first phase of the study**

PHASE II

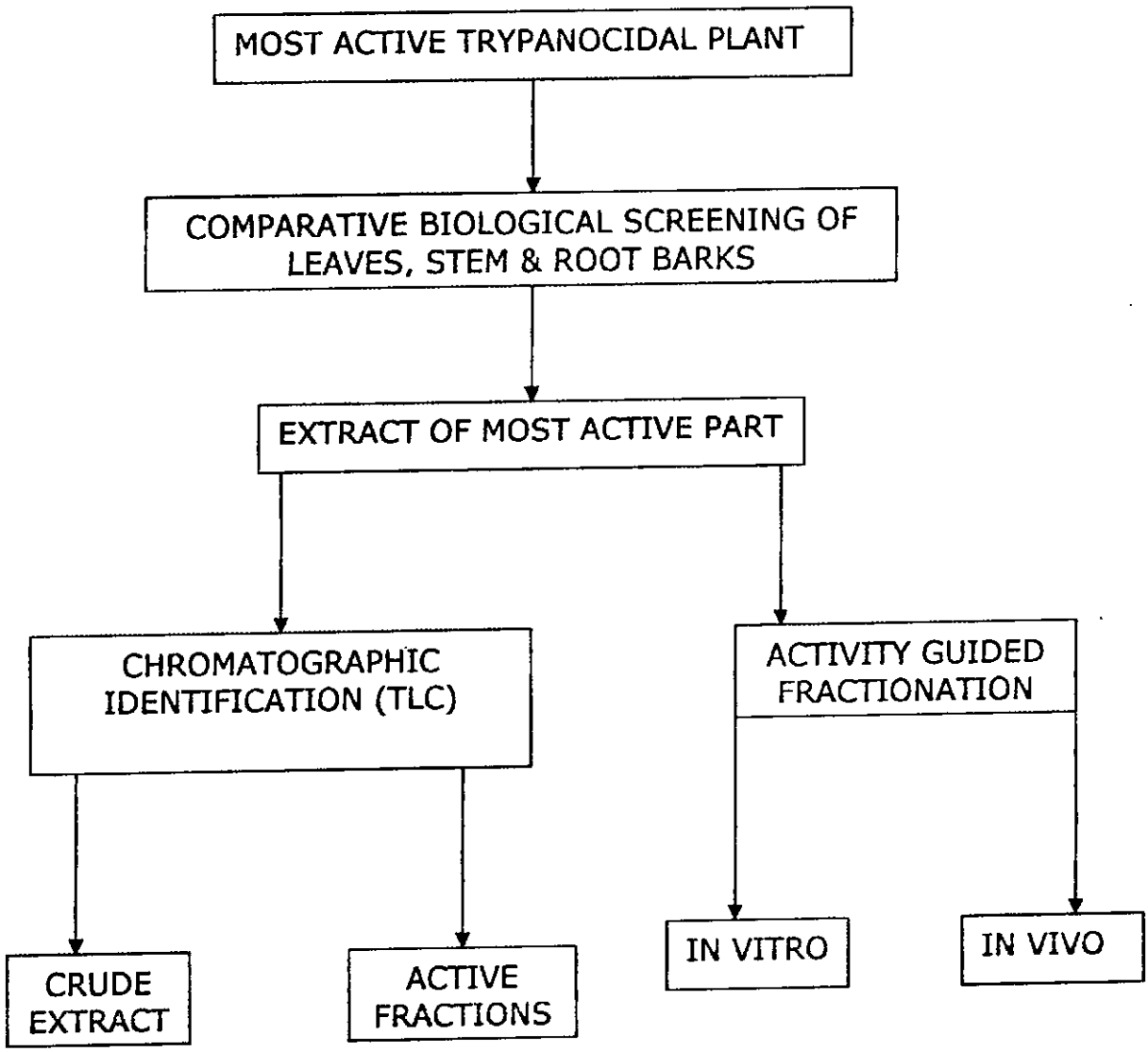



**Fig.1b: The second phase of the study**

**PHASE III**

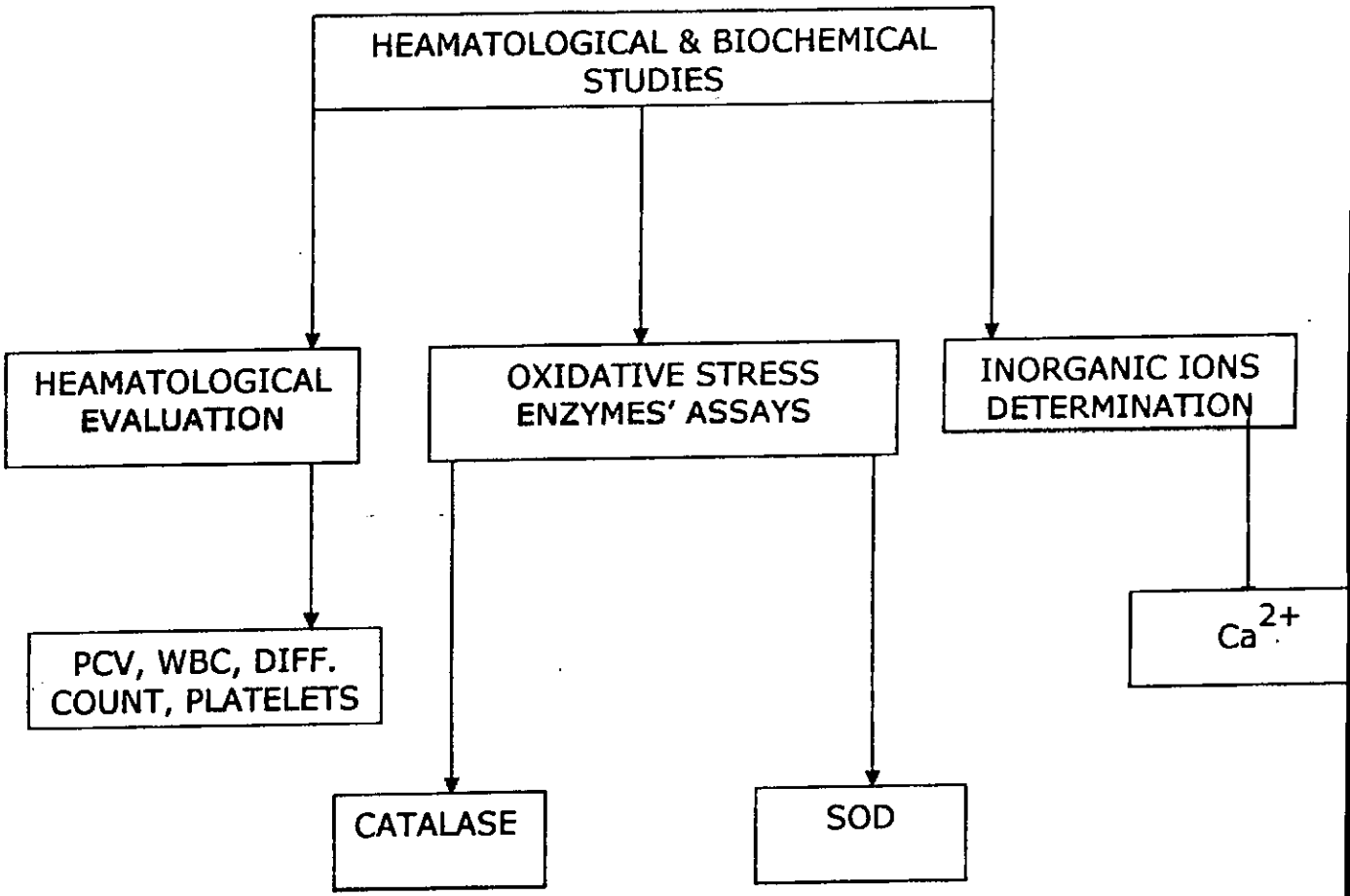



**Fig.1c: The third phase of the study**

# CHAPTER TWO

## 2.0 LITERATURE REVIEW

### 2.1 TRYPANOSOMES

Trypanosomes belong to a widespread group of flagellated protozoa known as the **kinetoplastids**. Members of this group are characterized by a single large mitochondrion containing a body that stains darkly in histological preparations. This body is known as kinetoplast from which the subphylum derived its name. Kinetoplast is a disc-shaped, DNA-containing organelle within the mitochondrion. The group parasitizes everything from human to plants. There are also free-living kinetoplatids that feed on bacteria in aquatic, marine and terrestrial environments. Kinetoplatida genera differ considerably in their host distribution, life cycles, and medical and veterinary importance.Three families are recognized: Bodonidae (coprozoic and free-living), Crytobidae (parasites of fish and invertebrates), and Trypanosomatidae, some members of which are important human and veterinary pathogens (Cox, 1987; Roberts and Janovy, 2000).

**TRYPANOSOMATIDAE**

All species of Trypanosomatidae have a single nucleus and are either elongated with a single flagellum or rounded with a very short, nonprotruding flagellum (fig. 2.1). Many members of the family are heteroxenous: During one stage of their lives they live in the blood and/or fixed tissues of all classes of vertebrates, and during other stages they live in the intestines of bloodsucking invetebrates. In addition, they are hemeoflagellates because laboratory culture of these parasites must contain blood (Roberts and Janovy, 2000).

Trypanosomes are divided into two broad groups based characteristics of their development in their insect hosts.

**Salivaria Group:** The species that develops in the anterior portion of the digestive tract of the insect host is termed anterior station development and the species belong to the salivaria group. Three subspecies of *Trypanosoma brucei*, which are morphologically indistinguishable, belong to this group – *T. b. brucei, T. b. gambience* and *T. b. rhodesiense* (Roberts and Janovy, 2000).

*T. brucei brucei* is fundamentally a parasite of the bloodstream form of native antelopes and other African ruminants (fig. 2.2), causing a disease called *nagana*. The parasite also infects livestock, including sheep, goats, oxen, horses' camels, pigs and mules. It is pathogenic to these animals, humans are however not susceptible.

*T. brucei gambiense* and *T. b. rhodesiense* are the etiological agents of African sleeping sickness. *T. brucei gambiense* causes a chronic infection and it is found in west central and central Africa whereas *T. b. rhodesiense* causes a more acute type of infection and occurs in central and east Africa (WHO, 2001).

**Stecoraria Group:** When a species develops in the hindgut of its invertebrate host, it is said to undergo posterior station development and is placed in the stecoraria group. *Trypanosoma cruzi* belong to this group and is distributed throughout South and Central America. *T. cruzi* cause Chagas disease also known as the American trypanosomiasis. *Leishmania* specie which causes Leishmaniasis is essentially a disease of the reticuloendothlial system. The spleen and the liver become greatly enlarged – hepatosplenogamy (Roberts and Janovy, 2000).

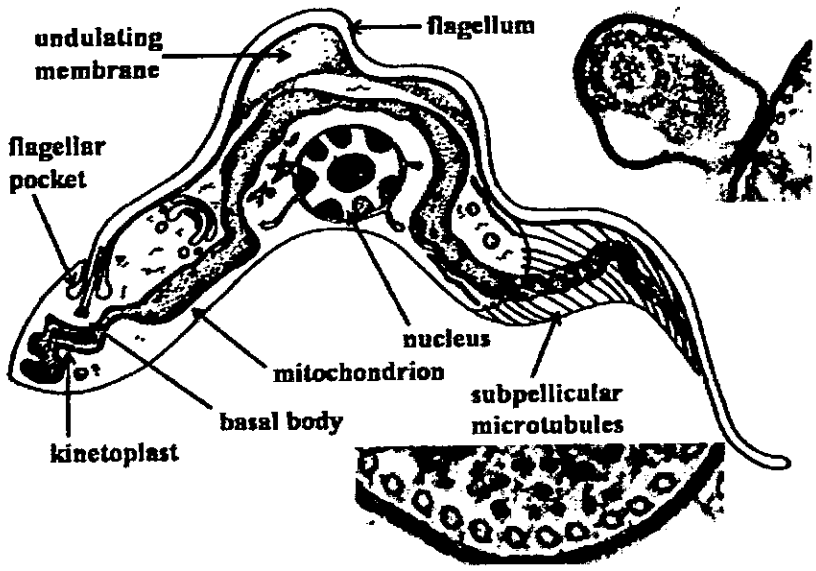
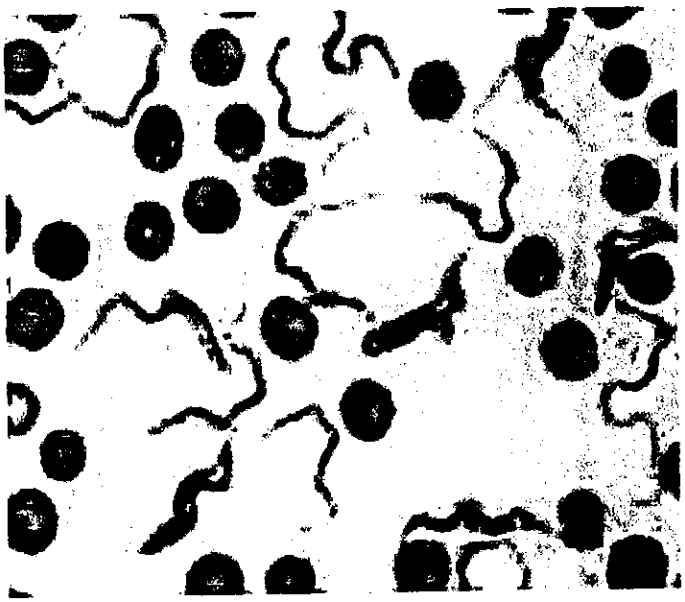



**Fig. 2.1: Diagrammatic Representation of Trypanosome**



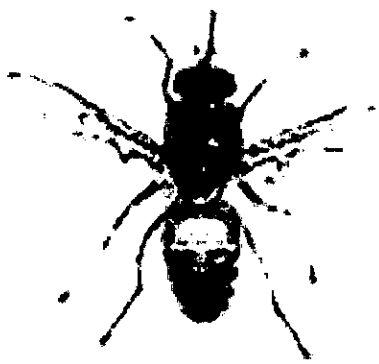
Source: WHO Trypanosomiasis info. (2001)

**Fig. 2.2: *Trypanosome brucei brucei* in thin blood film of infected animal**

## 2.2 TRYPANOSOMIASIS

Trypanosomiasis is a major debilitating and devastating tropical disease that is a target for the World Health Organization's special program for research and training in tropical disease (WHO/TDR) and most recently the drugs for neglected disease initiative (DNDI). Trypanosomatids cause severe diseases in both human and livestock resulting in considerable sufferings especially in developing countries (WHO, 1998).

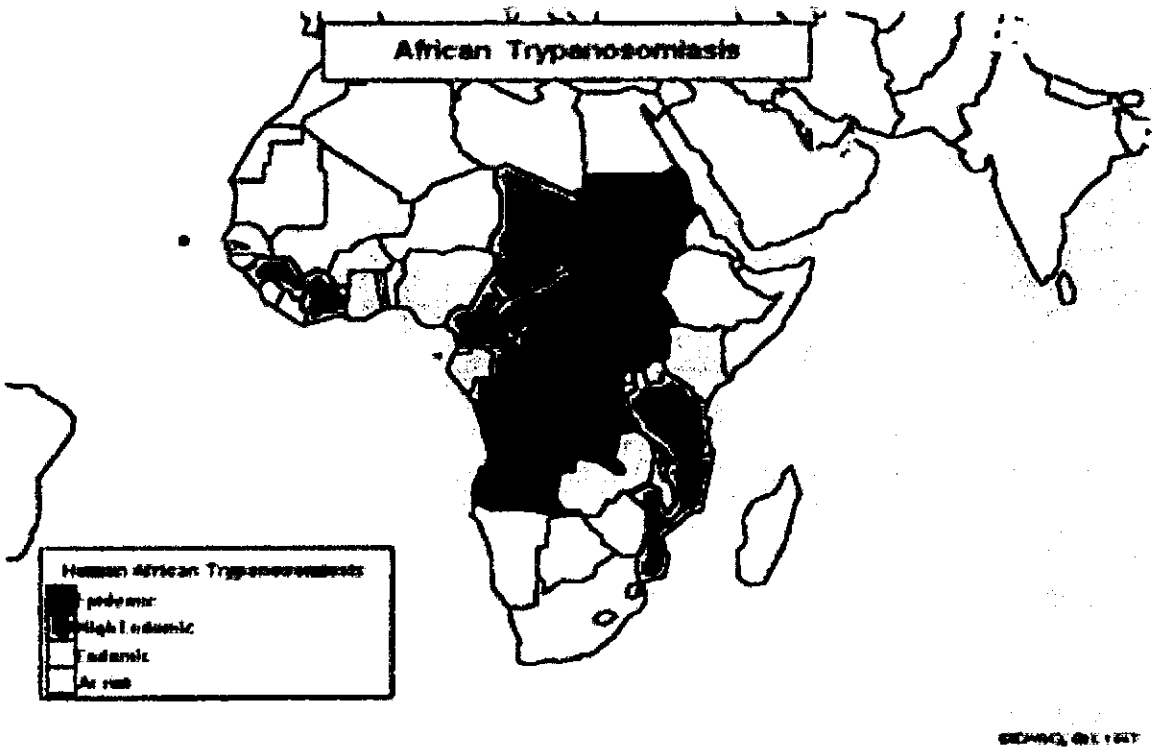
**African Trypanosomiasis:** African trypanosomiasis (sleeping sickness) occurs in 36 sub-Saharan countries (fig. 2.4), within the area of distribution of the tsetse fly (fig. 2.3). 60million people are at risk in these countries (WHO, 1998). The control of African trypanosomiasis is complicated by poverty, political instability, and civil wars often found in areas endemic for the parasites. *T. brucei* is a major pathogen for wild and domestic animals and has far reaching effects on raising of livestock. In fact, although trypanosomiasis can be a devastating human disease, the greatest impact of trypanosomiasis on human health is at the agricultural level. Large areas of Africa are unsuitable for raising cattle and other livestock due to the presence of the tsetse fly vector and the transmission of trypanosomes. This contributes to protein deficient diets among the indigenous population.



Source: WHO Trypanosomiasis info. (2001)

**Fig. 2.3: GLOSSINA SPECIE TSETSE FLY: TheVector of African Trypansomiasis.**

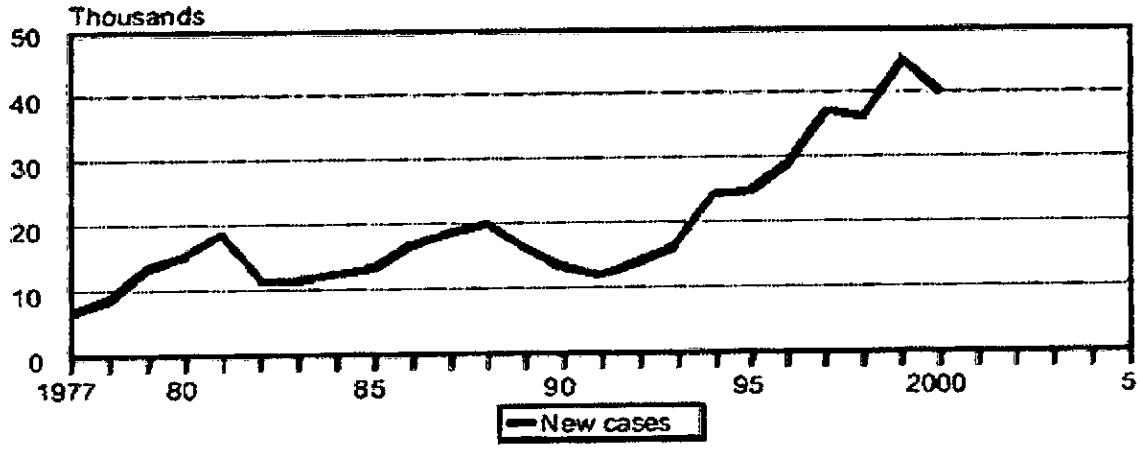
According to recent estimates, the disability adjusted life years (DALYs) lost due to sleeping sickness is 2.0 million (WHO Report 2000). The disease occurs in two forms: the acute and chronic. The chronic infection lasts for years, whilst the acute disease may last for few weeks before death occurs, if treatment is not administered (WHO Report 2000) .




**Source: WHO Trypanosomiasis info. 2001**

**Fig. 2.4: Geographical Distribution of Trypanosomasis**

The health problems caused by these protozoa parasites are currently reaching disastrous proportions (fig. 2.5) with the resurgence of sleeping sickness across central Africa (Suresh *et al.,* 2000; Mishina *et al.*, 2007). Recent estimates indicate that over 60 million people living in some 250 foci are at risk of contracting the disease and there are about 300 000 new cases each year (WHO, 1998). This disease is further complicated by the complex life cycle of these parasites, divided between the vector tsetse fly (*Glossina specie*) and its mammalian host.




**Source: Report of WHO Expert Committee, 2000**

**Fig. 2.5: Incidence of African Trypanosomiasis (1977 –1999)**

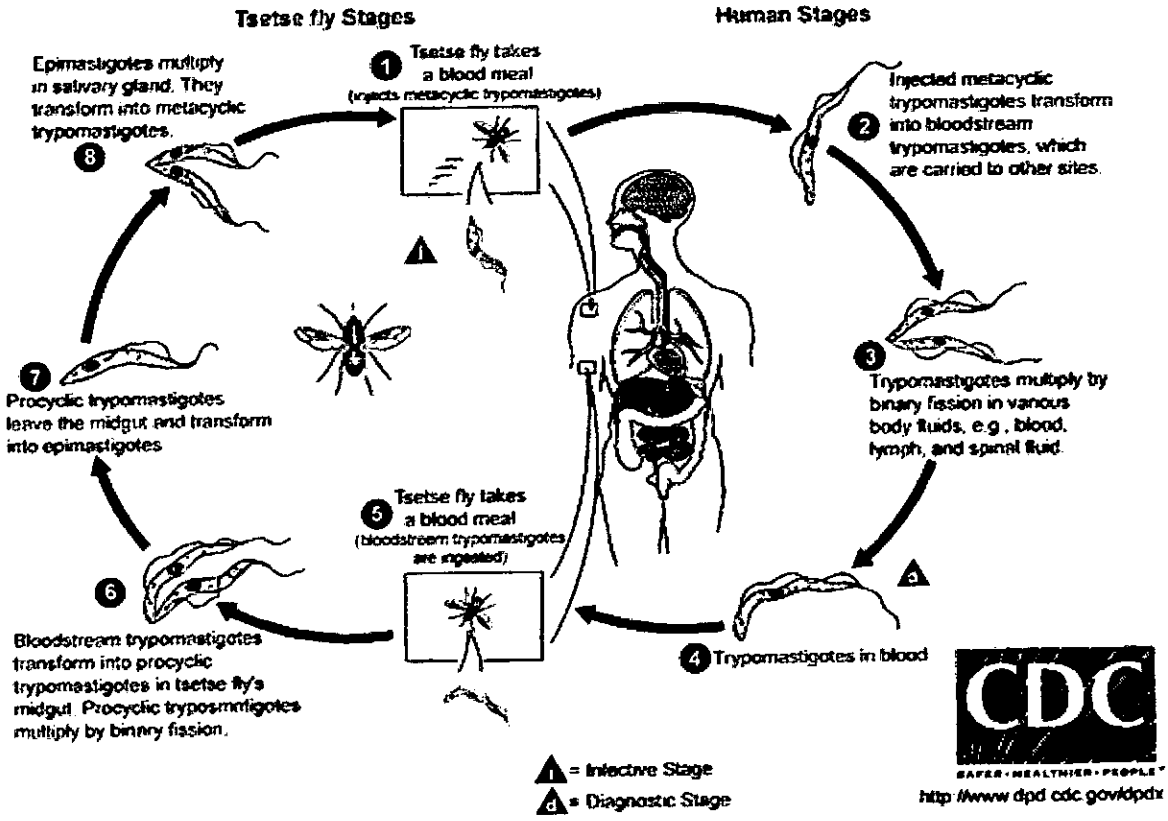
Despite the seriousness of the situation, the drugs in use are outmoded and grossly inadequate. For example, melarsoprol, the only drug against the late stage *T. brucei rhodesiense* infections, has such toxic side effects that 5-10% of patients treated die due to reactive encephalopathy (Suresh *et al.*, 2000). There is therefore an urgent need for better drugs, especially as the effectiveness of the few available drugs is being eroded by increasing resistance (FAO, 1998; Buchmore *et al.*, 2002).

