ANTI-TRYPANOSOMAL POTENTIALS OF *ANNONA SENEGALENSIS* AND *EUCALYPTUS CAMALDULENSIS*

BY

KABIRU, Yusuf Adamu

Ph.D/SSSE/1999/027

A Thesis submitted to the Post Graduate School of Federal University of Technology, Minna, in partial fulfilment of the requirement for the Degree of DOCTOR OF PHILOSOPHY

Department of Biochemistry

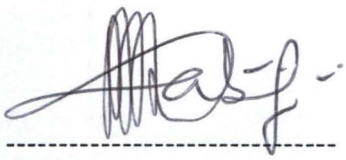
School of Science and Science Education

Federal University of Technology, Minna

July, 2010

DECLARATION

I hereby declare that this research project/thesis is my original work and to the best of my knowledge has not been presented in any form for the award of degree or any other certificate in any other institution.



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This thesis titled: "ANTI-TRYPANOSOMAL POTENTIALS OF *ANNONA SENEGALENSIS* AND *EUCALYPTUS CAMALDULENSIS*" by Kabiru, Yusuf Adamu (Ph.D/SSSE/1999/027) meets the regulations governing the award of the degree of Doctor of Philosophy in Biochemistry of Federal University of Technology, Minna, and is approved for its contribution to knowledge and literary presentation.

Dr. E. O. Ogbadoyi

Name of Major Supervisor

22/07/2010

Signature & Date

Prof. T. A. Gbodi

Name of Co-Supervisor (1)

22/07/2010

Signature & Date

Prof. J. I. Okogun

Name of Co-Supervisor (2)

22/07/2010

Signature & Date

Dr. A. A. Jigam

Name of Head of Department

22/07/2

Signature & Date

Prof. M. Galadima

Name of Dean of School (SSSE)

16/8/2010

Signature & Date

Prof. S. L. Lamai

Name of Dean, Post graduate school

6/9/2010

Signature & Date

DEDICATION

This work is dedicated to all those who stand for truth and justice.

ACKNOWLEDGEMENTS

It is by Allah's Mercy and Benevolence that all things are possible. All Praises and Gratitude are due to Him for enabling me go through this programme successfully. My team of supervisors was marvellous. I want to appreciate the invaluable assistance of my major supervisor, Dr E. O. Ogbadoyi who graciously provided the chemicals used in this work through a grant he secured from the International Centre for Genetic Engineering and Biotechnology (ICGEB), Italy. Thank you for the concern and assistance. I owe Professor J. I. Okogun my sincere appreciation for his fatherly encouragement and the provision of technical expertise in the area of medicinal chemistry. He was always there for me from the beginning to the end. The technical support of Mr. Andrew, Mrs. Menegbe and Anty Elizabeth of the Medicinal Chemistry Laboratory of National Institute for Pharmaceutical Research and Development is highly appreciated.

I am grateful to Professor T. A. Gbodi of Ibrahim Badamasi Babangida University Lapai and Dr. H. A. Makun of the Department of Biochemistry, Federal University of Technology, Minna, for their assistance in the aspect of toxicity studies. I cannot forget the active role played by my Head of Department, Dr. A.A. Jigam in ensuring the completion of this work. My other colleagues in the department, laboratory staff and my project students all played positive roles in the execution of this project. Thank you all.

I wish to specially acknowledge Professor A. J. Nok of the Department of Biochemistry, Ahmadu Bello University, Zaria, for guiding me through the techniques of cerebrospinal fluid infectivity test; Dr. U. I. Tiffin of General Hospital, Suleja, Niger state for interpreting the histopathology plates; and Mallam Abubakar Usman Zungeru for putting me through the statistical analysis of the results.

I must appreciate my late father, Alhaji Kabiru; my mother, Hajiya Habiba; and my guardian, Alhaji Yusuf Sadoh for their prayers. The positive role played by my wife cannot be overemphasized. She was always encouraging, and praying for a successful completion of this work. My wife and children were the immediate recipients of the pains of the enormous sacrifices that went into this long adventure. May Allah bless you for your patience and understanding. To my brothers and sisters, thank you all for your concern. May Allah bless you all.

Finally, I must express my sincere appreciation to my employer, Federal University of Technology, Minna, for granting me the opportunity to undertake this study; and theirs will be a great measure of the credit if, on going out into the world, the result of the work makes an impact.

ABSTRACT

Chemotherapy of African trypanosomiasis in both the human and animal forms has been confronted with multidimensional problems that include paucity of drugs, resistance, high cost, prolonged treatment protocol and adverse side effects. To explore alternatives, the leaves, stem and root barks of *Eucalyptus camaldulensis* and *Annona senegalensis* were sequentially extracted with hexane, ethyl acetate, methanol and water; and the extracts tested for antitrypanosomal activity. Mice infected with *Trypanosoma brucei brucei* were administered intraperitoneally doses ranging from 100-600mg/kgbodyweight/day of the extracts for 21 consecutive days. One control group was treated with 3.5mg/kg bodyweight of berenil while the other was left untreated. The methanol extract of *E. camaldulensis* (leaf), hexane and aqueous extracts of *A. senegalensis* (stem) produced complete cure for the animals in the different dose groups, and survived as long as those treated with the standard drug, berenil, although the clearance time was faster for the standard drug. Sub inoculation of healthy mice with the blood and cerebrospinal fluid (CSF) of the cured mice did not result in infection. Acute toxicity studies of the methanol extract of *E. camaldulensis* (leaf) confirmed the safety of the extract because no mortality was recorded even at 5000mg/kg bodyweight and sub chronic toxicity studies showed that the integrity of the liver and kidney was not compromised. However, the extract had no prophylactic activity, but in combination with methanol extract of *A. senegalensis* (leaf), one mouse was cured of *T. b. brucei* infection. The crude methanol extract also demonstrated antibacterial activity against *Klebsiella pneumoniae* and *Staphylococcus* aureus. Bioassay-guided fractionation of the crude methanol extract of *E. camaldulensis* (leaf) gave 10 fractions, with only fractions 8 and 9 exhibiting antitrypanosomal activities that were significantly different from that of the crude extract and the standard drug ($p \leq 0.05$). Phytochemical screening revealed the presence of terpenes, steroids, saponins, tannins, alkaloids and fatty acids in both the crude extract and fraction 9; while fraction 8 contained only terpenes, steroids and fatty acids. Data from GC-MS analysis, fractions 8 and 9 were found to contain 2-Chloro-N—(1, 3 - thiozol-2-yl) acetamide, 9-Octadecenamide, 1-Nonadecene, (Z)-9-Eicosene, Hexadecanol, 1-pentadecanol; and Methylhexadecanoate, Methyl cis-9-octadecenoate, Methyl-n-octadecanoate, 1-heptadecanol respectively. It can be concluded that the methanol extract of *E. camaldulensis* (leaf) has immense potential for the development of drugs against African trypanosomiasis; and in combination with *A. senegalensis* extract offer hope for the development of combination drugs capable of overcoming resistance to monotherapies.

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ABBREVIATIONS

AT -African Trypanosomiasis

AAT-Animal African Trypanosomiasis

ALT-Alanine Amino Transferase

A.S -*Annona senegalensis*

AST -Aspartate Amino Transferase

BW -Body weight

CC -Column Chromatography

CSF -Cerebrospinal Fluid

DMSO -Dimethyl Sulpho Oxide

E.C -*Eucalyptus camaldulensis*

Et. Ac. -Ethyl acetate

Ext. -Extract

GC-MS -Gas Chromatography-Mass Spectroscopy

HAT -Human African Trypanosomiasis

Hb -Haemoglobin

Hex. -Hexane

Inf. N T -Infected not treated

MeOH -Methanol

MCD -Minimum Curative Dose

PCV -Packed Cell Volume

SGOT-Serum Glutamate Oxaloacetate Transaminase

SGPT-Serum Glutamate Pyruvate Transaminase

TLC-Thin layer Chromatography

CHAPTER ONE

1.0 INTRODUCTION

Parasitic diseases remain a major public health problem affecting hundreds of millions of people, particularly in tropical developing countries. Economic and social conditions such as inadequate sanitation, ignorance and poverty may encourage the spread of these diseases. The immense suffering caused by these illnesses and the consequent loss of productivity is a major drain on the resources of the communities in which they occur.

Generally, diseases caused by protozoans are responsible for considerable mortality and morbidity throughout the world. There are an estimated 20 million people infected with *Leishmania species* (Goto *et al.*, 1998) and nearly the same number infected with *Trypanosoma cruzi* (Buckner *et al.*, 1998). There are 50 million cases of amoebiasis with up to 100,000 deaths each year (Huston and Petri, 1998). Current estimates indicate that 60 million people are at risk of infection with African Trypanosomiasis, with about 300,000 new cases each year (WHO, 1998), and 50 – 70 million animals are exposed to the infection (Ogbadoyi *et al.*, 2007a).

African trypanosomiasis is confined to tropical Africa between latitudes 15°N and 20°S, or from North of South Africa to South of Algeria, Libya, and Egypt. The prevalence of the disease differs from one country to another and varies within a single country. In 2005, major outbreaks were observed in Angola, the Democratic Republic of Congo and Sudan (WHO, 2006).

In Central African Republic, Chad, Congo, Côte d'Ivoire, Guinea, Malawi, Uganda, and United Republic of Tanzania, sleeping sickness remains an important public health problem. Countries such as Burkina Faso, Cameroon, Equatorial Guinea, Gabon, Kenya, Mozambique, Nigeria, Rwanda, Zambia, and Zimbabwe are reporting

fewer than 50 new cases per year. Transmission seems to have stopped and no new cases have been reported for several decades in countries such as Benin, Botswana, Burundi, Ethiopia, Gambia, Ghana, Guinea Bissau, Liberia, Mali, Namibia, Niger, Senegal, Sierra Leone, Swaziland, and Togo. African trypanosomiasis is distinct from American trypanosomiasis, which is caused by *Trypanosoma cruzi* and has different vectors, clinical manifestations, and therapies (WHO, 2003).

The limited availability and affordability of pharmaceutical medicines means that the majority of the world's population depend on traditional medical remedies, and it is estimated that some 20,000 species of higher plants are used medicinally throughout the world (Phillipson and Wright 1991). There are rather limited numbers of safe and effective antiparasitic drugs licensed for human use (WHO, 1990). Furthermore, increasing drug resistance has limited the usefulness of some existing compounds. Parasitic diseases occur mostly in poorer countries, where the technical expertise and financial resources necessary for new drug development are scarce. Despite efforts made by the World Health Organization (WHO) to help find new treatments, there have been few new drugs in recent times.

Traditional medical remedies for several diseases abound in most endemic regions. Many of these preparations have been used extensively and knowledge about them has been accrued by several generations of practitioners from experience, trial and error. Although formal toxicology studies are limited, most of the extensively used local remedies are unlikely to be severe toxins and are worthy of further evaluation for novel antiparasitic compounds. In the developed countries, where scientifically formulated drugs are readily available, there is increasing confidence in and growing use of non-prescription drugs from plant sources. In the USA, it has been estimated that $5 billion was spent on such preparations in 1997 (Swerdlow, 2000). Ethno medicine and ethno

botany have long been of interest to medical researchers, physicians, the pharmaceutical industry, anthropologists and botanists. The value of ethno medicinal and ethno botanical information in drug development is highly variable. There are many splendid texts cataloguing the ethno botanical uses of plant species by traditional peoples, and there are publications on this subject in specialist journals, like Journal of Ethnopharmacology. There are a number of databases that include over 150 genera with folklore reputations as antimalarials (Phillipson and O. Neill, 1986; Phillipson and Wright, 1991). There are however, relatively few publications of the ethno botanical uses of most other natural products. The need to investigate unexplored natural products for their medicinal properties cannot be over-emphasized with some urgency.

Various well-known drugs listed in modern pharmacopoeia have their origins in nature. These include digitalis and related cardiac glycosides isolated from the leaves of the foxglove plant (*Digitalis lantana* and *Digitalis purpurea*). These drugs are presently used to treat certain abnormal heart rhythms and chronic heart failure. The foxglove plant had been used as a topical preparation for the treatment of heart problems since the middle ages (Groves and Bisset, 1991). Ephedrine, a component of many treatments for respiratory disorders, is derived from the ephedra plant (*Ephedra sinica* and some related species). Morphine, a widely used potent analgesic is derived from the opium poppy (*Papaver somniferum*). These drugs were first used as traditional herbal remedies. Salicin, a natural product of the willow (*Salix sp.*), was discovered accidentally during attempts to use its bitter bark to treat malaria following the success of *Cinchona* bark. Salicylates were later developed as semi synthetic derivatives of salicin (Greenwood, 1992). Periwinkle extracts have been used traditionally to treat diabetes in many parts of the world (Noble, 1990). Paclitaxel (taxol) is a new drug derived from the pacific yew tree, *Taxos brevifolia*. The use of this drug has been a

major advance in the treatment of breast, ovarian and other cancers (Gotaskei and Andreassi, 1994).

With regard to parasitic diseases, natural product research has contributed to outstanding new drugs for treating some infections. These drugs include quinine and artemisinin for treating malaria and ivermectin for onchocerciasis and lymphatic filariasis. There continues to be increasing number of reports of antiparasitic activities of natural products which will undoubtedly be a major source of new medicines yet to be identified and developed. And there is a growing awareness by governments and the scientific and medical communities of the importance of medicinal plants in health-care systems in many developing countries. Greater importance is now being attached to the use of locally available medicines as a means of reducing reliance on expensive imported drugs (Bodeker and Willcox, 2000).

There are many problems associated with the currently available drugs for treating African trypanosomiasis. There is growing resistance to the few drugs available (De koning, 2001). Relapse rate with melarsoprol in Northern Uganda, Southern Sudan, and Northern Angola was as much as 30% (Ogbadoyi *et al.*, 2007a). Most of the available drugs are highly toxic with about 5% of those treated with melarsoprol dying as a result of high toxicity of the drug (Ogbadoyi *et al.*, 2007a). In addition to these drawbacks is the problem of non availability of the drugs in rural communities where the burden of the disease is more manifest and where available the cost is prohibitive. As a consequence of these developments, there is an urgent need to source for new, cheap, safe and effective drugs from natural products. Many plant products have undergone scientific evaluation for antiprotozoal activity, but the need to evaluate more plants cannot be over-emphasized. Following an initiative by the World Health Organization in August 2000, there arose the opportunity to evaluate scientifically many

more traditional medicines and other natural products in validated antiparasitic and toxicity screens, which will help establish which substances have potential for new pharmaceutical products.

Based on the fore-going, this study was designed to evaluate the antitrypanosomal activities of *Annona senegalensis* and *Eucalyptus camaldulensis* with a view to isolating, identifying and characterizing novel anti-trypanosomal agents. *Annona senegalensis* Pers.(Annonaceae) is used by traditional medicine practitioners in Nigeria for the treatment of various diseases, including sleeping sickness and cancer (Ogbadoyi *et al.*,2007a), while *Eucalyptus camaldulensis* (Myrtaceae), is used locally as a decoction for the treatment of malaria and typhoid fevers.

1.1 RESEARCH OBJECTIVES AND AIMS

1.1.1 Research Objectives

The main objective of this research work is to obtain ethno medicine capable of treating African trypanosomiasis and to obtain lead compound(s) that will form the basis for the chemical synthesis of modern pharmaceuticals.

1.1.2 Research Aims

- The research aims include the following:
- Screening of a wide range of indigenous medicinal plants for anti trypanosomal activities.
- Bioassay – directed fractionation of potentially active extracts
- Isolation and characterization of bioactive constituents

1.2 Justification for the research

Pathogenic trypanosomatids cause a plethora of diseases compounded by the lack of efficient vaccines and therapies.

These diseases, predominantly a problem of the poor and underdeveloped countries, have less than their deserved share of efforts towards the development of new drugs and vaccines (Croft, 1997). Most of the drugs in use were developed decades ago, and show variable efficacy, serious side effects, can require long-term treatment, and many have poor activity in immuno-suppressed patients. These picture/prospects clearly indicate the necessity to search for new chemotherapeutic agents.

The development of a conventional drug typically takes at least ten years and costs US$ 300 million to $.500 million in research and development expenditure for every new product that reaches the market. For this reason, many pharmaceutical companies are not interested in developing drugs against diseases in developing

countries, except as a by-product of drug development for the industrialized world (Merlin *et al.*, 2001).

The number of drugs available for the treatment of Human and Animal Africa Trypanosomiasis is very limited in addition to problems of toxicity and resistance. Up to 20% of patients with late stage trypanosomiasis show resistance to melarsoprol (Barrett, 1999). This is a particularly serious problem since melarsoprol and Difluoromethyl-ornithine (DFMO) are the only drugs commercially available for the treatment of late stage infections. The drugs for the treatment of early stages of trypanosomiasis are pentamidine and suramin. Both drugs when administered have side effects that include vomiting, abdominal pains, hypotension, hypoglycaemia, pyremia, joint pains, skin rash and proteinuria (Croft *et al.*, 1993).

The inherent expensive nature of current trypanocides, coupled with protracted treatment protocols and unbearable side effects necessitated the conception of this research which is targeted at sourcing for better, readily available, and cheaper trypanocidal agents from a variety of natural plant products with little or no side effects.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Historical perspective

Trypanosomatids are ubiquitous unicellular flagellated protozoa that are ancient survivors on planet Earth. They probably embarked on their own evolutionary branch more than 500 million years ago (Stevens and Gibson, 1999), prior to the origins of their present invertebrate and vertebrate hosts.

The first reports of human sleeping sickness were filed in the early 19th century, although the disease was described as 'sleeping sickness' by the Arab historian Ibn khaldun as early as the late 14th century. The disease has been known to slave traders and naval surgeons at least since the beginning of the 18th century (Williams, 1996). The disease is thought to be ancient enough for it to have influenced human evolution, as have malaria and other infectious diseases. The association between trypanosome and disease was reported about a hundred years ago by David Bruce when he discovered a species of trypanosome, *Tryponosoma evansi* in the blood of camels in India and *Trypanosoma brucei* in African cattle (Cross, 2001).

2.2 Trypanosomes

Trypanosomes are unicellular protozoans that belong to the family Trypanosomatidae. Not all trypanosomes are pathogenic. The best known pathogenic species include *Trypanosoma brucei gambiense (T. b. gambiense), Trypanosoma brucei rhodesiense (T. b. rhodesiense)* which infect humans, and *Trypanosoma congolense (T. congolense), Trypanosoma vivax (T. vivax), and Trypanosoma brucei brucei (T. b. brucei)* which infect animals. The genus *Trypanosoma* is divided into two groups: Stercoraria and salivaria. Species belonging to the first group (starcoraria) complete their developmental cycle in the terminal gut and are transmitted into the faeces of the

vector, while those belonging to the second group (salivaria) complete their developmental cycle in the anterior part of the digestive tract and are transmitted via the vector's saliva.

2.2.1 Morphology

A typical trypanosome is an elongated organism 5 – 30mm in length (including the flagellum) and 1.5 – 3.0μm in width, spindle-shaped, flattened body, which is usually curved although variations exist in different species, with a single nucleus containing a large central nucleolus (Karyosome) (fig.2.1). Movement is effected by a single flagellum, which arises at the posterior end of the cell body in an invagination of the cell surface known as the flagellar pocket and runs along the outer margin of the pellicle, being attached by a structure known as the Flagellum Attachment Zone (FAZ). There is a single kinetoplast (mitochondrial DNA), and a basal body in close association with the kinetoplast. Other structural features include the glycosomes, Golgi complex, Endoplasmic Reticulum, and a subpellicular corset of microtubules which envelopes the cell.

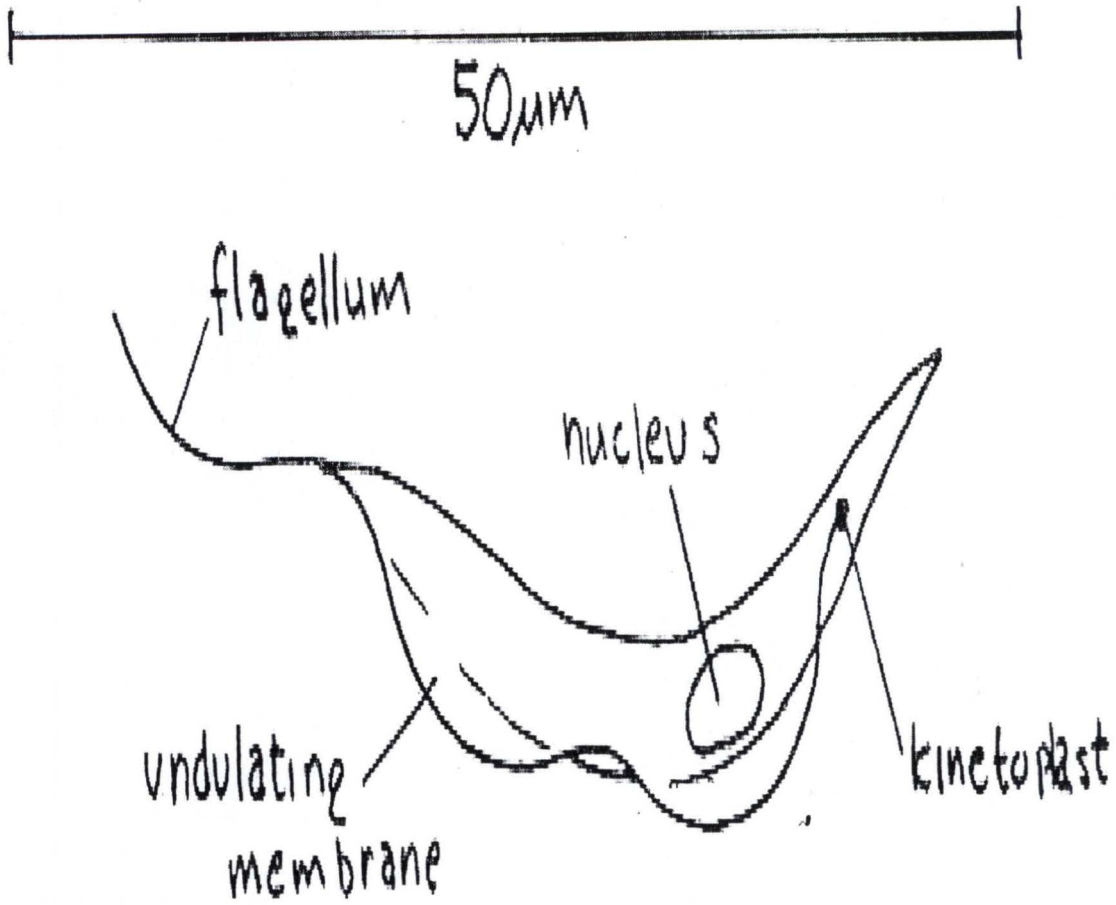


Fig.2.1: Cell Structure of Trypanosoma (**Source:** CDC, 2005)

The structure of the cell is fairly typical of eukaryotes. All major organelles are seen, including the nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus etc. Unusual features include the single large mitochondria with a condensed mitochondrial DNA structure, and its association with the basal body of the flagellum, unusually the cytoskeleton organization mechanism of the cell. The cell surface of the bloodstream form features a dense coat of variable surface glycoproteins (VSGs) which is replaced by an equally dense coat of procyclins when the parasite differentiates into the procylic in the tsetse fly midgut.

Trypanosomatids show specific cellular forms:

- Amastigote - Basal body anterior of nucleus, with a short, essentially non-functional, flagellum.
- Promastigote - Basal body anterior of nucleus, with a long detached flagellum.
- Epimastigote - Basal body anterior of nucleus, with a long flagellum attached along the cell body.
- Trypomastigote - Basal body posterior of nucleus, with a long flagellum attached along the cell body.

These names are derived from the Greek *mastig-* meaning whip, referring to the trypanosome's whip-like flagellum.

T. brucei is found as a trypomastigote in the slender, stumpy, procyclic and metacyclic forms. The procylic form differentiates to the proliferative epimastigote form in the salivary glands of the insect. Unlike Leishmania, the promastigote and the amastigote form does not form part of the *T. brucei* life cycle.

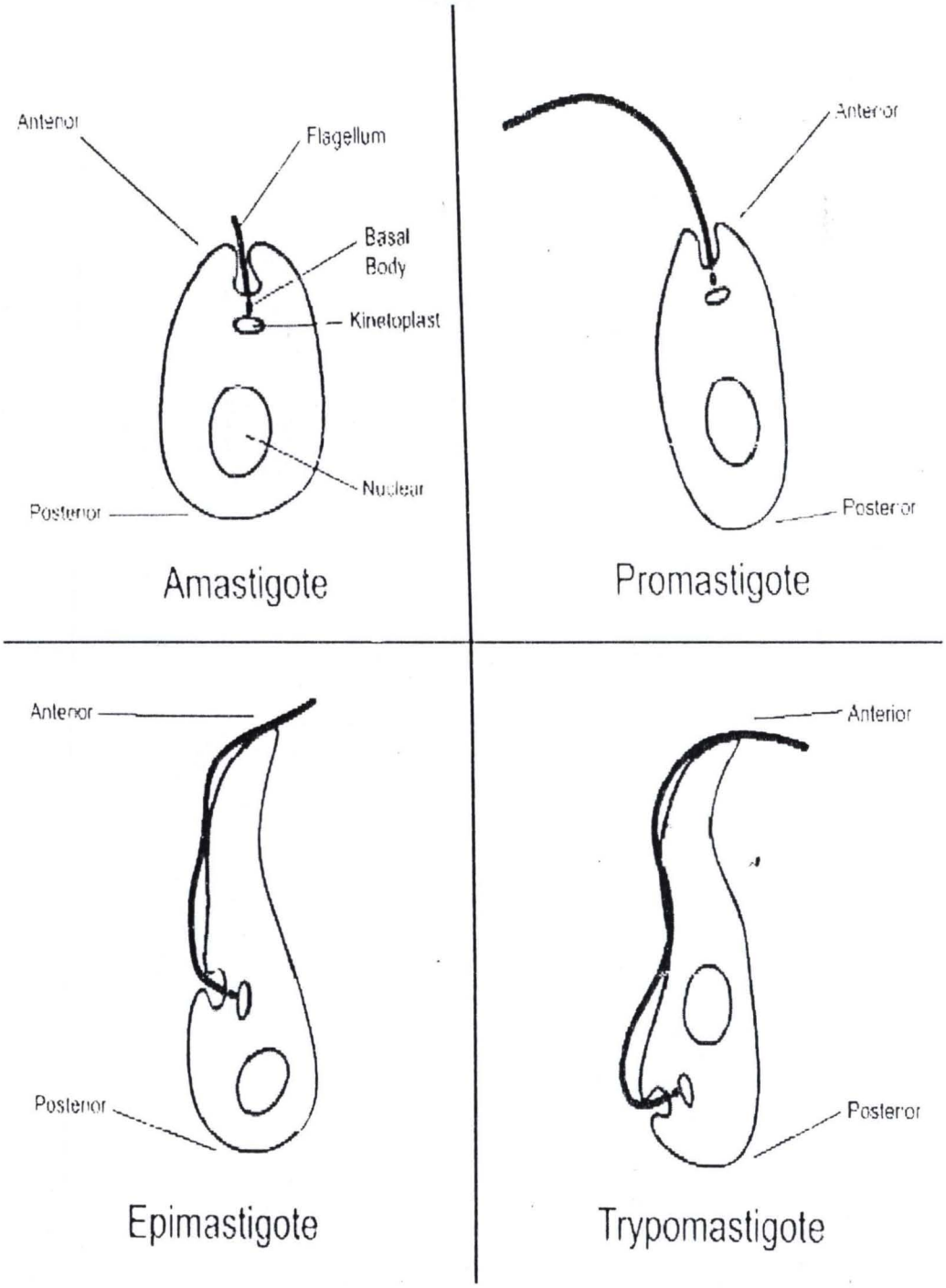


Fig. 2.2 Trypanosomatid cellular forms **Source: Vickerman, 1985**

2.2.2 The cytoskeleton

The trypanosome cytoskeleton is the subject of considerable research. The cytoskeleton, as the structure behind mitosis, locomotion and surface binding, is vital for viability and so is a target of interest for drug development. Much research on *Trypanosoma brucei* was first done on *Crithidia fasciculata*, a highly similar organism that is not dangerous to humans.

The cytoskeleton is predominantly made up of microtubules, forming a subpellicular corset. The microtubules lie parallel to each other along the long axis of the cell, with the number of microtubules at any point roughly proportional to the circumference of the cell at that point. As the cell grows (including for mitosis) additional microtubules grow between the existing tubules, leading to semi conservative inheritance of the cytoskeleton. The microtubules are orientated + at the posterior and - at the anterior.

Microfilament and intermediate filaments also play an important role in the cytoskeleton, but these are generally overlooked.

2.2.3 The Flagellum

The trypanosome flagellum has two main structures. It is made up of a typical flagellar axoneme which lies parallel to the paraflagellar rod, a lattice structure of proteins unique to the Kinetoplastida, euglenoids and dinoflagellates.

The microtubules of the flagellar axoneme lie in the normal 9+2 arrangement, orientated with the + at the anterior end and the - in the basal body. The cytoskeleton structure extends from the basal body to the kinetoplast. The flagellum is bound to the cytoskeleton of the main cell body by four specialized microtubules, which run parallel and in the same direction to the flagellar turbulent.

The flagellar function is twofold - locomotion via oscillations along the attached flagellum and cell body, and attachment to the fly gut during the procyclic phase.

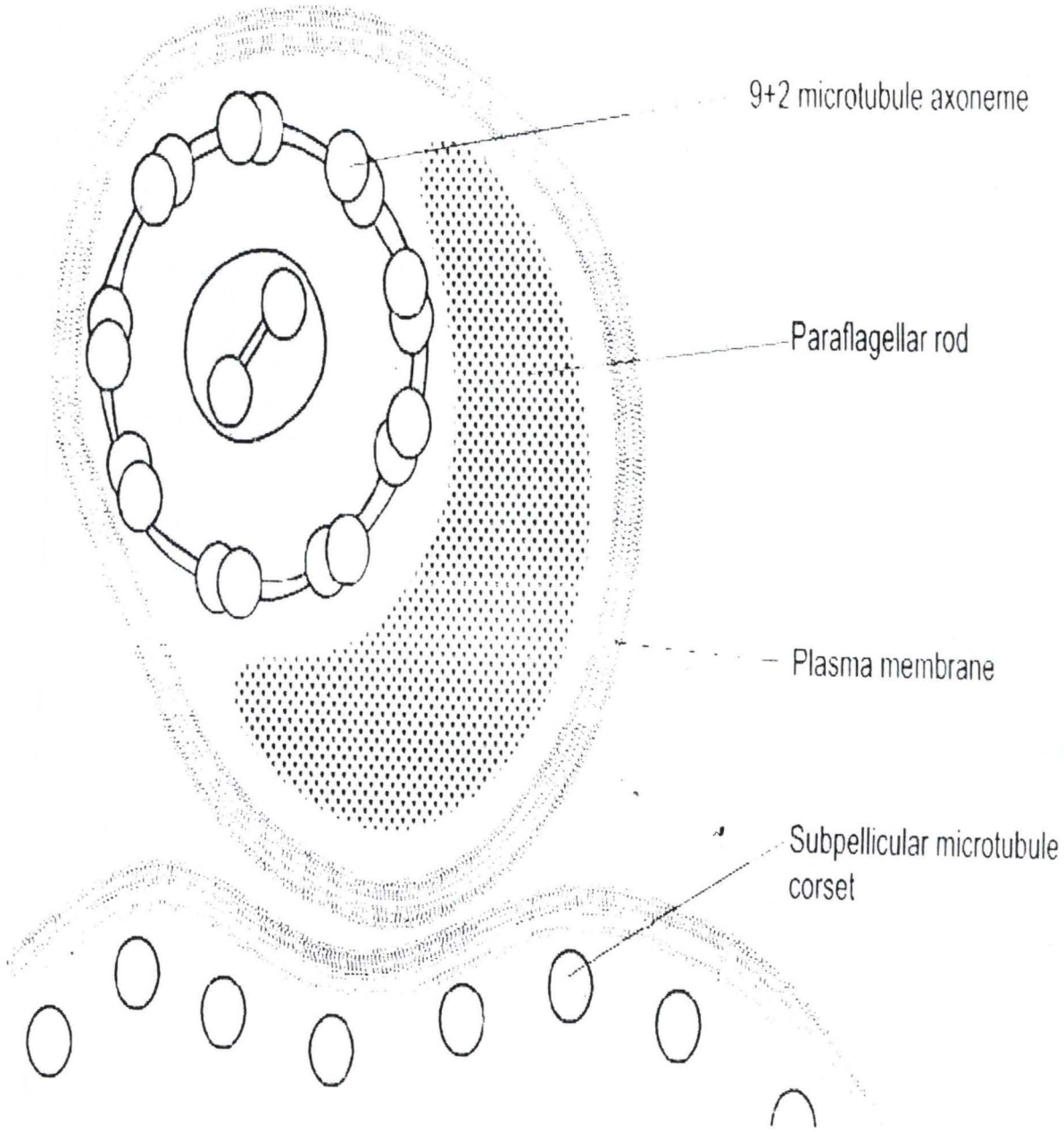


Fig 2.3. ***Trypanosoma brucei*** **flagella structure** **Source: Vickerman, 1985**

The flagellum contains microtubules in the 9+2 arrangement typical of other flagella. At the base the flagellum is the Kinetoplast which contains DNA in the form of about 6000 catenated circles. The kinetoplast. DNA is 10% of the total cellular DNA and is the important site of action of some anti-trypanosome drugs such as ethidium. The kinetoplast is part of the single long mitochondrion which changes morphology during various stages of the life cycle. Most other organelles are those typical of any other eukaryotic cells. At the surface of the cell are sub-membranous pellicular microtubules which give the trypanosome its shape. This underlies a typical plasma membrane which is often covered by an electron-dense surface coat.

2.2.4 The Basal Body

The basal body is a cylindrical centriole-like organelle which lies anterior to the kinetoplast (Steiger, 1973). The walls of the cylinder are made up of nine microtubule triplets. It is at the base of the flagellum within the cell and separated from the extracellular flagellum by an incomplete (basal) plate (Vickerman and Preston, 1976).

2.2.5 The mitochondrion

The mitochondrion is a tubular structure which is often branched to form a reticulum of canals, and in most cases, lying close to the pellicle (Vickerman and Preston, 1976). This reticulate form of the mitochondrion is usually found in the culture forms, while in the blood stream forms, the mitochondrion is a single linear canal where it is repressed, possessing few or no cristae, the cristae becoming more elaborate as the cell transforms into the procyclic forms in which the mitochondrion is well developed (Vickerman, 1962, 1965).

2.2.6 The kinetoplast

Kinetoplast is a DNA – containing structure that is found at the base of the flagellum. It is equivalent to the mitochondrial DNA of all other cells but makes up a very much greater proportion of the DNA of the cell than does the single circular mitochondrial DNA of other cells. The single circular mitochondria can code for a few of their own proteins (some cytochrome subunits and ribosome subunits) together with all of the mitochondrial ribosomal RNAs and all of the transfer RNAs. The kinetoplast DNA is much more elaborate. It codes for the same set of RNAs and proteins, but some of the tRNAs of the kinetoplast are not encoded in its DNA and have to be imported from the cytoplasm which is not the case with mammalian mitochondria.

In molecular terms, the kinetoplast exhibits the following features that are peculiar to it:

- contains 20 – 50 copies of a 22kb MAXI CIRCLE (same as in other mitochondria)
- An additional 10,000 1kb MINI-CIRCLES
- They form a network of catenated circles
- Several unidentified open reading frames exist on the maxi circle of DNA
- Changes with metabolism
- Bloodstream slender trypomastigotes: simple mitochondrion, few cristae are short and tubular.
- Bloodstream short-stumpy: elaboration of mitochondrion synthesis of mitochondrial enzymes.
- Fly midgut: elaborate array of plate-like cristae mitochondrion extends both anteriorally and posteriorally from kinetoplast.
- Fly metacyclic: degeneration of mitochondrion.

2.2.7 The Nucleus

The trypanosome interphase nucleus is bounded by a double-membrane nuclear envelope, the two membranes of the envelope being separated by a space of 20-30nm (Vickerman and Preston, 1970). The outer membrane carries a few ribosomes and is continuous with the cisternae of the granular reticulum. The nuclear envelope bears wide pores, 80-100nm in diameter. There is a large central or slightly eccentric, endosome (nucleolus), which is circular to oval in profile and composed of finely granular material of uniform density, without differentiation into recognisable fibrillar and granular components (Vickerman and Preston, 1976). Chromatin masses, about 20nm across are scattered within the electron-pale nucleoplasm and occasionally found in chains or aggregated in clumps close to the nuclear envelope.

2.2.8 Life Cycle

The life cycle of trypanosomes involves an invertebrate host (the insect vector), and a vertebrate host (the mammalian). There are two subdivisions of mammalian trypanosomes on the basis of the course of development in the vector. These are salivaria and stercoraria. In the stercoraria, the entire invertebrate cycle of development is confined to the alimentary canal of the vector. Some members of this group include *Trypanosoma Lewisi* of rodents, *Trypanosoma theileri* of ruminants and *Trypanosoma cruzi* of man.

In the salivaria group, the initial stages of development take place in the gut of the vector while the final stages occur in the proboscis or salivary gland in the fly's midgut; the parasites transform into procyclic trypomastigotes and multiply by binary fission. They leave the mid-gut, and transform into epimastigotes. The epimastigotes reach the fly's salivary glands and continue multiplication by binary fission. The cycle in the fly takes approximately 3 weeks.

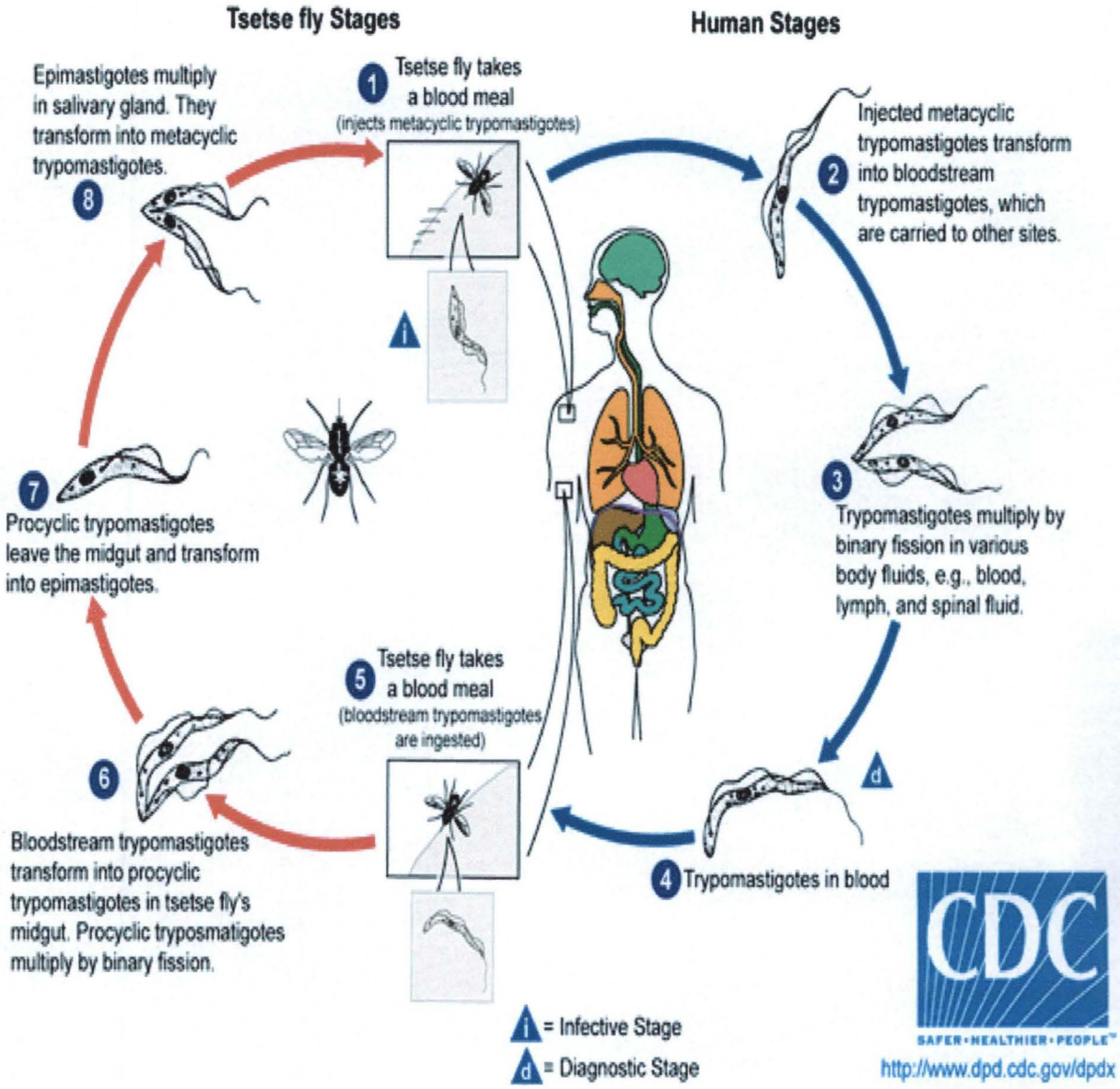
During a blood meal on the mammalian host, an infected tsetse fly injects metacyclic trypomastigotes into the skin tissue. The parasites enter the lymphatic system and pass into the blood stream. Inside the host, they transform into bloodstream trypomastigotes that are carried to other sites throughout the body (the lymph and spinal fluid) where they continue replication by binary fission. The entire life cycle of African trypanosomes is extracellular. The tsetse fly becomes infected with bloodstream trypomastigotes when taking a blood meal on an infected mammalian host.

The life cycle of trypanosomes is reflected in the four morphological stages highlighted above (fig. 2.2), the relative positions of the Kinetoplast, the nucleus and the extent of flagella apparatus (Vickerman, 1985).

The medically important hemoflagellates fall into three groups in two genera. *Trypanosoma gambiense* and related forms exhibit the trypaniform stage in vertebrate host and trypaniform and crithidal stages in the invertebrate.

Trypanosoma cruzi exhibits all four stages in the vertebrate host, trypaniform and crithidal stages in the invertebrate.

Members of the genus *Leishmania* exhibit only the Leishmaniform stage in the vertebrate and the leptomonad stage in the invertebrate host. Reproduction of all forms is by binary fission. The nucleus, blepharoplast and parabasal body divide, a new flagellum arises from the blepharoplast, and the cytoplasm divides longitudinally to produce two daughter cells (fig.2.4).



Source: CDC (2005)

Fig 2.4: The Life cycle of African Trypanosomes, alternating between the insect vector, the tsetse fly, and the mammalian host.

2.3.1.2 Compartmentation of Glycolysis in Trypanosomes

The glycolytic pathway in Kinetoplastida is organized in a way that majority of the enzymes are sequestered in organelles, called glycosomes. In most eukaryotic cells, glycolysis occurs in the cytosol. The compartmentation of glycolysis in glycosomes was first described for *T. brucei* (Opperdoes, 1987). In the organelles are those enzymes which are responsible for the conversion of glucose and glycerol into 3 – phosphoglyccerate. The last three enzymes of the pathway are present in the cytosol.

As a consequence, net ATP synthesis occurs in the cytosol, in the reaction catalyzed by pyruvate kinase (PYK), whereas the consumption and production of ATP are balanced within the glycosomes. Similarly, no net change in the redox state of NAD takes place in the organelles, because of the presence of a glycosomal glycerol – 3 – phosphate dehydrogenase (GDH) and the existence of a glycerol-3-phosphate/dihydroxy acetone phosphate (G-3-P/DHAP) shuttle between glycosomes and mitochondrion. This shuttle guarantees that the NADH, produced in the reaction catalyzed by glyceraldehyde-3-hosphate dehydrogenase (GAPDH), can be reoxidised by oxygen via a mitochondrial glycerol-3-phosphate oxidase. Thus respiratory process seems not to be involved in free-energy transduction.

Under anaerobic conditions, the glycosomal G-3-P is converted into glycerol, with the concomitant production of ATP, via the reversal of the glycerol kinase reaction. This would be possible under a condition of low phosphate potential and a high concentration of G-3-P inside the glycosome. The glycosomal ATP and NAD balance are thus also maintained under anaerobic conditions and one molecule of glycerol and one molecule of pyruvate are produced per molecule of glucose, instead of two molecules of pyruvate. Thus, under anaerobiosis, the net synthesis of ATP drops from two to one molecule per molecule of glucose.

2.3.1.3 Regulation and Control of Glycolysis in Trypanosomes

The regulation of glycolysis in Trypanosomatidae seems to differ substantially from its regulation in other eukaryotes. Most striking is the apparent lack of activity regulation of glycolytic enzymes in these organisms, while in most other eukaryotes the activities of particularly hexokinase (HK) and phosphofructokinase (PFK) are extensively regulated by the cellular levels of various glycolytic intermediates and other effectors. Either such regulatory mechanisms are redundant or they cannot work for the enzymes of the Kinetoplastida, because of their compartmentation and the low permeability of the organelles' membrane for most metabolic intermediates. In many eukaryotes, fructose 2, 6 - diphosophate stimulates PFK and inhibits the gluconeogenic enzyme fructose 1, 6 - diphosphatase. This prevents futile cycling. In contrast, trypanosomal PFK is insensitive to fructose 1, 6-diphosphate. Gluconeogenesis has never been reported in bloodstream *T.brucei*. In other Trypanosomatidae fructose-1,6-diphasphatase is probably localized in the cytosol, thus physically separated from PFK, so that futile cycling cannot occur (Michels and Hannaert, 1994). However, trypanosomal PYK is stimulated by fructose 2, 6-diphosphate. This is consistent with the cytosolic localization of both 6 – phosphofructo - 2-Kinase and fructose, 2, 6 - diphosphatase (Van Schaftingen *et al.*, 1987). In bloodstream form *T. brucei*, the effector concentration is saturating, so that PYK is maximally activated. It is expected that the fructose 2, 6 - diphosphate concentration is low in procyclic *T. brucei*, resulting in a strongly decreased activity of the enzyme.

In other eukaryotes, increasing (ATP)/ (ADP) or (ATP)/AMP) ratios strongly inhibit the activities of HK and PFK. This serves to buffer the cellular (ATP)/ (ADP) ratio, when the activities of ATP-utilizing reactions are varying. In trypanosomes, such a mechanism could not work, as HK and PFK are not in direct with cytosolic (ATP)/

(ADP) ratio, but with the distinct glycosomal (ATP)/ (ADP) ratio. Therefore, it is not surprising that these enzymes are not very sensitive to those compounds (Bakker *et al.*, 1995).

2.3.1.4 Metabolism of Polyamines in Trypanosomes

Polyamines are small molecules universally required for growth and division by prokaryotic and eukaryotic cells. The most important polyamines in eukaryotes are putrescine, spermidine and spermine, which are believed to have major roles in cell division, differentiation and the maintenance of essential cell functions. The molecular underpinnings of polyamine action are still shrouded, but it is clear that their presence (especially spermidine) is important for conformational regulation of DNA, elongation and initiation of protein synthesis and fidelity of translation. Polyamines appear to be essential for ribosome and transfer RNA (tRNA) structure and function, and exert dramatic control over protein such as topoisomerases (Marton and Morris, 1987).

Synthesis of polyamines is closely regulated, with the reactions catalysed by ornithine decarboxylase and S-adenosyl-methionine decarboxylase emerging as the critical steps. Ornithine decarboxylase (ODC) produces putrescine from ornithine; S-adenosyl-methionine decarboxylase, (SAM DC) synthesizes decarboxylated S-adensosyl-methionine (SAM), which is the aminopropyl group donor for spermidine and spermine synthesis. Aminopropyl groups are transferred to putrescine and spermidine by spermidine and spermine synthases, respectively.

2.3.1.5 Novel Aspects of Polyamine Synthesis in Trypanosomes

Despite apparent similarities between mammalian and trypanosome polyamine pathways, subtle differences exist. Trypanosomes synthesize putrescine from ornithine via ODC, and spermidine from methionine and putrescine by way of SAM synthase, SAM DC and speridine synthase. The origin of ornithine is unclear since *Trypanosoma*

brucei lacks arginase (Bacchi and McCann, 1987), a major source of ornithine in mammalian cells. African trypanosomes neither synthesize nor contain significant levels of spermine. Many cells avidly transport polyamines, but African trypanosomes do not, unless prior depletion of intracellular levels has occurred (Bacchi *et al.*, 1983).

A key aspect of trypanosome polyamine metabolism is the presence of a unique spermidine – containing peptide, trypanothione (N^1N^8-bis (glutathionyl) spermidine), which serves as co-factor for glutathione reduction and hence is essential in protecting trypanosomes from free radical damage and other oxidant stress (Fairlamb, 1989).

2.3.1.6 Metabolism and Functions of Trypanothione

In mammalian cells, one of the primary means of defence against damage by oxidants and other toxic substances involves the tripeptide glutathione gamma-glutamyl-cysteinyl glycine (GSH) and its ancillary enzymes glutathione reductase, glutathione peroxidase and glutathione-S-transferases. Trypanosomes and Leishmania are uniquely different from the mammalian host in that they do not contain glutathione reductase, an enzyme crucial to these processes. Instead, trypanothione (N^1,N^8-bis(glutathionyl) spermidine; $T\{SH\}^2$) which is the principal low-molecular weight thiol in logarithmically growing trypanosomatids acts in concert with a flavoprotein disulphide oxidoreductase, trypanothione reductase, in maintaining the correct intracellular redox balance in these organisms (Fairlamb, 1989).

Similarly, current evidence suggests that, at least in *Trypanosoma brucei* and *crithidia fasciculate*, the role of GSH and glutathione peroxidase in the removal of hydrogen peroxide (H_2O_2) and other alkyl peroxides has been replaced by $T\{SH_2)^2$ and trypanothione peroxidase. Indirect evidence suggests that this may be the case in *Leishmania* species and *T.cruzi* as well. Trypanothione and its precursor metabolite N^1-glutationylspermidine may also be involved in regulation of intracellular levels of

unconjugated spermidine in *C. fasciculate* (Fairlamb, 1989). The relevance of this finding to the medically important parasitic trypanosomatids is not clear at present.

2.3.2 Molecular Biology of Trypanosomes

2.3.2.1 Genomic Organization of *T. brucei*

African trypanosomes are primarily diploid organisms with a haploid DNA content of about $4x10^7$bp (Borst *et al.*, 1982). Their nuclear organization differs from that of higher eukaryotes in several ways, including the lack of chromatin condensation and the persistence of a nuclear membrane during cell division. The chromosomes do not condense during metaphase. Pulse-field gel electrophoresis (PFGE) has revealed that the nucleus of African trypanosomes has at least 120 chromosomal- associated-DNA molecules. These DNAs are grouped into two general size classes. One class contains about 20 DNA molecules whose sizes range from 200Kb up to 5700Kb. Homologous pairs of chromosomes in this class can differ in size by as much as 20%, presumably due in part to variable numbers of repetitive elements (Gottesdiener *et al.*, 1990). The other class consists of at least 100 micro chromosomal DNAs of 50 – 150Kb.

The chromosomal ends or telomeres of all these DNA molecules share the {GGGTAA}n repeats that also occur at human chromosomal telomeres. Variable surface glycoprotein (VSG) genes are adjacent to many, in not all, of the telomeres. These telomere-linked genes and other chromosomal-interior VSG genes are manoeuvred by DNA recombination events into special telomere-linked expression sites where one and only one VSG gene is expressed at a time by a given bloodstream trypanosome (Donelsen, 1995) . The micro chromosomal DNAs contain a highly repetitive 177bp sequence as well as unexpressed, tolemere-linked VSG genes. These

mini chromosomes may serve primarily as repositories for spare VSG genes (Weiden *et al.*, 1991).

Researchers have carried out studies on the genetic constitution of the African trypanosomes, towards establishing a trypanosome transcript map (El-Sayed *et al.*, 1995) and characterization of Expressed Sequence Tags (ESTs). The combined information from these studies will facilitate a detailed study of relevant genetic loci and the identification of specific genes involved in such important functions as drug resistance, pathogenesis, regulation of antigenic variation and interactions with the immune system.

2.3.2.2 The Trypanosome as a Model Organism for Cell and Molecular Biology

Trypanosomes have established themselves scientifically as the studied example of a 'differently evolved' eukaryote, although other organisms have emerged to challenge this role (Cross, 2001). Trypanosomes exhibit remarkable differences in basic cell functions compared to their mammalian hosts. Paramount among these is the RNA editing of mitochondrial genome transcripts, the unique compartmentation of glycolysis in organelles that encapsulate other pathways that are common to peroxisomes, which glycosomes resemble in this and other respects (Michels *et al.*, 2000), and the polycystronic and apparently unregulated transcription by RNA polymerase II.

2.3.2.3. Antigenic Variation in African Trypanosomes

The trypanosome parasite is very antigenic, thereby conferring on it the ability to overcome all the challenges posed by the host simultaneously. The presence of a densely-packed coat made of variable surface antigens or variable surface glycoproteins (VSGs) tends to inhibit the non-specific immune mechanisms and is thought to deny antibodies access to the invariant antigens. This dense surface coat surrounds the blood stream forms of the parasite and is the only major antigen recognized by the hosts'

immune system. When single cells from different infected animals or patients were cloned, the coats were found to be biochemically different. However, when cells from a defined wave of parasitemia in the same patient were taken, it was found that all of the trypanosomes in that wave of organisms expressed the same single surface antigen whereas in other waves, all of the parasites expressed a single but completely different antigen.

Thus, escape from the hosts' immune response depends on the ability to express a new VSG. Since hundreds of these waves of parasitemia can occur before the host dies (in a laboratory situation, normal number of waves is much fewer than this) and no antigen is repeated, there must be an equal number of VSG genes. About 10% of the cell's genome is devoted to genes that express these surface molecules that allow the organism to be one step ahead of the host's immune response (Hunt, 2004).

2.3.2.4 Structure of VSG

The VSG glycoprotein has a size of about 65KD, about 500 amino acids and has three domains. At the N-terminal is the signal sequence: the next 360 amino acids are usually very different from the similar sequence in other VSGs. The 120 C-terminal amino acids are quite similar in different VSGs. This latter part is hidden from the immune system by being next to the plasma membrane.

Protein and cDNA sequencing of the C-terminal part shows a typical transmembrane hydrophobic sequence that is used normally to attach the protein to the plasma membrane together with a short intracellular domain, but protein sequencing shows that the transmembrane part of the protein and the intracellular part are not there in the mature protein. They are replaced by a weird structure that contains sugars, ethanolamine, phosphoinositol and fatty acids. This structure is common to all VSGs

and is highly antigenic when purified but not *in vivo*. This suggests that the VSGs in the coat are tightly packed to exclude antibodies.

2.3.2.5 Sequential Expression of VSGs

When an African trypanosome expresses a new coat, this is frequently associated with the appearance of a new copy of the gene for that coat, called Expression-Linked Copy (ELC). This implies gene duplication but the phenomenon is temporary because when the trypanosome switches to a new VSG, the extra copy usually disappears and is replaced by a new expression-linked copy. It is the extra copy that is being transcribed into mRNA for translation into protein, not the copy that is permanently in the genome. The new copy is in an EXPRESSION SITE, which is always close to a TELOMERE (near the end of a chromosome).

An ELC and a permanent gene may be on different chromosomes, suggesting a copy/translocate mechanism for a cassette of information. Sometimes an ELC is not produced but the permanent copy is transcribed. These transcribed non-ELC genes are always at telomeres. The process of temporary copying of a gene and using the copy as observed in trypanosomes is very unusual.

The order of expression of VSGs is not absolute. At the beginning of the infective phase, VSGs are produced by parasites in the insect salivary glands. Subsets of the repertoire (about 12) of the VSGs are produced and remain during the first wave of parasitemia in mammals. The whole repertoire is then open to expression and there is preferred but not fixed order of expression. Each gene can be expressed only once during an infection. The fact that there is an initial subset of VSGs that are expressed gives hope for a vaccine.

Messenger RNAS in trypanosomes are also very unusual. Almost mRNA that has been observed starts with the same 35 nucleotides. This is coded by an exon far away from the rest of the gene (Hunt, 2004)

2.3.2.6. Shedding of the VSG Coat

A trypanosome-and VSG-specific phospholipase C is found in the bloodstream forms of the parasite. The VSG at the cell surface is attached through a glucolipid and not via a transmembrane protein sequence. Since all VSGs have the same attachment structure, only one enzyme is needed for rapid and complete cleavage. The shedding of the coat is important at a given time in order for the parasite to survive the developing immune response against its original VSG.

2.3.2.7 Extraordinary RNA editing by Trypanosomes

The trypanosomes have a very odd nuclear genome. The chromosomes do not condense in nuclear division thereby making the exact number of chromosomes involved unknown. The nuclear gene includes 1000 – 2000 genes that encode the variable surface antigens that allow the coat of the organisms to be changed regularly so that it can avoid hosts' immune response. The process is extraordinary because it involves the shifting of a new copy of a gene into an expression site when it is needed. Up to 10% of the genome is composed of all of these genes for variable surface antigens (Hunt, 2004).

2.3.2.8 The Enigma of the Cytochrome Oxidase of Some Trypanosomes

The cytochrome oxidase subunit 111 (CO 111) is encoded in the kinetoplast DNA of all trypanosomes except *Trypanosoma brucei.* The CO 111 genes are located in the same position within the DNA. Except for the absence of these genes, T.brucei K DNA looks very similar to that of other trypanosomes.

Investigators have looked for the CO 111 gene elsewhere in the KDNA maxi circle but not found it, but evidence was found for an mRNA transcript in *T.brucei* that had a sequence similar to the gene for CO 111 in the other trypanosomes. The correct open reading frame for the *T.brucei* transcript was determined and the following were established. Of the 181 amino acids predicted by the sequence, 135 were conserved amongst all three. Taking conservative replacements and those conserved in one other species, 160 out of 181 are conserved, amounting to 88% level of conservation. This means that there must be a gene for CO 111 protein in *T.brucei* but its exact location is not certain.