**American Trypanosomiasis:** American trypanosomiasis (Chagas disease) is a zoonotic infection caused by the protozoan parasite *Trypanosoma cruzi* which results in acute, sub-acute and chronic parasitemia with dissemination into various organs especially the heart, brain, esophagus and colon. The disease occurs in North and South America, mostly in the area from Argentina to Mexico. The vectors are large biting insects of the genus *Triatoma.* These insects are found in areas where there are unhygienic conditions associated with poverty.

## 2.3 LIFE CYCLE FORMS OF TRYPANOSOMES

Trypanosomes have a complex life cycle. The entirely parasitic species has two hosts – its vector tsetse fly (in the gut and salivary glands) and mammalian (in blood and other body fluids and tissues). Because of the large difference between these hosts, the cell undergoes complex changes in its life cycle (fig. 2.6) to facilitate its survival in the insect gut and the mammalian bloodstream. A variety of different forms appear in the life-cycles of trypanosomes distinguished mainly by the position of the flagellum. The **trypanomastigote** stage is characteristic of the bloodstream form of the genus *Trypanosoma* as well as the infective metacyclic stages in the tsetse fly vector. In trypanomastigote the kinetoplast and kinetsome are near the posterior end of the body, and the flagellum runs along the surface, usually continuing as a free whip anterior to the body (Roberts and Janovy, 2000). In the typical bloodstream form of trypanomastigote, a simple mitochondrion with or without tubular cristea runs anteriorly from the trypanosome's body. In the insect stage of the organism, the mitochondrion is much larger and more complex, with lamellar cristae.

A spheroid **amastigote** occurs in the life cycles of some species and is definitive in the genus *Leishmania.* The flagellum is very short, projecting only slightly beyond the flagellum pocket. In the **promastigote** stage the elongated body has the flagellum extending forward as a functional organele. The promastigote form is found in the life cycle of several species while they are in their insect hosts (Roberts and Janovy, 2000)




**Source: Safer Helthier People. http//www.dpd.cdc.gov/dpdx**

**Fig 2.6: Life cycle of *Trypanosoma brucei brucei***

## 2.4 SURVIVAL STRATEGY OF TRYPANOSOMES

Trypanosomes are unusual parasites in that they multiply extracellularly within the blood of the mammalian host where they remain fully exposed to continuous immune attack. Their success as pathogens is a consequence of their highly sophisticated strategy of evading the host's immune system through antigenic variation of their Variant Surface Glycoprotein (VSG) coat. Turner (1999) postulates that antigenic variation has evolved as a bifunctional rather than a unifunctional strategy: evasion of humoral immunity and depression of cellular immunity. As trypanosomes multiply in the bloodstream, the host eventually mounts an antibody response against trypanosomes expressing a given VSG. However, as trypanosomes can switch to new VSG coat variants not recognized by host

antibodies; they can evade antibody mediated lyses and form the next wave of infection. As trypanosomes have up to a thousand antigenically distinct VSGs, a chronic infection can be mounted lasting for years (Rudenko and Taylor, 2006). This ability to change their surface coat by trypanosomes has made development of vaccine against the disease difficult. An important mechanism for switching expressed VSG gene is the duplicative transposition of a silent VSG gene into one of the tolomeric VSG expression site of the trypanosomes resulting in the replacement of the previously expressed gene (McCulloch *et al.*, 1997).

### 2.4.1 VARIANT SURFACE GLYCOPROTEIN

The VSGs are a group of glycoproteins of MW 65,000 kilodaltons consisting of a single polypeptide chain and 15 to 30 monosaccharide units. The variant antigen forms a continuous surface coat on the trypanosomes plasma membrane. Trypanosomes VSG belongs to a group of proteins that are anchored in the plasma membrane by means of a phosphotidylinositol-containing glycolipids covalently attached by ethanolamine to the C-terminal end of the protein (Rifkin and Landsberger, 1990). Membrane proteins having such glycolipid anchor are able to partition between the different lipid environments of the donor membrane and the target membrane (Rifkin and Landsberger, 1990). The rate, direction and the extent of such intermembrane transfers depend on the relative lipid composition and fluidity of the donor and acceptor (target) membranes. Studies suggest that the transfer of VSG from trypanosomes to erythrocytes could lead to the pathological features associated with trypanosomes infection, that is, anaemia. This transfer of VSG may sensitize the cells to immune destruction and contribute to anemia associated with African trypanosomiasis (Rifkin and Landsberger, 1990).

### 2.4.2 HEAMATOLOGICAL CHANGES IN TRYPANOSOMAL INFECTION

African trypanosomiasis is characterized by heamatological changes, which drastically influence the pathogenesis of the disease. One major heamatological change is anaemia. Anaemia is a reduction in the quantity of red blood cells, or heamoglobin, in the bloodstream. It is a common feature of both animal and human trypanosomiasis. Indeed some farmers in Kenya treat their cattle with trypanocidal drugs as soon as they observe a sharp decline in packed cell volume (PCV), even before demonstrating the presence of trypanosomes (ILRAD, 1984). During the period of infection anaemia sets in acutely with the first appearance of trypanosomes. This period is also marked by an increase in the activity of macrophages, cells responsible for phagocytosis (ILRAD, 1984, Kagira *et al.*, 2006).

In livestock, most workers have reported many distinct heamatological changes in trypanosomiasis. These changes include leucocytopaenia (Lorne, 1986, Kagira *et al.*, 2006), leucocytosis in *T. brucei* infection in deer mice and rabbits (Emeribe and Anosa, 1991). Thrombocytopaenia (a decrease in the number of platelets) is another significant feature of trypanosomiasis observed in a number of studies (Kagira *et al.*, 2006). Severe microcytic anaemia has also been reported in *T. b. rhodesiense* infection in vervet monkey model of HAT (Kagira *et al.*, 2006)

### 2.4.3 THE ROLE OF CALCIUM IN TRYPANOSOMES

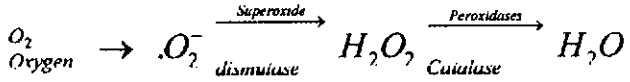
Calcium plays a very important role in trypanosomes. This is substantiated by the fact that calcium binding protein has been purified from trypanosomes where it plays a major role in many cellular functions including cell signaling (Decampo and Morenso, 2001). Calcium which is compartmentalized in organelles known as acidocalcisomes is pumped into these organelles by $Ca^{2+}/H^{+}$ - ATPase pumping system (Decampo *et al.*, 1995).

Calcium is required for association with the inner leaflet of the flagellar membrane; it is required for stable flagellar localization as well (Buchanan *et al.*, 2005). Numerous calcium sensor proteins have been identified in kinetoplastids. Flagellar Calcium Binding Proteins (FCaBP) are localized to the flagellum, a unique organelle that has many functions, including motility, chemotaxis and cell signaling (Buchanan *et al.*, 2005). These proteins undergo calcium-dependent membrane association by virtue of calcium-regulated extrusion or sequestration of a myristate moiety that mediates membrane binding (Buchanan *et al.*, 2005). FCaBP appears to undergo calcium-acyl switching, where association with the flagellar plasma membrane is mediated by calcium and two N-terminal acyl groups. Maldonado *et al.*, (1999) demonstrated that FCaBPs of trpanosomatids are distinct from other myristol switch proteins by virtue of its calcium binding sites. These workers stated that these proteins apparently have a novel mechanism by which they modulate flagellar membrane association.

### 2.4.4 FREE RADICALS IN LIVING SYSTEMS

It is known that the generation of the so-called reactive oxygen species (ROS) is a consequence of the appearance of aerobic life on earth. Thus, molecules such as hydrogen peroxide ($H_2O_2$), superoxide anion ($O_2^{-\bullet}$), and hydroxyl radical ($HO^{\bullet}$) constitute side effects of adopting an extremely efficient mechanism for energy generation through the oxidative metabolism pathway. In mammals, it has been estimated that almost 1–4% of the total oxygen consumption (OC) is converted into $O_2^{-\bullet}$ and $H_2O_2$ (Storey, 1996). The ROS generated must be intercepted or degraded to avoid oxidative damage to several macromolecules (protein, lipids, and DNA), a task accomplished by the antioxidant system (Halliwell and Gutteridge, 1999). This system includes enzymes such as superoxide

dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), where the first degrades $O_2^{-\bullet}$ and the other two are involved in $H_2O_2$ degradation (Sies, 1993).




Enzymatic Pathway for the Detoxification of Reactive Oxygen

**Superoxide Dismutase (SOD)**

Superoxide dismutase (EC.1.15.1.1) is probably the most important enzyme protecting against oxygen toxicity in the body (Aksnes *et al.*, 1980). This enzyme, which is found in all living cells utilizing molecular oxygen, converts superoxide anion ($O_2^-$) to hydrogen peroxide ($H_2O_2$) and oxygen ($O_2$), (Fridovich, 1989). SOD enzymes are present in almost all aerobic cells and in extra-cellular fluids. These SOD enzymes contain metal ion cofactors that can be copper, zinc, manganese or iron depending on the isoenzyme. (Landmesser and Drexler, 2002).

**Catalases (CAT):**

These are enzymes that catalyze the conversion of hydrogen peroxide to water and oxygen, using either an iron or manganese cofactor. These proteins are localized in the peroxisomes in most eukaryotic cells. Catalase is an unusual enzyme since, although hydrogen peroxide is its only substrate, it follows a "ping-pong" mechanism whereby its cofactor is oxidized by one molecule of hydrogen peroxide and then regenerated by transferring the bound oxygen to a second molecule of substrate. CAT and SOD have a close relationship. An increase in SOD activity may protect CAT against enzyme inactivation by superoxide radical as these radicals have been shown to inactivate CAT. Thus, the increase in SOD activity may directly play an important protective role in preserving the activity of CAT (Kono.Y. *et al.*, 1982)

### 2.4.5 FREE RADICALS DEFENCE MECHANISM IN TRYPANOSOMES

Protection against free radicals in trypanosomes is provided by an enzymatic defense system consisting of superoxide dismutase (SOD) and trypanothione. Both the mammalian and protozoan enzymatic systems have this enzyme in common. SOD catalyzes the dismutation of superoxide radical into hydrogen peroxide and oxygen. However, in the mammalian host catalase converts the toxic $H_2O_2$ to molecular oxygen and water. In trypanosomes, catalase is absent (Soulere *et al.*, 1999); this makes the antioxidant defense mechanism in trypanosomes weak, and essentially based on the spermidine-gluthathione conjugate – trypanothione (Khan and Omar, 2007). The reduced form of this enzyme is regenerated in its dithiol form by an NADPH-dependent flavoprotein, trypanothione reductase. As a consequence, most of the trypanosomes and parasitic protozoan are susceptible to free oxygen radical-induced oxidative stress and do not tolerate high concentration of oxygen.

## 2.5 CURRENT STATE OF CHEMOTHERAPEUTIC AGENTS FOR CONTROL OF TRYPANOSOMIASIS

Presently, there are only four approved drugs for Human African Trypanosomiasis (HAT); three of which were developed more than half a century ago: suramin, pentamidine, melarsoprol and eflornithine (Heet *et al.*, 2004). Other molecules such as homidium, isometamidium, and diminazene diaceturate are used in animal infection. Only melarsoprol and eflornithine, which are able to cross blood brain barrier, can be used for the second stage infection. Eflornithine is the only alternative registered drug for the treatment of *T. b. gambiense* sleeping sickness patients who do not respond to melarsoprol. However, apart from other drawbacks, it costs $US 300-500 per patient. Nifurtimox, although not registered for African trypanosomiasis, has been used experimentally and on

compassionate grounds for *T. b. gambiese* sleeping sickness patients who did not respond to melarsoprol, with varying results. New drugs that are safe, effective and affordable are needed. The mechanisms of action of these molecules remain poorly understood except for eflornithine, which inhibits polyamine biosynthetic pathway. All these drugs have to be administered by injection over a long period of time, thus requiring medical facility and specialized staff which often do not exist in rural areas. Adverse effects are often severe and there has been increasing reports of treatment failures especially with melarsoprol. The availability of these drugs has not always been guaranteed as drug companies periodically abandon production because of non-profitability. There is probably more information on the biochemistry and molecular biology of trypanosomes than any other non-mammalian cell type and a great deal is known about the differences between trypanosomes and mammalian cells (WHO/TDR, 2002), yet no drug has yet been designed rationally. Therefore, there is an urgent need to translate these findings into integrated approach for design of new molecules against sleeping sickness which are safe, effective, cheap, and easy-to-administer and for new leads with novel mechanism of action.

### 2.5.1 AVAILABLE DRUGS FOR THE TREATMENT OF TRYPANOSOMIASIS

**Suramin:** This drug is useful in the treatment of early infection of Human African Trypanosomiasis (HAT). Suramin is a sulphated naphthylamine which contains six negative charges at physiological pH. Its charge precludes its diffusion across biomembranes thus it cannot cross the blood brain barrier; therefore it is not effective against late stage trypanosomiasis involving the central nervous system. The mechanism of action of Suramin remains uncertain though it has been demonstrated to inhibit many different enzymes (de Koning, 2001). Suramin uptake into trypanosomes has been shown

to increase in the presence of serum proteins. Suramin because of its charged nature, easily binds to many serum proteins and at therapeutically attainable levels of 70-100μM (Collins et al, 1986). Some serum proteins such as transferrin and LDL are taken up by *T. b. brucei* through receptor mediated endocytosis and it was shown that suramin enters trypanosomes while bound to LDL (de Koning, 2001).

**Pentamidine:** This trypanocide is a highly charged di-cationic molecule. Due to its charge it is only useful in treatment of early stage infection because it has very slow diffusion rate across membrane. Thus it does not cross the blood brain barrier. The mechanism of action of pentamidine is still a matter for intense research but studies have shown that it inhibits transport of several amino acids (de Koning, 2001). It has also been shown that P2 transporter – an adenine/adenosine transporter is involved in the uptake of pentamidine in trypanosomes. Resistance to this drug is also on the increase.

**Melarsoprol:** Melarsoprol is a trivalent arsenical or trypasamide used in late stage trpanosomiasis control. The use of this drug caused blindness by damaging optic nerves in central Africa (de Koning, 2001). Melarsoprol has been shown to act by inhibiting P2 mediated adenosine uptake (Carter and Fairlamb, 1993).

**Eflornithine:** Eflornithine (α-difluoromethyornithine, DFMO) is used against last stage West African sleeping sickness, though availability and price has severely restricted its use. This drug is an inhibitor of ornithyl decarboxylase (ODC) (de Koning, 2001), which in most organisms is the rate-limiting enzyme for synthesis of polyamines from ornithine. DFMO inhibits both the host and trypanosome enzyme and its selective toxicity is a result of different turnover rates of ODC in the two species. Whereas the mammalian enzyme is degraded and replaced very rapidly, the *Trypanosome brucei* ODC is stable. Its irreversible

inhibition by DFMO seriously disrupts the biosynthesis of polyamines, and cause accumulation of *S*-adenosylmethionine metabolites.

**Isometamidium:** Isometamidium chloride (Samorin), a conjugate of homidium and part diminazine diaceturate is used exclusively as a veterinary trypanocide, and is used both prophylactically and therapeutically. Studies showed that it appeared that isometamidium freely crosses the plasma membrane, probably by facilitated diffusion, and is subsequently actively accumulated into the mitochondria, using mitochondria potential as a driving force (de Koning, 2001). Isometamidium action in trypanosomes is dependent on two sets of transporters: facilitated diffusion carriers in the plasma membrane and active transporters in the mitochondria (de Koning, 2001).

## 2.6 CHALLENGES THAT RESULTED IN THE TURN TO MEDICINAL PLANTS

Chemotherapeutic options for the treatment and control of trypanosomiasis are very limited and far from ideal. The increase in the incidences of drug resistance to known trypanocides (FAO, 1998); the toxic side effects of the available drugs (Hoet *et al., 2004);* the unavailability; long treatment regimen and cost of available trypanocides (Buchmore *et al.*, 2002) have led to the search for alternative therapies for the control and management of this disease. This search has focused on medicinal plants used in treatment of trypanosomiasis in traditional African medicine used for the treatment of the disease. This is especially important since trypanosmiasis is restricted to the poor countries of sub-Saharan Africa. Finding an alternative medicine from the abundant medicinal plants in the forests of this area will provide cheaper, simple to administer and easily available drugs for the management and treatment of the disease (Atawodi *et al*; 2003).

## 2.7 MEDICINAL PLANTS

The variety and sheer number of plants with therapeutic properties is quite astonishing. It is estimated that around 70,000 plant species, from lichens to towering trees, have been used at one time or another for medicinal purposes (Chevalier, 2000). The investigation of medicinal plants as potential sources of new drugs to treat parasitic diseases, cancer, cardiovascular diseases, diabetes and AIDS require the search of as many resources as possible. The discovery of phytochemical compounds to treat various diseases that plague man could lead to production of new drugs for the treatment of these diseases. Consequently, scientists have intensified the search for leads from medicinal plants used in ethno – medicine, for the treatment of various diseases.

### 2.7.1 ACTIVE PRINCIPLES IN PLANTS

The active principles in medicinal plants are chemical compounds known as secondary plant products or secondary metabolites. Some of these active principles perform protective functions in the plants, some discourage herbivores; others inhibit the growth and/or survival of bacterial or fungal pathogens. There are two major categories of these compounds – the alkaloids and the glycosides (Trease, 1996).

**Alkaloids:** More than 3000 alkaloids have been identified in 4000 plant species; most occur in herbaceous dicots and also in fungi. Alkaloids contain nitrogen, they are usually alkaline, and they have bitter taste. It is from plants containing alkaloids that a large number of drugs are derived (Evans, 1996). And they have marked pharmacological effects when administered to animals. Their most pronounced actions are on the nervous system, where they can produce physiological and/or psychological results (Evans, 1996).

**Glycosides:** Glycosides are so named because a sugar molecule is attached to the active component. Glycosides are generally categorized by the nature of the non-sugar or active component.

Cyanogenic glycosides release cyanide upon breakdown. The most abundant cyanogenic glycoside is amygdalin (Evans, 1996).

Cadioactive glycosides and Saponins respectively contain a steroid molecule as the active component. Cadioactive glycosides have their effect on the heart muscle and are used for the treatment of heart failure. Some of the deadliest toxins from plants such as the milkweed are cadioactive glycosides.

Saponins: These are glycosides which are characterized by their production of a frothing aqueous solution. They also have hemolytic property and can be highly toxic when injected into the blood. However, when taken by mouth, saponins are comparatively harmless. Sarsaparilla, for example, is rich in saponins but is widely used in the preparation of nonalcoholic beverages (Evans, 1996).

## 2.8 TRADITIONAL HERBAL MEDICINE

According to WHO (De Silva, 1997), about 80% of third world countries still use traditional medicinal plants and herbs for their primary health care due to inaccessibility and cost of modern medicine. Coupled with this, herbs are becoming popular throughout the developed world, as people strive to stay healthy in the face of chronic stress and pollution. Since about 80% of 6.1 billion people of the world live in developing countries, this means that more than 3.9 billion people will likely use medicinal plants on a frequent basis. Therefore, this underscores the need to study medicinal plants for their efficacy, safety and quality and also to search for potentially valuable bioactive agents from which novel therapeutic agents may be created for the benefits of humankind.

Much of what is currently known about pharmacological activity of medicinal plants results from its traditional use or its ethno -medical use. Traditional healing systems usually have active pharmacopoeia. Herbal medicine used by traditional medicine practitioners are so complex (usually a mixture of as many as fifteen plants or more) and variable that what is known scientifically is rarely definitive, but rather a sound pointer to how it works. Traditional use of herbs is based on the experience of the practitioner; herbal medicine is thus both a science and an art (Chevalier, 2000).

### 2.8.1 *MITRAGYNA CILIATA*

*Mitragyna ciliata Aubrev and Pellerg* (Rubiaceae) is an erect evergreen woody plant, found widely distributed in the forest of West African usually growing to a height of 30 meters. It serves as a major source of timber and its close species *M. speciosa* and *M. inermis* are also abundant. The leaves of *M. speciosa* have been chewed for many years under the local name 'Kratom' by the native population of Thailand as a stimulant. Ridley in 1897 reported that the leaves and bark of the same plant are used as a cure for opium addiction.

The different parts of the plant are claimed by traditional healers to possess various therapeutic properties. The general use of this plant in West Africa is as febrifuge and to cure malaria fever (Iwu, 1993, Dogmo *et al.*, 2003), manage hypertension and to facilitate delivery (Dogmo *et al.*, 2003). The decoction of the stem bark is also used for the treatment of broncho-pulmonary diseases (Iwu, 1993), dysentery, mental disorder and epilepsy (Abdulrahman, 1992), rheumatism and gonorrhoea (Jinju, 1990). The leaves of related species *M. inermis* is used for the treatment of hepatic disease in Mali (Adjanohoun *et al.*, 1985). Some of these claims by traditional healers have been investigated and substantiated by experimental evidence. Aji *et al*, (2001) reported that the methanolic extract of stem

bark of a related specie *M. inermis*, produced depressant effect on the central nervous system. Toure *et al.*, (1996) reported that the alkaloids from the leaves significantly increased bile flow in rats administered the extract. Dogmo *et al.*, (2003) reported the anti-inflammatory and analgesic properties of the methanolic stem bark extract of this plant. The alkaloids – mitraphylline and mitragynine are reported to possess a local anesthetics activity, induce a fall in blood pressure and increase cardiac frequency (Dogmo *et al.*, 2003).

The genus contains a mixture of indole alkaloids, the major ones being oxidoles mitraphylline (2-methoxycorynantheine) rynchophylline, mitractine (Iwu 1993, Shellard, 1983). Others are rotundifoline, isorotundifoline, ciliaphyline, rhynchociline, mitragynine and speciociliatine (Dongmo *et al.*, 2003)).

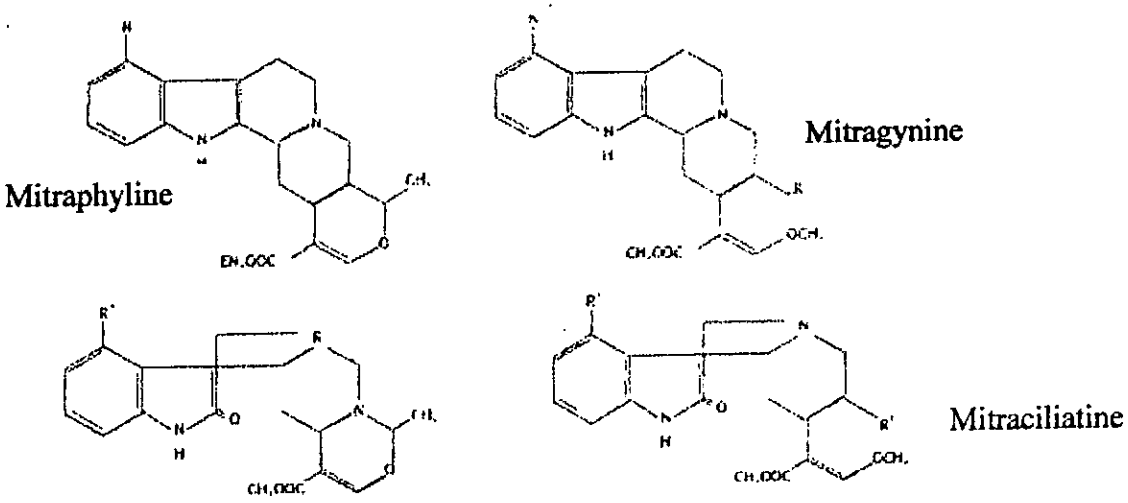



**Fig. 2.6: Structures of *Mitragyna* Alkaloids (Shellard, 1974)**

## 2.9 TOXICOLOGICAL STUDIES OF MEDICINAL PLANT EXTRACTS

Toxicological studies are aimed at finding out if a plant extract or a chemical agent administered to experimental animal has toxic effect. This is a very important aspect of screening and validation of plant products especially in Nigeria, where standardization and safety studies have not been carried out on most of the herbal remedies used by traditional medicine practitioners. These evaluations depend on several factors which include the dose of extract given, route of administration and weight of animal. The dose is of particular importance since all substances are poisons; the right dose differentiates between a poison and a remedy (Tietz, 2000).

In order to assess the toxicity of a plant extract, its effect on major body organs - liver, kidneys, heart, etc that carriy out vital body functions is often evaluated. The most common organs used for the study are the liver, the kidneys and the heart. There are various approaches to toxicity studies – acute, sub-acute and chronic toxicity test.

**Acute Toxicity Test:** Acute toxicity test describes the adverse effect of a substance, which results either from a single dose exposure or from multiple exposures in a short space of time (usually less than 24hours). For plant extracts various doses estimated to cover the range of 0 - 100% lethality is administered to groups of animals. The mortality in each group within 24hours is determined (Thorney, 1999).

**Sub-acute Toxicity:** In this approach various doses of the plant extract is given to a group of animals over a specified period of days, weeks or months to observe changes in physiological function, enzyme system as well as physical and behavioral changes (Yates, 2002).

### 2.9.1 The Liver Function Test

The liver is the major site of intermediary metabolism and synthesis of many important compounds, the site of conjugation and detoxification of natural and potentially toxic foreign substances and the site of storage of glycogen (Tietz, 2000). It is its role in detoxification of toxic substances (it is the seat of drug metabolism) that makes it the organ for assessing the toxic effect of a particular substance. A study of activity of a number of enzymes in the serum is a useful method of assessment. Many of these enzymes, such as alkaline phosphatase, alanine transaminase, aspatate transaminase are involved in intermediary metabolism and thus are present in high concentration in the liver. When the cells are injured or disrupted, as occurs in acute liver disease, these enzymes are released into the serum and their increased levels are often of diagnostic significance. Studies have shown that the serum concentration of enzymes such as alkaline phosphatase and the transaminases (ALT and AST), increases in obstructive jaundice and liver cell damage (Tietz, 2000). Alkaline phosphatase has different isoenzymes and the one originating from the liver has been shown to be the main component of the serum. The transaminases on the other hand, are rapidly released into the serum in cases of acute destruction of tissues such as in myocardial infarction or hepatocellular necrosis. Thus, the level of these enzymes in the serum of experimental animals administered a particular plant extract will give an indication of the effect of the extract on the liver, since the liver is the seat of drug/xenobiotics metabolism.

## 2.10 NATURAL PRODUCTS/MEDICINAL PLANTS WITH ANTITRYPANOSOMAL ACTIVITY AND THEIR MODES OF ACTION

Nature with its numerous plants, microorganisms and marine organisms is a potential source of new drugs for the treatment of trypanosomiasis. These organisms contain countless quantity of molecules with great variety of structures and pharmacological activities. Several established human antiprotozoa drugs have their origin in nature such as quinine, an alkaloid from *Chincona sp.* (*Rubiaceae*) and artemisinin a sesquiterpene lactone from *Artemisia annua* (*Asteraceae*) used to treat malaria. Additionally, these antiprotozoa plant-derived compounds have been used as leads to develop other semi-synthetic or synthetic drugs with better efficacy, safety or pharmacokinetics profile (Hoet *et al.*, 2004). Reports on plant extracts active against trypanosomes are not abundant compared to other protozoal diseases such as malaria. Isolated natural products (fig. 2.7) active against trypanosomes are even less abundant. Majority of the isolated compounds (e.g. tryptanthrins) have been tested *in vitro* (Scovil, 2001). Only a few natural products have been evaluated for *in vivo* activity in animals and none have been evaluated clinically in humans (Hoet *et al.*, 2004).

The modes of action of many natural products and medicinal plants have been studied. The action of these products on trypanosomes range from inhibition of specific enzymes, inducing oxidative stress, effect on protein synthesis to DNA intercalation.

**1. Alkaloids:** Several alkaloids have been tested on trypanosomes *in vitro.* Isoquinoline and quinoline alkaloids exhibited $IC_{50}$ values below 10μmM. However, *T. congolense* is less susceptible to these compounds than *T. b. brucei.* Among all compounds tested *in vitro* in various studies, the natural alkaloids themselves showed highest activities (Hoet *et al.*,

2004). Table 2.1 shows a list of natural alkaloids tested *in vitro* against trypanosomes and their $IC_{50}$s.

**TABLE 2.1: ALKALOIDS WITH ANTITRYPANOSOMAL ACTIVITY**

| ALKALOID | TEST ORGANISM | $IC_{50}$ (μM) |
|---|---|---|
| 1.Quinoline Alkaloids | | |
| Quinidine | *T.b. brucei* | 0.8 |
| Cinchomine | ,, | 1.2 |
| Quinine | ,, | 4.9 |
| Cinchomdine | ,, | 7.1 |
| 2.Isoquiinoline alkaloids | | |
| Emetine | ,, | 0.039 |
| ,, | *T. congolense* | 0.4 |
| 3.Benzylisoquiline alkaloids | | |
| Beberine | *T. b. brucei* | 0.5 |
| Sanguinarine | *T. b. brucei* | 1.9 |
| Berbamine | ,, | 2.6 |
| 4.Bisbenzylisoquinoline alkaloids | | |
| (BBIQ) | *T.b.brucei* trypomastigote | 1-2 |
| 5.Naphthylisoquinoline alkaloids | | |
| Dioncophylines | *T. brucei & rhodesiense* | 2-3 |
| Pancracine | *T. rhodesiense* | 2.4 |
| Fascaplyin | ,, | 0.6 |

**Source: Hoet *et al*, 2004.**

**Mode of action of alkaloids:**

Mechanism of action of various alkaloids especially isoquloline and quiloline such as the quiloline alkaloids from *Cinchoma* bark *(Rubiaceae)* have been studied for their trypanocidal activity. DNA intercalation in combination with the inhibition of protein

synthesis could be responsible for the observed trypanocidal effect (Hoet *et al.*, 2004). The activity of oxoaporphines isolated from *Cassytha filiformis* (*Lauraceae*) active against *T. brucei brucei* bloodstream forms is by intercalation with DNA, which seems to be related to their ability to stabilize the DNA helix against heat denaturation and to inhibit the catalytic activity of topoisomerase I. However no clear structure-activity relationship could be extracted from the study of several bisbenzylisoquinoline (BBIQ) alkaloids (Hoet *et al.*, 2004).

A well know antitumor agent – Camptothesin – a pentacyclic alkaloid isolated from *Campptotheca acuminate (Nyssaceae)*, inhibits the nuclear and mitochondrial topoisomerase I of *T. brucei* thus blocking DNA replication and inducing cell death of bloodstream trypanomastigotes ($IC_{50}$ = 1.6 μM).

**2. Phenolic Derivatives:**

Many derivatives of phenolic compounds have been found to be active against trypanosomes *in vitro*. Table 2.2 shows a list of phenolic derivatives that are active against trypanosomes and their $IC_{50}$.

**Mechanism of action of phenolic derivatives:**

Ascofuranone, a prenylated phenol isolated from phytopathogenic fungus, *Ascochyta visiae* is a potent inhibitor of the glycerol-3-phosphate-dependent mitochondrial oxygen consumption of *T. brucei* bloodstream forms. The specificity of this compound is due to the presence of a unique mitochondrial electron transport system. This system is composed of two enzymes, a glycerol-3-phosphate dehydrogenase and trypanosome alternative oxidase (TAO) - a ubiquinone: oxygen oxidoreductase. The two enzymes interact via ubiquinone, also known as coenzyme Q. The mechanism of action of Ascofuranone, which has a

structure analogous to that of coenzyme Q, is attributed to its binding at the coenzyme Q site of the ubiquinol oxidase, thus blocking the TAO (Hoet *et al.*, 2004).

Gallic acid and gallic acid esters have been suggested to exert their trypanocidal effect by the formation of reactive oxygen species (such as superoxide anion). In other words, it acts as a prooxidant (Hoet *et al.*, 2004). A certain flavonol has been shown to be able to inhibit $F_1$-ATPase of *T. cruzi* responsible for Chagas disease (Scovil *et al.*, 2001). Four methoxylated flavones were isolated from the leaves of *Ehretia amoena* (Boragiaceae) a plant used traditionally to treat sleeping sickness. The isolates were – chrysosplenetin, chrysosphenol D, retusin and artemetin. Chrysosplenetin and chrysosphenol D were most potent and retusin was shown to increase synergistically their activity (Hoet *et al.*, 2004).

**TABLE 2.2: PHENOLIC DERIVATIVES ANTITRYPANOSOMAL ACTIVITY**

| PHENOLIC DERIVATIVE | TEST ORGANISM | $IC_{50}$ (μM) |
|---|---|---|
| 1.Ascofuranone + glycerol | *T. b. brucei* | 0.03 |
| Ascofuranone – glycerol | ,, | 250 |
| Gallic acid | ,, | 15.6 |
| Ethyl gallate | ,, | 2.3 |
| n-Propylgallate | ,, | 1.5 |
| 2.Flavonoids | | |
| 7,8-Dihydrxyflavone | *T. b. rhodesiense* | 0.16 |
| Quercetagetin | ,, | 0.8 |
| Quercetin | *T. cruzi* | 13.2 |
| Chrysosplenetin | *T. b. rhodesiense* | 2.9 |
| Chrysosplenol | ,, | 4.7 |
| Cissampelflavone | ,, | 1 |
| Curcumin | *T. b. brucei* | 0.83 |
| Piscatorin | *T. b. rhodesiense* | 0.55 |

Source: Hoet *et al*, 2004

### 3. Quinones:

Quinones have been reported to induce oxidative stress in trypanosomes (*T. congolense* and *T. cruzi*). Many quinones have been isolated from plants and other organisms have been shown to possess antitrypanosomal activity. Table 2.3 shows quinones with *in vitro* trypanocidal activity and their $IC_{50}$.

**TABLE 2.3: QUINONES WITH ANTITRYPANOSOMAL ACTIVITY**

| QUINONE | TEST ORGANISM | $IC_{50}$ (µM) |
|---|---|---|
| Plumbangin | *T. b. brucei* | 1.5-6.5 |
| Diospyrin | „ | 50 |
| Diospyrin dimethyl ether | „ | 24 |
| Furanonaphthoquinone | *T. b. brucei & rhodesiense* | 0.12 |
| Isopinnatal | „ | 0.73 |
| 4'0-ß-D-Glucoside | „ | 1.2 |
| Azanthraquinone | *T. congolense* | 5 |

**Source: Hoet *et al.* (2004)**

**Mechanism of action of quinones:**

Studies have reported that quinones, especially 1, 4-naphtoquinones such as plumbagin can induce oxidative stress in trypanosomes (*T.congolense and T. cruzi*). This may be explained by the reduction to semi-quinone radicals by enzymes such as those present in the mitochondrial electron transport chain and the trypanothione reductase, a key enzyme of the trypanosomal antioxidant thiol metabolism (Hoet *et al.*, 2004). Azaanthraquinone isolated from *Mitracarpus scaber (Rubiaceae)* ethanolic extract inhibited motility and induced lysis of T. congolense bloodstream form *in vitro*. Nok (2002) showed that azaanthraquinone inhibits glycerol-3-phosphate mitochondrial oxygen consumption in T.

congolense bloodstream forms. It blocks the TAO of the mitochondrial electron transport system by apparently the same mechanism as ascofuranone.

**4. Terpenes:**

Workers evaluated the effect of several mono- and sesquiterpenes which are frequently present in essential oils, on the viability of bloodstream form of *T. b. brucei*. Only one monoterpene - terpinen-4-ol was active at $IC_{50}$ of 0.13μM. Helenalin and mexicanin, two sesquiterpenes had *in vitro* trypanocidal activity with $IC_{50}$ of 0.05μM and 0.32 μM against *T. b. rhodesiense*. A diastereoisomer of the diterpene Kolavenol had an $IC_{50}$ of 8.6 μM against *T. b. rhodesience* bloodstream trypanomastigotes (Hoet *et al*, 2004).

**Fig 2.7a Asofuranone**

**Fig 2.7b Camptothecin**

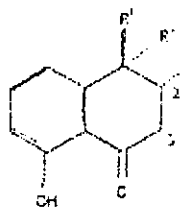


**Fig 2.7c Plumbangin**

**Fig 2.7:** Structures of Some Natural Products with Antitrypanosomal Activity (Hoet *et al*, 2004).

**Mechanism of action of terpenes:**

The mechanism of action of the trypanocidal effect of several terpenes evaluated suggests that stereochemistry plays an important role, since it was shown by workers that diastereomers of the same compound show differential activity (Hoet *et al.*, 2004). The workers postulated that the mechanism of action of these molecules towards trypanosomes might depend on interference with trypanothione metabolism (for example trypanothione reductase) leading to increased oxidative stress in parasites.

## 2.11 POTENTIAL CHEMOTHERAPEUTICS TARGETS FOR TRYPANOSOMES

In this genomic, proteomic, and bioinformatics era of target identification, scores of potential targets for antitrypanosomal chemotherapy will be emerging. Many of these potential targets have formed the basis for designing chemotherapeutic agents against trypanosomiasis. Some of these targets have been validated and others have not. Biological features of trypanosomiasis that can be turned to targets have to meet the following requirements: - (Khan, 2007).

(a) The target must be essential for the survival of the parasites.

(b) The target must be such that a counterpart in the mammalian host either does not exist or is sufficiently different to allow selective inhibition.

Some of the biological features extensively studied as targets are:-

**Glycolytic pathway:** Trypanosomal cells are completely energetically dependent on glycolysis which makes it a good target for chemotherapeutic drug design. Compartmentation of glycolytic enzymes is generally assumed to result in an enhancement of the rate of glycolysis in bloodstream trypanosomes, approximately 50 times (Khan,

2007). Trypanosomes need to replicate every 6-8hrs in mammalian blood and Variant Surface Glycoproteins (VSG) must be replaced frequently to evade a host immune response (Rodenko and Taylor, 2006). Thus, it has been suggested that inhibition of the glycolytic enzymes inside the glycosomes may block glycolytic activity and kill bloodstream trypanosomes (Michels *et al.*, 2000). The 3D structure of glycosomal glyceraldehydes-3-phosphate dehydrogenase (G3PDH) (Helfert *et al.*, 2001) could provide opportunities for designing selective inhibitors as it differs from the mammalian homolog (Khan, 2007). A second potential target in glycolysis is the trypanosomes hexose, transporters (THT). The genes encoding trypanosomal glucose transporters are two - THT 1 and THT2. THT 1-encoded glucose transporters preferentially expressed in a bloodstream form, have a moderate sensitivity to cytochalasin B and recognize D-fructose as a substrate, thereby distinguishing them from the human erythrocyte glucose transporter (Eisenthal and Cornish-Bowden, 1999).

**DNA topoisomerases:** There are two potential sites for DNA binding in trypanosomes: nuclear and kinetoplast DNA. It was assumed that binding to DNA-dependent processes, but it is now generally accepted that intercalating agents induce topoisomerase II – mediated strand breaks in DNA (Khan, 2007). Trypanosomal topoisomerase II inhibitors affect both nuclear and mitochondria DNA and may prove to be effective and safe antitrypanosomal drugs as they differ structurally from mammalian topoisomerase II (Khan, 2007).

**Ergosterol biosynthesis:** Ergosterol biosynthesis is a novel metabolic pathway essential for parasitic survival lacking a counterpart in the host. Several enzymes of this pathway e.g. squalene synthase, fernsylpyrophosphate synthase are capable of depleting endergonous sterols, and therefore represent viable chemotherapeutic targets (Khan, 2007).

**Purine salvage pathway**: Most parasites lack the *de novo* purine biosynthesis mechanisms and rely on salvage pathway to meet their purine needs. There are sufficient distinctions between enzymes of purine salvage pathway in the mammalian host and trypanosomes that can be exploited to design specific inhibitors for the parasitic enzymes. Furthermore, the specificities of purine transport, the first step in purine salvage, differ significantly between trypanosomes and their mammalian host to allow selective inhibitor design (Khan, 2007).

**Polyamine biosynthesis**: The ability to synthesize polyamines is vitally important for the proliferation of bloodstream trypanosomes in an environment deficient in polyamines. Ornithine decarboxylase (ODC), *S*-adenosyl-L-methionine and spermidine synthase in trypanosomes serve crucial functions (Cox, 1987) and may be potential targets for antitrypanosomal chemotherapy. Trypanothione - a conjugate of gluthathione and the polyamine spermidine is responsible for maintenance of intracellular redox environment. This polyamine component of the structure of trypanothione rationalized the actions of several antitrypanosomal drugs. DFMO, the first new drug licensed to treat trypanosomiasis over 40 years, inhibits ODC which catalyzes the initial step in polyamine biosynthesis decreasing trypathione pool (Barret, 1999).

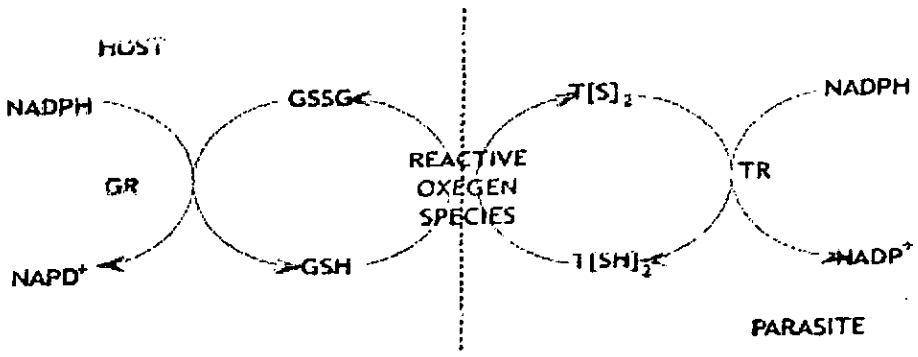



**Fig 2.8: Outline of gluthathione and trypanothione based redox defence (Khan, 2007).**

Trypanothione is unique to the parasites and its synthesis proceeds from gluthathione. In mammals, regeneration of protective GSH from GSSG is catalyzed by gluthathione reductase. In trypanosomes (fig. 2.8), this is catalyzed by a disulfide ($T[SH]_2$ trypanothione reductase (TR) which differs from GSSG by the presence of a spermidine crosslink between the two glycyl carboxyl groups. With this fundamental metabolic difference trypanothione reductase has been proposed as a target for rational design for antitrypanosomal (Khan, 2007).

# CHAPTER THREE

## 3.0 MATERIALS AND METHODS

### 3.1 MATERIALS

**i) Plant Materials**

The root of *R. longipedicellata* (*Capparidaceae* local name "Ologbe-kuyan") and *Mitragyna ciliata* (*Rubiaceae* local name "*abura*") were collected by Mr. Felix Isang of Forestry Research Institute (FRIN) Ibadan. The plants were collected from the Onigambari Forest Reserve and authenticated by Mr.T.K. Odewo of the same Institute. Voucher specimen of the plant with No FHI: 106997 and FHI: 106999 respectively, were deposited at the FRIN Herbarium.

**ii) Animals**

Animals of mixed sexes - Swiss albino mice weighing about 20 g and Wistar rats weighing between 200–250 g, purchased from the animal houses of College of Medicine. University of Lagos, Nigerian Institute of Medical Research (NIMR), Yaba-Lagos and University of Nigeria, Nsukka were used for the experiments during the research period. The animals were housed in standard mouse and rat cages and were maintained on standard commercial livestock feeds and water *ad libitum.*

**ii) Test organism**

The parasites - *T. brucei brucei* were obtained from Nigerian Institute of Trypanosomiasis Research (NITR), Jos and isolates from infected cow at the Department of Veterinary Parasitology and Entomology, University of Nigeria, Nsukka. They were authenticated by Dr. Ikenna Eze of the same department. They were maintained by continuous passaging in mice in the laboratory until needed.