Heterogenous hybridization using *L. tarèntolae* and *C. Fasciculata* CO 111 probes could not detect CO 111 sequences in *T.brucei* genes. This was confirmed by southern blot analyses using probes that were predicted from the sequence that was obtained for the transcript.

The probes do not hybridize to KDNA or to total DNA. The possibility that have been put forward to explain this unusual feature is that:

The transcript may be made by splicing together small fragments of RNA transcribed from multiple sites. Mini-exons are present in the trypanosome system. There is severe editing of the transcript after or during transcription so that the final transcript is nothing like the gene from which it was transcribed.

Investigations have revealed that most of the uridines of the DNA are left out and are put into the transcript after transcription to mRNA.

- Of the 626 nucleotides sequenced initially in the *T.brucei* CO 111 transcripts, 347 are uridines that are not coded for by the gene. They are added in 121 different sites.

- At one of those sites, the addition of a U creates a stop codon exactly where the native stop codon occurs in the other CO 111 genes.
- In addition, 16 uridines, predicted by the genomic sequence at 7 sites appear to be deleted.
- The protein coding protein of the transcript contains 315 additions and 15 deletions in 546 nucleotides.
- Thus 58% of the coding part of mRNA results from editing and is not in the original gene.

This editing accounts for the inability of cDNA probes to hybridize to the genomic DNA (Hunt, 2004).

2.3.2.9 RNA trans-splicing

Component parts of a biochemical pathway which occurs exclusively in trypanosomatids have been identified. The 5' end of all trypanosome messenger RNAs contain a spliced leader (SL) sequence, which is transferred along with its cap, from the SL RNA by a process called trans-splicing. Methylation of the cap structure is essential for use of the SLRNA in transplicing. The cap structure in *Trypanosoma brucei* and *crithidia fasciculate* (a non-pathogenic trypanosome) has been shown to have an unusual structure, which has not yet been identified in any other eukaryotic organism.

While isolating the enzymes (RNA methyltransferases) responsible for the modifications to the cap structure, researchers also identified a 75KD a protein which had capping activity, and subsequently cloned the corresponding gene from *C. fasciculata*. This protein was found to contain the signature motifs of known capping enzymes in its C-terminus, but also contains an extra N-terminal domain, which interestingly has a consensus sequence for a phosphate binding loop. This loop is a motif commonly found in ATP-and GTP-binding proteins. Thus, the structure of *C.*

fasciculata capping enzyme suggests that the trypanosome capping enzyme is mechanistically different from the capping enzymes of other eukaryotes, and as such may be an ideal drug target (TDR, 1998).

2.3.3 Research Models

Genome analysis is the most important thing happening at the present time, for *T.bucei, T.cruzi* and *Leishmania major*, which can be one of the most enabling activities for the better design of future experimental research (Cross 2001). Among eukaryotes, trypanosomes are almost ideal for genome analysis. Genes are tightly packed on the chromosomes; there is, so far, only one example of an intron (Mair *et al.*, 2000), but it seems reasonable to assume that there will be others. The genes appear to be orientated unidirectionally over long regions, although this organization needs to be reviewed with care lest important genes be overlooked. Several negative issues and important technical obstacles need to be overcome so as to maximize the impact of the genome project.

2.3.3.1. Vaccine targets

Few researchers of African trypanosomes remain optimistic about vaccination. Antigenic variation represents a formidable obstacle that has apparently evolved to evade the immune response. The understanding of the mechanisms of antigenic variation and trypanosome multiplication may envision strategies to intervene therapeutically in these processes (Cross, 2001).

Recent results from a search for trypanosome antigen that could be used to provide protection against infection, suggest that the *T. brucei* hsp 60 genes warrants further investigation.

High levels of *anti-T. brucei* hsp 60 antibodies have been detected in *T. brucei*-infected mice throughout almost the whole course of parasitemia. Significant peptide similarities between mouse and trypanosome hsp 60 proteins have been shown. As

to rivers and lakes; and *G. fusea*, which favours the high dense forest areas (Hide, 1999).

Trypanosomiasis is also mechanically transmitted by tsetse and other biting flies through the transfer of blood from one animal to another. The most important mechanical vectors are flies of the genus *Tabanus*, but *Haematopola*, *Liperosia*, *stomoxys*, and *chrysops* flies have also been implicated. In Africa, both *T. vivax* and *T. b. brucei* have spread beyond the "tsetse-fly belts" (Leak, 1998), where transmission is principally by *tabanid* and *hippoboseid* flies.

The vector for *T. vivax* in the western hemisphere remains unknown, but several species of hematophagous (especially *tabanid* and *hippoboseid)* flies are believed to serve as mechanical vectors. Figure 2.5 is a diagram showing one of the species of *Glossina.*

The significance of wild animals in the spread of African sleeping sickness has long been disputed. Various large animals, especially the sitatunga antelope, harbour the flagellates, usually without symptoms, and they have been shown to maintain the infection for long periods in the absence of human reservoirs. However, most authorities agree that man is usually the source of infection.

2.4.4 Clinical Manifestations

The incubation period following an infective tsetse-fly bite varies from two to three weeks to several months. The first stage of the human disease is a systemic infection in which the parasites are found chiefly in the blood but also in the lymph. This phase entails bouts of fever, headaches, pains in the joints and itching. Somewhat later, as the parasites come to predominate in the lymph nodes, these organs and the spleen are enlarged, and anaemia and wasting are seen. Cardiac injury may be prominent, and oedema is often present (WHO, 2008)).

This stage gradually gives way to the sleeping sickness stage when the parasite crosses the blood-brain-barrier and infests the central nervous system. This stage is known as the neurological phase. The parasites are now most abundant in the cerebrospinal fluid, occurring less commonly in the lymph. This is when the characteristic signs and symptoms of the disease appear: lesions in the central nervous system (CNS), somnolence, confusion, apathy, sensory disturbance and poor coordination; disturbance of the sleep cycle, which gives the disease its name, is the most important feature. Without treatment, coma and death are the final outcome.

Typically, the course of the disease is of several month's duration, but in parts of East Africa it develops more rapidly, invasion of the CNS occurs early, and deaths frequently results from cardiac injury before the typical sickness stage is reached (WHO, 2008).

2.4.5 Diagnosis and detection of Trypanosomes

Trypanosomes may be found in the chancre, blood, the lymph glands, the cerebrospinal fluid or the bone narrow. Dry smears stained with blood stains, such as Wright's, are used, either of the whole fluid as obtained or of centrifuged specimens. In the case of blood, thick or thin films may be used. Advanced concentration techniques which enhance the detection of trypanosomes in blood are now available. These are the micro haematocrit centrifuge technique, in which trypanosomes are concentrated in the buffy coat and the minimum exchange column.

several of these peptides in mouse hsp 60 protein are responsible for the induction of strong anti-self reactions during various infectious diseases, the trypanosomal hsp 60 may account for the development of autoimmunity during the course of trypanosomiasis. Synthetic peptides of the trypanosome hsp 60 protein could be used to evaluate their possible role in parasite – induced immuno-suppression and their protective potential (TDR, 1998).

2.4 TRYPANOSOMIASIS

Trypanosomiasis is a class of diseases caused by the protozoan parasites, trypanosomes which affects both man (in the form of sleeping sickness in sub-Saharan Africa, and Chagas disease in South America) and his domestic animals (e.g. nagana in cattle). The ravages of sleeping sickness, which is the final phase of trypanosome infection, have appreciable health and economic importance in African and South America.

The diseases resulting from infection are made up of syndromes ranging from mild, virtually asymptomatic forms lasting for years, to fulminating diseases of a few days' duration. Although similarities exist between the diseases caused by different species in some of the clinical and pathological manifestations, many of the underlying host-parasite interactions are significantly different. The similarities and differences in the pathogenesis are of practical importance not only in the diagnosis and control, but also in research (Losos, 1986).

In Africa, the effects of the disease are manifested in the form of depopulation by death, induced sterility, high infant mortality and displacement of population.

2.4.1 African Trypanosomiasis (AT)

African trypanosomiasis refers to the class of diseases caused by trypanosomes and limited to sub-Saharan Africa. Two forms exist; the Human African Trypanosomiasis and African Animal Trypanosomiasis.

2.4.1.1 Human African Trypanosomiasis (HAT)

Human African Trypanosomiasis (HAT), also known as sleeping sickness is vector-borne parasitic diseases caused by hemoflagellates of the genus Trypanosoma, subgenus Trypanozoon which classically include three subspecies: *Trypanosoma brucei brucei*, *Trypanosoma brucei gambiense*, and *Trypanosoma brucei rhodesiense*. The three sub-species are morphologically identical but differ in their infectivity. Only two of these parasites are known to infect humans.

* *T. b. gambiense* is found in Central and West Africa. It causes chronic infection, which does not mean benign. A person can be infected for months or even years without obvious symptoms of the disease emerging. When symptoms do emerge, the disease is already at an advanced stage.

* *T. b. rhodesiense* is found in East and Southern Africa; it causes acute infection that emerges after a few weeks. It is more virulent than the other strain and develops more rapidly, which means that it is more quickly detected clinically.

T. b. gambiense is transmitted by the riverine *Glossina* species while *T .b. rhodesiense* is transmitted by the savannah species of *Glossina.* In both cases, the disease results from complex interactions between vertebrate hosts (humans and animals), the parasite and its tsetse fly vector (Smith *et al.*, 1995).

2.4.1.2 African Animal Trypanosomiasis

African animal trypanosomiasis (AAT) is a disease complex caused by tsetse-fly transmitted *Trypanosoma congolense*, *Trypanosoma Vivax*, or *Trypanosoma brucei*

brucei or simultaneous infection with one or more of these trypanosomes. The disease is most important in cattle but cause serious losses in pigs, camels, goats, and sheep. Infection of cattle by one or more of the three African animal trypanosomes results in sub acute, acute, or chronic disease characterized by intermittent fever, anaemia, occasional diarrhoea and rapid loss of condition and often terminates in death. In Southern Africa the disease is widely known as nagana, which is derived from a Zulu term meaning "to be in low or depressed spirits" – a very apt description of the disease.

2.4.2 Epidemiology of African Trypanosomiasis (AT)

The normal transfer of infection occurs by the bite of tsetse flies. Two species of flies are of most importance, *Glossina palpalis* in West Africa and *Glossina morsitans* in East Africa, where the more virulent Rhodesian form of the disease occurs. Other species of importance are *Glossina tachinodes, Glossina swynnertoni, Glossina pallidipes,* and *Glossina austeni.*

These insects are relatives of the common housefly. They are limited to tropical Africa and a small part of southern Arabia, and the human disease is confined to their range. Both male and female flies bite and can transmit the disease. They bite exclusively by day. The larva develops completely in the body of the female and is deposited singly on loose soil to pupate. After four to eight weeks, the adults emerge. *G. palpalis* breeds almost entirely near water and is thus more limited in local distribution than *G. morsitans*, which is less dependent on shade and moisture.

2.4.3 Transmission

Trypanosomes replicate in the tsetse-fly and are transmitted through tsetse-fly saliva when the fly feeds on an animal. The three main species of tsetse-flies for transmission of trypanosomes are *Glossina morisans*, which favours the open wood land of the savanna; *G. palpalis* which prefers the shaded habitat immediately adjacent

Fig.2.5 Tsetse fly; the vector of African trypanosomiasis Source: (WHO, 2006)

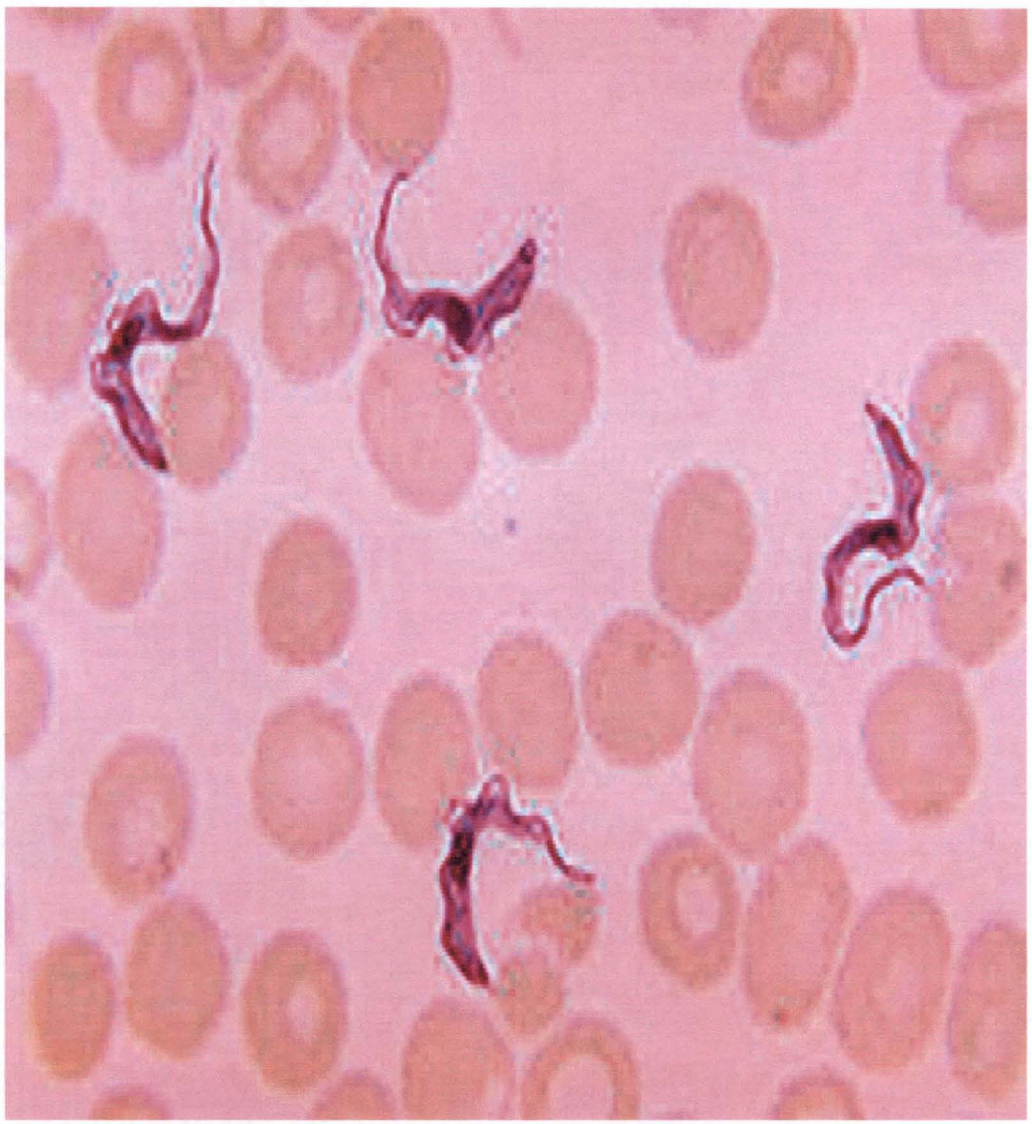


Fig.2.6 Trypanosoma forms in a blood smear

Source: (CDC:http://www.dpd.cdc.gov/dpdx)

2.4.6 Disease Burden

Detection of the disease calls for major human and material resources, such as well-equipped health centres and qualified staff. Because such resources are lacking, most people with sleeping sickness die before they can ever be diagnosed.

At the turn of the 20th century, epidemics of human sleeping sickness threatened to decimate Equatorial Africa. Today, the disease is as prevalent as ever, but its relative importance has been greatly diminished in comparison with HIV-induced decimation, amounting to millions of deaths per year in Africa, the epicentre of acquired immune deficiency syndrome (AIDS) and malaria.

Animal trypanosomiasis is still regarded as a major problem, causing economic, social and protein deficiency because of the massive effect on animals. The presence of animal reservoir and the Tsetse, the ever-present human to animal contact, and the breakdown of infrastructure in many parts of Africa means that trypanosomiasis remains a serious threat (Barrett, 1999; Hide, 1999).

Sleeping sickness threatens over 60 million people in 36 countries of sub-Saharan Africa (WHO/TDR, 2004), and 50 – 70 million animals are exposed to the infection (Ogbadoyi *et al.*, 2007a). Only 3 to 4 million people at risk are under surveillance, with regular access to a health centre that can provide screening (WHO, 1998). Effective chemotherapeutic and chemo prophylactic control has been elusive due to high cost of available drugs, long treatment protocols, adverse side effects and increasing resistance of parasites to available drugs (Ogbadoyi *et al.*, 2007a). Current research efforts in the field of trypanosomiasis chemotherapy are directed at sourcing for antitrypanosomal agents (from natural products) that are cheap, readily available, shorter treatment protocols, and with little or no adverse side effects.

There have been three severe epidemics in Africa over the last century, but the most recent one was in Uganda in 2008, the first in this century (WHO/TDR World Health Report., 2009).

2.4.7 Geographical Distribution of African Trypanosomiasis

The tsetse-fly infested areas of Africa extend from the southern edge of the Saharan desert (lat. 10°N.) to Angola, Zimbabwe, and Mozambique (lat. 20°S).

Of the three African animal trypanosomes, only *T. vivax* occurs in the western hemisphere in at least 10 countries in the Caribbean, South and Central America.

Cases of Human African Trypanosomiasis have been reported in Abraka (Delta State) by the National Institute of Trypanosomiasis Research (NITR). The Nigerian Society of Parasitology (NSP) Conference report, 2002, indicated that Abraka in Delta State is an active focus. Other active foci, which have not been properly documented due to lack of screening and surveillance, include Agaie in Niger State and Katsina- Ala in Benue State. African Animal trypanosomiasis has also been reported in the Abraka focus.

2.5 PREVENTION AND CONTROL OF AFRICAN TRYPANOSOMIASIS

Prevention and control focus on, where it is possible, the eradication of the parasitic host, the tsetse fly. Two alternative strategies have been used in the attempts to reduce African trypanosomiasis. The first strategy is generally entomological and intends to disrupt the cycle of transmission by reducing the number of flies. The second tactic is primarily medical or veterinary and targets the disease directly using monitoring, prophylaxis, treatment, and surveillance to reduce the number of organisms which carry the disease.

2.5.1 Vector Control

Several approaches to fly control have been used with varying degrees of success.

Discriminative bush clearing: This approach has been used extensively in local settings in early tsetse-fly eradication campaigns. It eliminates the breeding places of the tsetse. But to be completely effective, ecologically unacceptable destruction of vast areas of bush and forest is undertaken.

(i) Game elimination – involves the elimination of the main source of blood meals for the tsetse. This was used in early eradication campaigns, but was an ineffective and wasteful procedure.

(ii) Application of the sterile male technique. This approach received considerable attention in the 1980's. Early problems with breeding of the male flies have been overcome, and field trials have been done in both East and West Africa to determine the effectiveness of this technique in vector control. This method has succeeded in reducing fly populations in limited trials.

(iii) Ground and aerial spraying with insecticides and the use of synthetic pyrethroids on cattle have lowered fly densities in some areas, but widespread use would require considerable international cooperation and expense. Widespread application of insecticide has the tremendous disadvantage of also eradicating many other arthropods, several of which are desirable. The recent introduction of odour-baited targets impregnated with insecticides is proving promising as a means of reducing the tsetse-fly.

2.5.1.1 Personal measures to reduce exposure to tsetse flies

The following measures can be taken at a personal level to avoid contact with the flies.

(i) Protective clothing which involves wearing thick clothing that reduces the amount of exposed skin, such as heavy long-sleeved shirts, long pants, socks and

shoes. Wearing bright or dark-coloured clothing as these colours attract tsetse flies should be avoided

(ii) Screens and bed nets: Vulnerable individuals should sleep inside screened areas, under a net or in an air-conditioned room. Bed nets that are rectangular in size, impregnated with permethrin every 6 months are encouraged and after use, they should be tucked tightly under the mattress before dusk.

(iii)Other measures: In areas where tsetse flies are predominant, people should avoid riding in the back of open vehicles as tsetse flies are attracted to dust churned up by moving vehicles or animal herds and the inside of all vehicles should be checked for tsetse flies before getting in. In addition to this, bushy areas should be avoided especially during the heat of the day. Tsetse flies rest in bushes during high heat periods and can bite if disturbed. Insect repellent has not been proven to prevent tsetse fly bites.

2.5.2 Chemotherapy and Chemoprophylaxis of AAT

The use of drugs for the prevention and treatment of trypanosomiasis has been important for many decades, but the rapidity with which the trypanosomes have developed resistance to each drug introduced has tremendously complicated this approach to controlling the disease. Inspite of this problem, some of the older chemoprophylactic drugs such as the quinapyramine derivatives, antrycide and antrycide Prosalt are still used and give effective protection against *T. b. brucei* infection in horses, camels, and cattle for up to 3 months. The drug, pyrithidium bromide (Prothidium and AD2801) is useful in the prophylaxis of *T. vivax* and *T. congolense* infections in cattle, sheep, and goats and can give protection for up to 6 months. The most widely used of the newer chemoprophylatic drugs (and also the least expensive) is isometamidium chloride. This drug, in use for over 20 years and sold

under the trade names Samorin, Trypanidium, and M & B 4180A, is excellent for the prophylaxis of all three African animal trypanosomes, and gives protection for 3 – 6 months (Bitonti *et al.*, 1986). The development of resistance to this drug has been reported in both East and West Africa. Homidium bromide has also been found to be an effective chemoprophylatic drug in Kenya, and the newly introduced arsenical, clymelarsan is effective in treatment of *T. b. brucei* infection.

A widely used chemotherapeutic drug is diminazine aceturate (Berenil), which is effective against all three African animal trypanosomes. The isometamidium drugs are also excellent chemotherapeutic agents as are the quaternary ammonium trypanocides, Antrium trypanocides, Antride, Ethidium and Prothidium. Although extensively used in trypanosomiasis control, chemoprophylaxis is an expensive, time-consuming, and thus unsatisfactory long-term solution to the problem of African animal trypanosomiasis.

2.5.3 Immunization

There is presently no vaccine available for African animal trypanosomiasis (Murray *et al.*, 1979). It has long been recognized that certain breeds of African cattle are considerably more resistant to African trypanosomiasis than others (Murray *et al.*, 1979). This is especially true of the West African short-horned cattle (Muturu, Baoule, Laguna, Samba, and Dahomey) and the N'Dama, which is also of West Africa. These cattle existed in the region for over 5,000 years. Susceptibility studies have shown the N'dama to be the most resistant breed followed by the smaller West African short-horned cattle, but the large and more introduced Zebu is the most susceptible (Murray *et al.*, 1979). The mechanisms of trypanotolerance have been extensively studied, and it is now well established that trypanotolerance has a genetic basis (Moulton and Sollod, 1976, and Murray *et al.*, 1984). Trypanotolerance in sheep and goats has also been

described, but the mechanisms of the tolerance phenomenon have not been fully understood.

2.5.4 Chemotherapy and Chemoprophylaxis of Human African Trypanosomiasis (HAT)

Chemotherapy and chemoprophylaxis form the most important and major aspect of the control and eradication of Human African Trypanosomiasis especially in the African countries. The successes of these measures depend largely on the correct use of drugs (Finelle, 1973). Effective application of chemotherapy and chemoprophylaxis on the field depends on several factors that include the species of trypanosome causing infection, severity of challenge, and lastly the occurrence of resistant strains.

The dosage used, within the recommended safety limits, depends on the species of trypanosome, local and systemic tolerance of the drug by the species of animal, and the possibility of occurrence of partial resistance in the infecting trypanosomes. The first-line drugs for the treatment of African trypanosomiasis were developed over 30 years ago. The efficacy of these drugs is blighted by the problems of resistance, variable efficacy between strains or species of causative organisms, toxicity, and difficulty of administration or a combination of these factors. If the disease is diagnosed early, the chances of cure are high. The type of treatment depends on the phase of the disease: initial or neurological. Success in the latter phase depends on having a drug that can cross the blood-brain barrier to reach the parasite. Four drugs have been used until now and Table 2.1 describes the drugs and their draw backs.

Table 2.1 Drugs for treatment of Human African Trypanosomiasis and their drawbacks

Drug	Use	Drawbacks
Melarsoprol **-discovered in 1949**	First line drug for late stage *gambiense* and *rhodesiense*	Adverse side effects especially encephalopathy -Fatal in 1-5% of cases
Eflornithine **-registered in 1990** **-production ceased in 1990** **-Aventis gave licence to WHO in 2003**	Effective against late stage *gambiense* disease	-high cost -not effective against *rhodesiense* disease -non – oral route -has to be given intravenously for 14 days -requires hospitalisation
Pentamidine **-discovered in 1949**	Effective against early stage *gambiense* disease	-has adverse effects -non – oral route
Suramin **-discovered in 1921**	Effective against early stage *gambiense*	-has adverse effects -non – oral route

2.5.5 The Need for Research and Development

Human African trypanosomiasis re-emerged in the 1980s. However, little progress has been made in the treatment of this disease over the past decades. The first-line treatment for second-stage cases is melarsoprol, a toxic drug in use since 1949. High therapeutic failure rates have been reported recently in several foci. The alternative, eflornithine, is better tolerated but difficult to administer. A third drug, nifurtimox, is a cheap, orally administered drug not yet fully validated for use in human African trypanosomiasis (Legros *et al.* 2002). No new drugs for second-stage cases are expected in the near future. Because of resistance to and limited number of current treatments, there may soon be no effective drugs available to treat trypanosomiasis patients, especially second-stage cases.

Additional research and development efforts must be made for the development of new compounds, including: testing combinations of current trypanocidal drugs, completing the clinical development of nifurtimox and registering it for trypanosomiasis, completing the clinical development of an oral form of eflornithine, pursuing the development of DB 289 and its derivatives, and advancing the pre-clinical development of megazol, eventually engaging firmly in its clinical development. Partners from the public and private sector are already engaged in joint initiatives to maintain the production of current drugs (Legros *et al.*, 2002).

This network should go further and be responsible for assigning selected teams to urgently needed research projects with funds provided by industry and governments. At the same time, on a long term basis, ambitious research programmes for new compounds must be supported to ensure the sustainable development of new drugs, especially from natural products.

2.5.6 Cases of Treatment Failures

Treatment of Human African Trypanosomiasis (HAT or sleeping sickness) relies on a few drugs which are old, toxic and expensive. The most important drug for the treatment of second stage infection is melarsoprol. During the last 50 years treatment failures with melarsoprol were not a major problem in *Trypanosoma brucei gambiense* patients. Commonly a relapse rate of 5-8% was reported, but in recent years it has increased dramatically in some important foci of *T. b. gambiense* sleeping sickness.

Treatment failures for *T. b. rhodesiense* are much less of a problem apart from some reports between 1960 and 1985 of refractoriness in *T. b. rhodesiense* patients in East Africa. Analysis of those isolates revealed that *in vitro* sensitivity to melarsoprol was one-tenth that of sensitive isolates, and that complete failure to cure the infection in acute mouse model was comparable with those in human patients.

There was very little indication of resistance in *T. b. gambiense* isolates from Cote d'Ivoire and North West Uganda. The *in vitro* melarsoprol sensitivities for populations from relapsing and from curable patients were in the same range.

Melarsoprol concentrations in the plasma and cerebrospinal fluid of patients 24 h after treatment did not show any difference between patients who relapsed and those who could be cured. The reasons for relapses in the recent *T. b. gambiense* epidemics are not known. Other parasite-related factors might be involved, e.g. affinity to extra vascular sites other than the CNS which is less accessible to the drug. In conclusion, a combination of factors rather than a single one may be responsible for the phenomenon of melarsoprol treatment failures in *T. b. gambiense* patients (Brun *et al.*, 2001).

2.5.7 Combination therapy as a solution to treatment failures

Currently, the drug most commonly used to treat sleeping sickness is melarsoprol. A derivative of arsenic developed more than 50 years ago. Treatment from

this drug is excruciatingly painful and potentially fatal. Often described by patients as "fire in the veins," between 5 and 20 percent of those treated die of complications from the injected drug.

Recent research suggests that a safe, alternative treatment is available through the combination of two drugs, eflornithine and nifurtimox. While this combination represents an improved therapy for patients, it is not ideal. The treatment is complicated to administer and requires close patient monitoring—something frequently unavailable in sub-Saharan Africa. Hence, major efforts are needed to bring truly innovative drugs into the pipeline (WHO, 2006).

2.5.8 Attempts at new drug designs using new targets

Researches in various laboratories are now targeted at developing chemotherapeutic agents that will identify specific targets in the cell cycle mechanisms of trypanosomatids. Some of these attempts are targeted at the following processes: cellular trafficking in trypanosomatids, the mitochondrion, the glycosomes, flagellum, enzymes of polyamine metabolism and enzymes of carbohydrate metabolism.

Any strategy to identify novel anti-protozoan drugs must take into consideration the metabolic differences between stages of the life cycle and that there is often a reduction in the complexity of metabolic pathways as parasites are bathed in a nutritional environment (Fairlamb, 1989).

Carbohydrate metabolism and energy production in the bloodstream forms of African trypanosomes are reliant upon glycolysis organized in a unique pattern between a reduced mitochondrion, the cytoplasm and glycosomes (Opperdoes, 1987). This pathway is probably the target for suramin and forms the basis for several novel approaches (Clarkson *et al.*, 1989; Michelis *et al.*, 2000).