**iii) Chemicals and Solvents**

Laboratory solvents and chemicals obtained from Sigma-Aldrich Germany were used for the experiments. Reference drug diaminazine diaceturate manufactured by Pfizer Parmaceutical Company, Lagos, was purchased at Eagle Agricultural and Veterinary Services, Allen Avenue, Ikeja.

iv). Equipment

General laboratory glassware, Glass column, syringes, cannular, Specimen and sample bottles, Silica gel GF pre-coated (250 microns) fluorescence alumina plates (10x20), (Sigma-Aldrich, UK) Spectrophotometer (Thermospectronic Genesys 4001/1, USA; Spectonic Genesys TMS, USA), Light Microscope, (Carl Zeiss, Germany, Swift Instruments International, South Africa) Camera Microscope (Carl Zeiss, Germany, Swift Instruments International, South Africa) , Micro-hematocrit centrifuge (Uniscope Sm 112, Spingfriend Medicals, England), Hematology analyser (Coulter $A^c$. T.diff., Beckman coulter, Miami, USA.), Centrifuge (Uniscope Sm 112, Spingfriend Medicals, England). Freeze Drier (Thermoservant, Microduodyo, USA).

## 3.2 METHODS

### 3.2.1 PRELIMINARY STUDIES

### 3.2.2 Extraction of Plant Materials

500 g each of air-dried crushed (with local manual grinder) plant materials (root) was macerated with 2.5 L EtOH-$H_20$ (8:2 v/v) for 24 hours (by cold extraction method). The process was repeated several times to remove extractable components. The extract solution was filtered with Whatman filter paper, concentrated by open air evaporation on the bench, freeze-dried and stored in the refrigerator until needed.

### 3.2.3. Phytochemical Analysis

Phytochemical analysis of hydroethanolic extract of *R. longipedicellata* and *M. ciliata* were performed according to the method of Sofowora (1982). The presence of alkaloids, glycosides, reducing sugars, tannins, saponins and flavonoids etc. were determined.

**Test for Alkaloids:**

0.5 g of the extracts was stirred with 5 ml of 1 % aqueous HCl on a steam bath, 1ml of the filtrate was treated with a few drops of Mayer's reagent and a second 1 ml portion was treated similarly with Dragendoff's reagent. Turbidity or precipitation indicated the presence of alkaloids in the extracts.

**Test for Anthraquinones:**

Bontrager's test for anthraquinones was employed. 5 g of each extract was shaken with 10 ml benzene, filtered and 5 ml of 10 % ammonia solution added to the filtrate. The mixture was shaken and the presence of a pink red or violet colour in the ammonical (lower) phase indicated the presence of anthraquinones.

**Test for Phlobatannins:**

Aqueous extracts (2 ml each) were boiled with 1 % aqueous HCl and the appearance of red precipitate indicated the presence of phlobatannins.

**Test for Saponnins:**

0.5 g of each extract was shaken with water in a testtube. Frothing which persists on warming was taken as an indication of the presence of saponins.

**Test for Tannins:**

About 5 g of each extract was stirred with 10 ml of distilled water, filtered, and $FeCl_2$ reagent added to the filtrate. A blue-black, green or blue-green precipitate was taken as evidence for the presence of tannins.

**Test for Glycosides:**

**Legal Test:**

The extract was dissolved in pyridine and a few drops of 2 % sodium nitroprusside together with a few drops of 20 % NaOH were added. A deep red colour which faded to brownish-yellow indicated the presence cardenolides.

**Libermann's Test:**

0.5 g of the extracts was dissolved in 2 ml of acetic anhydride and cooled well in ice. Sulphuric acid was then carefully added. A colour change from violet to blue to green, indicate the presence of steroidal nucleus/aglycone portion of cardiac glycosides.

### 3.2.4. Toxicological Studies

**Acute Toxicity Test:** Determination of the dose of hydroethanolic root extract of *M. ciliata* and *R. longipedicellata* that would kill 50 % of animal population ($LD_{50}$) was undertaken. Five groups of 5 mice each of male albino mice weighing approximately 20 g were used for the study. They were fasted for 12 hrs but were allowed water freely. Doses of 20 g/kg, 15 g/kg 10 g/kg, 5 g/kg and 2.5 g/kg were administered orally with the aid of a cannular. Animals were observed for 24 hrs and mortality was recorded. The $LD_{50}$ was calculated by probit analysis method (Rane *et al*; 1976).

**Sub-acute Toxicity Test:** Determination of the effect of the hydroethanolic extracts of *M. ciliata and R. longepedicellata* on liver enzymes of rats was carried out. 30 rats, divided into groups of 10 rats each were used for this test. Two groups (test groups) were administered the extracts dissolved in 5 % carboxy-methyl cellulose (CMC), at a dose of 800 mg/kg (selected from $LD_{50}$) for 21 days. The third group served as control and was given 5 % CMC. The animals were fasted overnight after the last administration, and then bled at the end of the day 22. Alkaline Phosphatase (ALP), Alanine Transaminase (ALT),

Aspartate Transaminase (AST), Creatinine and Bilirubin levels were determined using commercially available kits supplied by Randox Industries, UK. PCV levels were determined by the micro heamatocrit method.

### 3.2.5. Biological Screening and Selection of Trypanocidal Plant

Due to the ability of animal trypanosomes (*T. brucei brucei*) to infect all categories of animals, not being species' specific (Roberts and Janovy, 2000), the anti-trypanosomal screening of hydroethanolic extract of *M. ciliata* and *R. longipedicellata* and their combination (1:1 v/v) was carried out using the rodent model - mice specifically (Sofowora, 1993; Dutta, 1996; Mokhorjee, 2007). The potential of both plants to suppress and clear parasites from bloodstream of *T. brucei* -infected mice was investigated.

**Suppressive and Therapeutic Activities of Crude Plants Extracts**

Thirty six mice of both sexes were used for this experiment and were inoculated with $10^6$parasites/ml of blood. Screenings of suppressive and therapeutic activities were carried out according to the method of Dutta (1996). For suppressive activity, extracts were administered orally 2 hrs after infection for five days at doses of (50 & 100) mg/kg for *M. ciliata* and (400 & 800) mg/kg for *R. longipedicellata*. The combination of *M. ciliata* and *R. longipedicellata* at a ratio 1:1 (v/v) and at doses (200 & 400) mg/kg were also administered. For therapeutic activity, extracts and their combination were administered at the same doses after 5 days of infection (after expression of about 0.2 % parasitemia). Reference drug (diminazine diaceturate) was administered at 3.5 mg/kg for both activities. The levels of parasitemia of each mouse and rat were monitored during the administration of the extracts and drug for a period of 28 days. The parasiteamia levels were estimated by Rapid Matching Method of Herbert and Lumsden (1976). Parasiteamia levels were compared with untreated control animals and percent inhibition of parasites assessed.

Assessment of the extracts and fractions was also based on survival periods and survival rates of the animals.

### 3.2.6 Evaluation of Selected Plant

The most active plant was subjected to further screening, to determine which of its part posess the greatest trypanocidal activity. The rodent model - rats were employed for this comparative study (Mokhorjee, 2007).

#### *In vivo* Comparative Studies of Parts

Hydroethanolic extracts of *M. ciliata* parts: the leaf, stem and root barks were screened for comparative trypanocidal activity. Rats were inoculated with $10^{o}$ trypanosomes per ml of blood. Two groups served as positive control (reference drug - diminazine diaceturate) and negative control (untreated) respectively and a third group served as the test group. The test groups were administered orally with the ethanolic crude extract of the leaves, stem bark and root bark. Doses chosen from the acute toxicity test studies were administered at 500 mg/kg for leaves and stem bark, 50 mg/kg for root while the reference drug was at a dose of 3.5 mg/kg. Administration was for 5 days. Parasitemia levels were monitored for 28 days.

### 3.2.7 Further Studies of Active Plant Part

The hydroethanolic extract of the most active plant was evaluated for free radical scavenging activity; for its constituents; its *in vitro* anti-trypanosomal activity; for identification of its active fraction by activity-guided fractionation and for its effect on the some biochemical parameters in infected rats.

**i) *In vitro* Free Radical Scavenging Activity**

The *in vitro* free radical scavenging activity of 1, 1 - Diphenyl-2-picrylhydrazyl (DPPH) free radical by *M. ciliata* hydroethanolic extract was determined according to the method reported by Gyamfi *et al.*, (1999). *M. ciliata* hydroethanolic root extract (50 µL) was prepared in methanol, to yield a series of extract concentrations of 0.02, 0.04, 0.08 and 1.0 mg/ml. Each concentration was mixed with 1000 µL of 0.1 mM DPPH-ethanol solution and 450 µL of 50 mM Tris-HCl buffer (pH 7.4). Methanol (50 µL) alone was used as a control for this experiment. The reaction mixture was incubated for 30 mins at room temperature. The reduction of the DPPH free radical was measured by reading the absorbance at 517nm. Vitamin E was used as positive control. The inhibition (%) was calculated and noted.

**ii) Thin Layer Chromatography (TLC) Separation**

Thin layer chromatography was employed to separate the compounds present in the crude hydroethanolic root extract of *M. ciliata.* Normal phase silica gel GF pre-coated (250 microns) fluorescence alumina plates (10 x 20) were employed for the analysis. The solvent system used for the separation was chloroform/butanol/xylene in the ration v/v/v (2:2:3). Spots were detected by visualizing at UV-365nm and spraying with Dragendorff's reagent (Wagner and Blandt, 1996).

**iii) Column chromatographic Separation**

Column chromatography of *M. ciliata* root extract was performed using silica gel G60, using gradient elution of increasing polarity. Flow rate was 10 ml / 5 min. Eluted fractions were concentrated, dried under controlled temperature and refrigerated until needed.

**iv) *In vitro* Trypanocidal Evaluation of Extract**

Column chromatography fractions of *M. ciliata* root extract prepared with phosphate buffer saline to give effective concentrations of 20 µl and 10 µl respectively and were evaluated for trypanocidal activity. 20 µl of infected blood ($10^6$ parasites/ml of blood) was mixed with 5 µl of extract solutions to give effective concentration of 2 and 4 µg/ml respectively. Positive and negative controls were mixed with diminazine diaceturate and phosphate buffer saline only. Each mixture was incubated at 37°C. Parasitemia was monitored every 5 min for 60 min under x40 magnification (Herbert and Lumsden, 1976).

**v) Fractionation of the Plant Extract**

*M. ciliata* hydroethanolic root extract (25 g) was fractionated by the method of Wu *et al.*, (2005). This method involved successive extraction by increasing polarity with n-Hexane, chloroform, ethyl acetate, saturated n-butanol and water. The hydroethanolic extract was dissolved in 200 ml of MeOH/$H_2O$ (9:1) mixture and shaken with n-hexane (3 x 100 ml). Combined extract was left to dry on the bench to yield 'Hexane fraction'. MeOH was evaporated from the remaining extract and diluted with distilled $H_2O$ to 200 ml and further fractionated by successive solvent extraction with chloroform (4 x 100 ml), ethyl acetate (2 x 100 ml) and n-butanol saturated with $H_2O$ (3 x 100 ml). Each fraction was left to evaporated to dryness on the bench to yield 'n-Hexane fr' (4.16 g), '$CHCl_3$ fr' (5.59 g) 'EtOAc fr' (6.90 g), 'BuOH fr' (1.46 g) and '$H_2O$ fr' (2.0 g).

**vi) *In vivo* Trypanocidal Evaluation of Fractions**

The n-Hexane, $CHCl_3$, EtOAc, BuOH and $H_2O$ fractions were evaluated for trypanocidal activity. Seven groups of five rats each were inoculated with$10^6$ trypanosomes per ml of blood using phosphate buffer saline. Two groups served as positive (reference drug -

diminazine diaceturate) and negative (untreated) controls. Five groups served as test groups. n-Hexane and chloroform fractions were dissolved in 1 % tween 80, while butanol, ethyl acetate and water fractions were dissolved in 5 % carboxyl-methyl cellulose. After allowing the establishment of parasitemia for 5 days, the fractions and drug were administered orally, to the infected rats at doses of 100 mg/kg and 3.5 mg/kg for 5 days. Parasitemia levels were monitored for 28 days or until death of animals. Parasitemia levels were compared with that of untreated control animals. The survival period for each group of animals was also noted.

### 3.2.8 Hematological Evaluation of Active Plant Part

Hematogical studies of n-Hexane, $CHCl_3$, EtOAc, BuOH and $H_2O$ fractions evaluated for trypanocidal activity in **(v)** was performed. The hematogical analysis of infected and treated animals was done using automated hematology analyzer (Coulter $A^c$. T.diff., Beckman coulter, Miami, USA.). The packed cell volume (PCV) was determined using standard micro-haematocrit method. Differential white blood cell and platelets count were determined by haemocytometer method.

### 3.2.9 Biochemical Investigation of Active Plant Part

n-Hexane, $CHCl_3$, EtOAc, BuOH and $H_2O$ fractions evaluated for trypanocidal activity in **(v)** were used to study some biochemical enzymes activities.

**Determination of Superoxide Dismutase (SOD) Activity**

Whole blood superoxide dismutase was assayed utilizing the technique of Fridovich as described by Usoh *et al.*, (2005). 1 ml of whole blood was diluted with 9 ml of distilled water to make a one in ten dilution of whole blood. An aliquot of 2.0 ml of the diluted blood was added to 2.5 ml of 0.05 M carbonate buffer (pH 10.2) and left to equilibrate in the spectrophotometer. The reaction started by the addition of 0.3ml freshly prepared 0.3

mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5 ml buffer, 0.3 ml of substrate (adrenaline) and 0.2 ml of water. The increase in absorbance at 480nm was monitored every 30 sec. for 150 sec. SOD activity was calculated and expressed in specific enzyme activity (UI).

**Determination of Catalase (CAT) Activity**

Serum catalase activity was determined according to Beers and Sizer by measuring the decrease in absorbance at 240nm in a UV recording spectrophotomer at 240nm by monitoring the decomposition of $H_2O_2$ as described by Usoh *et al.*, 2005. The reaction mixture (3 ml) contained 0.1 ml of diluted serum in phosphate buffer (50 mM, pH7.0) and 2.9 ml of 30 mM $H_2O_2$ in phosphate buffer (pH 7.0). An extinction coefficient for $H_2O_2$ at 240nm of $40.0M^{-1}cm^{-1}$ was used for the calculation. The specific activity of catalase was expressed as moles of $H_2O_2$ reduced per minute per mg protein.

### 3.2.10 Further Studies on Active Fraction

The active butanol was subjected to further investigation by screening for the constituents, the serum calcium concentration in infected rats treated with the fraction and comparative studies of two routes of administration of the fraction to infected rats.

**Screening of constituents of Active Fraction**

The active butanol fraction was evaluated for its constituents with the Mayer's reagent as described by Sofowora (1992).

### 3.2.11 Determination of Calcium ($Ca^{2+}$) Concentration

Active butanol fraction of ethanol root extract of *M.* ciliata was evaluated for its effect on serum calcium concentration in rats administered with the fraction. Calcium concentration in mmol/L was estimated by the method of Biggs and Moorehead as described by Yakubu *et al.*, 2007; using commercially available kit supplied by Biolab S. A. UK.

### 3.2.12 Comparative Studies on Routes of Administration of Active Fraction

The effect of the active butanol extract when administered through two different routes of administration was investigated. Two groups of five rats were inoculated with T. brucei approximately $10^6$ parasites/ml of blood and left for 5 days. The active butanol fraction was administered at doses 100 mg/kg orally and 50 mg/kg intraperitonally for 5 days. Parasitemia was monitored for 28 days or until death of animals.

### 3.2.13 Statistical Analysis

The results of this study were reported as mean ± SEM from three repeated determinations ($n = 6$) and evaluated with the analysis of Student's t-test. Differences were considered to be statistically significant at $p < 0.05$. Correlation test was by Rank's correlation. The data was also evaluated with one way ANOVA. The analysis was performed using Medcalc statistical package.

# CHAPTER FOUR

## 4.0 RESULTS

### 4.1 PRELIMINARY STUDIES

#### 4.1.1 Extraction of Plant Material

The percentage crude extract yield was 25 % and 17 % respectively for *M. ciliata* and *R. longipedicellata*. The *M. ciliata* extract was dark brown in colour and was soluble in water, while *R. longipedicellata* was reddish brown and was also soluble in water

#### 4.1.2 Phytochemical Screening

Phytochemical analysis revealed that *M. ciliata* hydroethanolic root extract contained alkaloids, saponins, flavonoids and glycosides while *R. longipedicellata* extract contained alkaloids, cardiac glycosides, flavonoids, phlobatanins, anthraquinones and reducing sugars.

#### 4.1.3. Toxicological Studies

##### 4.1.3.1 Acute Toxicity Test ($LD_{50}$)

The $LD_{50}$ of *R. longipedicellata* hydroethanolic root extract was 3.65 g/kg and that of *M. ciliata* was 2.5 g/kg.

##### 4.1.3.2 Sub-acute Toxicity Test

*M. ciliata* and *R. longipedicellata* hydroethanolic root extracts-treated animals showed a decrease ($p < 0.05$) in Alkaline Phosphatase (ALP), Alanine Transaminase (ALT), Aspartate Transaminase(AST) and Creatinine while there was an increase ($p < 0.05$) in bilirubin levels in the serum of *M. ciliata* treated rats (Table 4.1). Furthermore, *M. ciliata* treated animals showed a lower PCV ($p < 0.05$); while *R. longipedicellata* had a significantly higher (PCV) in the treated animals than the untreated control.

**TABLE 4.1: Effect of Administration of Extracts of *M. ciliata* and *R. longipedicellata* on Some Biochemical and Heamatological Parameters**

| Parameter | Control | *M. ciliata* | *R. longipedicellata* |
|---|---|---|---|
| PCV (%) | 36.50±0.28 | 34.6±0.73* | 38.8±0.16* |
| ALP (IU) | 37.66±0.37 | 32.2±0.28* | 34.6±0.50* |
| ALT (IU) | 14.43±0.21 | 9.23±0.12* | 12.3±0.03* |
| AST (IU) | 20.50±0.22 | 13.7±0.78* | 15.6±0.76* |
| Creatinine (µmol/L) | 185.0±1.15 | 146.1±0.3* | 162.3±0.2* |
| Bilirubin (µmol/L) | 27.80±0.23 | 41.0±0.25* | 26.46±0.44 |

**Values represent Mean ± SEM**

***significant at p < 0.05 vertically**

## 4.1.4 BIOLOGICAL SCREENING & SELECTION OF TRYPANOCIDAL PLANT

### 4.1.4.1 Trypanocidal activity: Suppressive

Fig. 4.1a-d summarises the results of the suppressive activities of hydroethanolic root extract *M. ciliata, R. longipedicellata* and their combination.

Fig. 4.1a shows the suppressive activity of *M. ciliata at* 50 mg/kg and 100 mg/kg. At 50 mg/kg dose, the infected and treated rats exhibited average parasitemia of $0.19 \pm 0.11 \times 10^6$ parasites/ml of blood compared to untreated rats with $0.9 \pm 0.28 \times 10^6$ parasites/ml of blood. This dose also cleared parasites from the animals' bloodstream by day 7. The rats treated with 100 mg/kg dose, had average parasitemia of $0.87 \pm 0.7 \times 10^6$ parasites/ml of blood. The rats administered this dose did not exhibit any difference in average parasites/ml of blood from the untreated control. The same characteristic cyclic parasitemia of trypanosomes observed in the untreated rats, was also observed in the rats administered 100 mg/kg dose. The results showed that parasitemia was significantly lower ($p < 0.05$) in animals treated with the extract at 50 mg/kg dose, as compared to the 100 mg/kg dose and untreated control. The reference drug (diminazine diaceturate) exhibited complete suppression of parasites, with all animals surviving like the ones treated with the 50 mg/kg dose, while no animal survived at the 100 mg/kg dose.

Fig. 4.1b showed the suppressive activity of ethanolic root extract of *R. longipedicellata.* The 400 mg/kg dose treated rats had average parasitemia of $3.95 \pm 0.84 \times 10^6$ parasites/ml of blood and at 800 mg/kg it was $5.54 \pm 1.23 \times 10^6$ parasites/ml of blood, while untreated rats had $0.9 \pm 0.28 \times 10^6$ parasites/ml of blood. The extract at both doses revealed a significant increase ($p < 0.05$) in parasitemia and there was distinct exhibition of cyclic parasitemia of trypanosomes especially at the 800 mg/kg dose. The rats administered the 400 mg/kg dose lived as long as the untreated control (19 days), while the animals at 800

mg/kg dose survived longer than the untreated (23 days). No animal survived in these groups.

Fig. 4.1c is the result from the suppressive activity of ratio1:1 (v/v) of the combination of *M. ciliata* and *R. longipedicellata* extracts. The rats administered the mixture at 200 mg/kg and 400 mg/kg had average parasitemia of $0.28 \pm 0.3 \times 10^0$ and 0 parasites/ml of blood respectively. Parasites were cleared from bloodstream of animals at 200 mg/kg dose by day 7, same as clearance at 50 mg/kg of *M. cliata*, Parasitemia at 200 mg/kg was significantly lower ($p < 0.05$) than the untreated control ($0.9 \pm 0.28 \times 10^0$ parasites/ml of blood). At 400 mg/kg dose there was complete suppression of parasites' growth, similar to that of the reference drug. All animals survived through out the 28 days monitoring period in these groups.

Fig. 4.1d shows the inhibition (%) of parasites growth by the ethanolic root extracts of *M. ciliata, R. longipedicellata* and their combination. *M. ciliata* extract exhibited good suppressive activity with average inhibition of 78.84 % at 50 mg/kg dose comparable to the reference drug (100 %). *M. ciliata* at 100mg/kg dose and *R. longipedicellata* at both doses did not inhibit parasites' growth, with average inhibition of 3.3 % and 0 % respectively. The combination of both plants exhibited average inhibition of parasites' proliferation of 33.33 % and 100 % at 200 mg/kg and 400 mg/kg respectively. The combination of both plants at 400 mg/kg completely inhibited parasites' proliferation same as the reference drug.

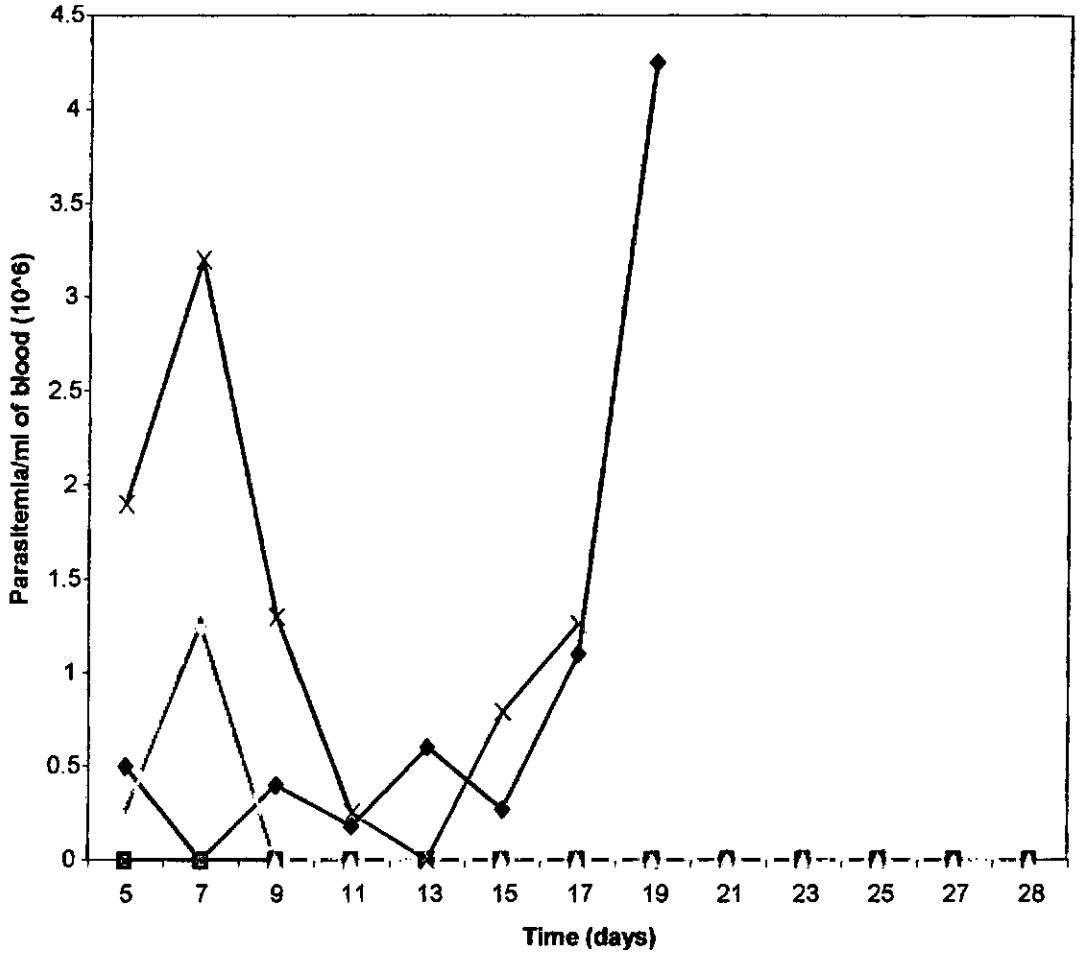



**Fig. 4.1a: Suppressive Activity: Effects of *M.cilliata* root extract on parasitemia/ml of blood for 28 days**

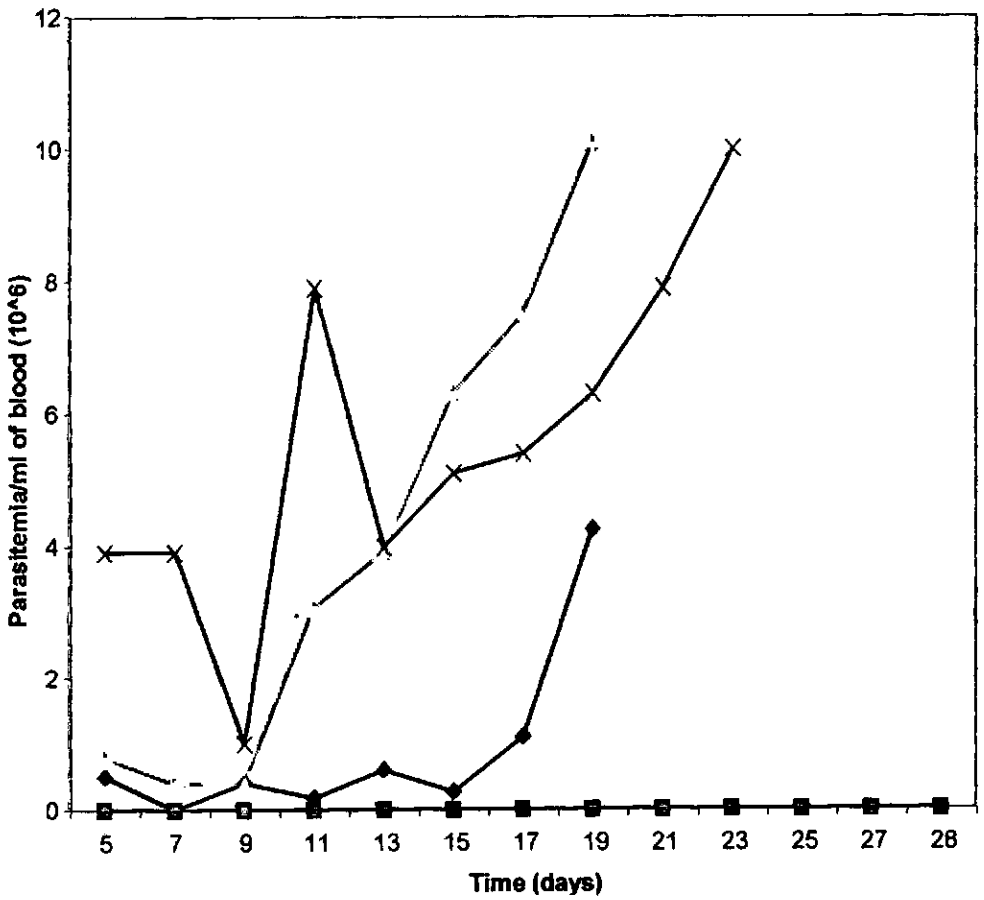



**Fig 4.1b: Suppressive Activity: Effects of *R.longipedicellata* root extract on parasitemia/ml of blood for 28 Days**

Untreated
Ref drug
R.longipedicellata (400mg/kg)
R.longipedicellata (800mg/kg)

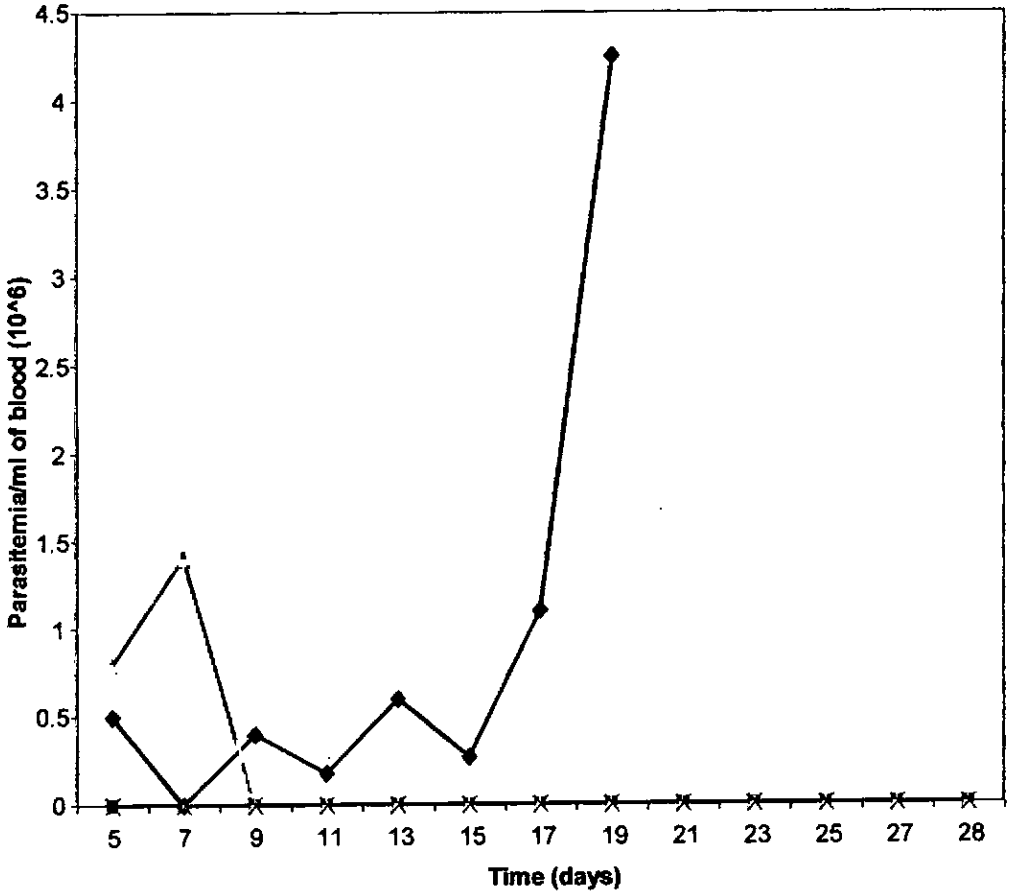



**Fig. 4.1c: Suppressive Activity: Effects of combination of *M.cilliata* and *R. longipedicellata* on parasitemia in infected rats for 28 days**

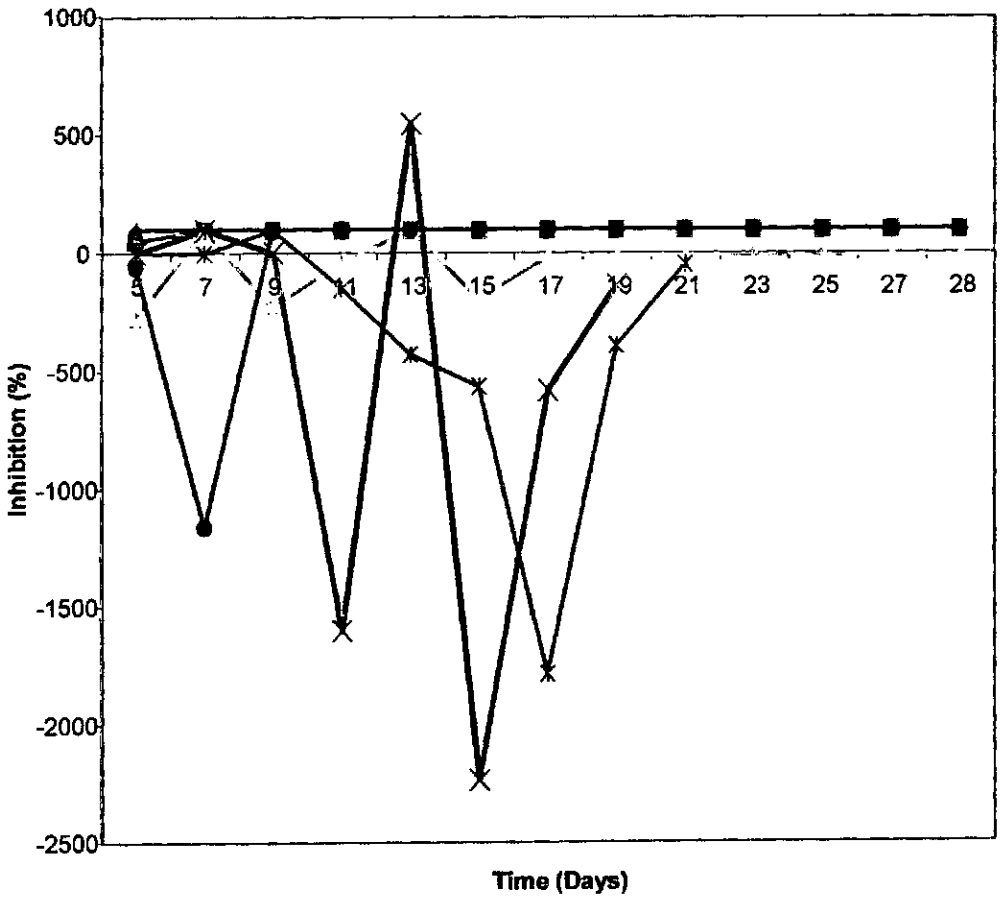



**Fig 4.1d: Suppressive Activity: Inhibition of parasites growth (%) by *M.cilliat* and *R.longipedicellata* root and their combination for 28 days**

### 4.1.4.2 Trypanocidal Activity: Therapeutic

Fig. 4.2a-d summarises the results for the therapeutic activities for *M. ciliata, R. longipedicellata* and their combination. Figure 4.2a shows the results of the therapeutic activity of ethanolic root extract of *M. ciliata.* At 50 mg/kg, the extract treated rats had average parasitemia of $0.51 \pm 0.175 \times 10^0$ parasites/ml of blood and at 100 mg/kg they had $4.05 \pm 0.06 \times 10^0$ parasites/ml of blood. The untreated control animals had $1.1 \pm 0.3 \times 10^0$ parasites/ml of blood, while the reference drug had $0.035 \pm 0.06 \times 10^0$ parasites/ml of blood. Results revealed a significant decrease ($p < 0.05$) in parasitemia in 50 mg/kg dose of the extract and parasites were cleared from the bloodstream of the animals by day 13 resulting in survival of the animals throughout the monitoring period similar to the reference drug. The 100 mg/kg dose showed no activity and did not exhibit the characteristic cyclic parasitemia of trypanosomes. Parasitemia was significantly higher ($p < 0.05$) in the 100 mg/kg dose, than untreated control and all animals died by day 13 earlier than the untreated control animals (day 19).

Figure 4.2b shows the results of the therapeutic activity of *R. longipedicellata* extract. The rats administered 400 and 800 mg/kg doses had average parasitemia of $2.08 \pm 0.27 \times 10^0$ and $5.30 \pm 0.22 \times 10^0$ parasites/ml of blood respectively, while the untreated control rats had $1.1 \pm 0.3 \times 10^0$ parasites/ml of blood. The reference drug had $0.035 \pm 0.06 \times 10^0$ parasites/ml of blood. The extract at both the tested doses exhibited no activity as there was significantly ($p < 0.05$) higher parasitemia in the extract treated rats than the untreated control and the reference drug treated. The characteristic cyclic parasitemia of trypanosomes was distinctly absent in *R. longipedicellata* treated rats, as there was sustained increase in parasitemia. However, the extract treated rats had an increased

survival period from 19 to 25 days at 800 mg/kg dose, but animals at 400 mg/kg dose died earlier (17 days) than the untreated rats (19 days).

In Figure 4.2c the therapeutic activity of the combination of *M. ciliata* and *R. longipedicellata* is shown. The rats treated with the combination (1:1 v/v) of both plants' extracts had average parasitemia of $3.78 \pm 0.47 \times 10^{0}$ and $0.0025 \pm 0.0075 \times 10^{0}$ parasites/ml of blood at 200 mg/kg and 400 mg/kg doses respectively. The untreated control rats had $1.1 \pm 0.3 \times 10^{0}$ parasites/ml of blood, while the reference drug produced $0.035 \pm 0.06 \times 10^{0}$ parasites/ml of blood. There was a significant decrease ($p < 0.05$) in parasitemia at 400 mg/kg of the mixture and parasites clearance from the bloodstream of the rats was by day 9. This was earlier than the reference drug that was by day 11. The mixture exhibited activity same as the reference drug at the 400 mg/kg dose and there was no occurrence of cyclic parasitemia at the 200 mg/kg dose.

In Figure 4.2d, the percentage inhibition of growth of the parasites by ethanolic root extract of *M. ciliata*, *R. longipecellata*, and their combination is shown. The *M. ciliata* extract inhibited the growth of the parasites by 54.54 %, while *R. longipedicellata* and the combination of both plants at 200 mg/kg dose did not inhibit parasites growth. But, the mixture completely inhibited the parasites growth at 400 mg/kg dose, which was comparable to the reference drug. Table 4.2 show the survival rate and survival period of the animals in therapeutic activity. Results indicated that all animals survived at 400 mg/kg dose of the combination and the reference drug. *M. ciliata* had 60 % of the animals surviving at 50 mg/kg dose which was comparable with the reference drug. Results also showed that the combination at 400 mg/kg dose produced the longest (> 28days) surviving animals as was observed for the reference drug. The animals treated with 200 mg/kg dose of the mixture died earlier than the untreated animals.

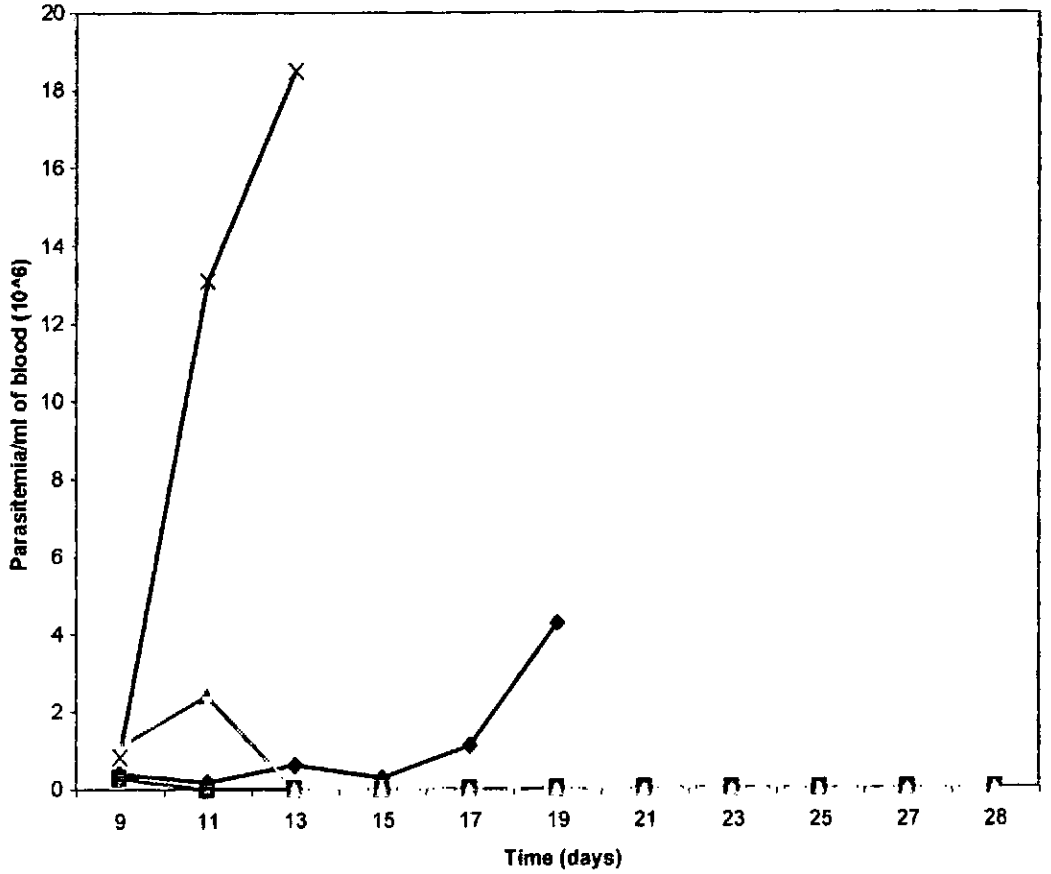



**Fig 4.2a: Therapeutic Activity: Effects of *M.cilliata* root extract on parasitemia of blood for 28 days**

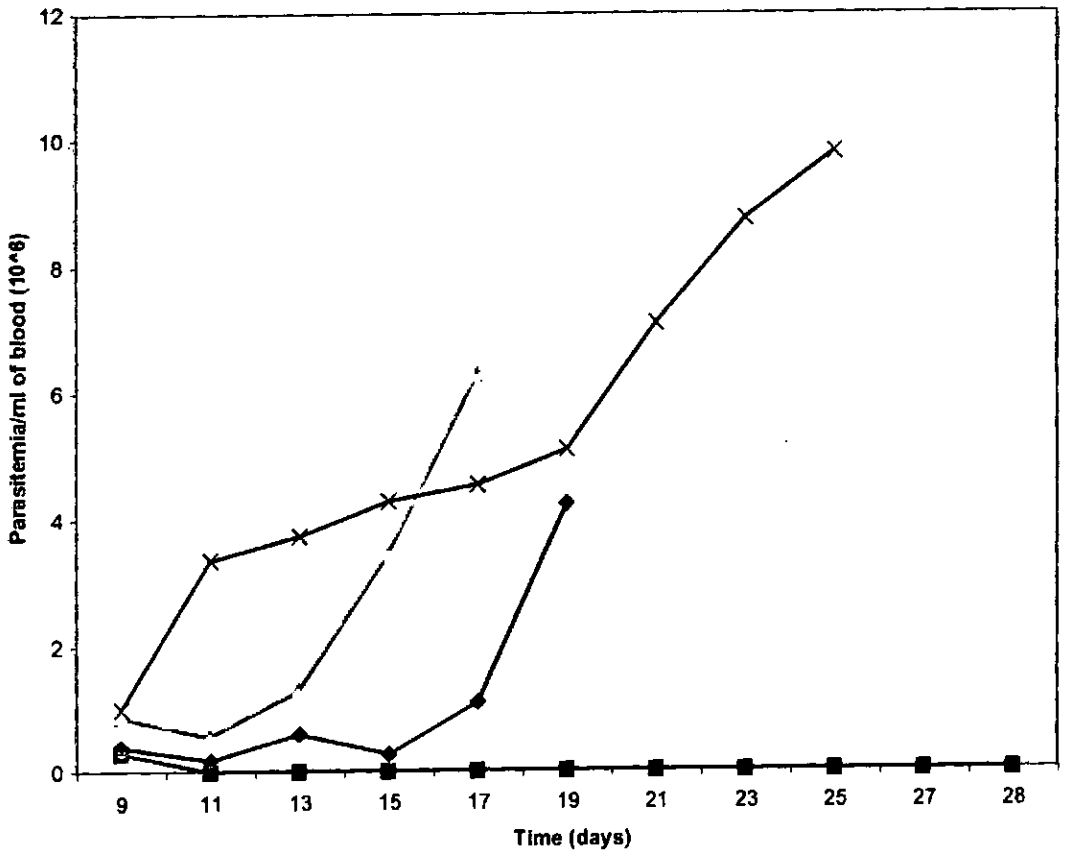



**Fig. 4.2b: Therapeutic Activity: Effects of *R.longipedicellata* root extract on parasitemia/ml of blood for 28 days**

- Untreated
- Ref drug
- M.cilliata (50mg/kg)
- M.cilliata (100mg/kg)