Nucleic acid synthesis in trypanosomatids also has unique characteristics. These

organisms, being unable to synthesize purines *de novo*, have evolved a special salvage pathway with enzymes that have a higher affinity for disruptive purine analoques than natural precursors. Nuclear and Kinetoplast DNA – dependent processes are inhibited by many standard trypanocides that intercalate (phenanthridines) or bind externally to DNA (diamidines). This may interfere with the functioning of DNA and RNA polymerases as well as DNA topoisomerases I and II.

The metabolic pathways of polyamine synthesis have been most productive in the last decade for the identification of new anti-trypanosomal compounds, such as eflornithine (Bacchi, 1989) because they have a wide range of regulatory functions (Fairlamb, 1989).

Microtubules, an integral part of the trypanosomatid pellicle, flagellum and mitotic nucleus, are sensitive to phenothiazines and inhibitors of microtubular disassembly such as taxol.

A cellular regulatory and productive process, mainly unique to trypanosomatids has also been exploited either by using drugs that produce toxic metabolites or radicals, or by lowering the defence mechanisms of superoxide dismutase or glutathione. Current interest is focused on the novel intracellular trypanothione as a potential target because it plays a protective and regulatory role in trypanosomatids (Fairlamb, 1989). Biochemical studies have revealed many more potential targets, but there is one final area of cellular metabolism to be considered. The drug uptake process includes not only transport but also the intracellular metabolism, binding and distribution of a drug. This is critical for selective toxicity and for some trypanocides that have been studied extensively (Fairlamb, 1989).

2.6 DRUG TARGETS AND DRUG DISCOVERY

The genome of the parasite has been decoded and several proteins have been identified as potential targets for drug treatment. The decoded DNA also revealed the reason why generating a vaccine for this disease has been so difficult. *T. brucei* has over 800 genes that manufacture proteins that the disease mixes and matches to evade immune system detection (Berriman *et al.*, 2005). Recent findings indicate that the parasite is unable to survive in the bloodstream without its flagellum. This insight gives researchers a new angle with which to attack the parasite (Williamson, 2005). A new treatment based on a truncated version of the apolipoprotein L-1 of high density lipoprotein and a nanobody has recently been found to work in mice, but has not been tested in humans (VanNieuwenhove *et al.*, 1985)

The cover story of the August 25, 2006 issue of Cell Journal described an advance in which Dr. Lee Soo Hee and colleagues, working at Johns Hopkins, investigated the pathway by which the trypanosomes make myristate. It was discovered that they use a novel fatty acid synthesis pathway involving fatty acid elongases to make myristate and other fatty acids, thus making it a potential target for drug development. Myristate (a 14-carbon length fatty acid) is a component of the variant surface glycoprotein (VSG), the molecule that makes up the trypanosome's outer layer. This outer surface coat of VSG is vital to the trypanosome's avoidance of immunological capture.

2.6.1 Targeting the polyamine biosynthetic enzymes

Trypanosomatids depend on spermidine for growth and survival. Consequently, enzymes involved in spermidine synthesis and utilization, i.e. arginase, ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (AdoMetDC), spermidine synthase, trypanothione synthetase (TryS), and trypanothione reductase (TryR), are

promising targets for drug development. The ODC inhibitor alpha-difluoromethylornithine (DFMO) is about to become a first-line drug against human late-stage gambiense sleeping sickness. Another ODC inhibitor, 3-aminooxy-1-aminopropane (APA), is considerably more effective than DFMO against Leishmania promastigotes and amastigotes multiplying in macrophages. AdoMetDC inhibitors can cure animals infected with isolates from patients with *rhodesiense* sleeping sickness and leishmaniasis, but have not been tested on humans. The antiparasitic effects of inhibitors of polyamine and trypanothione formation, reviewed here, emphasize the relevance of these enzymes as drug targets. By taking advantage of the differences in enzyme structure between parasite and host, it should be possible to design new drugs that can selectively kill the parasites (Heby *et al.*, 2007). Using polyamine metabolism as potential therapeutic target, alpha Difluoromethyl ornithine (RMI 71,782), a specific irreversible inhibitor of the first step in polyamine biosynthesis, that is, the formation of putrescine from ornithine by ornithine decarboxylase, cured mice infected with a virulent, rodent-passaged strain of *Trypanosoma brucei brucei*. This parasite is closely related to the trypanosomes that cause human sleeping sickness. The drug, which is remarkably nontoxic, was effective when administered in drinking water or by intubation. The ability of the compound to inhibit ornithine decarboxylase *in vitro* was demonstrated by the reduced amounts of putrescine synthesized from tritiated ornithine in *Trypanosoma brucei* suspensions. These observations direct attention to polyamine metabolism as a target for chemotherapy of parasitic diseases (Janice *et al.*, 2008).

2.6.2 Trypanothione as a target in the design of anti-trypanosomal and anti-leishmanial agents

Trypanothione is the key molecule in the defence mechanism of *Trypanosoma* and *Leishmania* against oxidative stress. The uniqueness of trypanothione makes the

metabolism of this molecule an attractive target in anti trypanosomal and anti leishmanial drug design. It became clear that this antioxidant cascade can be considered as the "Achilles heel" of these parasites. Some of the target enzymes include trypanothione synthetase, gamma-glutamylcysteine synthetase, and Ornithine decarboxylase (Augustyns *et al.*, 2001).

2.6.3 Proline Metabolism in Procyclic *Trypanosoma brucei is* Down-regulated in the Presence of Glucose

Proline metabolism has been studied in procyclic form of *Trypanosoma brucei*. These parasites consume six times more proline from the medium when glucose is in limiting supply than when this carbohydrate is present as an abundant energy source. The sensitivity of procyclic *T. brucei* to oligomycin increases by three orders of magnitude when the parasites are obliged to catabolise proline in medium depleted in glucose. This indicates that oxidative phosphorylation is far more important to energy metabolism in this latter case than when glucose is available and the energy needs of the parasite can be fulfilled by substrate level phosphorylation alone. A gene encoding proline dehydrogenase, the first enzyme of the proline catabolic pathway, was cloned. RNA interference studies revealed the loss of this activity to be conditionally lethal. Proline dehydrogenase defective parasites grew as wild-type when glucose was available, but, unlike wild-type cells, they failed to proliferate using proline.

In parasites grown in the presence of glucose, proline dehydrogenase activity was markedly lower than when glucose was absent from the medium. Proline uptake too was shown to be diminished when glucose was abundant in the growth medium. Wild-type cells were sensitive to 2-deoxy-D-glucose if grown using proline as the principal carbon source, but not in glucose-rich medium, indicating that this non-catabolizable glucose analogue might also stimulate repression of proline utilization. These results

indicate that the ability of trypanosomes to use proline as an energy source can be regulated depending upon the availability of glucose (Nadia *et al.*, 2005).

2.6.4 Targeting proteins to the glycosomes of African trypanosomes

Glycosomes are micro bodies found in trypanosomes. These highly specialized organelles compartmentalize most of the glycolytic enzymes normally located in the cytosol of other eukaryotic cells. The recent success in expressing foreign proteins in Trypanosoma brucei has permitted a detailed analysis of glycosomes protein targeting signals in these organisms. These studies have revealed that the previously identified C-terminal tripeptide peroxisomal targeting signal also functions in the import of proteins into the glycosomes of *T. brucei*. However, the glycosomal and peroxisomal targeting signals differ in a few important ways. The C-terminal tripeptide sequence requirements for glycosomal protein targeting are comparatively relaxed. Of the three C-terminal amino acids, the first can be any small, neutral amino acid; the second should be capable of forming hydrogen bondings, whereas the third is a hydrophobic amino acid. This degenerate tripeptide sequence differs significantly from the more stringent requirements observed for the import of proteins into mammalian peroxisomes and thus represents an opportunity for designing peptide analogues that specifically block the glycosomal protein import for a possible anti-trypanosomal chemotherapy.

A recently described N-terminal signal that targets thiolase to the mammalian peroxisomes does not appear to function in import into the glycosomes. However, a novel internal targeting signal has tentatively been identified in at least one of the glycosomal proteins that can target a reporter protein to the glycosomes of *T. brucei*. Glycosome-deficient mutants have been isolated recently, which will aid in the identification of genes involved in the biogenesis of the glycosomes (Sommer and Wang, 1994).

2.7 MEDICINAL PLANTS IN DRUG DISCOVERY AND TREATMENT OF DISEASES

From primordial times, the healing power of herbs had since been recognized and botanic medicine is one of the oldest practiced professions by mankind. The plant kingdom has long served as a prolific source of useful drugs, foods, additives, flavouring agents, colourants, binders and lubricants. In fact, it has been estimated that about 25% of all prescribed medicines today are substances derived from plants. Such drugs are in use today, including the analgesic drug aspirin, from filipendula, the antimalarial agent, and quinine from *Cinhona spp*, the anti-hypertensive principles, reserpine from *Ratowolfia serpetina*, as well as the anti neoplastic alkaloids, vircristine and vinblastine from *Catharantus roseus*. Hence, the need for continued research into medicinal plants, especially those that are used in traditional medicine across the developing countries of Africa, Asia and South America, cannot be over-emphasized (Gamaliel, 2000).

Most medicinal plants have not been thoroughly evaluated for their toxicity profiles. This notwithstanding, it is generally agreed that medicinal plants and their products are relatively safer than their synthetic counterpart drugs. The reasons for this may not be far -fetched. First, medicinal plant constituents mimic more closely the natural constitution of the human somatic system, and following the lock and key hypothesis, it is expected that they will fit better into such system. For instance, digitalis leaf is still preferred to the pure compounds (digoxin or digitoxin) as a worldwide remedy for Congestive Heart Failure (CHF) due to the higher toxicity profile of the pure compound (Gamaliel, 2000). Secondly, in terms of environmental friendliness, medicinal plants and their products are far more advantageous than

orthodox medicines since they constitute a lesser form of pollution menace and are renewable.

However a blanket assumption should not be made about the safety of medicinal plants and their products. It is necessary to ensure a thorough and detailed pharmacological and toxicological assessment of these plants and their products before approving them for therapeutic purposes. These assessments should be paramount in order to avoid "Medicinal plants misadventuring" of the past when seemingly innocuous plants may turn out to be toxic (Gamaliel, 2000).

Medicinal herbs are moving from fringe to mainstream use with a greater number of people seeking remedies and health approaches free from side effects caused by synthetic chemicals. Recently, considerable attention has been paid to utilize eco-friendly and bio- friendly plant-based products for the prevention and cure of different human diseases.

Considering the adverse effects of synthetic drugs, the Western population is looking for natural remedies which are safe and effective. It is documented that 80% of the World's population has faith in traditional medicine, particularly plant-based remedies for their primary healthcare (Gijtenbeek *et al.*, 1999).

The importance of traditional medicines was recognised by the World Health Organisation (WHO) since the late 1970s, when its Traditional Medicine Programme was established (Merlin *et al.*, 2001). This programme has developed guidelines for the assessment of herbal medicines and has sponsored clinical trials in Ghana, Nigeria and Kenya. In August 2000, the UNDP-World Bank-WHO Special Programme for Research and Training in Tropical Diseases (TDR) convened a meeting in Geneva, Switzerland, to explore how best to develop natural products for treatment of tropical diseases. This was a follow-up to a meeting that TDR helped to organise in Moshi,

Tanzania, in 1999, in partnership with the Global Initiative for Traditional Systems (GIFTS) of Health based at Oxford University (Wilcox and Bodeker, 2000). Discussions revolved around medicinal chemistry, pre-clinical and clinical evaluation of the safety and efficacy of herbal medicines, commercialisation and regulatory issues.

This initiative is presently being spear headed by the African Network for Drugs and Diagnostics Innovation (ANDI) which was launched in Abuja, Nigeria, in October, 2008 to promote and sustain African-led product R&D innovation through the discovery, development and delivery of affordable new tools, including those based on traditional medicines. The network has so far succeeded in bringing together African scientists and those in the Diaspora to exchange and synergise ideas in the areas of medicinal chemistry, pre-clinical and clinical evaluation of medicinal plants and herbal products.

In November 2008, a workshop on developing novel strategies for natural product-based drug discovery for tropical diseases was held at the University of Cape Town, South Africa under the auspices of The Global Institute for Bio Exploration-Africa (GIBEX – Africa); a R&D network that promotes ethical, natural product-based pharmacological bio exploration to benefit human health in Africa. The network is a component of the Global Institute for Bio exploration (GIBEX) established in 2004 by Rutgers, the State University of New Jersey and the University of Illinois at Urbana-Champaign. GIBEX-Africa is guided by the pioneering "Reversing the flow" paradigm intended to bring pharmacological screens to Africa (screens-to-Nature technology) and reverse the human "brain drain". The network intends to equip scientists in Africa with innovative drug- discovery tools designed to transform forests, savannas, deserts and marshlands into modern pharmacological laboratories.

Based on the recognition accorded traditional medicines by the World Health Organization (WHO) when its traditional medicine programme was established, it has been argued that traditional medicines should be evaluated as they are used traditionally, in the hope of finding remedies that can be prepared and used locally at very low cost (Bodeker, 1998).

Traditional medicines have been used for a very long period, thereby making clinical studies a warranted first step, with close monitoring for side effects. This could be followed by *in vitro* and *in vivo* studies to elucidate the mechanisms of action of the herbal preparations and to further refine efficacy towards setting a therapeutic index. Traditional health systems have developed methods of preparing medicinal plants to maximize clinical effects, but the understanding of medicinal chemistry will help to modify natural products to improve their pharmacokinetics and pharmaco-dynamic properties and reduce toxic effects.

2.7.1 Trypanocidal potentials of some medicinal plants

Several medicinal plants, used locally in traditional herbal medicare, have been evaluated by many workers and reports indicate very encouraging results for most of the *in vitro* studies (Freiburghaus *et al.*, 1997; Kiuchi *et al.*, 1998; Atawodi *et al.*, 2003; Kamanzi *et al.*, 2004; Sara *et al.*, 2004; Wurochekke and Nok., 2004; Atawodi, 2005; Essam *et al.*, 2009; Nibret *et al.*, 2009).

Kiuchi *et al.*, (1998) used hot water and methanol extracts of 17 natural medicines from South-East Asia to test for anti-trypanosomal activities against *Trypanosoma cruzi*. The methanol extracts of the woods of *Cedrus deodara*, seeds of *Psoratea carylifolia*, leaves of *Laurus nobilis* and a resin of *Styrax sp.* showed strong *in vitro* anti-trypanosomal activities. Nibret *et al.*, (2009) evaluated the anti-trypanosomal and cytotoxic activities of pyrrolizidine alkaloid-producing plants of Ethiopia. The

dichloromethane extracts of *Solanecio angulatus* flowers and *Crotalaria phillipsiae* twigs were found to exhibit a pronounced *in vitro* anti-trypanosomal activity. Kamanzi *et al.*, (2004) tested one hundred and one (101) crude ethanol extracts derived from 88 medicinal plants from Cote d Ivoire for *in vitro* anti-trypanosomal and anti-plasmodial activity. Eight of the extracts showed good activity against *T. b. rhodesiense* (IC_{50} values $\leq$ 8µg/ml). Essam *et al.*, (2009) evaluated the anti-plasmodial and anti-trypanosomal activity of the methanol extracts of 42 plants collected from the Kingdom of Saudi Africa and some fractions obtained thereof. The methanol extracts of *Solanum schimperianum* had a very high *in vitro* activity (IC_{50} 0.061µg/ml) against *T. b. brucei* (GUTat 3.1 strain). They also demonstrated that the petroleum ether-soluble fraction (IC_{50} 0.5µg/ml) and the chloroform-soluble fraction (IC_{50} 3.5µg/ml) of the methanol extract of *Caralluma tuberculata* were moderately active against *T. b. brucei.*

The *in vitro* trypanocidal effects of the methanol extracts of some Nigerian savannah plants have been investigated by Atawodi *et al.*, 2003. Some of the extracts were found to be strongly trypanocidal to either *T. b. brucei* or *T. b. congolense.* In another study by Atawodi, 2005, the trypanocidal activities of petroleum ether, chloroform, methanol and aqueous extracts of some Nigerian savannah plants were compared. Most of the plants were found to have differential trypanocidal activities.

The *in vitro* antitrypanosomal activity of some medicinal plants used in the treatment of trypanosomiasis in Northern Nigeria was investigated by Wuruchekke and Nok, 2004, and three of the thirteen plants screened exhibited anti-trypanosomal activity. These were aqueous and methanol extracts of *Guira senegalensis* (root, bark, and leaf), aqueous extract of *Tamarindus indica* (roots, bark, and leaf), and the aqueous and methanol extracts of *Khaya senegalensis* (bark).

The anti-trypanosomal activities of some rare Tanzanian medicinal plants were evaluated by Freiburghaus *et al.*, 1997. Two of the 37 extracts investigated were found to have strong activity with IC_{50} values below 1µg/ml. The same type of *in vitro* studies was carried out on ethno pharmacologically selected Beninese plants was investigated by Sara *et al.*, 2004 and Methylene chloride extracts of *Hymenocardia acida* twig and leaf, *Strychnos spinosa* leaf, and *Trichilia emetica* leaf exhibited profound activity at MIC values ≤ 19µg/ml. It is very clear from the foregoing that so many plants have been investigated in vitro and so the need to ascertain their efficacy in vivo cannot be over emphasised because it is only when this is done that the extracts can be purified, characterised and packaged as ethno medicine or serve as templates for synthetic drug production.

The number of *in vivo* studies on the anti-trypanosomal activities of medicinal plants and natural products is very limited compared to *in vitro* studies probably due to the involvement of live animals, which have to be used for under a strict regulatory protocol. However, a few medicinal plants have been evaluated *in vivo* for anti-trypanosomal activities (Igwe and Onabanjo, 1989; Nok *et al.*, 1993; Ogbadoyi *et al.*, 2007a; Ogbadoyi *et al.*, 2007b).

The Neem tree, *Azadirachta indica,* has been exploited for a long time as a potential source of traditional drugs. Several parts of the plant are known to have extensive medicinal application (Okpanyi and Ezekwu, 1973). This plant grows wild and thrives well even in harsh environments.

Aqueous extract of defatted leaves of *Azadirachta indica* was found to possess trypanocidal activity against *Trypanosoma brucei brucei* (Nok *et al.*, 1993). Preliminary experiments showed that a dosage of 60mg/kg/day of the crude extract

resulted in parasitological cure of at least 65% of mice infected with 10^5 trypanosomes/ml of blood.

Column chromatography of the crude extract and subsequent treatment of infected mice with the different fractions showed that the active component(s) were present in fraction III. A little above sixty three percent (63.3%) of infected mice were completely cured when given a dose of 25mg/kg/day of fraction III for 12 days. The leaf extract of the plant has been shown not to have any toxicity in mammals (Nok *et al.*, 1993). The need to undertake more *in vivo* studies using the vast natural products, both evaluated (*in vitro*) and un-evaluated cannot be over-emphasized.

There are three main reasons for doing screening studies i.e. to find new lead compounds for developing pharmaceuticals, to confirm the ethno medicinal use of plants or to develop phyto- medicines for use as herbal medicine. Apparently, little effort has gone into developing low technology techniques to use medicinal plants in rural communities. In many screening studies, activities are reported non-quantitatively. There is the need to undertake more *in vivo* screening studies, with quantitative expression of results such that will allow for comparison of efficacies of different plants based on the results presented (Shale *et al.*, 1999; Eloff *et al.*, 2004) . The same principle holds when bioactive compounds are isolated by bioassay-guided fractionation. If the total activity is calculated at each step it will be easy to determine if there is a loss of biological activity along the way. Even more importantly, this approach makes it easier to discover the presence of synergistic effects.

2.8 EUCALYPTUS SPECIES

Eucalyptus (Myrtaceae), an Australian native, represented by around 700 species is a genus of tall, evergreen and magnificent trees cultivated the world over for its oil, gum, pulp, timber, medicine and aesthetic value. Among the various wood and non-

wood products, essential oil found in its foliage is the most important one and finds extensive use in food, perfumery and pharmaceutical industry. In addition, the oil possesses a wide spectrum of biological activity including anti-microbial, fungicidal, insecticidal/insect repellent, herbicidal, acaricidal and nematicidal.

The River Red Gum (*Eucalyptus camaldulensis*) usually grows up to 45 metres tall; its thick (30mm) spongy, the bark is dappled with red, grey, green and white. Most Eucalypts germinate within 2-5 weeks of sowing (Batish *et al.*, 2008)..

2.8.1Geographical Distribution

This is said to be the most widely distributed eucalypt, ranging over 23° lat. in most of arid and semi-arid Australia but not the humid eastern and south western coasts. It is regarded as one of the most widely planted eucalypts in the world (ca 500,000 ha planted). Plantations occur in Argentina, Arizona, California, Egypt, Kenya, Morocco, Nigeria, Pakistan, Senegal, Sierra Leone, Spain, Sri Lanka, Sudan, Tanzania, Upper Volta, Uruguay, and Zimbabwe (Butcher, 2001).

2.8.2 Economic Importance of *E.camaldulensis*

Eucalyptus has many uses which have made them economically important trees. Perhaps the Karri and the Yellow box varieties are the best known. Due to their fast growth, the foremost benefit of these trees is the wood. They provide many desirable characteristics for use as ornament, timber, firewood and pulpwood. Fast growth also makes eucalypts suitable as windbreaks. Eucalypts draw a tremendous amount of water from the soil through the process of transpiration. They have been planted (or re-planted) in some places to lower the water table and reduce soil salination. Eucalypts have also been used as a way of reducing malaria by draining the soil in Algeria, Sicily and also in Europe and California. Drainage removes swamps which provide a habitat for mosquito larvae, but such drainage can also destroy ecologically productive areas.

This drainage is limited to the soil surface only since the eucalyptus roots have up to 2.5m length, not reaching the phreatic zone, meaning that rain or irrigation can wet the soil again.

Eucalyptus oil is readily steam distilled from the leaves and can be used for cleaning, deodorising, and in very small quantities in food supplements; especially sweets, cough drops and decongestants. Eucalyptus oil has insect repellent properties and is an active ingredient in some commercial mosquito repellents (Doran and Brophy, 1990).

The nectar of some eucalyptus produces high quality mono floral honey. Eucalyptus is also used to make the digeridoo, a musical wind instrument made popular by Australian aborigines. In the Adirondack Mountains it has been used for years to kill dust mites in bedding.

All parts of the *eucalyptus* may be used to make plant dyes that are substantive on protein fibres (silk and wool) simply by processing the plant part with water. Colours to be achieved range from yellow and orange through green, tan, chocolate and deep rust red. The material remaining after processing can be safely used as mulch or fertilizers. It is traditionally used in rot resistant application like stumps, fence posts and sleepers more recently; it has been recognised in craft furniture for its spectacular deep red colour and typical fiddle back figure. Popularly used as firewood, the wood makes fine charcoal and it is successfully used for bee-keeping in Brazil and Australia (Doran and Brophy, 1990).

2.8.3 Folk Medicine

The plant has been reported to be anaesthetic, antiseptic, astringent, and is a folk remedy for colds, colic, coughs, diarrhoea, dysentery, haemorrhages, laryngalgia, laryngitis, pharyngitis, sore throat, spasm, trachalgia, and wounds. Medicinally it has been used in the treatment of some ailments like fever, malaria and also wounds.

2.8.4 Chemistry

The leaves of *Eucalyptus* contain 0.1–0.4% essential oil, 77% of which is cineol. There is some cuminal, phellandrene, aromadendren (or aromadendral), and some valerylaldehyde, geraniol, cymene, and phellandral. It also contains 5–11% tannin. The Kino contains 45% kinotannic acid as well as Kino red, a glucoside, catechol, and pyrocatechol. The leaves and fruits have tested positive for flavonoids and sterols, while the bark contains 2.5–16% tannin, the wood 2–14%, and the Kino 46.2–76.7% (Atawodi, 2005).

In a preliminary study conducted by Ogbadoyi *et al.*, (2007b), the anti-trypanosomal potentials of *Eucalyptus camaldulensis* was demonstrated *in vivo* at different doses. The aqueous extract of *E. camaldulensis* administered at 400mg/kg bodyweight prolonged the lifespan of infected mice by 40 days. This study however did not go beyond the preliminary stage, and the effects of the extract on the haematology and histopathology of the infected and treated mice were not evaluated. In addition, there is the need to screen for activity using non-polar extracts and possibly get lower effective doses to allow easy extrapolation for higher animals and humans; and the safety margin (acute and sub chronic toxicity) of the extract was not determined, thus warranting a detailed study of the *E. camaldulensis* extracts to address these apparent gaps and possibly proceed to purifying and identifying the active anti-trypanosomal components present in this medicinal plant that is widely used locally to treat malaria

and typhoid fevers, and for the management of respiratory diseases (Duke and Wain, 1981).

2.9 *Annona* species

Annona is the second largest genus, after *Guatteria*, in the plant family Annonaceae, containing approximately 110 species of mostly neo-tropical and Afro tropical trees and shrubs. The name derives from the Taíno *annon* (NRC, 2008). Paleo-ethnobotanical studies have dated *Annona* exploitation and cultivation in the Yautepec River region of Mexico to approximately 1000 BC (Warrington and Warrington, 2008).

Currently, seven *Annona* species and one hybrid are grown for domestic or commercial use mostly for the edible and nutritious fruits. Many of the species are used in traditional medicines for the treatment of a variety of diseases. Several annonacaeous species have been found to contain acetogenins, a class of natural compounds with a wide variety of biological activities (Pilar-Rauter *et al.*, 2002). *Annona senegalensis* or African custard-apple is a species of plant in the Annonaceae family. This fruit is mostly found from Senegal to South Africa. A traditional food plant in Africa, this little-known fruit has the potential to improve nutrition, boost food security, foster rural development and support sustainable land care (NRC, 2008).

2.9.1 Description

Tap rooted evergreen or semi-deciduous tropical trees or shrubs, thin bark that has broad and shallow depression or fissures which join together and are scaly. Slender, stiff, cylindrical and tapering shoots with raised pores and naked buds (NRC, 2008). The leaf blades can be leathery or thin and rather soft or pliable, bald or hairy. The flowering stalks rise from an axil, or occasionally from axillary buds on main stem or older stems, or as solitary flowers or small bundle of flowers. Usually three or four deciduous sepals that is smaller than the outer petals that do not overlap while in bud.

Six to eight fleshy petals in two whorls, the petals of the outer whorl are larger and do not overlap; inner petals are ascending, distinctively smaller and nectar glands are darker pigmented. Numerous stamens that are ball, club-shaped, or curved and hooded or pointed beyond anther sac. Numerous pistils, attached directly to the base, partially united to various degrees with distinct stigmas. One or two ovules per pistil; style and stigma club-shaped or narrowly conic (Gottsberger, 1988).

One fleshy, ovate to spherical fruit per flower. Each fruit consisting of many individual small fruits or syncarps; one syncarp and seed per pistil. Seeds are beanlike with tough coats. *Annona* are generally disease free. They are susceptible to some fungus and wilt. Ants are a problem since they promote mealy bugs on the fruit (Robert and Gardner, 2008).

Fig. 2.8: A branch from *A. senegalensis* (African Custard-apple) plant bearing leaves and a fruit (Herbarium number: NIPRD/H/5868)

Annona senegalensis, of the family Annonaceae, locally called 'Abo' in Western Nigeria, 'Gwandar daji', in Northern Nigeria, "Uburu 0cha" in Ibo land and "Ikpokpo" among the Idoma people of Benue state, is a small tree commonly found in the savannah area and near streams. The plant decoction has been reported to be used traditionally in the treatment of sleeping sickness (Igweh and Onabanjo, 1989). It is also used in folk medicine for the treatment of cancer (Gbile and Adesina, 1985). Among the Idoma people, the plant is used singly, and in combination with other medicinal plants and herbs in the treatment of some sexually transmitted diseases (Ogbadoyi *et al.*, 2007a).

The trypanocidal activities of the aqueous extracts of *A. senegalensis* leaf and root have been demonstrated (Igweh and Onabanjo, 1989; and Ogbadoyi *et. al.*, 2007a). Parasites were cleared from circulation and no relapse was observed for over 60 days. The extracts were found to be inactive against late stage infection and had no prophylactic activity. There is need to further evaluate the plant parts using different solvents of varying polarities for extraction.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Plant Materials

Two plants, namely *Annona Senegalensis* and *Eucalyptus camaldulensis* were screened for antitrypanosomal activities.

Annona Senegalensis (NIPRD/H/5868) was obtained fresh from the bush along Minna – Bida road between the months of May and June.

Eucalyptus Camaldulensis (NIPRD/H/6263) was collected from the Education Resource Center/ETF ground in Minna, Niger State, between the months of June and July. The two plants were identified and assigned specimen voucher numbers at the herbarium of the National Institute for Pharmaceutical Research and Development, Abuja, Nigeria.

3.2 Experimental Animals

Albino mice, used for screening, were purchased from the Biochemistry and Chemotherapy division of the National Institute for Trypanosomiasis and Onchocerciasis Research, Vom, plateau State, Nigeria. The animals were bred in our laboratory for subsequent use.

Wistar rats, used for sub chronic toxicity studies, were purchased from Jos, Plateau State, Nigeria. All experiments involving the animals were conducted in compliance with the internationally accepted principles for laboratory animal use and care as contained in the Canadian Council on Animal Care (CCAC, 1997) guidelines on animal use protocol review (1997).