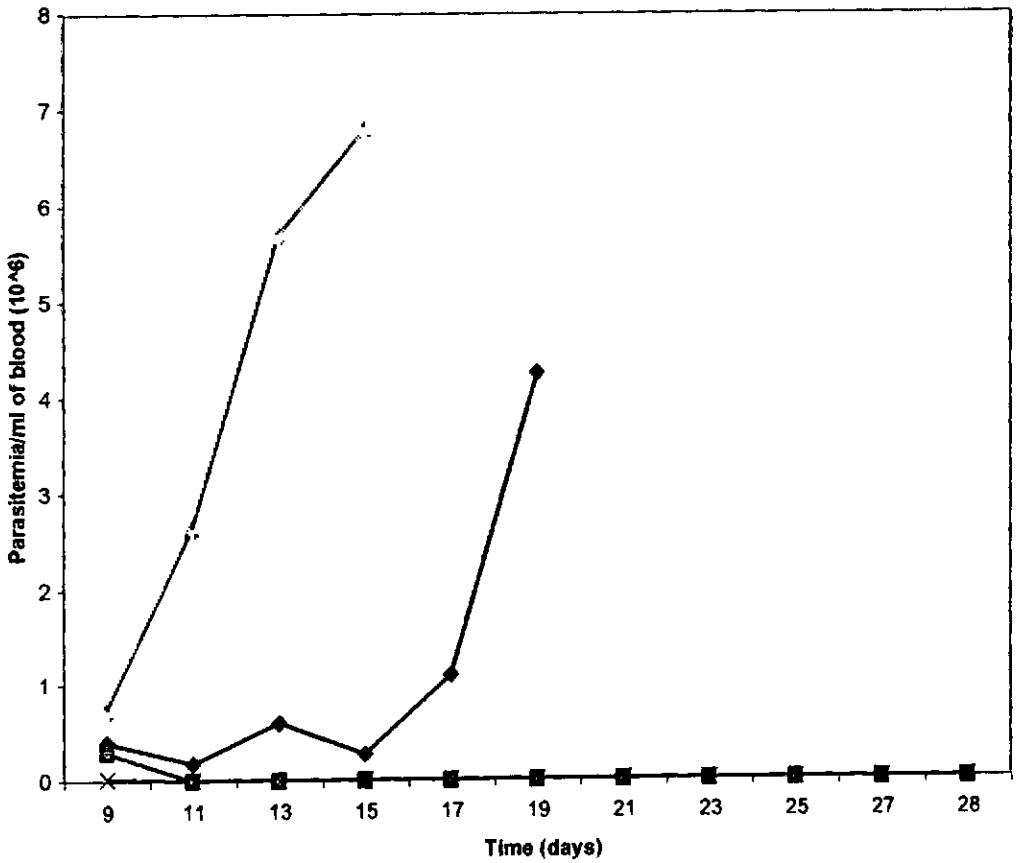



**Fig 4.2c: Therapeutic Activity: Effects of combination of *M.cilliata* and *R.longipedicellata* On parasitemia/ml of blood for 28 days**

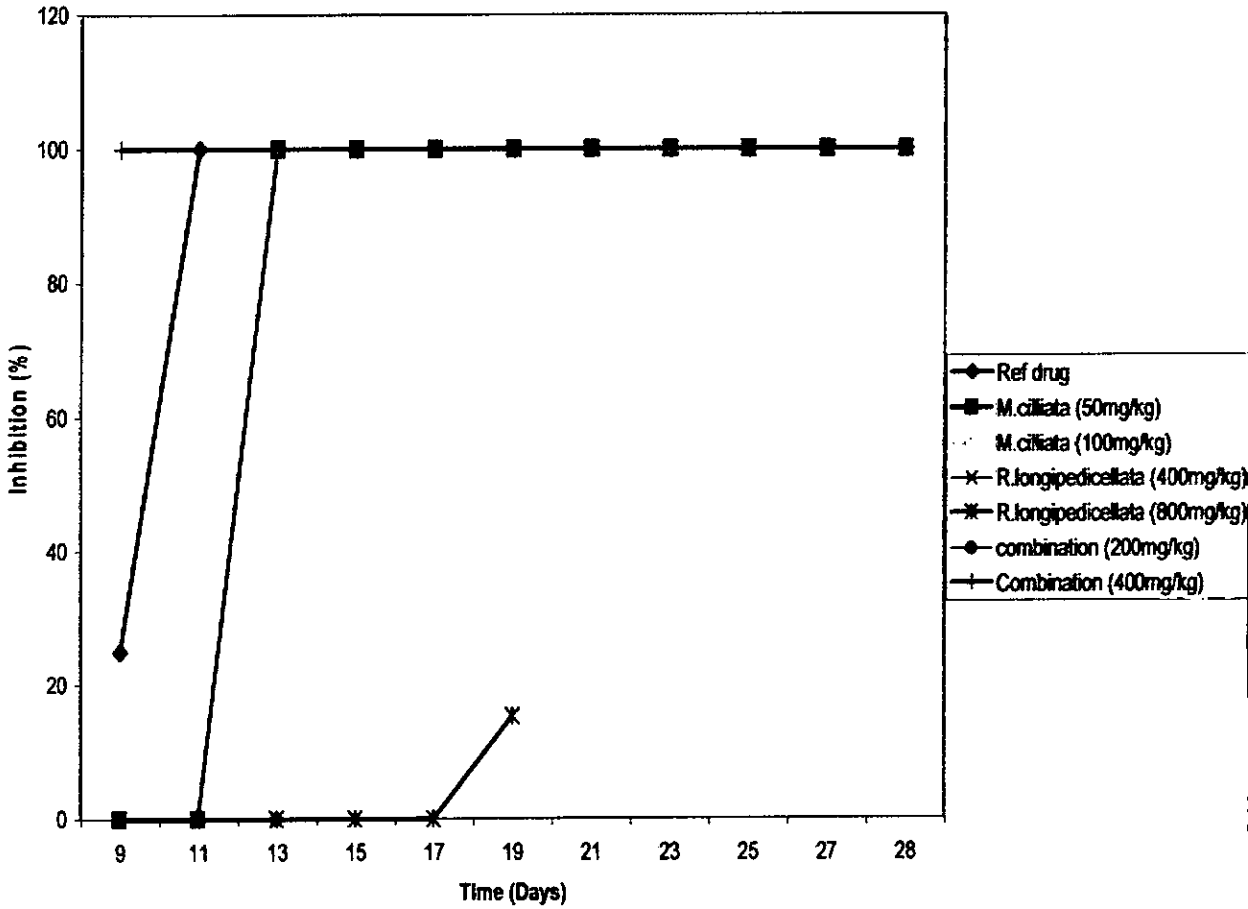



**Fig 4.2d: THERAPEUTIC ACTIVITY: INHIBITION OF PARASITES GROWTH (%) BY *M.ciliata* AND *R.longipedicellata* AND THEIR COMBINATION FOR 28 DAYS**

**Table 4.2: Dose, Survival Rate and Survival Period for Rats Treated With *M. Ciliata, R. Longipedicellata* and Their Combination in Therapeutic Activity**

| Compound | Untreated -ve Control | Ref. Drug +ve Control | *M .ciliata* | | *R. long* | | Combination | |
|---|---|---|---|---|---|---|---|---|
| Dose (mg/kg) | - | 3.5 | 50 | 100 | 400 | 800 | 200 | 400 |
| Surv. Rate (%) | 0 | 100 | 60 | 0 | 0 | 0 | 0 | 100 |
| Surv. Period (days) | 19 | 28 | 28 | 13 | 17 | 25 | 15 | 28 |

Surv = Survival

## 4.2 Evaluation of Selected Plant Parts

### 4.2.1 *In vivo* Comparative Screening of Parts of Selected Plant: M. ciliata

Table 4.3 shows the results from the comparative studies of the ethanolic extract of *M. ciliata* parts. The roots bark extract exhibited the highest activity (54.54 %) of the *M. ciliata* extracts. The leaves on the other hand, exhibited slight activity (21.6 %) when compared to the reference drug which showed complete inhibition, while the stem produced no activity.

## 4.3 Further Studies of Active Plant Part

### 4.3.1 *In vitro* Free Radical Scavenging Activity

Figure 4.2 shows the *in vitro* free radical scavenging activity of *M. ciliata* roots extract. The average free radical scavenging activity of the extract was 25.25 %, while Vitamin E had 82.83 %. The roots extract of the plant exhibited significantly lower antioxidative property.

### 4.3.2 Thin Layer Chromatography (TLC) Analysis

Figure 4.3 shows the TLC plate with the eluted fractions of *M. ciliata* root extract using solvent system chloroform:butanol:xylene (2:2:3) v/v/v as mobile phase and detected at UV-365nm. 5 fractions were detected on the plate. The eluted spots were positive to Dragendoff's reagent spray indicating the presence of alkaloids. Table 4.4 shows the retention factors ($R_f$) of the spots.

**Table 4.3: Anti-Trypanosomal Activity of Leaves, Stem Bark and Root Bark of *M. Ciliata***

| Parameter | Controls | | Part of Plant | | |
|---|---|---|---|---|---|
| | Untreated (-ve control) | Reference drug (+ve control) | Leaf | Stem bark | Root bark |
| Dose(mg/kg) | - | 3.5 | 500 | 500 | 50 |
| Average parasiteamia (x $10^6$/ml of blood) | 118.0 ± 20 | nil | 223.9 ± 50 | 42.87 ± 32 | 5.37 ± 24 |
| Inhibition (%) | 0 | 100 | 21.6 | 0 | 54.54 |
| Survival rate (%) | 0 | 100 | 0 | 0 | 60 |

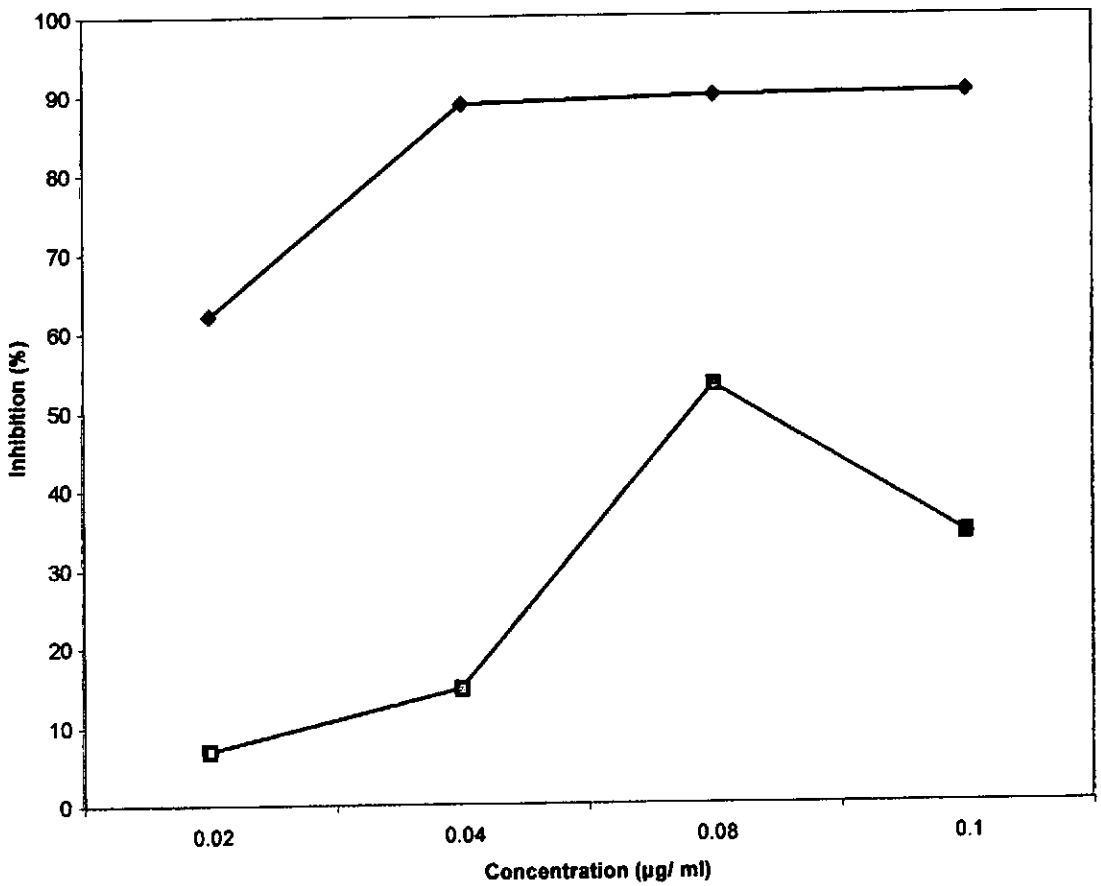



**Fig. 4.3: Free radical scavenging activity of *M.cilliata* roots extract**

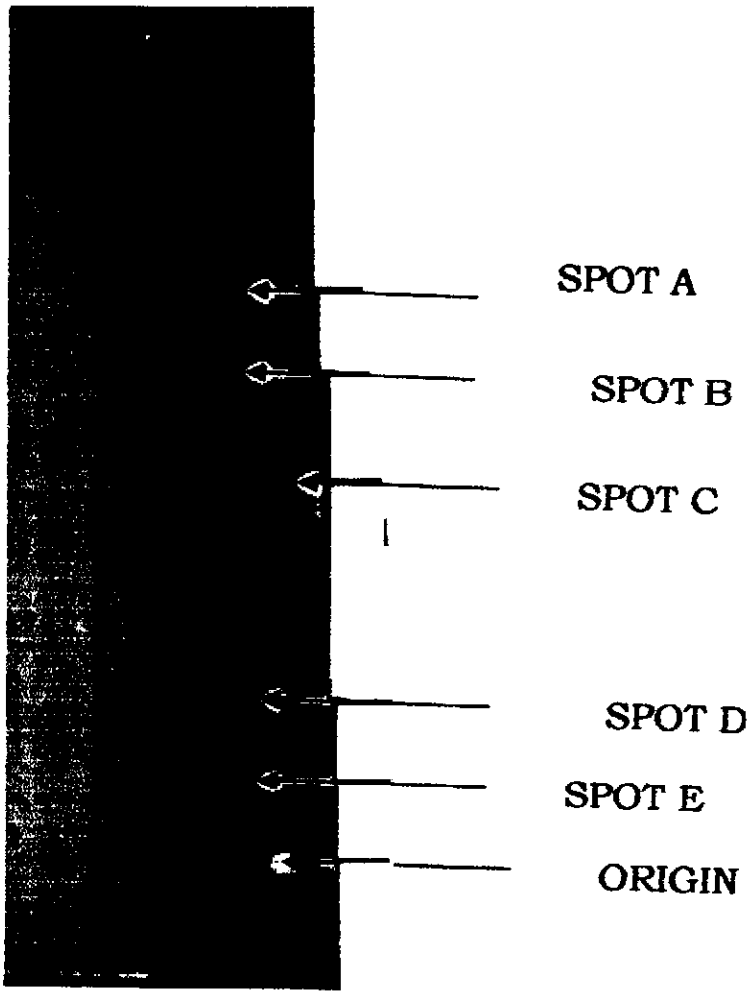



**Fig 4.4: TLC Plate showing eluted fractions from *M. ciliata* roots extracts detected at UV-365nm.**

**Table 4.4: Eluted fractions and their $R_f$ values in TLC separation of crude extract of *M. ciliata***

| Spot | A | B | C | D | E |
|---|---|---|---|---|---|
| $R_f$ Values (cm) | 0.83 | 0.69 | 0.58 | 0.24 | 0.10 |

### 4.3.3 *In vitro* Trypanocidal Evaluation of Fractions

Results from the *in vitro* studies of fractions from column chromatographic separation of *M. ciliata* are shown on Table 4.5. The effective inhibitory concentration (IC) that resulted in drastic reduction of motility for *M. ciliata* crude extract and the active butanol fraction was 4 μg/ml in 5 min. The reference drug IC value was 20 and 15 min at both the tested concentration of 2 and 4 μg/ml, respectively and all the other fractions showed no inhibition at both concentrations.

### 4.3.4 *In vivo* Trypanocidal Evaluation of Fractions (Therapeutic Activity)

Figure 4.5 is a synopsis of results from the activity-guided fractionation of the therapeutic activity of *M. ciliata*, showing parasites/ml of blood in the fractions treated rats during administration of fractions for seven days. Butanol fraction had average parasites/ml of blood $2.60 \pm 0.19 \times 10^6$ comparable to reference drug with $2.57 \pm 0.18 \times 10^6$ parasites/ml of blood. Parasitemia was significantly ($p < 0.05$) lower in the butanol fraction than untreated control ($5.09 \pm 0.4 \times 10^6$) and the other fractions with chloroform fraction exhibiting the highest parasitemia $10.15 \pm 3.2 \times 10^6$ parasites/ml of blood.

In Figure 4.6, the results indicate that the butanol fraction had a significant ($p < 0.05$) inhibitory effect on parasites proliferation and growth (66.61 %) in the infected animals. Water fraction had the least activity, while ethyl acetate fraction showed slight activity.

Table 4.6 shows inhibition and survival period of rats treated with fractions of *M. ciliata* extract. Animals treated with n-Hexane fraction had the shortest survival period while the butanol fraction had the same survival period as the reference drug group (> 28 days).

**Table 4.5: *In vitro* Trypanocidal activity of fractions from column chromatography of root extract of *M. Ciliata***

| Compound | Cessation of Motility (minutes) | Description Of Motility | Average Parasiteamia ($x10^6$ /ml of blood) | Average % Inhibition |
|---|---|---|---|---|
| **Concentration(2µg/ml)** | | | | |
| Untreated (negative Control) | >60 | Quick, Jerky (+++) | 118.00 | 0 |
| Reference Drug (+ve Control) | 20 | Slow (+) | 86.00 | 72.20 |
| Crude Ethanolic Extract | 10 | Slight | 31.62 | 73.21 |
| Saturated n-Butanol Fractn | 20 | Moderate (++) | 5.05 | 95.72 |
| Ethyl acetate/Ethanol Fraction | >60 | Quick, Jerky (+++) | 102.40 | 13.22 |
| Pet ether Fraction | >60 | Quick, Jerky (+++) | 128.00 | - 8.47 |
| **Concentration(4µg/ml)** | | | | |
| Reference Drug (positive Control) | 15 | Slight | 16.10 | 86.36 |
| Crude Ethanolic Extract | 5 | Drastic reduction | 0 | 100 |
| Saturated n-Butanol Fraction | 5 | Drastic reduction | 0 | 100 |
| Ethyl acetate/Ethanol Fraction | >60 | Quick, Jerky | 98.5 | 16.52 |
| Pet ether Fraction | >60 | Quick, Jerky | 148.0 | - 25.42 |

+ = Slow ++ = Moderate +++ = Quick

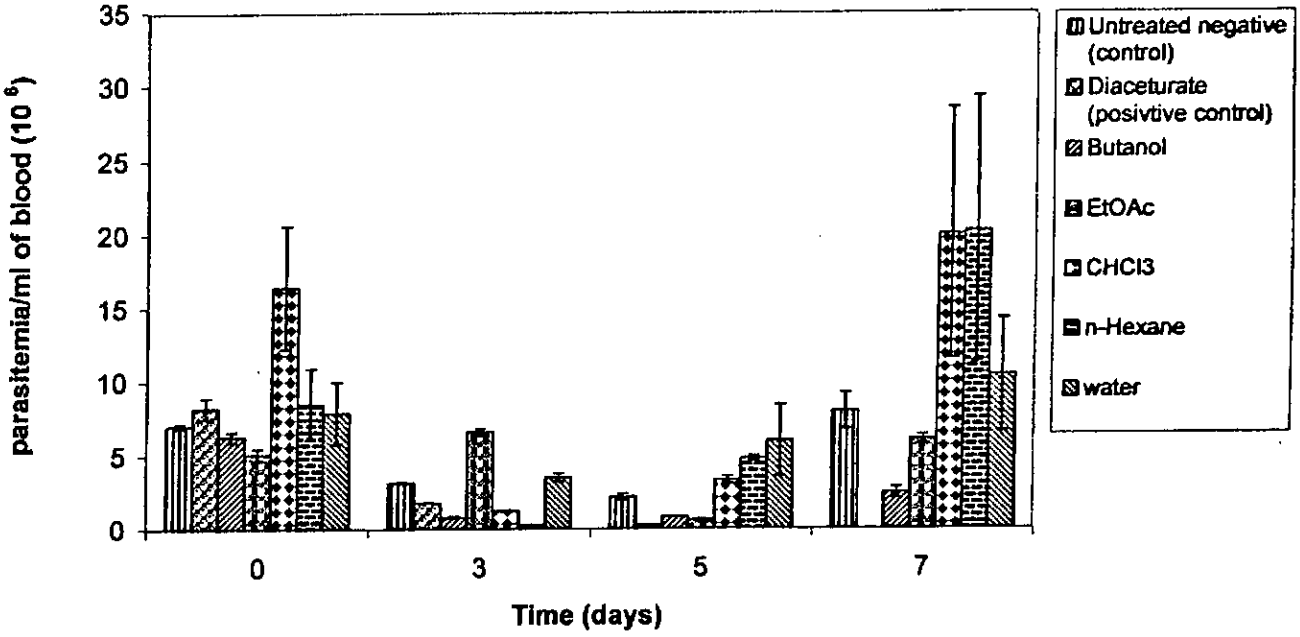



**Fig. 4.5: Parasitemia / ML of blood in infected rats treated with fractions of *M ciliata***

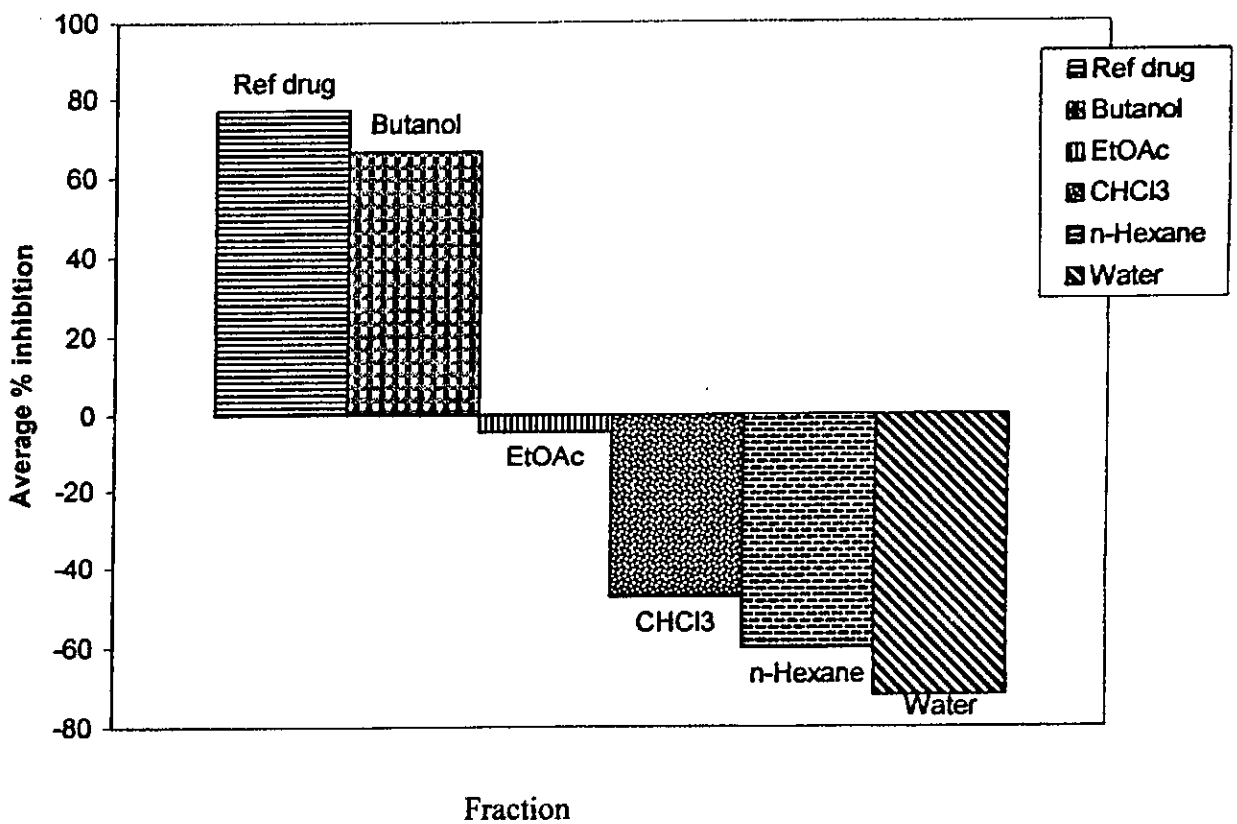



**Fig. 4.6: Average inhibition (%) for M. ciliata roots extract fractions**

**Table 4.6: Parasites' Inhibition (%) and Survival period of rats treated with fractions of *M. ciliata***

| DAY | Reference drug | Butanol | EtOAc | $CHCl_3$ | n-Hexane | Water |
|---|---|---|---|---|---|---|
| 3* | 42.86 | 74.82 | -109.20 | 60.65 | 91.40 | -12.83 |
| 5* | 88.23 | 53.33 | 71.38 | -52.51 | -118.59 | -172.56 |
| 7# | 100.00 | 68.68 | 23.75 | -150.52 | -153.75 | -30.63 |
| Average % Inhibition | 77.03 | 66.61 | - 4.69 | - 47.46 | -60.31 | - 72.0 |
| Survival # Period(Days) | >28 | >28 | 8 | 5 | 3 | 8 |

***During administration of extracts**

**#After administration of extract**

### 4.4 Heamatological Evaluation of *M. ciliata* Active Part

From results on Table 4.7, there was a significant decrease in packed cell volume (PCV) and hemoglobin concentration (Hb) ($p < 0.05$), in all the infected fractions'- treated animals indicating anemia. There was a significant increase in leucocytes or WBC count in all fractions revealing leucopenia and a significant decrease in platelets count indicating thrombocytopenia. There was a decrease in and an increase in lymphocytes and neutrophils count respectively, in all fractions compared to untreated/uninfected (control) but this decrease and increase were not significant.