3.3 *Trypanosoma brucei brucei*

A stabilate of *trypanosoma brucei brucei*, 8/18 strain was obtained from the National Institute for Trypanosomiasis and Onchocerciasis Research, Vom, Plateau State,

Nigeria, and subsequently maintained in the laboratory of the Biochemistry Department, Federal University of Technology, Minna Niger State Nigeria, by serial passage in mice.

3.4 Preparation of Plant Samples

About 1 kg each of the leaves, stem and root barks of the two plants were removed fresh, washed with running tap water and dried at room temperature.

The dried plant samples were blended into powdered form using mortar and pestle, and sometimes an electric blending machine where necessary. The powdered samples were stored in clean polythene bags until required for use.

3.5 Preparation of crude extracts

The extracts were prepared and screening carried out using the method described by Ogbadoyi *et al.*, (2007a). In this method, fifty grams (50g) of the dried powdered samples of each of the leaves, stem and root barks of the two plants were extracted sequentially under reflux with 400mls of hexane, ethyl acetate, methanol and water in that order for 2hrs in each case. The marc was dried after each extraction process for subsequent use. Extracts were filtered hot using cheese cloth and solvent was removed using rotary evaporator for organic solvents and freeze-drier for water extracts. The extracts, now in relatively dry form, were transferred into sterile sample bottles for storage in the refrigerator until required for use.

3.6 Infection of animals

Blood from a highly infected mouse was obtained by cardiac puncture and collected with EDTA coated syringe. The blood was appropriately diluted with physiological saline to serve as inoculum. Healthy mice of weight ranging from 25 - 35 g were infected intraperitoneally with 0.1ml of the inoculum containing about 10^3 trypanosomes.

3.7 Administration of Crude Extracts and Monitoring the Course of Parasitemia

Crude extracts (dissolved in physiological saline or Dimethyl sulphooxide, DMSO) in varied concentrations were administered on infected animals via the intraperitoneal and oral routes. The presence of parasites in infected animals was monitored by making a blood smear on a glass slide and observing under a microscope set at 40X magnification. The number of parasites in 1 ml of blood was estimated using the method of Herbert and Lumsden (1976).

3.8 Initial Screening of *A. senegalensis* and *Eucalyptus camaldulensis* extracts for Antitrypanosomal activity

For *A. senegalensis*, the hexane, ethyl acetate, methanol and aqueous extracts of the leaf, stem and root barks were screened for antitrypanosomal activity. For each extract, seven groups, each consisting of three *T. b. brucei* – infected mice. Groups one to five were administered 100, 200, 300, 400 and 500 mg/kg bodyweight per day intraperitoneally for 21 consecutive days, while the sixth group was left untreated and the seventh group administered a single dose of 3.5mg/kg bodyweight of berenil.

For *E. camaldulensis*, the hexane, ethyl acetate, methanol and aqueous extracts of the leaf, stem and root barks were also screened for antitrypanosomal activity but with a different format. For each extract, three groups; A, B and C were infected and administered intraperitoneally 200, 400 and 600mg/kg body weight per day of the extracts respectively for 21 consecutive days. Administration of extracts commenced 48 hours after infection. Group D was infected but not treated, while Group E was infected and administered a single dose of 3.5mg/kg bodyweight of berenil to serve as controls.

3.9 Confirmatory screening with effective doses of *E. camaldulensis* and *A. senegalensis*

In order to ascertain the efficacy and reproducibility of the doses that demonstrated appreciable antitrypanosomal activities in the initial screening, ten groups of mice, each containing three mice, were set up. Groups A, B and C were intraperitoneally administered 200mg/kg body weight per day of hexane extract, 200mg/kgbodyweight/day of ethyl acetate extract and 600mg/kgbodyweight/day of methanol extract of *E. camaldulensis* (leaf) respectively for 21 consecutive days. Groups D, E, F, G and H were administered 200mg/kg bodyweight of the hexane extract, 500mg/kg bodyweight of the methanol extract and 200mg/kg body weight of the aqueous extract of the leaves; 400mg/kg bodyweight of the hexane extract and 300mg/kg bodyweight of the aqueous extract of the stem bark of *A. senegalensis* respectively for three weeks. Group I was left untreated, while group J was administered a single dose of 3.5mg/kgbodyweight of berenil to serve as controls. Parasitemia was monitored three times weekly.

3.10 Determination of Minimum Curative Dose of the methanol extract of *E. camaldulensis* (leaves)

The methanol extract of *E. camaldulensis* (leaf), based on the results obtained in the confirmatory screening, was subjected to determination of the minimum dose that will produce a complete cure for animals infected with *T. b. brucei.*

Six groups of mice, each group containing three mice, were set up. Groups A, B, C and D were infected with *T. b. brucei* and treated with 50,100, 150 and 200mg/kg bodyweight per day of the extract for 21days. Group E was infected and left untreated, while Group F was infected and administered a single dose of 3.5mg/kg body weight of berenil, Parasitemia was monitored for four weeks.

3.11 Determination of the relationship between time of commencement of treatment and efficacy of the methanol extract of *E. camaldulensis* (leaf)

In order to determine the best time to start treatment with the effective dose, five groups of mice, each group containing three mice, were infected with *T. b. brucei*. Groups A, B, C and D were intraperitoneally administered 200mg/kg body weight per day of the methanol extract at 0, 24, 48 and 72 hours post infection respectively for 21 days. Group E was infected and left untreated.

3.12 Combination Therapy

The possibility of synergistic action of different extracts in varying combinations was investigated using a modified method of Gerardo *et al.*, (2007). To establish this, different extracts used in the initial screening were combined in varying ratios and used to test for antitrypanosomal activity in *T. b. brucei* infected mice.

Water and hexane extracts of *A. senegalensis* (leaf) in ratios 1:1, 2:1, 1:2, 3:1 and 1:3 were used to treat five groups of infected mice.

Methanol extracts of *A. senegalensis* (leaf) and *E. camaldulensis* (leaf) in ratios 1:1, 1:2, and 2:1 were used to treat infected mice in five groups.

Methanol and ethyl acetate extracts of *E. camaldulensis* (leaf) in rations 1:1, 1:2 and 2:1 were used to treat groups of infected mice.

In all cases, the maximum treatment period was 21 days and in the course of treatment, for animals that survived beyond the treatment period, the parasitemia, initial and terminal weight and % PCV were determined.

3.13 Blood and Cerebrospinal (CSF) infectivity tests

Both tests were carried out according to the method described by Nok *et al.*, (1993) and Ogbadoyi *et al.*, (2007a). Animals that survived after treatment with single crude extracts viz, methanol extract of *E. camaldulensis* leaf and hexane extract of *A.*

senegalensis stem bark and a combination of extracts (methanol extracts of *A. senegalensis* and *E. camaldulensis* leaves) were used to test for blood and CSF infectivity.

3.13.1 Blood Infectivity Test

The mice were sacrificed 12 weeks post-treatment and 0.02ml of diluted blood samples obtained from the punctured heart of individual mouse was sub-inoculated into three groups of clean parasite-free mice (two in each group), and parasitemia was monitored three times a week under the microscope over a 2-month period.

3.13.2 Cerebrospinal Fluid (CSF) Infectivity Test

Briefly, the mice that survived after treatment with crude extract and a combination of extracts had the hair on the back carefully shaved and were positioned such that the head touched the limbs. This made the vertebrae conspicuous for easy insertion of needle.

The lumbar was then punctured by the insertion of a clean micro needle and syringe, into which a clean, clear, and transparent fluid (CSF) flowed.

This was done under a mild anaesthesia and gentle handling. For each of the mouse from the treated groups, two clean, parasite-free mice were sub-inoculated with 0.02ml of the CSF, and parasitemia was monitored for over a 2- month period.

3.14 Screening for prophylactic activity of extracts

Two extracts, namely methanol extract of *E. camaldulensis* (leaf) and aqueous extract of *A. senegalensis* at 200mg/kgbodyweight that demonstrated activity against *T. b. brucei* at the acute stage of infection were screened for prophylactic activity. Three groups of mice, each group consisting of five mice, were set up. Groups A and B were administered the curative doses of the two extracts, 200mg/kgbodyweight of methanol extract of *E. camaldulensis* and aqueous extract of *A. senegalensis*, respectively for five

consecutive days. Group C was administered 0.1ml of physiological saline. On the sixth day, animals in the three groups were inoculated with 0.1ml of diluted parasite-infected blood (about 10^3trypanosomes/ml) intraperitoneally. Parasitemia was thereafter monitored twice weekly.

3.15 Test for antibacterial activity of the *E. camaldulensis* (leaf) extracts

Three extracts, namely, hexane, ethyl acetate and methanol extracts of *E. camaldulensis* (leaf) were tested for antibacterial activity using the agar well diffusion method described by Apak and Olila (2006).

Principle:

When an antibiotic-impregnated disk is placed on agar previously inoculated with the test bacterium, the disk picks up moisture and the antibiotic diffuses radially outward through the agar, producing an antibiotic concentration gradient. The antibiotic is present at high concentrations near the disk and affects even minimally susceptible microorganisms (resistant organisms will grow up to the disk). A clear zone or ring is present around antibiotic disk after incubation if the agent inhibits bacterial growth.

Procedure

A sterilized cork borer (7mm in size) was used to drill holes in Nutrient Agar plates. An inoculating loop was used to inoculate the test organisms on the plates and incubated for a few hours at 35°C until it became slightly turbid and was diluted to march a turbidity standard. Using sterilized forceps, the extracts were dispensed into the holes on the plates. The plates were immediately placed in an incubator at 35°C. After 24 hours of incubation, the diameters of the zones of inhibition were measured to the nearest mm. The test organisms used were: *Klebsiella pneumonia, Staphylococcus aureus, and salmonella typhi.*

3.16 Acute toxicity studies of methanol extract of *E. camaldulensis* (leaf)

The acute toxicity of the methanol extract of *E. camaldulensis* (leaf) was evaluated using the method described by Wallace (2001).

Six groups, consisting of three mice each, were set up. Groups A, B, C, D and E were administered single doses of 1000, 2000, 3000, 4000 and 5000 mg/kg body weight of the extract respectively. Group F was however administered 0.4ml of physiological saline. Signs of acute toxicity were monitored over a two-week period. The following signs of toxicity were evaluated.

- Physical activity and general appearance
- Changes in the rate and depth of breathing
- Changes in frequency and nature of movement
- Convulsion: marked involuntary contraction or seizures of contraction of voluntary muscle
- Salivation
- Gastrointestinal Signs: Dropping (Faeces)
- Mortality
- Post-mortem analysis of the organs of dead animals

3.17 Sub-Chronic Toxicity Studies of *E. camaldulensis* (leaf)

The minimum curative dose was used to evaluate sub-chronic toxicity of the extract according to the format described by Gamaliel, (2000). Two groups, A and B, consisting of three Wistar rats each, were set up. Group A was administered 200mg/kg body weight per day repeatedly for three weeks. Group B, which served as control, was administered physiological saline for three weeks. Both groups were monitored for another three weeks.

Blood samples from both groups were collected at two intervals; before treatment and 6 weeks post treatment. Blood samples were analysed for percentage packed cell volume (%PCV), haemoglobin concentration, and differential leukocyte counts. The body weights, organ weights and organ-body weight ratios were also determined.

3.17.1 Liver function test

Animals used for sub-chronic toxicity test were sacrificed at the end of study period and blood was withdrawn from the punctured heart into clean, sterile centrifuge tubes. The serum obtained after centrifugation was used to determine the activities of alanine amino transferase (ALT) or serum glutamate-pyruvate transaminase (SGPT) and Aspartate amino transferase (AST) or serum glutamate-oxaloacetate transaminase (SGOT).

3.17.2 Determination of Alanine Amino Transferase (ALT) or Serum Glutamate-Pyruvate Transaminase (SGPT) Activity

Principle:

The assay method used for the determination of SGPT activity was based on the one described by IFCC (1980). The working reagent was reconstituted by mixing Reagent 2 (R2) with the volume of Reagent 1 (R1) as indicated on the vial label. To 0.1ml of the serum sample, 1.0ml of the working reagent was added. The mixture was incubated at 37°C for 1 minute, after which the change in absorbance per minute (OD/min.) at 340nm was measured during the next 3 minutes.

Calculation:

SGPT activity (U/L) = ($\triangle$OD/min) × 1768

3.17.3 Determination of Aspartate AminoTransferase (AST) or Serum Glutamate – Oxaloacetate Transaminase (SGOT) Activity

Principle:

The method employed in this determination is a modified formulation for the assay of GOT, as recommended by the IFCC (International Federation of Clinical Chemistry) (1980). The IFCC reference method includes pyridoxal phosphate (PP), which functions as a coenzyme in amino acid (AA) transfer. This results in increased enzyme activity, avoiding falsely low values in samples containing insufficient endogenous PP, for example from patients with myocardial infarction, liver disease and intensive care patients.

In this method, 1.0ml of Reagent1 (R1) was added to 0.2ml of the serum sample and allowed to incubate at 25^0C for approximately 5 minutes. Then, 0.25ml of Reagent2 (R2) was added to the test tube and allowed to mix. After 1min., the absorbance was read against air at 340 nm. The absorbance was again measured after exactly 1, 2, and 3 minutes.

Calculation:

Light path- 1 cm

SGOT activity (IU/L) = $\triangle$ OD/min. × 1151

3.17.4 Kidney Function test

The serum obtained above (3.17.1) was used to determine the following kidney function parameters:

- Creatinine level
- Urea level
- Potassium (K^+) level
- Sodium (Na^+) level

- Chloride (Cl^+) level
- Bicarbonate (HCO_3) level

3.18 Preparation of Tissues for Histopathological Studies

The tissues were prepared according to the method described by Wallace, (2001). Only animals that had their lifespan extended beyond two months (methanol extract of *E. camaldulensis* and combined therapy treated groups) and animals that were exposed to chronic dose of the methanol extract of *E. camaldulensis* were subjected to histopathological studies. Animals were sacrificed and their livers and kidneys were carefully removed. These organs were processed, sectioned and stained according to standard laboratory methods. They were fixed in 10% formalin- saline and 3-4mm thick tissue was cut from each organ for processing. The cut tissues were transferred to the automatic tissue processor where the tissues were further fixed in 10% buffered formol-saline for two hours and dehydrated for two hours in each ascending grades of alcohol (70%, 90%, and 100% v/v). The dehydrated tissues were then cleared in xylene for two hours and the tissues impregnated in molten paraffin wax for another two hours and left to cool. The sections were then trimmed and sectioned on the microtome at 5 microns (μ). The sections were floated out in a warm water bath, then attached to slides, and dried on a hot plate and stained. The slides were viewed on a microscope with the assistance of a pathologist to enable interpretation of morphological changes in the tissues. The organ body weight ratios were also determined.

3.19 Bioassay- guided fractionation of methanol extract of E.camaldulensis (Leaf)

3.19.1 Column chromatography

Principle

Column chromatography is a separation technique in which the stationary bed is within a tube. The particles of the solid stationary phase or the support coated with a

liquid stationary phase may fill the whole inside volume of the tube (packed column) or be concentrated on or along the inside tube wall leaving an open, unrestricted path for the mobile phase in the middle part of the tube (open tubular column). Differences in rates of movement through the medium are calculated to different retention times of the sample (Still *et al.*, 1978). Column chromatography is categorized into two categories, depending on how the solvent flows down the column. If the solvent is allowed to flow down the column by gravity, or percolation, it is called gravity column chromatography. If the solvent is forced down the column by positive air pressure, it is called flash chromatography.

3.19.2 Column chromatography of crude methanol extract

In column chromatography, the stationary phase, a solid adsorbent (Silica gel-mesh 70-230) soaked in hexane, was placed in a vertical glass column and the mobile phase, hexane, was added to the top and allowed to flow down through the column (by gravity). Only the methanol extract of *E. camaldulensis* (leaf) was subjected to column chromatography and subsequent determinations because it was the only one that produced the desired anti-trypanosomal activity. The *A. senegalensis* extracts did not go beyond the preliminary stage.

Procedure

About 40g portion of the crude methanol extract of *E. camaldulensis* (leaf) was thoroughly mixed with140g of silica gel (Mesh 70- 230) and 5ml of acetone was added to ensure complete mixing after which the acetone was allowed to evaporate. The dried extract/silica gel mixture was added carefully to the top of the column already packed with silica gel in hexane. First, the tap was opened to allow the solvent (hexane) already in the column to drain so that it was level with the top of the packing material. The dried extract/silica gel mixture was then added carefully to the top of the column. The tap was

again opened to allow for complete absorption of the coloured extract into the top of the column.

Next, the solvent mixture, made up of hexane, ethyl acetate and methanol in increasing ratios of polarity was added to the top of the column, trying to disturb the packing material as little as possible. Then the tap was opened so that the solvent can flow down through the column, collecting it in a beaker or flask at the bottom. As the solvent ran through, fresh solvent was added to the top so that the column never dried out. Fractions were then collected sequentially in bottles and evaporated at between 60- 80^0 C on a rotary evaporator. The residues were kept in stoppered bottles until required for screening.

The Adsorbent

Silica gel (SiO_2) (mesh 70-230) was used in the fractionation of the methanol extract of *E. camaldulensis* (leaves). The mesh number refers to the mesh of the sieve used to size the silica, specifically, the number of holes in the mesh or sieve through which the crude silica particle mixture is passed in the manufacturing process. If there are more holes per unit area, those holes are smaller, thus allowing only smaller silica particles go through the sieve. The relationship is: the larger the mesh size, the smaller the adsorbent particles.

Adsorbent particle size affects how the solvent flows through the column. Smaller particles (higher mesh values) are used for flash chromatography; larger particles (lower mesh values) are used for gravity chromatography. For example, 70–230 silica gel is used for gravity columns and 230–400 mesh for flash columns.

The Solvent

In this fractionation process, a series of increasingly polar solvent systems were used to elute the column. A non-polar solvent, hexane, was first used to elute less-polar

compounds, and subsequently the polarity was gradually increased by varying the ratios of hexane, ethyl acetate, and methanol in the solvent mixture. The last elution was done with 100% methanol as shown in the Table below:

Collection and Analysis of Column Eluents

Small fractions of the eluents were collected sequentially in labelled tubes and the composition of each fraction was analyzed by thin layer chromatography. Forty eight (48) fractions obtained at the end of the column chromatography were subjected to thin layer chromatography in order to pool similar fractions together.

Analysis of Column Eluents

If the compounds separated in a column chromatography procedure are coloured, the progress of the separation can simply be monitored visually. More commonly, the compounds to be isolated from column chromatography are colourless. In this case, small fractions of the eluents were collected sequentially in labelled bottles and the fractions were pooled together using thin layer chromatography.

3.19.3 Thin Layer Chromatography

Principle

Thin layer chromatography (TLC) is a chromatography technique used to separate mixtures. It involves a *stationary phase* consisting of a thin layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose immobilized onto a flat, inert carrier sheet. A *liquid phase* consisting of the solution to be separated is then dissolved in an appropriate solvent and is drawn up the plate via capillary action, separating the experimental solution based on the polarity of the components of the compound in question (Jonathan *et al.*, 2007).

Plate preparation

TLC plates were made by mixing the *adsorbent*, silica gel, with a small amount of inert binder, calcium sulphate (gypsum) and water. This mixture was spread as thick slurry on an unreactive carrier sheet, glass, the resultant plate was dried and activated by heating in an oven for thirty minutes at 110 °C. The thickness of the adsorbent layer was typically around 0.1–0.25 mm for analytical purposes and around 1–2 mm for preparative TLC. Every type of chromatography contains a mobile phase and a stationary phase. The mobile phase was made up of hexane, ethyl acetate and methanol (4: 4: 1).

Sample Application

A small spot of the eluents were applied to the plate using capillary tube, about one centimetre from the base. The plate was then dipped into a mixture of solvents, hexane, ethyl acetate, and methanol (4: 4: 1) placed in a sealed container. The solvent mixture moved up the plate by capillary action and interacted with the fractions and were carried up the plate by the solvent. Different fractions travelled at different rates due to the differences in their attraction to the stationary phase, and because of differences in solubility in the solvent.

Pooling Together of Fractions

Using the RF values of the fractions after visualization of spots with iodine vapour, fractions with same values were pooled together as single fractions.

3.20 Screening for antitrypanosomal activity of fractions

Ten fractions obtained from column chromatography were screened for antitrypanosomal activity. For each of the fractions, three groups of three mice each were set up. Doses of 50, 100 and 150mg/kg body weight were administered on animals in groups 1, 2, and 3 respectively, and parasitemia was monitored for two weeks.

3.21 Phytochemical Analysis of the crude methanol extract of *E. camaldulensis* (Leaf)

The crude methanol extract of *E. camaldulensis* (leaf) was subjected to phytochemical analysis using standard analytical methods.

a. Test for flavonoids: About 3g of the crude extract was transferred into a clean test tube and 5ml of water was added. The mixture was boiled for 3 minutes and then filtered cool. Few drops of magnesium chips were added to the filtrate in a clean test tube and allowed to dissolve on addition of concentrated HCl. A green colour change indicates the presence of flavonoids.

b. Test for Tannins: A small amount of the extract was transferred into a test tube and 3ml of distilled water was added. To this mixture, a few drops of 10% ferric chloride were added. A deep brown coloration indicates the presence of tannins.

c. Test for Saponins: To 3g of the extract, 5ml of distilled was added. The tube was covered with the thumb and shaken vigorously for 30 minutes. The presence of saponins is confirmed by the persistence of frothing on warming.

d. Test for Terpenes: To 5mls of chloroform in a clean test tube, 3grams of the extract was added and mixed. The mixture was filtered and the filtrate was transferred into a clean test tube. Acetic anhydride was added to the filtrate followed by concentrated sulphuric acid. The formation of a ring at the interphase between the two immiscible liquids is a preliminary evidence for the presence of terpenes.

e. Test for steroids: 1ml of the extract was dissolved in 2mls of chloroform, and then concentrated H_2SO_4 was added to form a lower layer. The presence of a reddish violet ring at the interphase and a green chloroform upper layer indicates the presence of steroids.

f. Test for Anthraquinones: 1ml of the extract was dissolved in 10ml of benzene in a test tube. The mixture was filtered and to the filtrate, 5ml of 10% NH_3 was added and shaken. The presence of anthraquinones is indicated by the appearance of a pink red/violet colour in the ammonia phase.

g. Test for Alkaloids: 1ml of the extract was stirred with 5ml of 1% aqueous HCl in a steam bath. The mixture was filtered and 1ml of the filtrate was treated with few drops of Wagner's reagent.

h. Test for fatty acids: 1ml of the extract was made alkaline with 5mls of 25% NH_3. The mixture was exhaustively extracted with ether, and then acidified with concentrated HCl. The acidic aqueous solution was then shaken with ethyl ether in a separating funnel and subsequently the ether was evaporated to dryness. The presence of higher fatty acids is indicated by the oily nature of the residue.

3.22 Phytochemical Analysis of fractions 8 and 9 resulting from the crude methanol extract of *E.camaldulensis* (Leaf)

Fractions 8 and 9 which demonstrated some antitrypanosomal activity were screened to establish their phytochemicals contents. The two fractions were subjected to the tests carried out in 3.23 above.

3.23 Thin Layer Chromatography of Fractions 8 and 9

Using the method described above (3.21.3), the two fractions (8 and 9) were subjected to thin layer chromatography in order to have an idea of the number of components in the two fractions. The fractions were developed in the following solvents respectively: hexane: ethyl acetate (3:1); hexane: ethyl acetate (3:2); and hexane: ethyl acetate: methanol (4:4:1). The components were then located using (i) iodine tank (ii) spraying with vanillin in sulphuric acid.

3.24 Structural Elucidation of active fractions

Fractions 8 and 9 were subjected to Gas Chromatography – Mass Spectroscopy (GC-MS) in order to elucidate the structures of their components. The GC-MS spectrum of the fractions were obtained using GCMS-QP2010 PLUS (SHIMADZU, JAPAN).

Gas chromatography-mass spectrometry (GC-MS) is a method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample. The GC-MS is composed of two major building blocks: the gas chromatograph and the mass spectrometer. The gas chromatograph utilizes a capillary column which depends on the column's dimensions (length, diameter, film thickness) as well as the phase properties (e.g. 5% phenyl polysiloxane). The difference in the chemical properties between different molecules in a mixture will separate the molecules as the sample travels the length of the column. The molecules take different amounts of time (called the retention time) to come out of (elute from) the gas chromatograph, and this allows the mass spectrometer downstream to capture, ionize, accelerate, deflect, and detect the ionized molecules separately. The mass spectrometer does this by breaking each molecule into ionized fragments and detecting these fragments using their mass to charge ratio (Niessen, 2001).

3.25 Statistical Analysis of Experimental data

All data obtained in this work were statistically analyzed using analysis of variance (ANOVA). Data obtained were subjected to a one-way analysis of variance to derive mean values of parasitemia which were compared with least significant difference. Mean values among the treated groups were deemed to be different if the level of probability was < 0.05.

CHAPTER FOUR

4.0 RESULTS

4.1 Antitrypanosomal activity of *E.camaldulensis* (leaf) extracts

The results of screening with hexane, ethyl acetate, methanol and aqueous extracts of *E.camaldulensis* (leaf) are presented on figures 4.1, 4.2, 4.3 and 4.4. The hexane extract at doses of 200 and 600mg/kg bodyweight/day administered for 3weeks cleared trypanosomes from circulation but only one animal from each of the groups survived for more than 40 days. Mice in the 400mg/kg bodyweight/day group and the infected but untreated group did not survive beyond the treatment period of 3 weeks. Average number of parasites/ml.of blood before the death of animals was 3×10^6 and 7.77×10^6 respectively as shown on fig.4.1.

Fig.4.2 shows the course of parasitemia in infected mice treated with ethyl acetate extact at 200,400 and 600mg/kg bodyweight/day. Two mice in the group treated with 200mg/kg bodyweight survived for 30 days without parasites in circulation while animals in the other dose groups including the untreated animals died before the 25^{th} day post-infection with average parasites/ml of blood ranging between $1.0 - 6.5 \times 10^6$.

Fig.4.3 represents the course of parasitemia in mice treated with methanol extract of *E.camaldulensis* (leaf). All animals in the three dose groups (200,400 and 600mg/kg bodyweight/day) had parasites cleared from circulation about 2 weeks into the treatment period and this was maintained beyond 40 days. Animals in the untreated group started dying on day 10 post-infection with parasitemia rising to about 0.3×10^6/ml. of blood. A repeat screening with these active doses demonstrated the same efficacy against the parasites. Animals in the three groups that survived for three months post treatment when subjected to blood and cerebrospinal fluid infectivity tests proved negative indicating that parasites were cleared completely from circulation.

Treatment with the aqueous extract did not clear parasites from circulation. All animals in the different dose groups, including the untreated ones died on or before the 26^{th} day post-infection with a peak parasite count of 4.0×10^6/ml. of blood in the group treated with 200mg/kgbody weight/day on the 20^{th} day as shown on fig.4.4. Other dose groups had lower parasite counts. In all cases, the group treated with 3.5mg/kg bodyweight of berenil had parasites cleared from day three post infection and continued to survive throughout the period of screening.

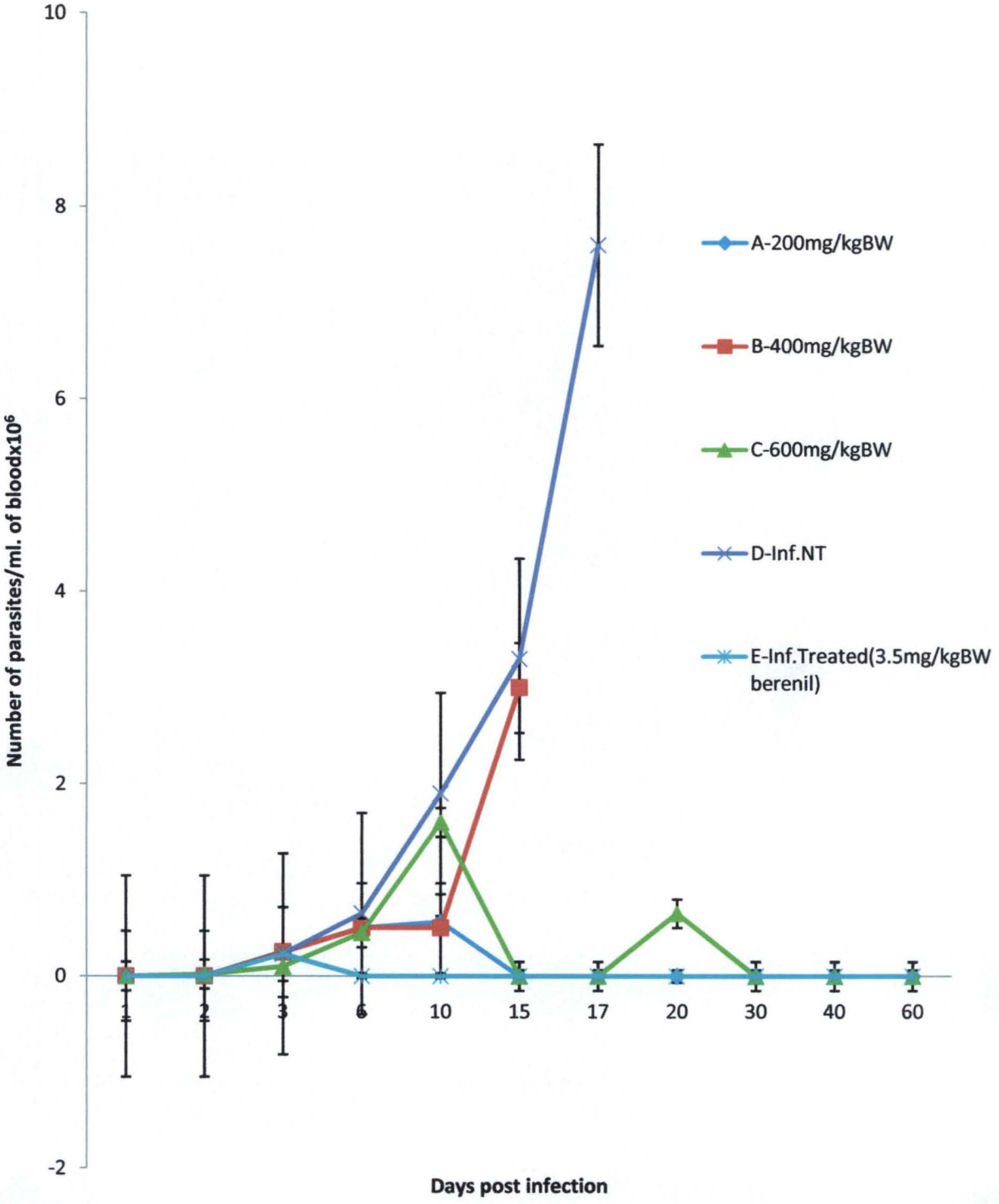


Fig.4.1: Course of parasitemia in infected mice treated with hexane extract of *E. camaldulensis* (leaf)

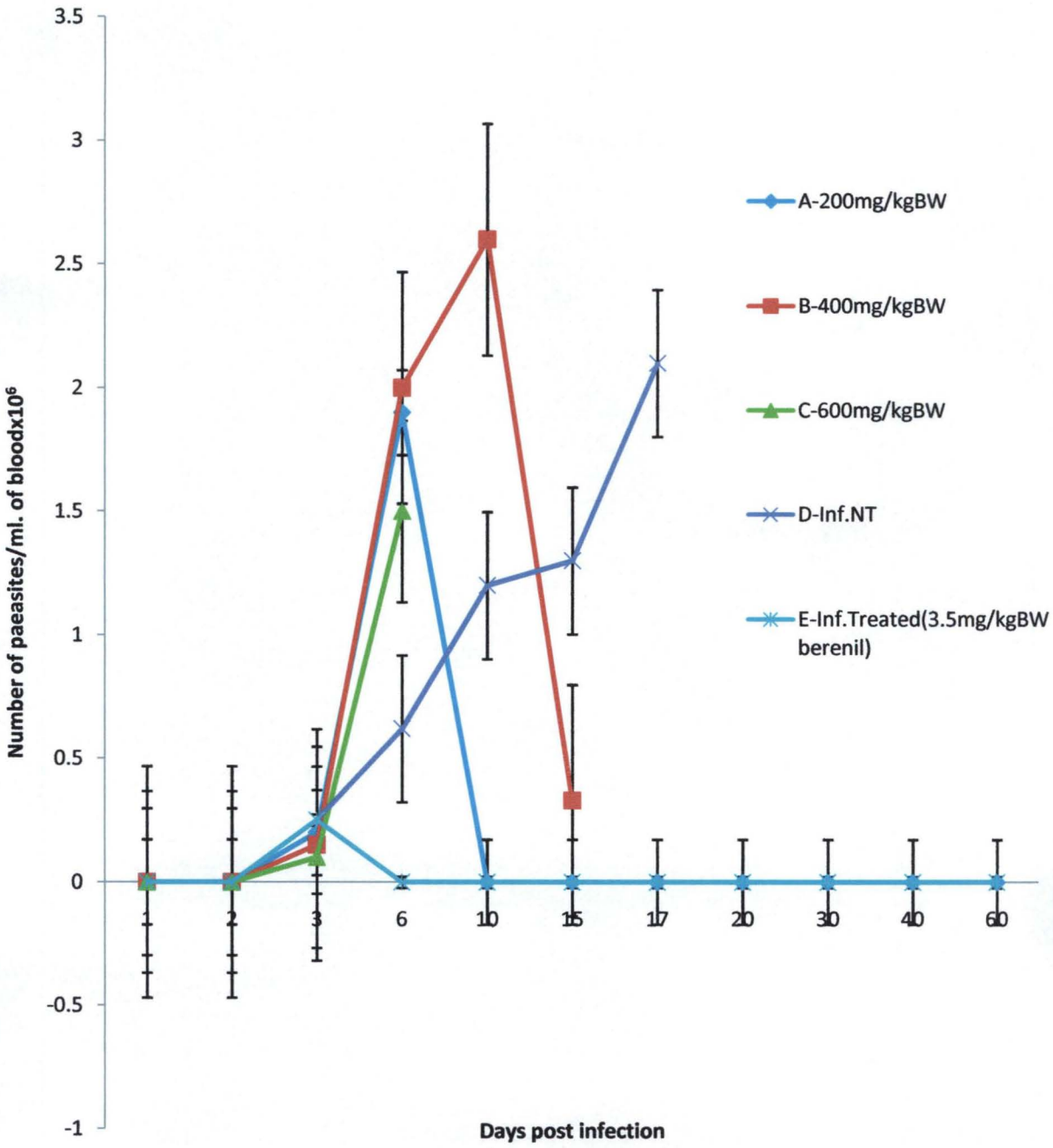


Fig.4.2: Course of parasitemia in infected mice treated with Ethyl acetate extract of *E. camaldulensis* (leaf)

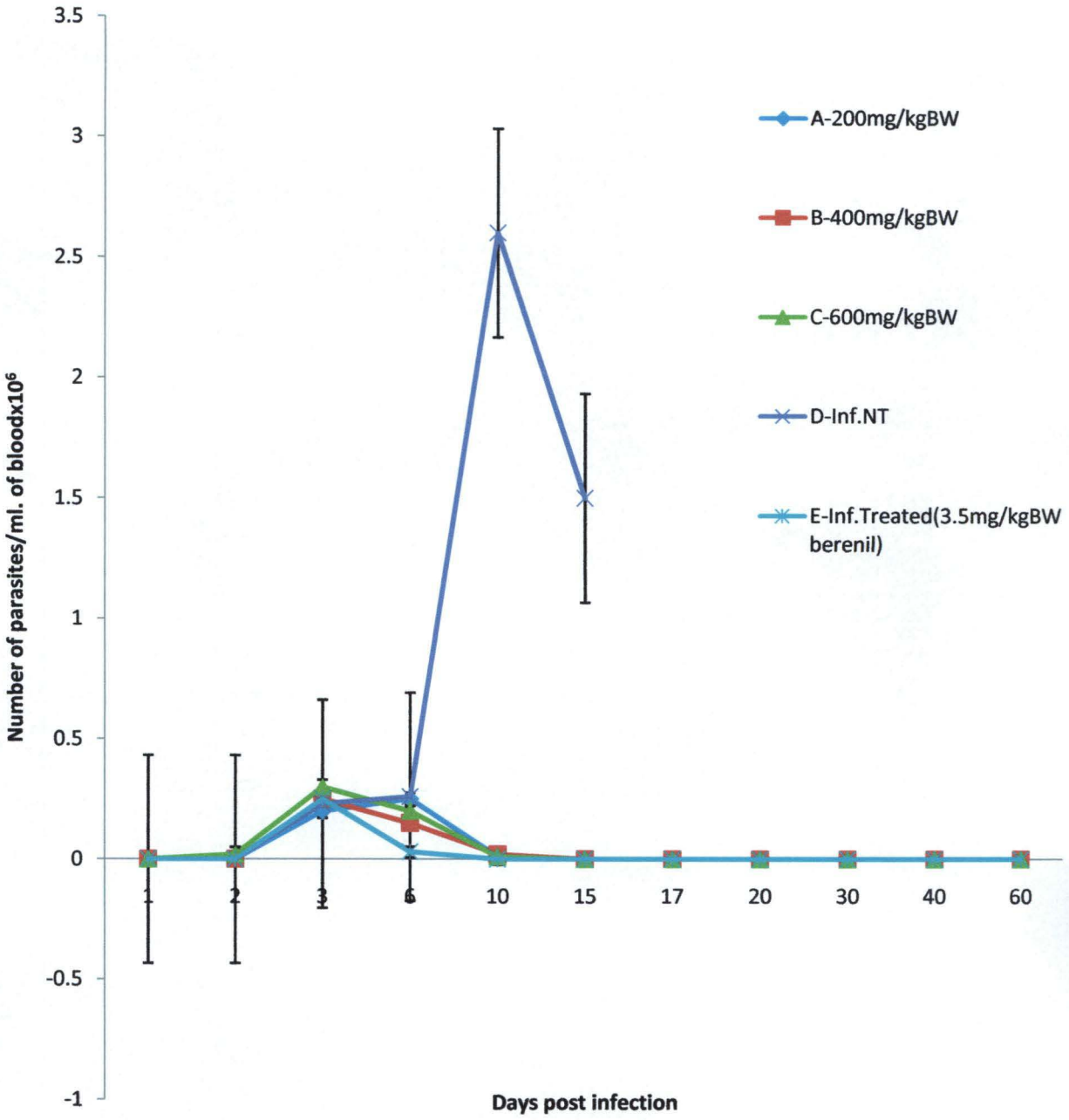


Fig.4.3: Course of parasitemia in infected mice treated with Methanol extract of *E. camaldulensis* (leaf)

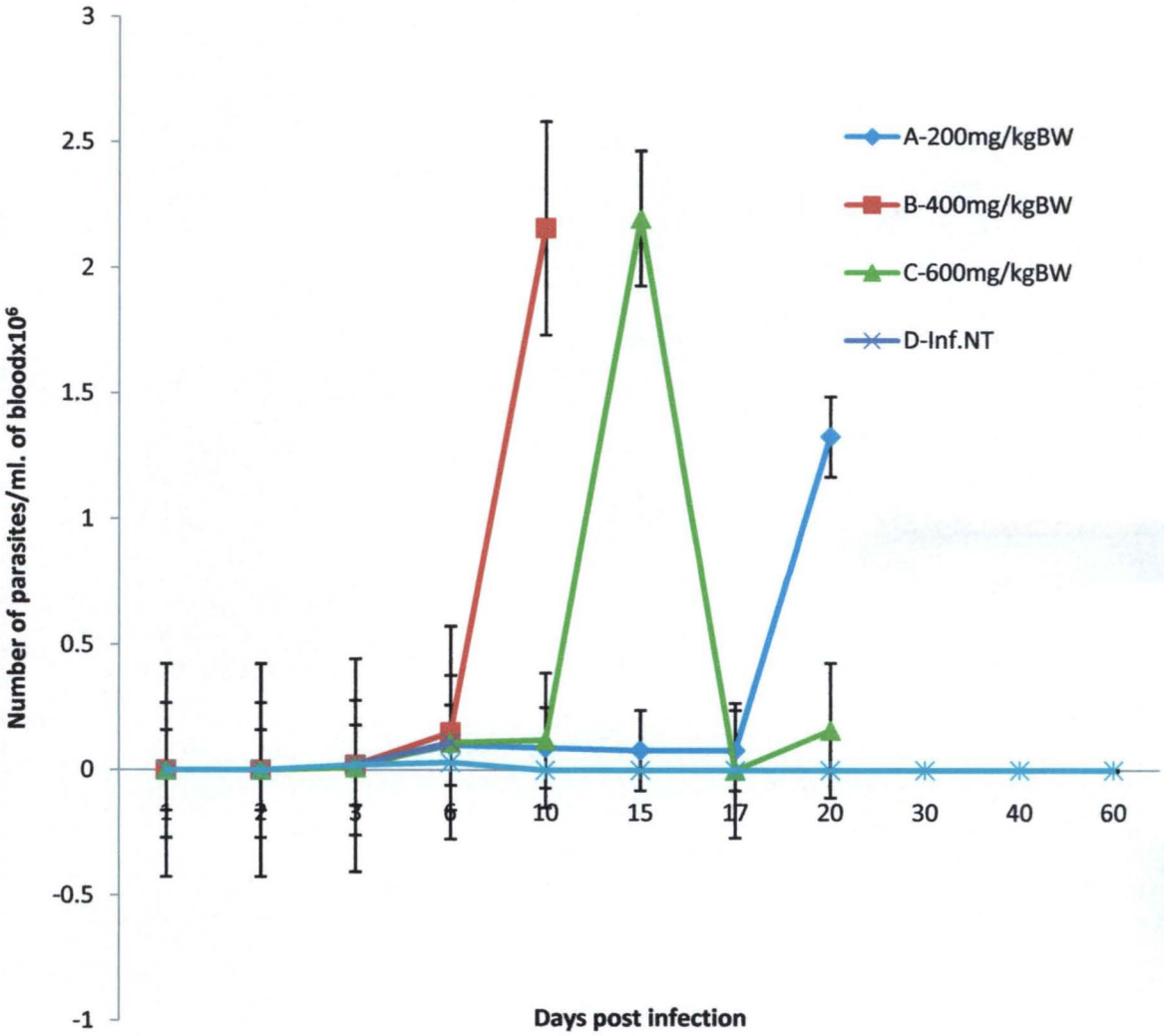


Fig.4.4: Course of parasitemia in infected mice treated with Ethyl acetate extract of *E. camaldulensis* (leaf)

4.2 Antitrypanosomal activity of E. camaldulensis (stem bark) extracts

The course of parasitemia in *T. b. brucei* - infected mice treated with hexane, ethyl acetate and methanol extracts of the stem bark are presented on figures 4.5, 4.6, and 4.7 respectively. Mice in the groups treated with 200, 400 and 600mg/kg bodyweight of hexane and ethyl acetate extract did not survive beyond the 10^{th} day post infection with average parasites/ml of blood ranging between 0.02×10^6 and 3.90×10^6 as shown on figures 4.5 and 4.6. Animals in the untreated group died four days earlier.

Treatment with the methanol extract produced a better result because animals in the group treated with 200mg/kg bodyweight survived for 17 days post infection, that is, 13 days beyond the untreated animals. The average number of parasites/ml of blood on the 17^{th} day was 1.90×10^6 (fig.4.7). The groups treated with 400 and 600mg/kg bodyweight died on the 10^{th} and 6^{th} day respectively.

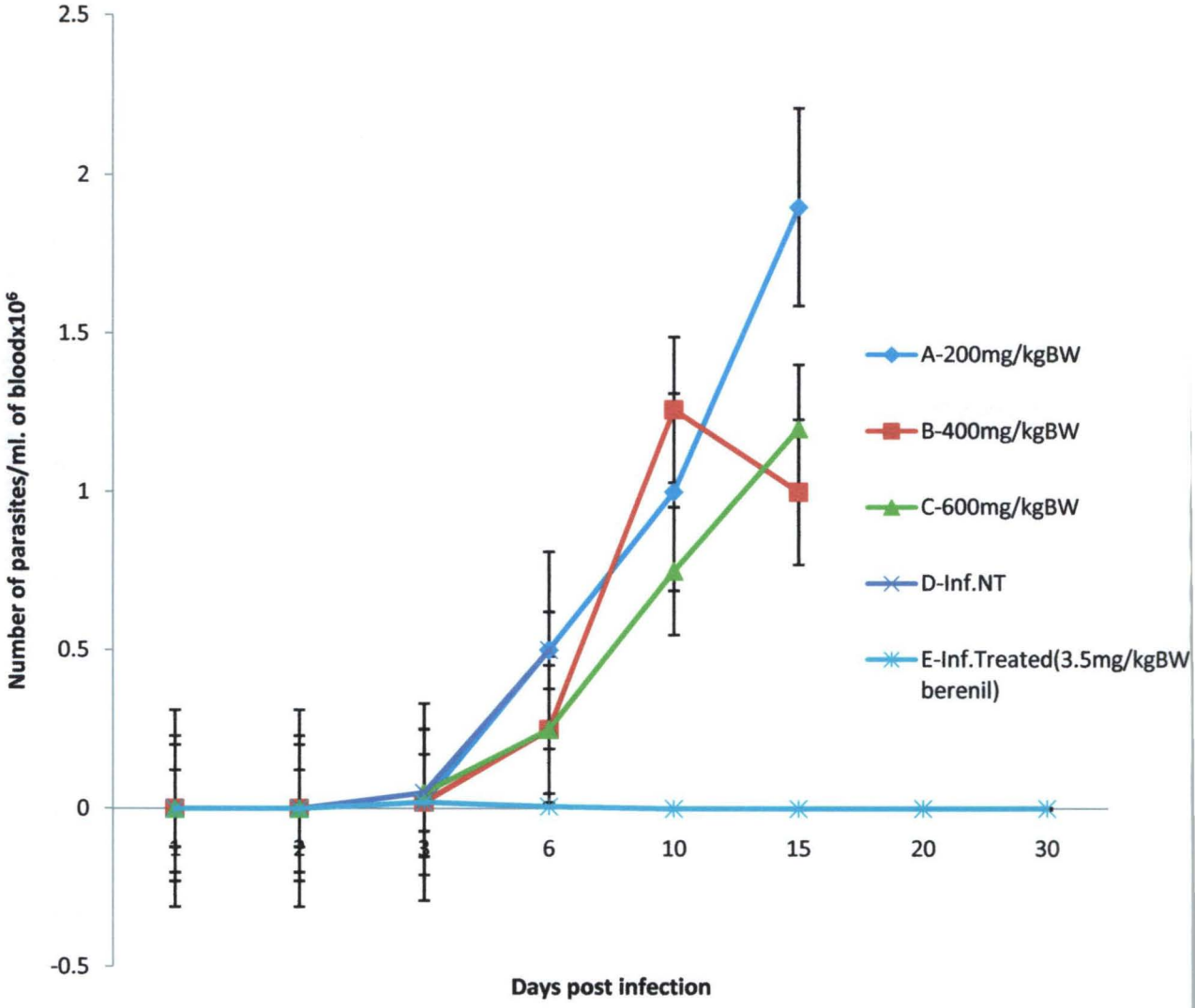


Fig.4.5: Course of parasitemia in infected mice treated with hexane extract of *E. camaldulensis* (Stem bark)

4.3 Antitrypanosomal activity of *E. camaldulensis* (Root bark) extracts

Figures 4.8, 4.9, and 4.10 show the course of parasitemia in infected mice treated with hexane, ethyl acetate, and methanol extracts of *E. camaldulensis* (Root bark). The hexane extract at doses of 200, 400, and 600mg/kg bodyweight prolonged the lifespan of animals in the groups by 10 days beyond that of the untreated group which recorded death on day 6. The average number of parasites /ml of blood in treated groups ranged between 1.0×10^6 and 1.9×10^6 on day 15 as seen on fig.4.18.

In the groups treated with ethyl acetate extract, one animal in the group administered 200mg/kg bodyweight/day survived for 20 days post infection, that is, 2 weeks after the death of the untreated animals (fig.4.9). Average number of parasites at the last count was 0.01×10^6/ml of blood.

Treatments with methanol extract using 200, 400, and 600 mg/kg bodyweight/day only prolonged life by 5 days in the 200mg/kg bodyweight group. Animals in the other dose groups died on the same day (day 6) with the untreated group as shown on fig.4.10.

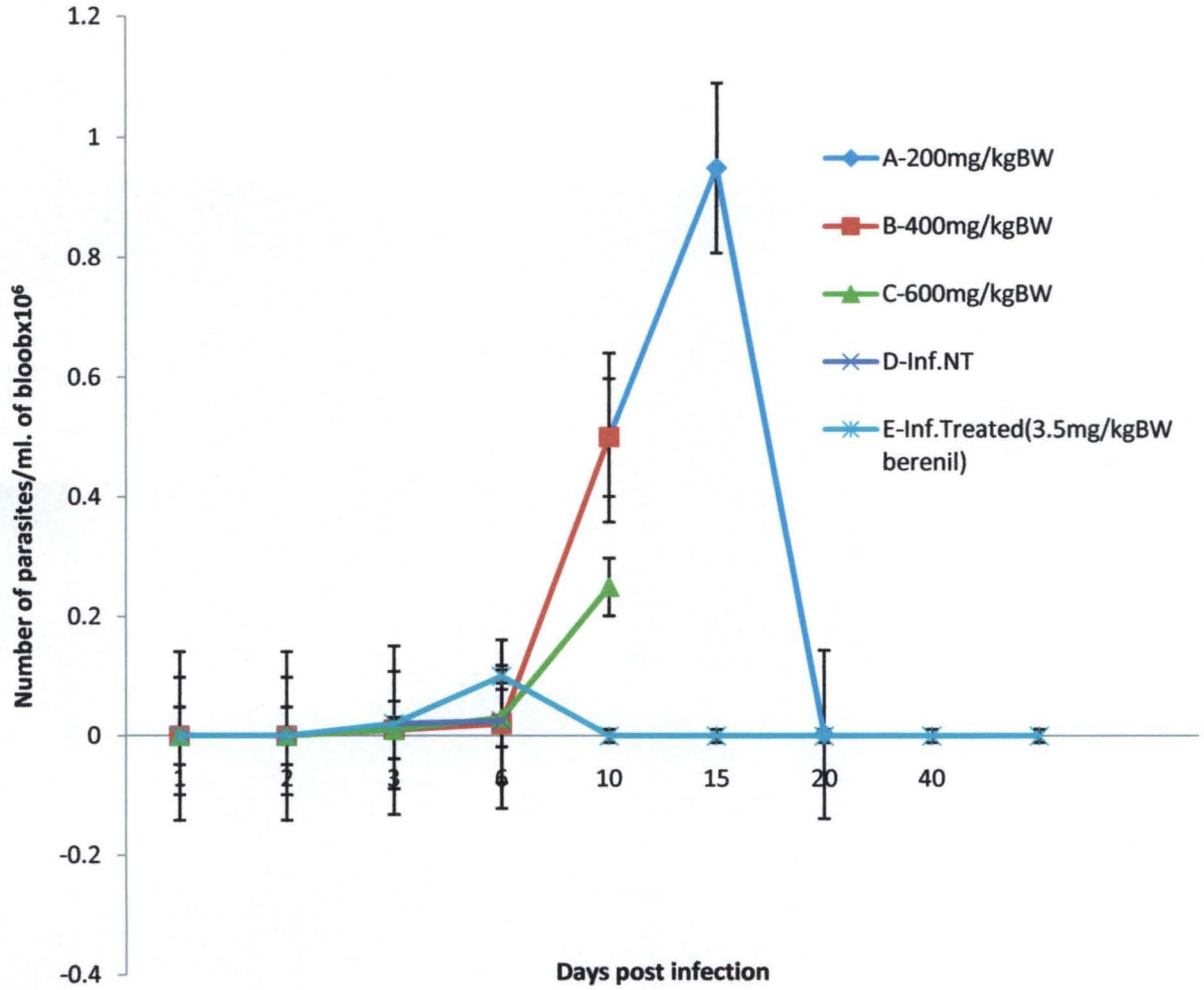


Fig.4.6: Course of parasitemia in infected mice treated with Ethyl acetate extract of *E.camaldulensis* (Stem bark)

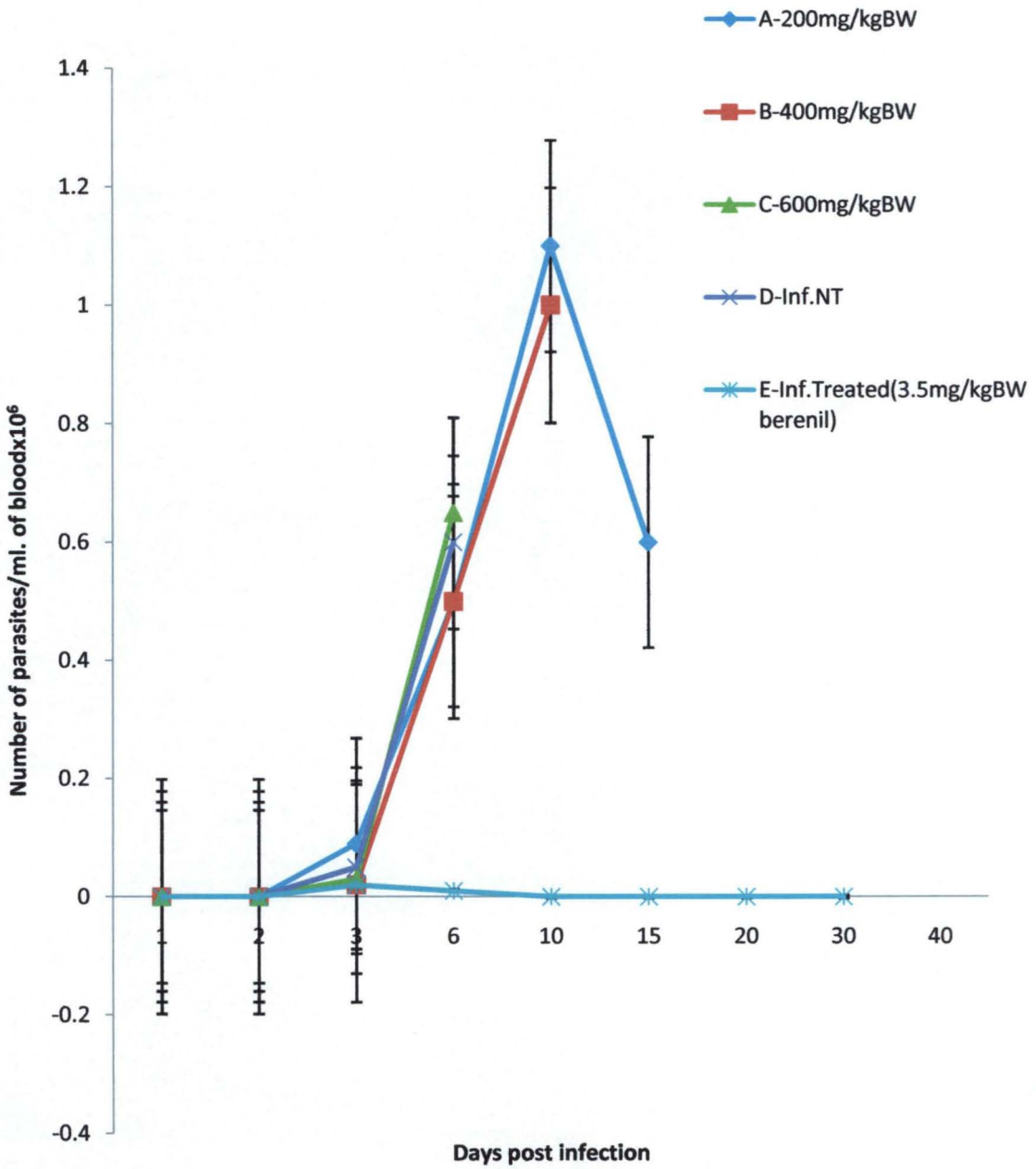


Fig. 4.7: Course of parasitemia in infected mice treated with Methanol extract of *E. camaldulensis* (Stem bark)

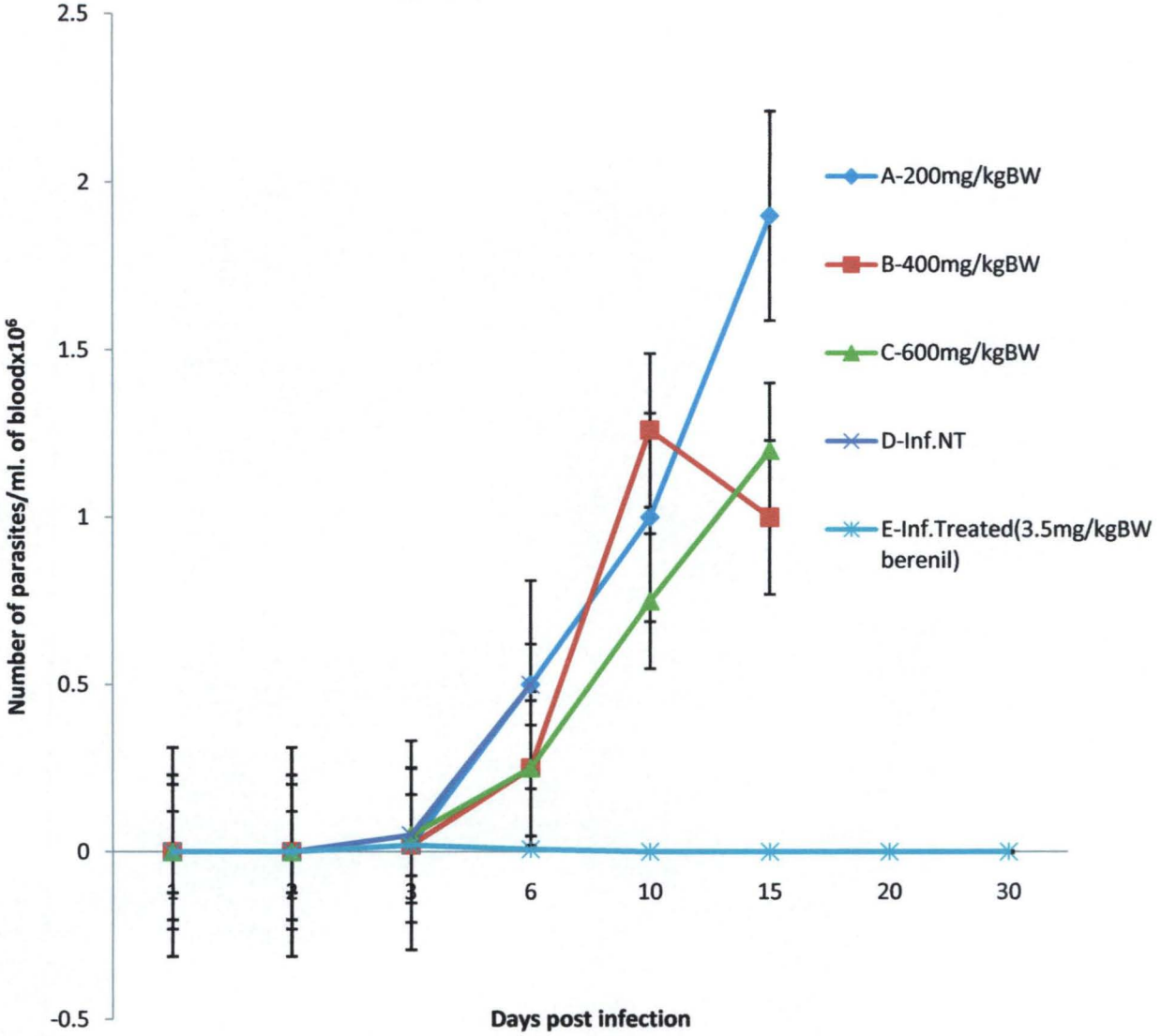


Fig.4.8: Course of parasitemia in infected mice treated with hexane extract of *E.camaldulensis* (Root bark)