### 4.5 Biochemical Investigation of Active Plant Part

#### 4.5.1.1 Specific activity of serum Superoxide dismutase (SOD) in fraction-treated rats

Figure 4.7 shows the activity of serum superoxide dismutase (SOD) in infected rats treated with fractions of *M. ciliata.* The activity of SOD was significantly increased ($p < 0.05$) in all fractions, except ethyl acetate fraction when compared to the untreated control. n-Hexane produced the greatest increase in activity, while chloroform also showed a significant increase. Comparison between the groups' fraction-treated rats did not show any significant difference in the increased activity of superoxide dismutase, except ethyl acetate that was lower than all the other groups.

#### 4.5.1.2. Specific activity of serum Catalase (CAT) in fraction-treated rats

The activity of serum catalase was significantly depressed ($p < 0.05$) in butanol and chloroform fractions of *M. ciliata*-treated rats as compared to untreated control (Fig 4.8). Catalase activity was not significantly different from the untreated control in n-hexane, ethyl acetate and water fractions treated rats. Furthermore, the activity of the enzyme between the groups was significantlyhigher in ethyl acetate, water and n-hexane fraction-treated groups when compared to the other groups.

**Table 4.7: Heamatological indices of fractions for *M. ciliata* in *T. brucei* infected rats**

| Fraction | PCV (%) | HB (g/dl) | WBC ($mm^3$) | Platelets ($X10^3$) | Lymphocytes ($mm^3$) | Neutrophils ($mm^3$) |
|---|---|---|---|---|---|---|
| **Uninfected/ Untreated** | 38.00 ± 2.40 | 13.8 ± 2.3 | 2900 ± 132.4 | 200.07 ± 2.30 | 50.02 ± 3.40 | 49.08 ± 2.9 |
| **Untreated** | 21.00 ± 2.35* | 6.98 ± 0.77* | 4300 ± 254.9* | 170.20 ± 4.90* | 46.4 ± 1.44 | 53.6 ± 1.44 |
| **n-Hexane** | 19.20 ± 2.20* | 6.40 ± 0.73* | 3360 ± 156.88* | 138.00 ± 2.55* | 47.4 ± 1.60 | 52.4 ± 1.63 |
| **Chloroform** | 25.00 ± 2.15* | 8.34 ± 0.72* | 4100 ± 291.55* | 115.00 ± 7.07* | 42.2 ± 2.27 | 57.4± 2.46 |
| **Ethyl acetate** | 22.40 ± 1.54* | 7.47 ± 0.51* | 3400 ± 187.0* | 117.00 ± 7.68* | 43.8 ± 2.63 | 56.2 ± 2.63 |
| **Butanol** | 19.20 ± 2.20* | 6.47 ± 0.29* | 3940 ± 337.05* | 118.00 ± 9.03* | 43.4 ± 2.10 | 56.6 ± 2.06 |
| **Water** | 18.40 ± 1.57* | 6.13 ± 0.52* | 3340 ± 187.10* | 146.60 ± 1.57* | 45.6 ± 2.06 | 54.4 ± 2.46 |

*Significant at $p<0.05$ vertically

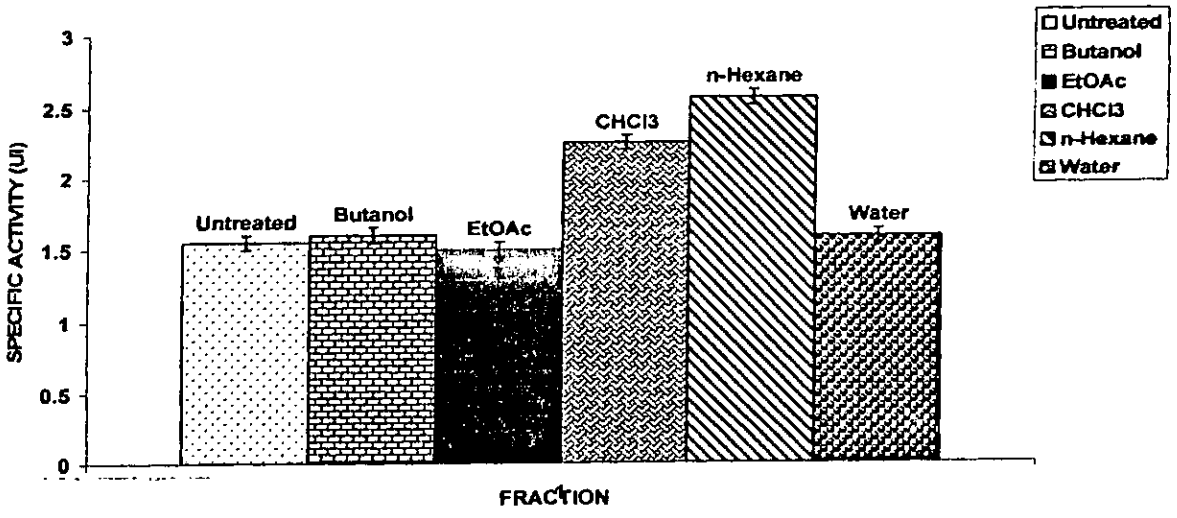



**Fig. 4.7: Specific activity (IU) of superoxide dismutase (SOD) in infected rats after administration of fractions of *Mitragyna ciliata***

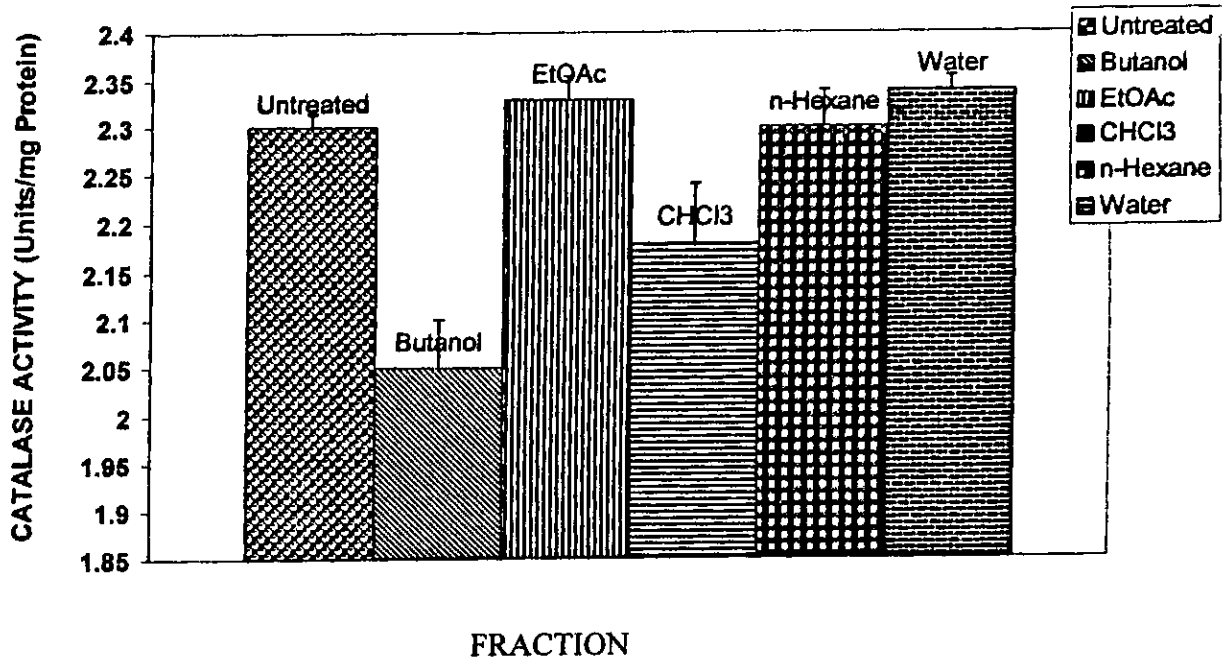



**Fig.4.8: Specific activity of catalase (UI) in infected rats after administration of fractions of *M. ciliata***

## 4.6: Studies on Active Fraction

### 4.6.1 Phytochemical Screening

Results from the test for alkaloids with Mayer's reagent indicated the presence of alkaloids in the active fraction.

### 4.6.2 Determination of $Ca^{2+}$ Concentrations

From the results of the effect of *M. ciliata* on serum $Ca^{2+}$ concentration (Fig. 4.9), a significant increase in concentration of $Ca^{2+}$ in the negative control (infected /untreated rats) was observed. The butanol fraction-treated rats exhibited a significantly reduced concentration of $Ca^{2+}$, (compared to the negative control: 17 mol/L to 2.53 mol/L). There was no significant difference between $Ca^{2+}$ concentration in the crude extract treated rats and the positive control (untreated/uninfected).

### 4.6.3 Comparative Studies of Routes of Administration of Active Fraction

Results from comparative studies of oral and intraperitonal routes of administration (Table 4.9) indicated that intraperitonal administration enhanced the activity of the butanol fraction (from 65.61-72 %). Survival rate and survival period of the test animals were also improved from 62-75 % and from 19 -21 days respectively. Figure 4.10 shows wet preparations of the blood sample of infected rats before treatment with active butanol fraction of *M. ciliata*. The picture taken at x40 magnification exhibits parasites per field which translates to $2.5 \times 10^8$ parasites per ml of blood. The red blood cells were lysed and clumpy indicating aneamia. Figure 4.11 on the other hand, shows the blood sample of same animal after treatment, at 100 mg/kg for 7 days, exhibiting parasites per field which translates to $7.9 \times 10^5$ parasites/ml of blood. The parasitemia was significantly lower than in the blood of the rats after treatment was administered (as shown in the picture). The red cells were also lysed and clumpy but few whole red cells are seen (red circle) indicating reduced aneamia.

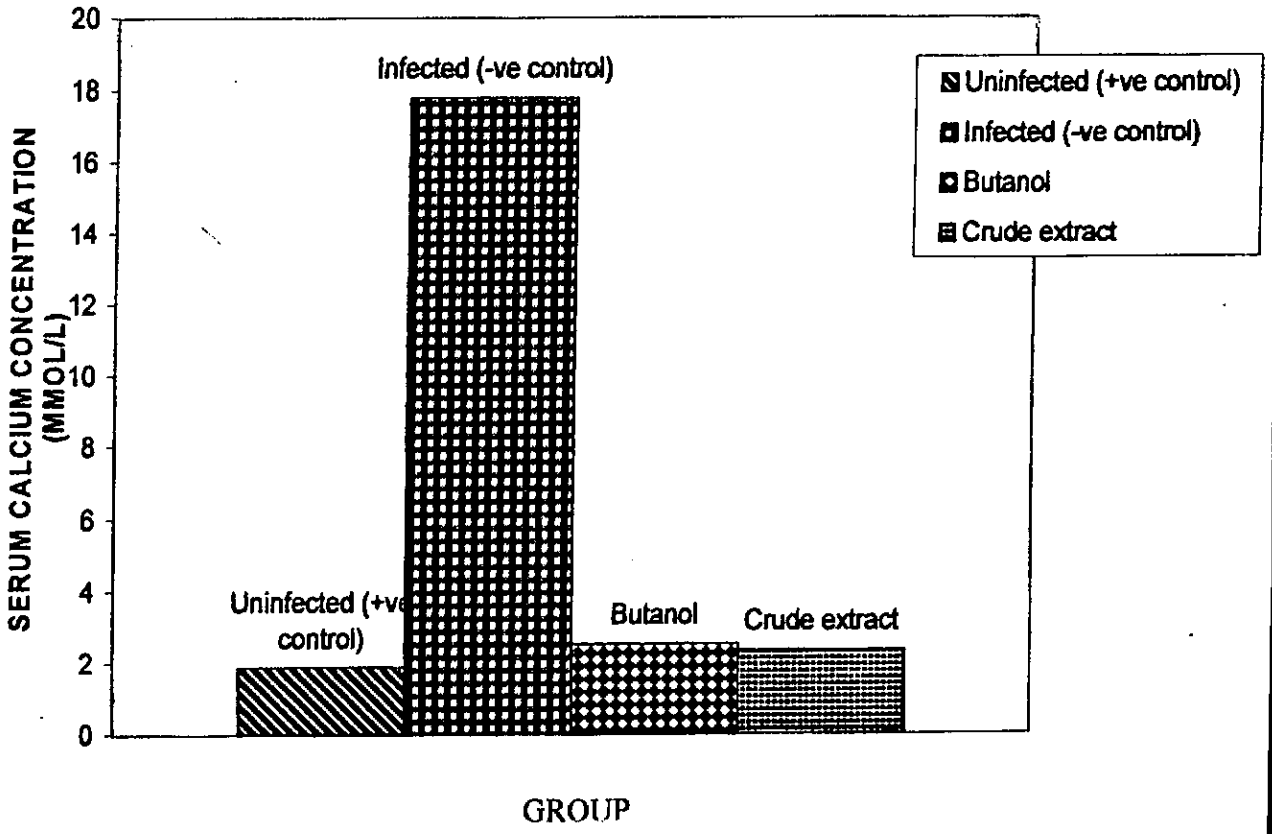



**Fig.4.9: Serum Calcium Concentration (mmol/L) in intected and *M. ciliata* treated rats**

**Table 4.9: Comparative evaluation of two routes of administration of active fraction in *T.b. brucei* infected rats**

| Parameter | Route of administration | | | |
|---|---|---|---|---|
| | Oral | | Intraperitonal | |
| | Ref Drug | Butanol fraction | Ref. drug | Butanol fraction |
| Dose (mg/kg) | 3.65 | 100 | 3.65 | 50 |
| Average Parasitemia (x $10^6$/ml of blood) | 39.9 | 2.6 | 2.56 | 87.60 |
| Average Inhibition (%) | 77.03 | 65.61 | 98.9 | 72 |
| Survival Rate (%) | 100 | 62 | 100 | 75 |
| Survival Period (Days) | > 28 | 19 | > 28 | 21 |

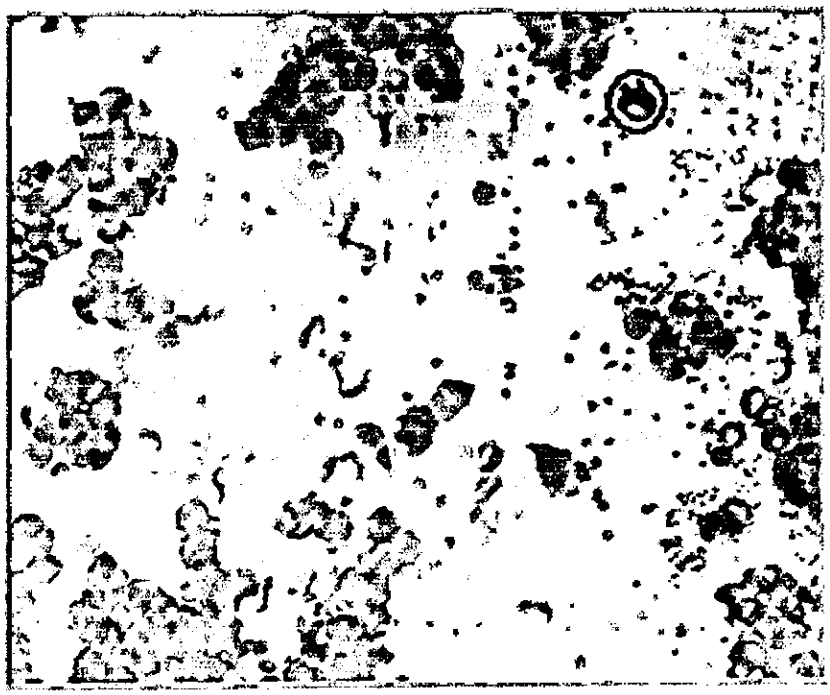


**Fig. 4.10: Blood of infected rats before administration of active fraction as seen under x40 magnification**

- Yellow arrows show trypanosomes

- Red circle show lysed red blood cells

**Fig 4.11: Blood of infected rats after administration of active fraction as seen under x40 magnification**

- Red circle shows whole red blood cell

# CHAPTER FIVE

## 5.0 DISCUSSION

This work was designed to investigate the antitrypanosomal activity of *Mitragyna ciliata Aubrev and Pellegr* (*Rubiaceae*), claimed by traditional medicine practitioners to be effective for the treatment of trypanosomiasis. Validation of this claim, evaluation of the therapeutic effects of other parts of the plant and determination of mode of *M. ciliata*-induced trypanocidal activity are necessary in order to fully understand and exploit the therapeutic potential of this plant.

### PRELIMINARY STUDIES

#### Phytochemical Screening

The preliminary studies started with phytochemical screening of hydroethanolic extract of *M. ciliata* and *R. longipedicellata* which revealed secondary metabolites that are usually the common constituents of other plants. The results indicated the presence of alkaloids, glycosides, saponins, flavonoids, phlobatannins and anthraquinones. These metabolites have been shown to exhibit various pharmacological effects such as antimalarial activity and central nervous system effects ascribed to alkaloids, antioxidant activity of flavonoids, purgative activity of anthraquinones, etc. (Evans, 1996).

#### Toxicological Studies

#### Acute Toxicity and Sub-acute Toxicity Tests

In the acute toxicity test, $LD_{50}$ of 3.65 g/kg for *R. longipedicellata* extract and 2.5 g/kg for *M. ciliata* indicated a good safety margin for both plant extracts. The death of the animals at the stated doses which was 100 times higher than recommended therapeutic drug dosage

(Singh and Devkota, 2003), was an indication of the lack of toxic effect of both plant extracts.

The single oral administration (sub-acute toxicity test) of *M. ciliata* at 800 mg/kg for 21 days produced some physical manifestations such as drowsiness and anesthesia (sleep) – which support previous work that *M. ciliata*'s extract has an effect on the central nervous system in rats (Aji *et al.*, 2001). On the other hand, *R. longipedicellata* for the same test at the same dose produced enhanced physical activity, corroborating its use as a tonic in traditional medicine.

The liver function test of both plant extracts, after a single dose administration for 21 days, showed a significant decrease ($p < 0.05$) in serum activity of liver enzymes. These enzymes are used as markers of liver toxicity. The decreased activity of these enzymes - alkaline phosphatase (ALP), Aspartate aminotransaminase (AST) and Alanine aminotransaminase (ALT) is an indication of lack of toxicity of the plant extracts (Singh and Devkota, 2003). These findings might be indicative of hepatic cellular activity without cellular necrosis (Adjanohoun *et al.*, 1985).

Among the different isoenzymes of alkaline phosphatase, the one originating from the liver has been shown to be the main component of the serum (Teitz, 2000). Alanine and aspartate transaminases, on the other hand, are localized within cells of the liver and other organs and are of major importance in monitoring and assessing liver cytolysis (Smith *et al.*, 2002, Yakubu *et al.*, 2005). Because the liver is the seat of protein synthesis and drug metabolism, the measurement of the activities of marker and diagnostic enzymes in liver tissues plays a significant role in diagnosis and disease investigation and in the assessment of drug or plant extract safety or toxicity. It is well known that an increase in the activity of liver enzymes in the serum is indicative of cellular leakage and loss of functional integrity

of cellular membrane of the liver, thus, the decreased activity of these liver enzymes in the test rats is suggestive of a positive hepatic effect of the extract (Iweala and Okeke, 2005). Furthermore, previous studies have shown that certain plant extracts significantly lowered serum liver enzymes in humans with liver cirrhosis (Scot, 1998). Smith *et al.*, (2002) also reported that serum levels of transaminases return to normal with healing of hepatic parenchyma and regeneration of hepatocytes. The foregoing results further justify the use of the leaves and root of this plant by traditional medicine practitioners for the treatment of liver disease in West Africa (Toure *et al.*, 1996). Moreover, research has confirmed traditional experience by discovering the mechanisms and modes of action of these plants as well as reaffirming the therapeutic effectiveness of certain plants or plant extracts in clinical studies (Scot, 1998).

However, the elevated level of bilirubin ($p < 0.05$) in the *M. ciliata* extract-treated rats is consistent with the decreased PCV levels observed in this group. This is because elevated level of bilirubin as is the case in this study could be due to red cell destruction (Kagira *et al.*, 2006). The low antioxidative activity of the extract might have caused red cell destruction by hemolysis which resulted in the elevated bilirubin levels in the serum of the rats. Elevated bilirubin, AST, and ALT are markers for severe liver disease and are independent prognostic factors in severe drug-induced liver disease (Smith *et al.*, 2002; Iweala and Okeke, 2005). It is to be noted however, that the elevated bilirubin levels in the *M. ciliata*-treated rats was not accompanied by increased ALT and AST, thus, this increase might not be due to injury to the Smith *et al.*, (2002) liver by the extract.