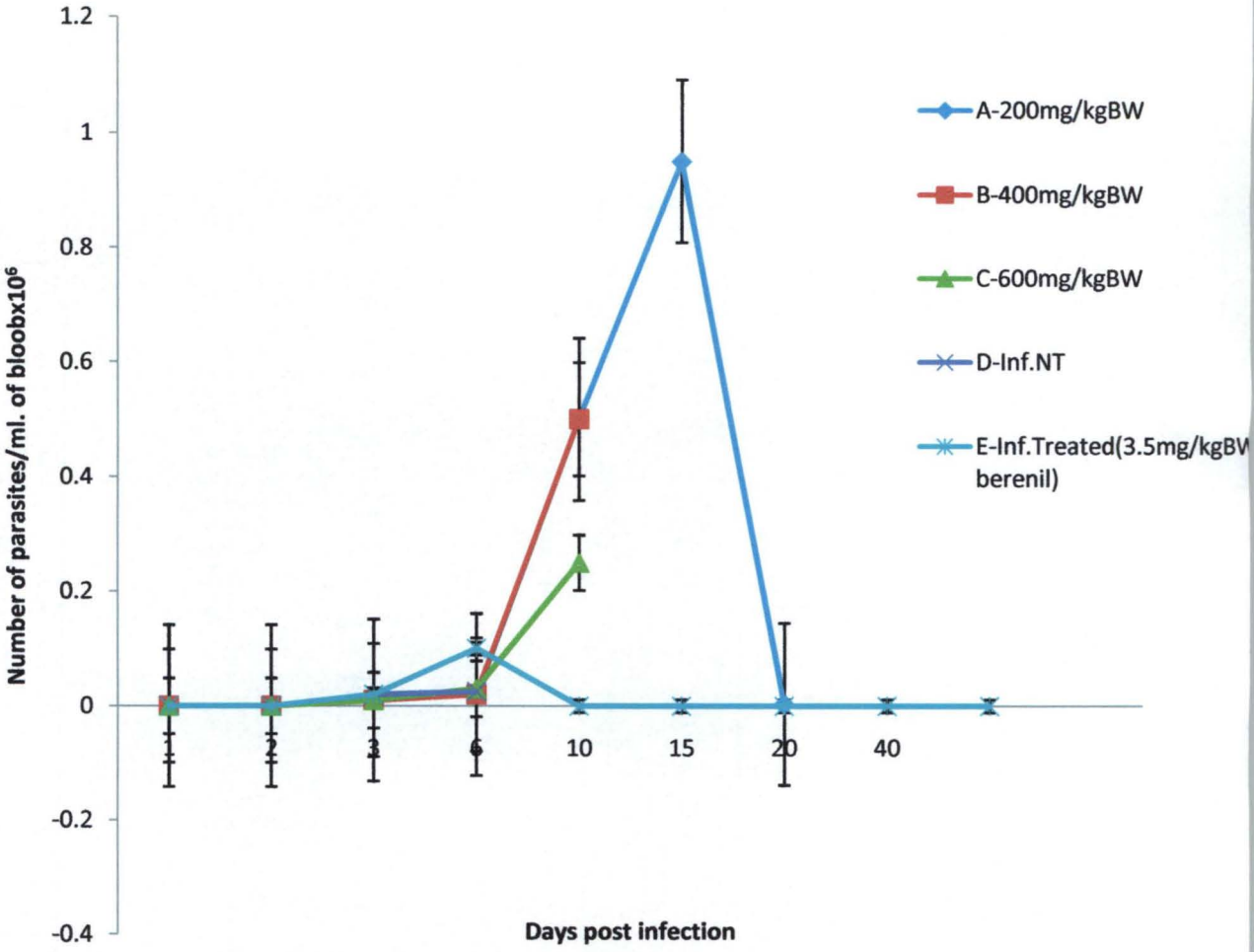


Fig.4.9: Course of parasitemia in infected mice treated with Ethyl acetate extract of *E.camaldulensis* (Root bark)

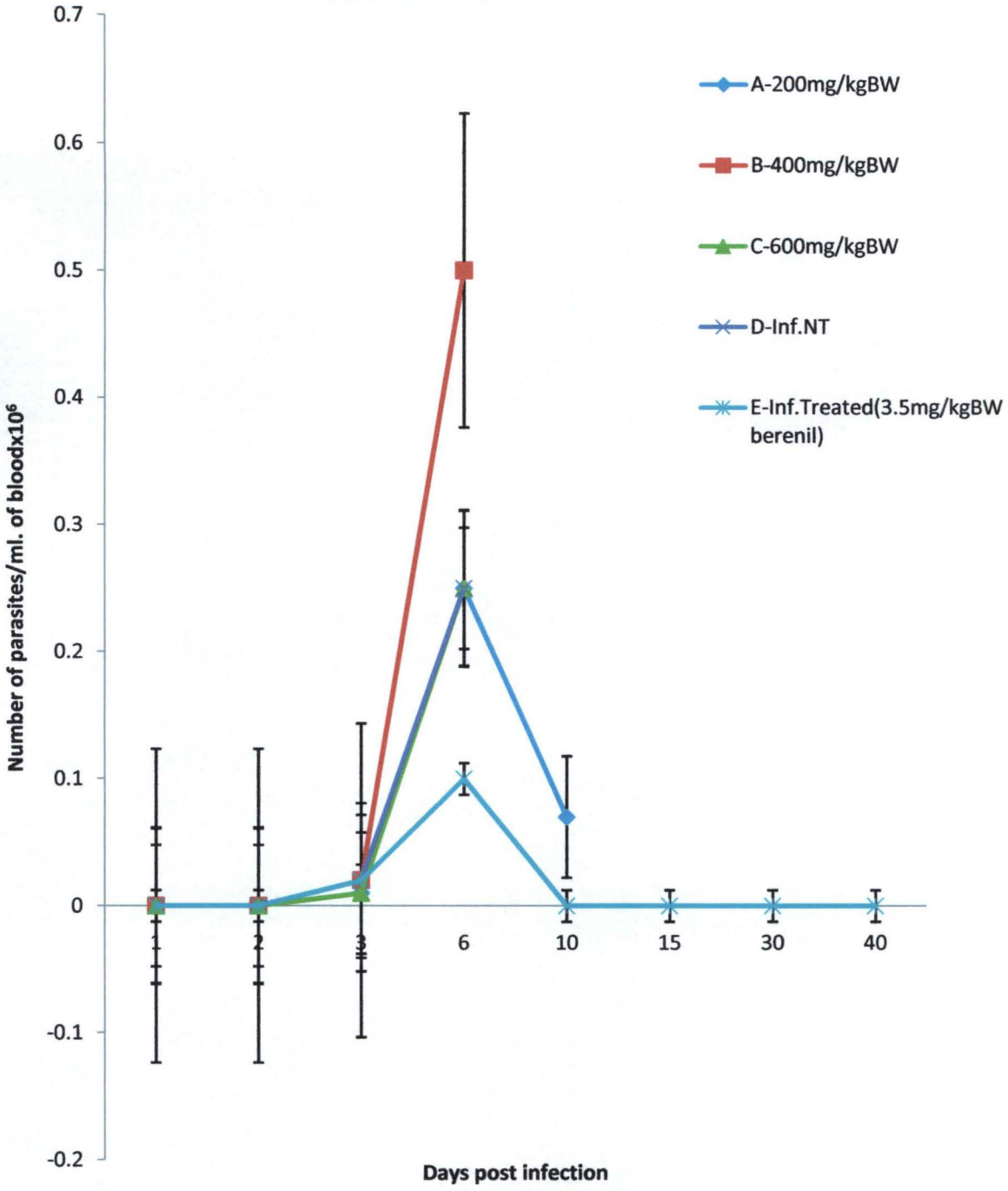


Fig. 4.10: Course of parasitemia in infected mice treated with methanol extract of *E.camaldulensis* (Root bark)

4.4 Confirmatory screening with effective doses of *E. camaldulensis*

Only the group treated with the methanol extract and the standard drug had parasites cleared from their circulation and their life span extended beyond that of the control. The other extracts did not clear parasites from circulation as shown on fig.4.11.

4.5 Screening for antitrypanosomal activity in infected mice treated for two and three weeks with 200mg/kg bodyweight of *E. camaldulensis* (leaf) methanol extract

The effective dose (200mg/kg bodyweight) of *E. camaldulensis* (leaf) methanol extract when used to treat infected mice for two and three weeks respectively in order to ascertain the effect of duration of treatment on the antitrypanosomal activity of the extracts showed that one mouse in the two weeks treatment group survived for 30 days with average number of parasites/ml ranging between 60 x 10^6 and 100 x10^6, while two mice in the three weeks treatment group had parasites completely cleared from circulation and they survived beyond 30 days as shown on fig. 4.12. The infected and untreated group survived for only 7 days.

4.6 Determination of minimum curative dose for methanol extract of *E. camaldulensis* (leaf)

Infected mice were treated with 50, 100, 150 and 200mg/kg bodyweight/day for three weeks. The course of parasitemia as presented on fig.4.13 shows that 200mg/kg bodyweight/day was most effective because animals in the group had parasites cleared from circulation on the 22nd day post infection and continued to survive beyond 30 days. The groups treated with 50, 100, and 150mg/kg bodyweight/day had parasites in circulation up to the 26th day but average number of parasites/ml of blood was highest in the 50mg/kg bodyweight group (500 x10^6), indicating that it had the least antitrypanosomal activity.

4.7 Determination of the best time to commence treatment using 200mg/kg bodyweight/day of methanol extract of *E. camaldulensis* (leaf)

Only groups A and B treated, 0 and 24 hours after inoculation had parasites cleared from circulation from day 16 and continued to survive beyond 30 days. Groups C and D, treated 48 and 72 hours after inoculation did not survive beyond 22 and 20 days respectively as shown on fig.4.14. The untreated group survived for only 10 days.

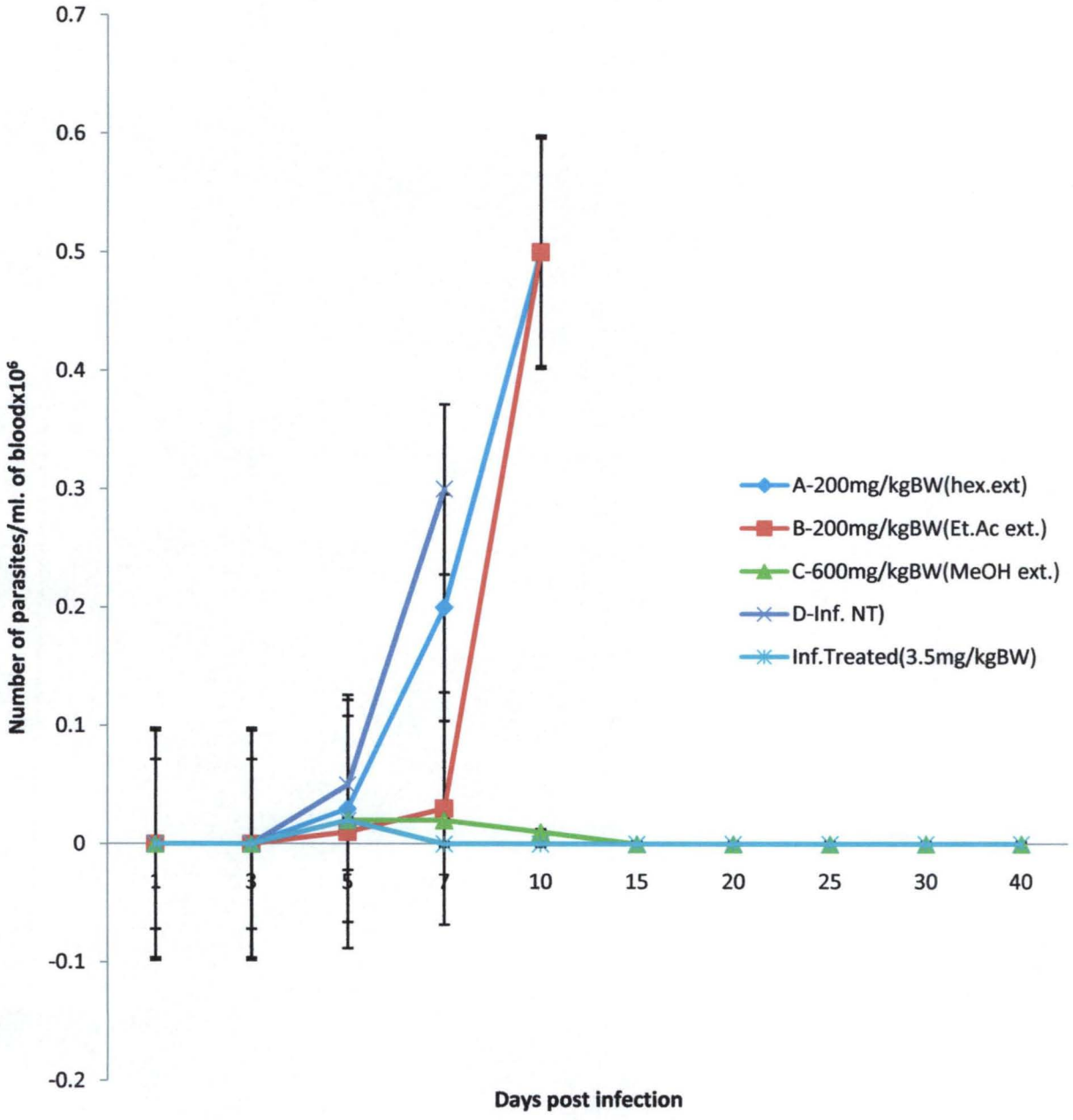


Fig.4.11: Course of parasitemia in infected mice treated with selected extracts of *E. camaldulensis* (leaf)

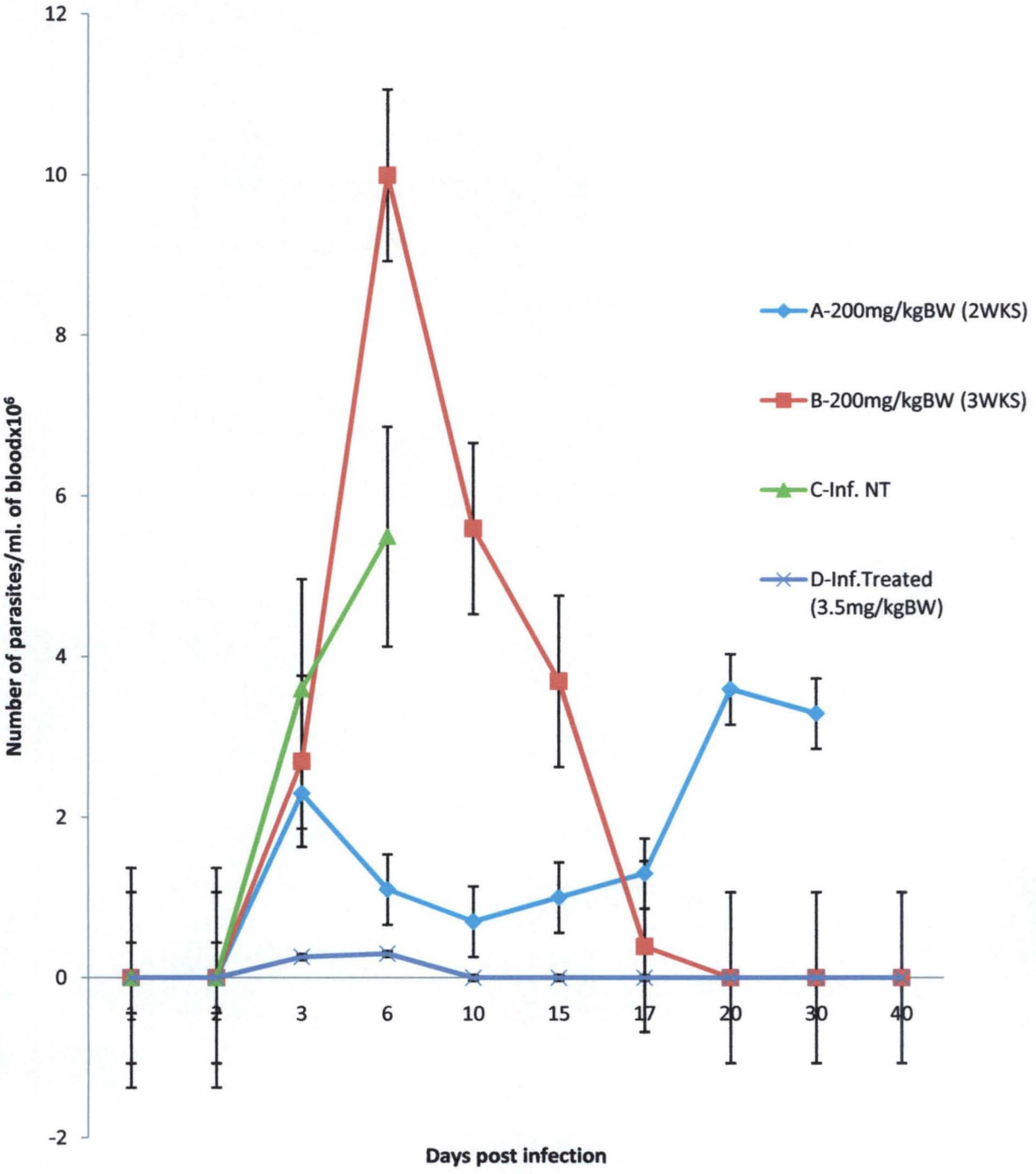


Fig.4.12: Course of parasitemia in infected mice treated for 2 and 3 weeks (WKS) with effective dose (200m/kgBW) of *E. camaldulensis* (leaf) methanol extracts

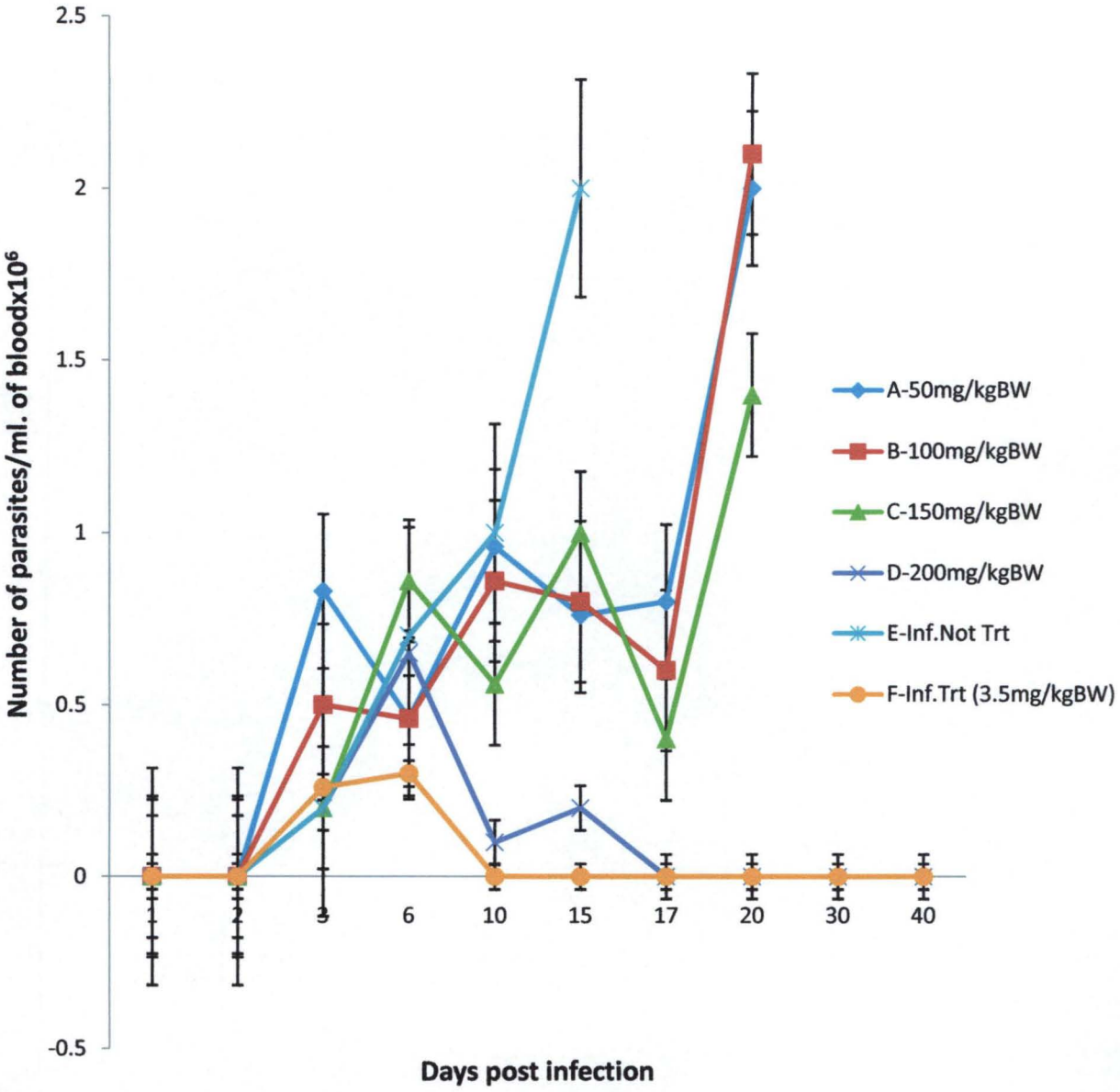


Fig.4.13: Determination of minimum curative dose for methanol extract of *E. camaldulensis* (Leaf)

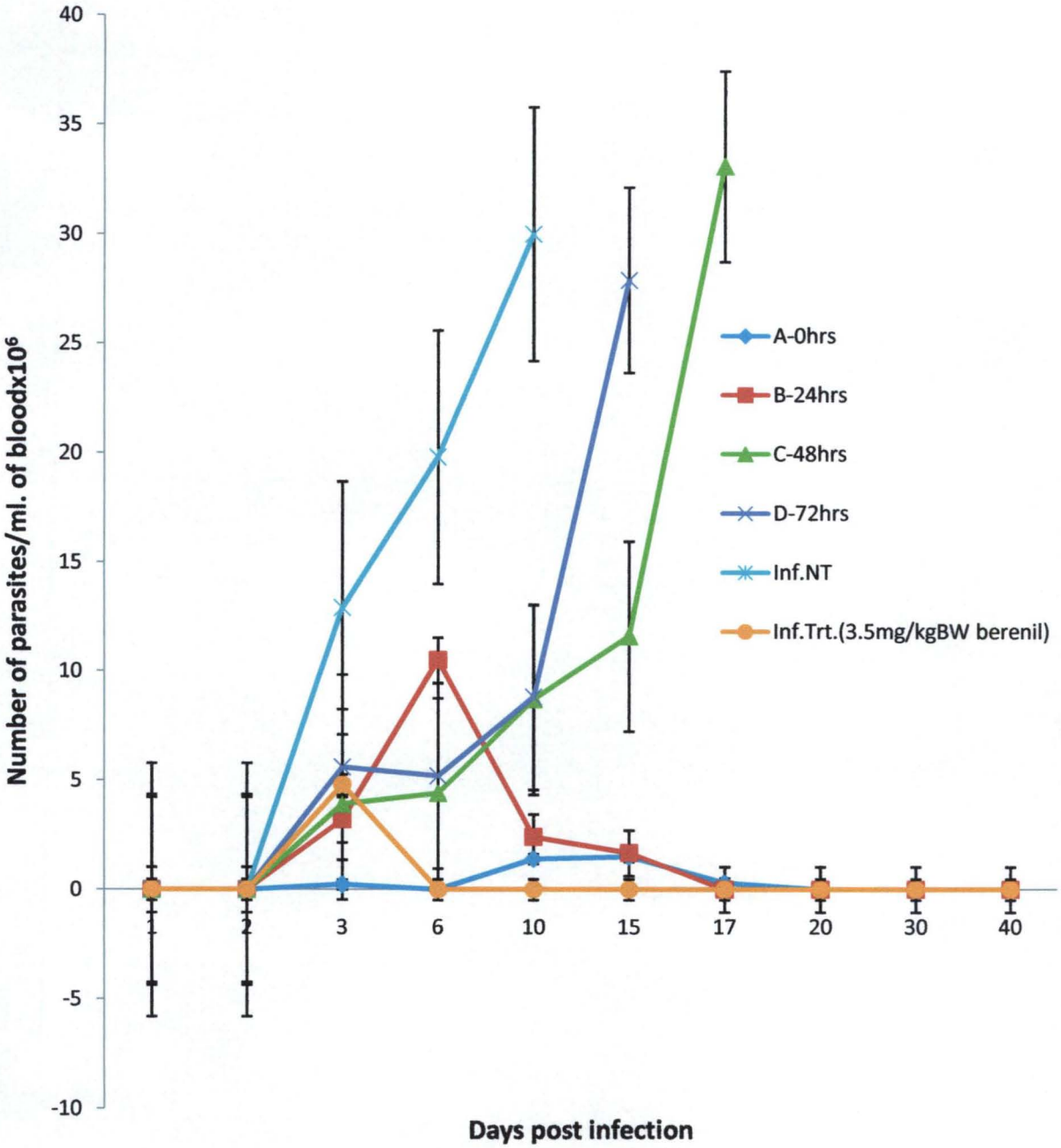


Fig.4.14: Determination of the best commencement time for therapy using 200mg/kg bodyweight /day of methanol extract of *E. camaldulensis* (leaf)

4.8 Confirmatory screening with effective *A. senegalensis* (stem bark) extracts

Initial screening with the extracts at different doses and a subsequent repeat screening based on the results obtained from the initial screening showed that 400mg/kg bodyweight of hexane extract and 300mg/kg bodyweight of aqueous extract of the stem bark after treating for three weeks were effective in clearing parasites from the circulation of *T. b. brucei* - infected mice and two animals in the hexane group and one in the aqueous group survived beyond two months, like the group treated with 3.5mg/kg bodyweight of berenil although the standard drug cleared parasites faster and the survival rate was higher. Infectivity tests with the blood and CSF of the surviving animals was negative because parasites did not appear in circulation after two months of monitoring. Other groups treated with 100, 200 and 500 mg/kg bodyweight of the extracts did not survive beyond three weeks. The control animals that were untreated died one week post infection. The results for the antitrypanosomal activities of the two extracts at the active doses are shown on figures 4.15 and 4.16.

4.9 Combination Therapy

Of all the combinations used to treat infected mice, only the combination of methanol extracts of *A. senegalensis* (leaf) and *E. camaldulensis* (leaf) (1:1) resulted in the clearance of parasites from the circulation of one animal in the group as shown on fig.4.17. The animal survived for more than two months and was used for blood and CSF infectivity tests. Other combinations used for treatment of T.b.brucei – infected mice did not clear parasites from circulation. The weight of the survived animal appreciated after an initial fall in the first two weeks. Fig.4.18 represents the comparative performance of the effective combination and the standard drug (berenil). There was no significant difference between their performances ($P \leq 0.05$).

4.10 Blood and Cerebrospinal Fluid (CSF) infectivity tests

Parasitemia was monitored for two months in animals that were inoculated with blood and CSF withdrawn from mice that survived the first screening (these are animals from the groups treated with methanol extract of *E. camaldulensis* (leaf), hexane extract of *A. senegalensis* (stem bark), and a combination of methanol extracts of *A. senegalensis* (leaf) and *E. camaldulensis* (leaf). Parasites were permanently absent from the blood throughout the period of monitoring and the animals continued to survive thereafter.