**Biological Screening of Selected Plant**

As a starting point, the ethanolic root extract of both plants – *Mitragyna ciliata Aubrev and Pellegr* (*Rubiaceae*) *and Ritchea longipedicillata Gilg (Capparidaceae)* claimed by

traditional healers to be effective against trypanosomiasis, were screened for toxicity and anti-trypanosomal activity.

**Anti-trypanosomal Activities**

Of the two plants – *R. longipedicellata* and *M. ciliata* screened for trypanocidal activity, *M. ciliata* exhibited both suppressive and therapeutic activities at 50mg/kg dose. In the suppressive activity, the plant extract produced a distinct biphasic response (Onasanwo and Elegbe, 2006). In other words, the extract exhibited an inhibitory activity at 50 mg/kg dose and did not show the same activity at 100 mg/kg dose. This might be due to the fact that the effective dose of the extract as a trypanocidal agent might be 50 mg/kg. This might also be due to other factors such as certain components of the extract playing an antagonistic role to that of the active constituents of the plant extract. (Chevallier, 2000). The characteristic cyclic parasiteamia of *T. brucei* infection (Kennedy, 2004) was absent in the animals in this group. This suggests that *M. ciliata* extract at 50 mg/kg inhibited the capacity for antigenic variation in the parasites (Rudenko and Taylor, 2006). Furthermore, the same dose produced inhibitory therapeutic effect, still exhibiting the biphasic property. The survival of all animals in these groups further buttress the potency of *M. ciliata root extract* at this dose.

However, *R. longipedicellata* at both doses did not exhibit both suppressive and therapeutic activities, but survival period of the animals increased at 800 mg/kg dose. This prolonged life, 6 days for suppressive activity and 8 days for therapeutic activity, point to a positive effect on the immune system of the animals. Trypanosomiasis is known to increase immunosuppressive activity (Askonas and Brancroft, 1964), and this extract might have ameliorated this feature of the disease. This observation supports the use of this plant as a blood tonic in traditional medicine (Iwu, 1993). In addition, there was a distinct absence of

cyclic parasitemia in *R. longipedicillata* extract treated animals, a consequence of a sustained increase in parasitemia. This observation might have resulted from the inhibitory effect of the extract on the capacity for antigenic variation and/or enhanced growth and proliferation of the parasites. That *R. longipedicellata* showed no activity should be taken within the context of the method of extraction and type of solvent used for extraction, as studies by Atawodi, (2005) has shown that plant extracts show differential activity due to the method of extraction and type of solvent used. Moreover, traditional healers often use combination of plants for preparation of their remedies. *R. longipedicellata* might have been used in combination with other plants based on its blood building capacity (significantly increased PCV, Table 4.2) and immune boosting ability (increased survival period Table 4.1). This line of thought is further supported by the observed results from the effect of the combination of its extract and that of *M. ciliata* (Figures 4.1 -4.4), on parasitemia in treated rats. This could have resulted in the potency claim of *R. logipedicellata* extract in their practice.

That the combination (1:1) of both plant extract exhibited good trypanocidal activity for both suppressive (at both 200 mg/kg and 400 mg/kg) and therapeutic (at 400 mg/kg) activities corroborates the practice by traditional doctors of combining a number of plants for treatment of various diseases (Iwu, 1993, Sofowora, 1982). Combination of both plants extracts might have produced additive or synergistic antitrypanosomal activity against the *T. b. brucei* infection in the animals. This is reported by Hoet *et al,* (2004) who observed that total alkaloids from *M. inermis* (close specie of *M. ciliata*) and methanolic fraction from *F. apodanthera* or tetrahydroharman from *G. senegalensis* had synergistic effect on $W_2$ and $D_6$ strain of *Plasmodium falciparium*.

In conventional medicine, combination therapy has received much attention in recent times. It has been accepted as a rational approach to management of diseases such as malaria and cancer (White *et al*, 1999, Arav-Boger & Shappiro, 2005). This above observation also finds support in earlier studies that standardized combined plant extracts was therapeutically equivalent to synthetic drugs (Wagner, 2005). A general observation from both suppressive and therapeutic screening activity screening is that parasitemia in the extracts'-treated rats were higher than the untreated control. It seemed that challenging the parasites with the extract at certain doses enhanced their proliferation. This observation was more pronounced in *R. longipedicellata* than *M. ciliata*. This suggests that the extacts might have inhibited the capacity for antigenic variation at certain doses and/or that it is because trypanosomiasis promotes immunosuppression Askonas and Bancroft, 1984; Turner, 1999; Rudenko and Taylor, 2006).

**Further Studies on *Mitragyna ciliata* Active Part**

Consequent upon the results from the biological screening and toxicological studies of the hydroethanolic root extract of the two selected plants, *Mitragyna ciliata* extract which exhibited a better trypanocidal activity over *Ritchea longipedicellata*, was selected for further investigation since the focus of this research is a search for trypanocidal agent from plants used in folk medicine for the treatment of trypanosomiasis. The combination of the extracts that exhibited potent trypanocidal activity was not selected for further studies because these plants' extract needed to be studied seperately.

**Evaluation of Selected Plant**

To start with the different parts (leaf, stem bark and roots) of the selected plant *Mitragyna ciliata* were investigated for their trypanocidal potential. The data obtained revealed that *M. ciliata* extract exhibited differential trypanocidal activity, with the root producing the

greatest trypanocidal activity. This finding is supported by previous reports that the trypanocidal activity of a particular plant has to be taken within the context of the plant parts (Atawodi *et al.*, 2005).

**Further Studies of Active Plant Part**

**i) Antioxidative Property:**

The rationale for investigation of the *in vitro* free radical scavenging property of *M. ciliata* roots extract is based on earlier reports that certain natural products effect their trypanocidal activity by creation of oxidative stress *in vivo* (Sepulveda-Boza *et al.*, 1996). Thus, the low *in vitro* free radical scavenging activity (25.25 %) of the extract might suggest the mode of its trypanocidal action *in vivo*. The extract might have acted as a prooxidant *in vivo;* altering the oxidative status of the *T. b. brucei* infected animals to which the extract was administered. Additionally, the low antioxidative property of the extract is further highlighted in the significantly decreased PCV value (34.6 ± 0.73) of the extract treated group compared to untreated control (36.50 ± 0.28). This might be due to increased oxidative stress in the animals which might have caused hemolysis of red cells.

**ii) TLC Analysis of *M. ciliata* Crude Roots Extract:**

Alkaloids are the major constituents identified in *M. ciliata* root which agrees with the separated fractions that was positive for Dragendorff's reagent. This is consistent with earlier work on this plant extract that reported the plant as being rich in alkaloids (Iwu, 1993). *Mitragyna* species alkaloids are those with conjugated double bond like the indoles and oxidoles. Many of these alkaloids such as mitraphylline, mitragynine, ciliaphyline, rotundifoline have been isolated. (Dongmo *et al.*, 2003, Mastumoto *et al.*, 2005). Alkaloids are known to possess strong pharmacological activity especially on the central nervous system, the heart and as antiprotozoan agent. Many alkaloids especially isoquloline and

quiloline from *Cinchoma* bark *(Rubiaceae)* have been reported to possess antitrypanosomal activity (Hoet *et al.*, 2004, Evans, 1993). Many have been used for years as antimalarial drugs.

**iii) *In vitro* Studies:**

The observations from the *in vitro* studies agree with the *in vivo* studies that the active constituent (butanol fraction) is a hydrophilic agent, exhibiting potent activity at 4 μg/ml. The extract and its active fraction exhibited a better *in vitro* inhibition than all other fractions. It is noteworthy that the crude extract exhibited a better activity with cessation of motility in10 min. at the 2 μg/ml. This observation is corroborated by previous studies that plant extracts act synergistically and produce enhanced activity over their isolated constituents or synthetic drugs (Chevallier, 2000).

**iv) *In vivo* Trypanocidal Evaluation of Fractions:**

The activity-guided fractionation of *M. ciliata* roots extract using solvents of increasing polarity showed that bioactive agent occurred in the saturated butanol fraction as the fraction showed the greatest activity ($p < 0.05$) This makes it a hydrophilic bioactive agent and indicates that the bioactive agents might be alkaloids, saponins, phenols, and other hydrophilic metabolites since these metabolites are characteristically soluble in polar solvents. The results that also showed that the non-polar, hydrophobic fraction of n-Hexane and chloroform possess no trypanocidal effect, supports that the active component is not a hydrophobic agent. The ethyl acetate fraction exhibited slight activity which might be due to the phenolic compounds extractable with the solvent (Wu *et al*, 2005). These compounds and/or their derivatives have been shown to be potent trypanocidal agent (Hoet *et al.*, 2004). However, the low activity of this fraction might be due to the antioxidative property credited to most phenolic natural products from plants (Wu *et al.*, 2001). Furthermore,

different species in the plant extracts of the *Rubiaceae* family have been reported to possess various pharmacological activities including trypanocidal. However, this is the first report on the *in vivo* effect of the root extract of *M. ciliata* on *T. brucei brucei*.

**iv) Heamatological Evaluation of Active Part:**

a) Heamatological indices observed in this study are consistent with the major heamatological characteristics of trypanosomiasis- anaemia, leucocytopeania and thrombocytopeania. Anemia is the most important heamatological condition in trypanosomiasis. Other changes, such as WBC, platelet and plasma factors occur simultaneous with anaemia of trypanosomiasis (Naessens *et al.*, 2005). The observed anaemia in the infected and treated rats, as reported by ILRAD (1984) and Naessens *et al.*, (2005) is not due to red cell destruction associated with other diseases i.e. where the red cell simply burst in the circulatory system leading to elevated bilirubin levels and jaundice. Anaemia in trypanosomiasis has been hypothesized to be caused by several factors. These include haemodilution i.e. increased plasma volume; immunological reasons such as autoantibodies; direct damage by biologically active molecules and breakdown in the capacity of the infected animals to regenerate red blood cells (ILRAD, 1984). 'Immunological factors' is postulated to involve antigen-antibody complement complexes, deposited on the surface of erythrocytes of infected animals, resulting in their immune elimination leading to clinical anemia (Kabayashi *et al.*, 1976). There is also a report that a relationship exists between sialidase enzyme and occurrence of anaemia in trypanosomiasis. Nok and Balogun (2003) reported that as serum sialidase activity increased in mice, low packed cell volume (PCV) and increase in parasitemia were observed. Sialidase is an enzyme that cleaves the terminal sialic acid residue in proteins,

which then marks the proteins for destruction. Therefore, its increased activity indicates increased protein destruction in the system, in this case increased red cell destruction.

There has been inconsistent report on the pattern of leucocytes changes in African trypanosomiasis. The report on leucocyte changes in this study is consistent with the report of most workers that a distinct leucocytopenia occurs in trypanosomiasis (Lorne, 1986). These changes are due to reduced lymphocytes and neutrophils (Odo and Omeke, 1999), although this change was not statistically significant in this study. During infection, macrophages become activated with changes in receptor expression and mediator release; so that there is for example spontaneous IL-1 release (with a role in T- and possibly B- cell proliferation) (Askonas and Bancroft, 1984). As mentioned earlier, these increased phagocytosis results in increased cell destruction especially of red cell with a concomitant increase in serum bilirubin levels which is a by-product of hemoglobin catabolism. Some other workers on the other hand have reported leucocytosis (Emeribe, 1991, Kagira *et al.*, 2006).

A number of studies have reported (as was also observed in this work) thrombocytopeania (decreased platelets count) as a significant feature of trypanosomiasis (Kagira *et al.*, 2006). This is due to enhanced splenic trappings of platelets (Greenwood and Whittle, 1976,), and/or shortened platelet life span (Robin-Browne *et al.*, 1975, Odo and Omeke, 1999).

**Enzyme Studies**

The evaluation of the effect of *M. ciliata* ethanolic root extract on the infected animals that might have resulted in its trypanocidal activity was based on the low free radical scavenging (25.25 %) ability of the crude extract. Thus, the oxidative stress enzymes - superoxide dismutase (SOD) and catalase (Cat), of the infected rats were assayed.

The significantly elevated activity of superoxide dismutase in all fractions, except ethyl acetate agrees with previous studies that SOD is inducible in mammals and microorganism (Elchuri *et al*, 2005), and the level of this enzyme will increase with increasing need to protect against toxic oxidation (Muller *et al.*, 2006; Sentman *et al.*, 2006). This elevation indicated an increase in superoxide free radicals in the system of the infected rats (Usoh *et al.*, 2005), which might be due to the low antioxidative property of the fractions and/or due to the formation of superoxides. Subsequently, these free radicals are converted by SOD to hydrogen peroxide. Catalase in turn catalyses the conversion of hydrogen peroxide to water and molecular oxygen (Yoruk *et al.*, 2005). The significantly depressed level of catalase activity ($p > 0.05$) in the butanol fraction-treated rats as compared to untreated and the other fractions treated rats, suggested that this fraction permitted $H_2O_2$ (produced from the elevated activity of SOD), to act more as its own inhibitor than as its own substrate (Aksoy *et al.*, 2004).

The host (rats) and the trypanosomes have in common superoxide dismutases in their enzymatic defence systems against free radicals (Soulere *et al.*, 1999), but the parasite lacks catalase (Wang *et al.*, 1999). In the absence of catalase the parasite depends solely on a spermidine-gluthathione conjugate named trypanothione whose oxidative form is regenerated by trypanothione reductase that is very sensitive to alteration in redox equilibrium (Atawodi *et al.*, 2003, Hoet *et al.*, 2004, Khan and Omar, 2007). Therefore, the depression in catalase activity in the infected rats, which might have arisen from its inhibition by accumulated $H_2O_2$, altered the oxidative status of the animals creating oxidative stress which undermined the integrity of the parasites' membrane. Consequently, the parasites became vulnerable as their membranes were disrupted resulting in their clearance from the bloodstream. Studies have shown that elevated cellular levels of free

radicals cause damage to biological macromolecules including membrane lipids (Halliwell, 1996, Wang and Jiao, 2000, Usoh *et al.*, 2005). From the physical manifestation and survival of the rats, the deleterious effect of the oxidative stress created by the active fraction seemed to be more pronounced on the parasites than the rats (even though PCV was lower ($p > 0.05$) in the active fraction than other fractions). The observation that depression in catalase activity coincides with clearance or inhibition of parasites' proliferation is supported by earlier work that *T. brucei* clearance from the blood of Cape buffalo coincided with the time catalase activity was depressed (Wang *et al.*, 1999). In addition, parasitic protozoa are known to be susceptible to free oxygen radical-induced oxidative stress (Elchuri *et al.*, 2005). This is supported in conventional medicine by the use of antimalarials such as primaquine, which act by creating oxidative stress (Clark and Hunt, 1983). Furthermore, these results are consistent with earlier reports that some natural products exert their trypanocidal activity by creating oxidative stress *in vivo.* (Sepulveda-Boza *et al.*, 1996; Hoet *et al.*, 2004).

**Further Investigation *M. ciliata* Active Fractions**

The presence of mainly alkaloids in the active fraction supports the trypanocidal activity of the fraction which is consistent with reports that some alkaloids possess trypanocidal activity (Hoet *et al.*, 2004). The role of calcium in *T. brucei brucei* infection led to the investigation of the effect of the active fraction on serum calcium concentration in the infected animals that might explain the most probable mode of action of the active fraction.

**Calcium Concentration**

The 8-fold increase in $Ca^{2+}$ concentration in the infected rats compared to uninfected, *M. ciliata* crude extract and active fraction treated rats underscores the important role $Ca^{2+}$ (which is compartmentalized in organelles called acidocalsisomes) plays in the parasites

during *T. b. brucei* infection (Eintracht *et al.*, 1998; Decampo and Morenso, 2001). Indirect lines of evidence implicate $Ca^{2+}$ as an important component in the control of *T. brucei* infection (Decampo and Morenso, 2001). Thus, the effect of the butanol fraction and the crude extract on serum $Ca^{2+}$ concentration in the infected animals might partly explain its trypanocidal activity. This is further substantiated by previous experimental evidences that $Ca^{2+}$ potentiate the lethal effect of certain trypanocides such as melarsoprol, salicylhydroxamic acid (SHAM) and HDL (Decampo and Morenso, 2001). Haghghat and Ruben, 1992, Buchanan *et al.*, 2005 have reported the purification of $Ca^{2+}$ binding proteins from *T. brucei,* where they serve as intracellular $Ca^{2+}$ buffers and mediate cellular response to $Ca^{2+}$ signals. Additionally, studies have shown that $Ca^{2+}$ signaling is required for transversal of blood-brain barrier in trypanosomes (Nikolskaia *et al.*, 2006). Furthermore, according to Hool and Corry, (2007), oxidative stress could affect calcium channels in mammals. Hydrogen peroxide which accumulates due to the decline in catalase activity is believed to interact with cell signaling pathway. This interaction is by way of modification of key thiol groups on proteins that possess regulatory function (Hool and Corry, 2007). Therefore the oxidative stress created in the animals treated with the active butanol fraction due to a decline in catalase activity in these animals, may have affected the $Ca^{2+}$ channels in them. Precisely, hydrogen peroxide regulates the catalytic activity of enzymes by redox mordification of cysteine residues (Barford, 2004). This is further buttressed by a weak correlation ($r = 0.400$, $p = 0.488$) between calcium concentration and parasiteamia in the active fraction treated animals and a moderate correlation ($r = -0.60$, $p = 0.29$) between calcium concentration and parasiteamia in the infected/untreated. This effect on the $Ca^{2+}$ metabolism produced a deleterious effect on the parasites.

It is of note that $Ca^{2+}$ levels in the crude extract treated rats were not significantly different ($p < 0.05$) from those of uninfected control rats. This finding suggests that the crude extract had a more pronounced effect on the $Ca^{2+}$ metabolism of the parasites, indicating that the combined constituents as they occur in the plant, produced better effect than the isolated constituents, further buttressing previous studies that the activity of some plant extracts diminishes with isolation and purification of the crude extract (Ezekwesili, 2007).

**Route of Administration**

The intraperitonal administration of the active fraction which produced a better activity (even at a lower dose) than the oral administration is consistent with the intraperitonal administration of most synthetic trypanocides, which exhibit greater therapeutic effect. The reason might be due to the fact that the parasites, *T. brucei,* occur extracellularlly, thus any drug that is administered into the extracellular fluids of the infected animals distributes to/and produces the same effects in the parasites that is produced in the animals. It is noteworthy that the oral administration of the crude extract prolonged the lives of the animals > 28 days (Table 4.2), and it had 60 % of the animals surviving (Table 4.3), while the oral/intraperitonal administrations of the active fraction left no survivals, with death occurring on 19 and 21 days respectively. This is consistent with previous studies, extracts of plants are thought to produce synergistic effect and they are therapeutically equivalent to synthetic drugs and have been shown to possess less toxic side effects (Chevalier, 2000; Wagner, 2005).

# CHAPTER SIX

## CONCLUSION

This study revealed that *M. ciliata* root extract possesses antitrypanosomal activity showing both suppressive and therapeutic activities, while *R. longipedicellata* exhibited no activity. Toxicological studies of the extract suggested the lack of toxicity and therefore the safety of the plant extract, indicating a hepatoprotective effect. *M. ciliata* extract showed low antioxidative property. Studies also showed that administration of extract at certain doses (100m/kg for *M. ciliata* and 800mg/kg for *R. longipedicellata*) seems to enhance the growth and proliferation of *T. b. brucei* in infected animals. Activity-guided fractionation identified butanol fraction of *M. ciliata* as the active fraction. Administration of active fraction to infected rat did not produce any major changes to hematological indices of trypanosomiasis. The active fraction altered the oxidative status of treated rats, resulting in lower parasitemia levels. The active fraction also had a marked effect on $Ca^{2+}$ levels in infected rats. In conclusion, this study has corroborated the use of *Mitragyna ciliata Aubrev and Pellegr (Rubiaceae)* in the treatment of trypanosomiasis in traditional medicine in Nigeria. Furthermore, the study provides the biochemical basis for the trypanocidal action of this plant which might be through creating oxidative stress in the *T. b. brucei* infected rats and/or its effect on $Ca^{2+}$ metabolism of the infected rats.

# CONTRIBUTIONS TO KNOWLEDGE

- The study established that the biochemical basis for the potency of the active fraction of *M. ciliata* is the creation of oxidative stress and effect on calcium metabolism *in vivo*. This aspect is as yet in dearth in the study of medicinal plants with antitrypanosomal activity.

- It provided the first report and documented scientific evidence of the trypanocidal activity of the root of *Mitragyna ciliata Aubrev and Pellegr (Rubiaceae)* and substantiates its use in traditional medicine for the treatment of trypanosomiasis.

- It established the lack of toxic effect of *M. ciliata* and *R. longipedicellata* extracts and provides data for both plants on safety studies of medicinal plants used in ethno medicine.

- It also identified the active fraction (consisting mainly of alkaloids) of *M. ciliata* root which is as yet unreported and is lacking in plants used in traditional medicine for the treatment of trypanosomiasis.

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# APPENDIX

## FORMULAS FOR CALCULATION OF BIOCHEMICAL PARAMETERS

**Alkaline Phosphatase (ALP)**

Enzyme Activity = $\frac{\text{Absorbance of test}}{\text{Absorbance of Control}}$ x Concentration of standard

Standard value = 50IU/L

Alaline Transaminase (ALT) and Aspatate Transaminase (AST) activities were read of tables provided.

**BILIRUBIN**

**Total Bilirubin (TB) mg/100ml =10.8 x Absorbance of Sample**

**CREATININE**

Result = $\frac{\text{(A2 – A1) Assay}}{\text{(A2 – A1) Standard}}$ X Standard concentration

A1 = Absorbance after 30 seconds

A2 = Absorbance 2 minutes after first reading

Absorbance taken at 490nm

**Enzyme Activity = $\frac{\text{OD/min x V}}{\varepsilon \text{ x v}}$**

where V = total volume of reaction mixture

v = volume of sample

ε = extinction coefficient

ε for Superoxide Dismutase (SOD) = $4020m^{-1}\ cm^{-1}$

Absorbance taken at 480nm

Units of enzyme activity = μg/min/mg protein

ε for Catalase (Cat.) = $40.0m^{-1}\ cm^{-1}$

Absorbance taken at 240nm

Units of enzyme activity = moles of $H_2O_2$ reduced per min per mg protein (Kat f)

**CALCIUM CONCENTRATION**

Absorbance taken at 570nm

$$\text{Calcium concentration in mmol/L} = \frac{\text{Abs (Test)}}{\text{Abs (Standard)}} \times 2.5\text{mmol/L}$$

**DOSAGE CONTROL FORMULA**

Ml of extract/drug given =

$$\frac{\text{mg of drug/extract per kg body weight}}{1000} \times \frac{\text{Weight of animal (g)}}{\text{Concentration (mg/ml)}}$$

**PER CENT INHIBITION**

$$\text{Inhibition (\%)} = 100 - \frac{\text{Parasiteamia of Test (Pt)}}{\text{Parasiteamia of Control (Pc)}} \times 100$$