4.11 Test for prophylactic activity o f extracts

Both the methanol extract of *E. camaldulensis* (leaf) and the aqueous extract of *A. senegalensis* (leaf) used to test for prophylaxis did not protect the animals from infection thus indicating no prophylactic activity against *T. b. brucei* infection. Parasites appeared in the blood almost at the same time with the control and death occurred at the same rate. The result is presented on fig.4.19.

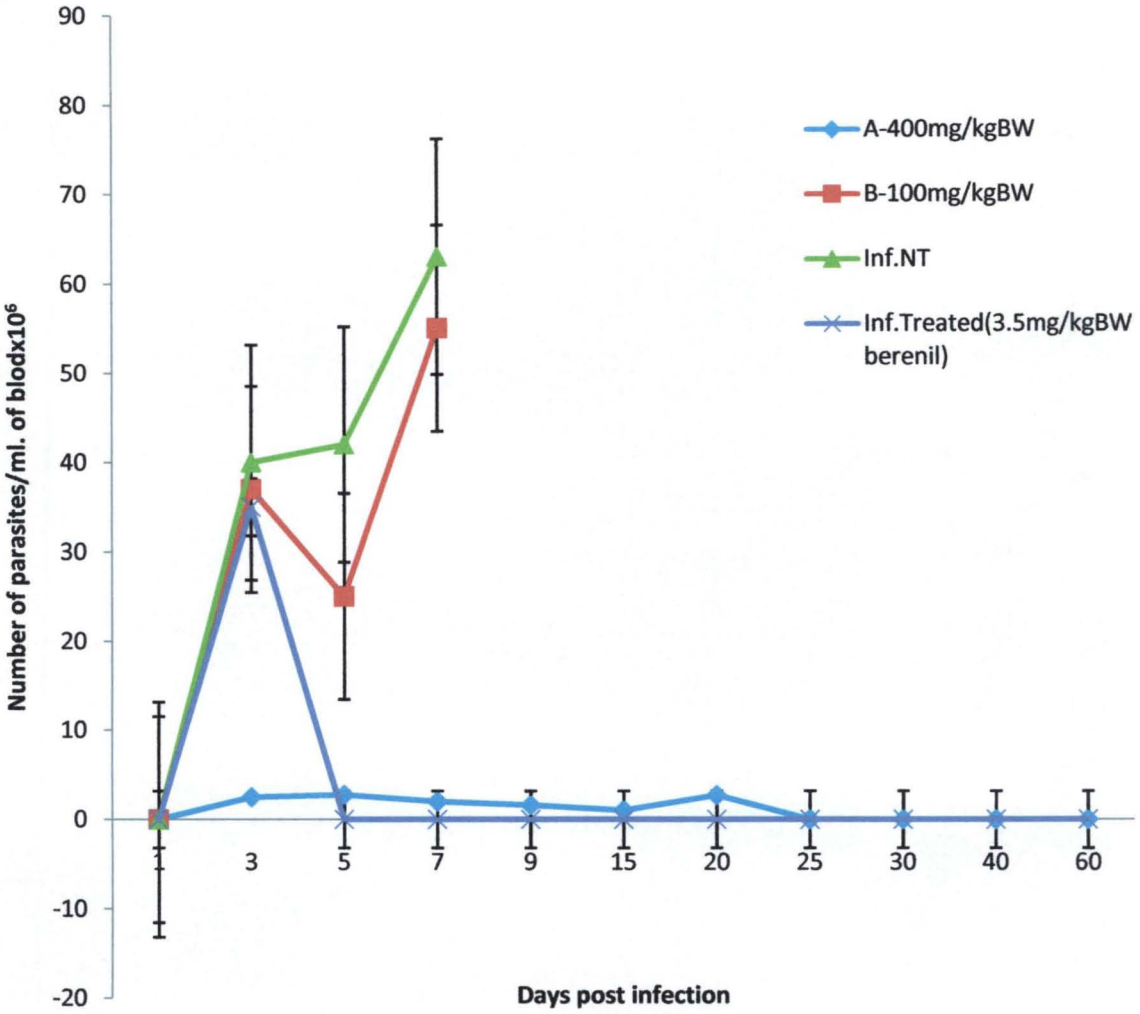


Fig. 4.15: Course of parasitemia in infected mice treated with hexane extract of *A. senegalensis* (stem bark) and berenil

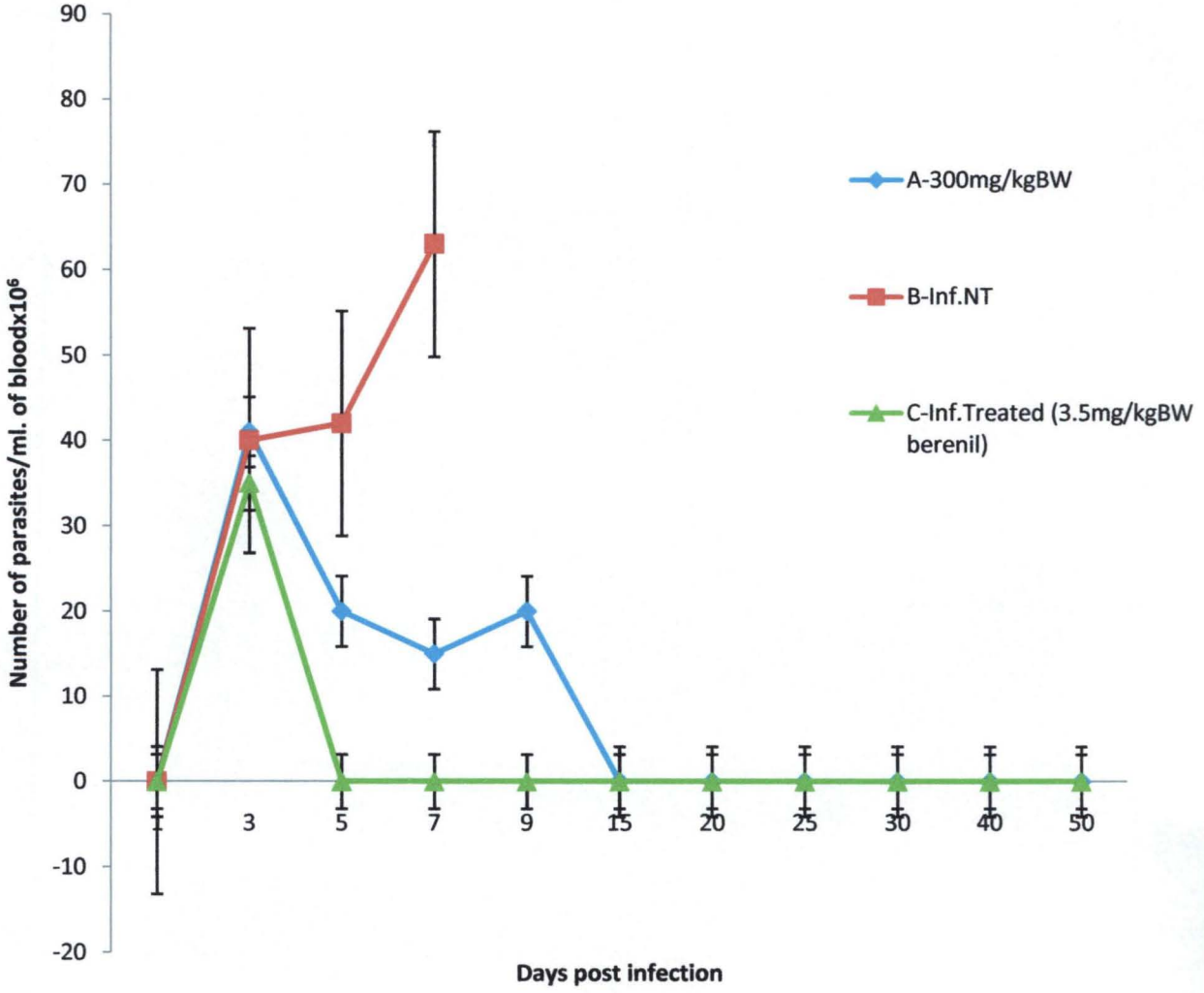


Fig. 4.16: Course of parasitemia in infected mice treated with aqueous extract of *A. senegalensis* (stem bark) and berenil

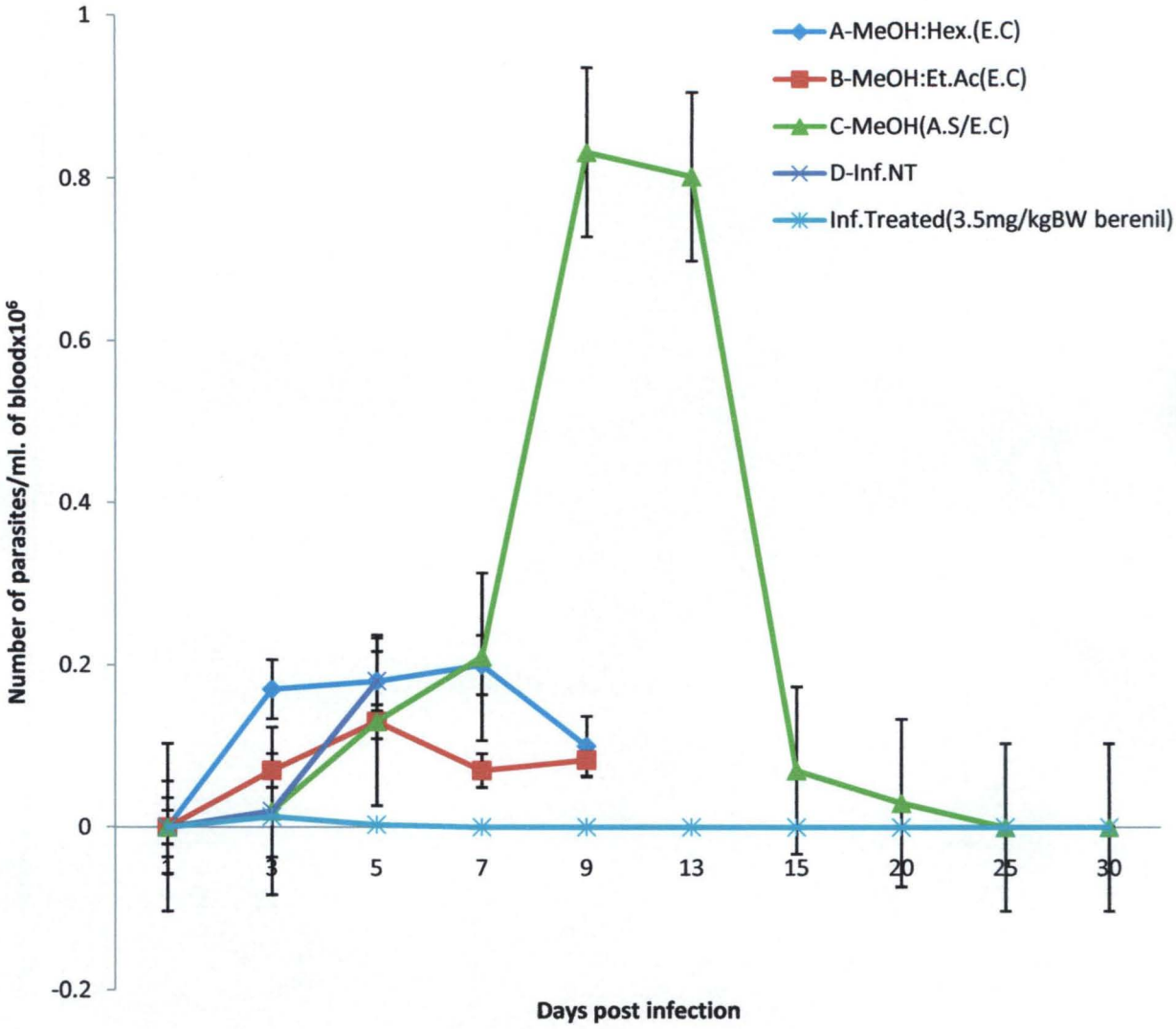


Fig. 4.17: Course of parasitemia in infected mice treated with combined therapy (200mg/kg bodyweight for three weeks at 1:1) of different extracts

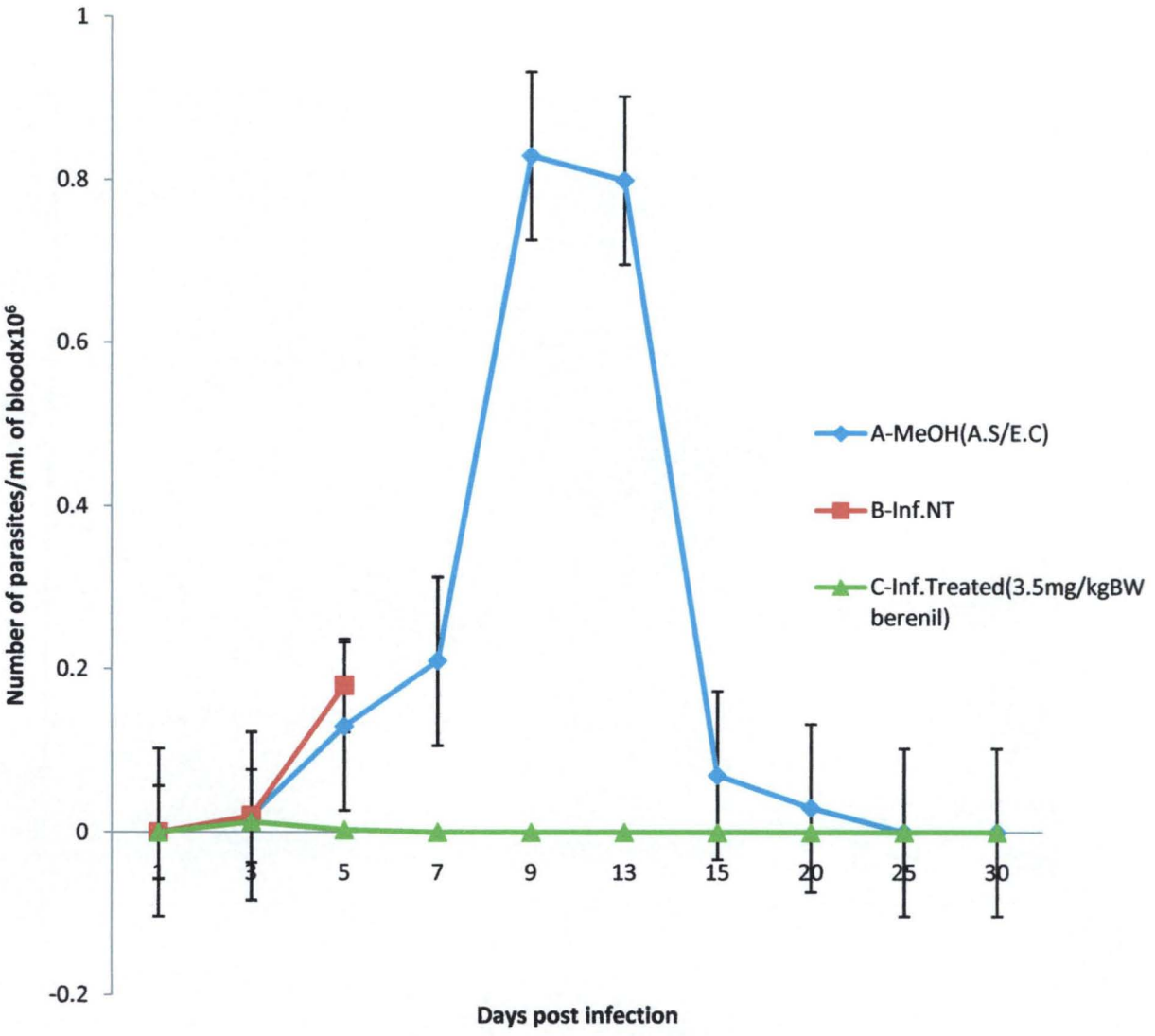


Fig. 4.18: Course of parasitemia in infected mice treated with a combination of *A. senegalensis* (A.S): *E. camaldulensis* (E.C) (1:1) compared with berenil.

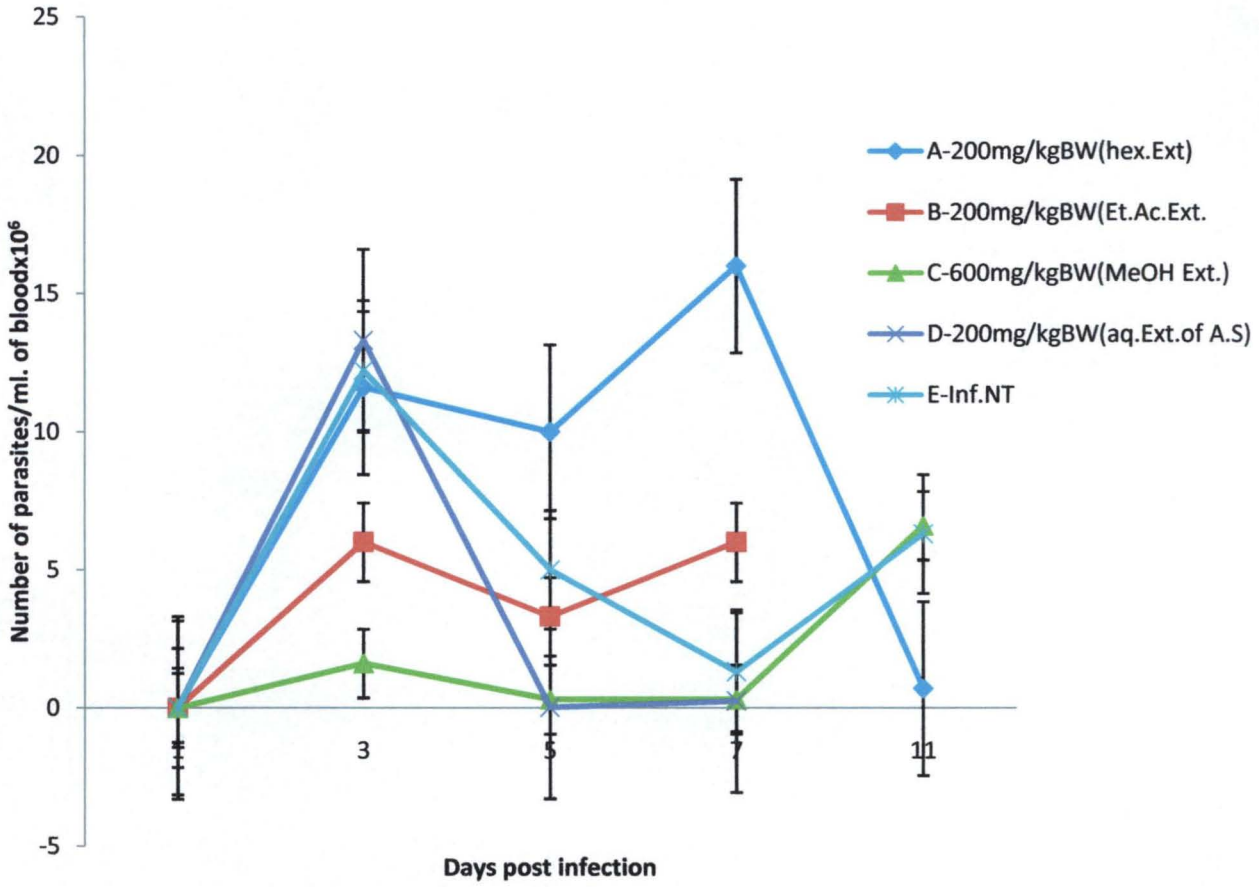


Fig. 4.19: Screening for prophylactic activity of selected extracts of *E. camaldulensis* (leaf) and *A. senegalensis* (A.S) leaf

4.12 Changes in weight and % PCV of infected mouse that survived after treating with combined therapy (Methanol extracts of A. senegalensis and E. camaldulensis) (1:1)

After treating for three weeks and the mouse surviving for more than 60 days, the terminal weight and % PCV were compared with the initial values as shown on fig.4.20. The weight increased by about 62.5%, from 25g to 40.45g. The %PCV on the other hand increased appreciably from 40 to 43 %. There was significant difference between the initial and final weights of the animal, but no significant difference between the % PCV values ($p \leq 0.05$).

4.13 Changes in the Weight and % PCV of infected mice treated with 200mg/kg bodyweight/day of methanol extract of E. camaldulensis (leaf) for three weeks

The mean values of weight and % PCV for the initial and terminal stages are shown on fig. 4.21. The mean weight increased from 26.4±5.0 to 34.63±7.0, while the % PCV increased from 42±5.0 to 44.33±3.0, indicating a significant difference between the initial and final weights of the animal, but no significant difference between the % PCV values ($p \leq 0.05$).

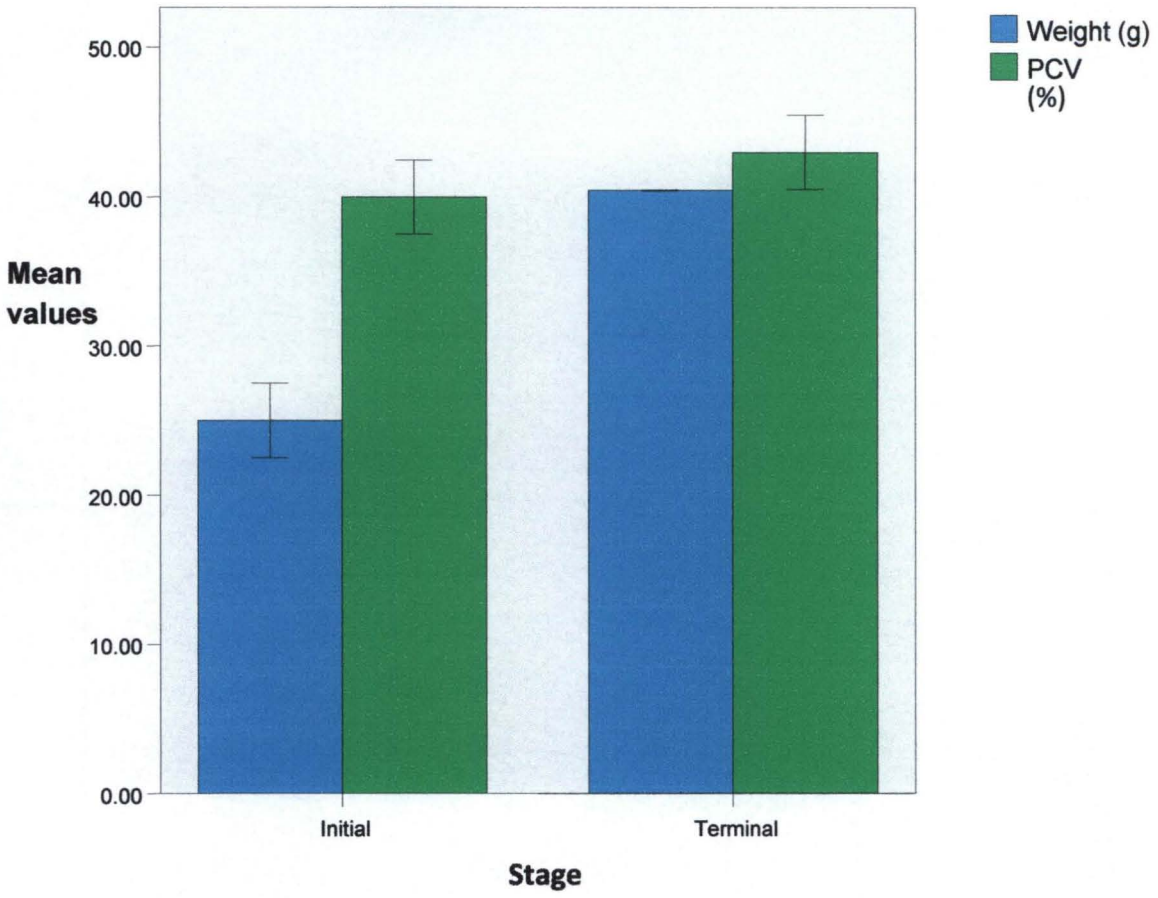


Fig.4.20: Initial and Terminal values for weight (g) and %PCV of infected mouse that survived combination therapy (methanol extracts of *E.camaldulensis* leaf and *A.senegalensis* leaf)

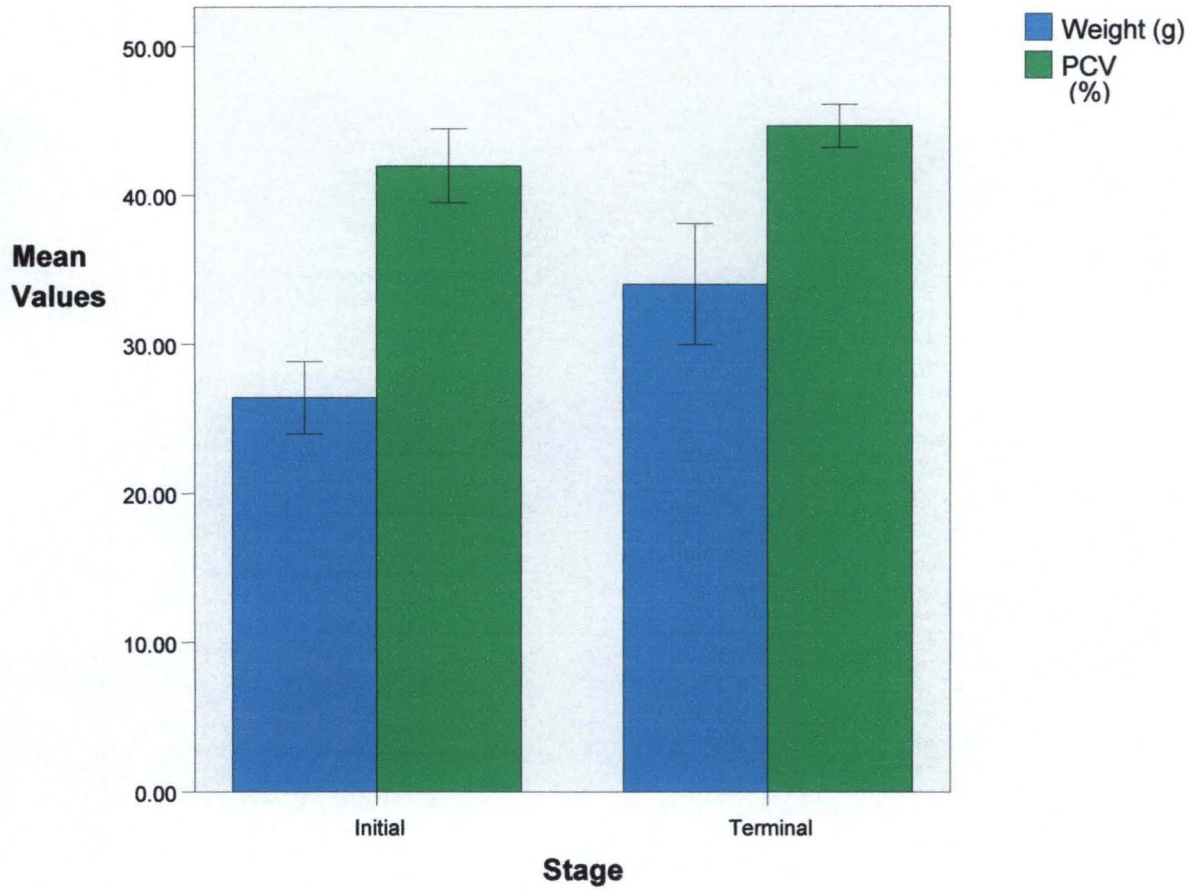


Fig.4.21: Initial and Terminal values of weights and %PCV for animals that survived after treating with 200mg/kg body weight of methanol extract *(E. camaldulensis)* for 3 weeks.

4.14 Bioassay-directed fractionation of crude methanol extract of *E. camaldulensis* (leaf)

Column chromatography of the crude methanol extract gave rise to ten (10) fractions which were tested for antitrypanosomal activity at 50, 100, and 150mg/kg bodyweight/day. Table 4.14.1 below represents the weights of the 10 fractions collected from column chromatography of the crude methanol extract.

Table.4.14.1: Weight of fractions obtained from column chromatography

Fraction	Weight (g)
1	0.218
2	0.288
3	0.528
4	0.628
5	0.718
6	0.378
7	0.348
8	0.638
9	3.588
10	18.078

4.15 Course of parasitemia in infected mice treated with fractions at 50, 100 and 150 mg/kg bodyweight/day

Treatment with fractions 1 - 10 at 50, 100 and 150 mg/kgbodyweight/day did not produce very outstanding results in terms of life elongation except for the two groups treated with fractions 8 and 9 at 150 mg/kg bodyweight/day, both of which extended the lifespan of animals by six (6) days, and within the treatment period parasitemia was reduced considerably, while the animals treated with the crude methanol extract of *E. camaldulensis* and the standard drug cleared parasites from circulation (fig. 4.22). Animals treated with the other fractions died on the same day as the untreated animals with relatively higher levels of parasitemia. There was no significant difference between the activities of the crude extract and the standard drug, but both were significantly different from the activities exhibited by the two fractions ($p \leq 0.05$).

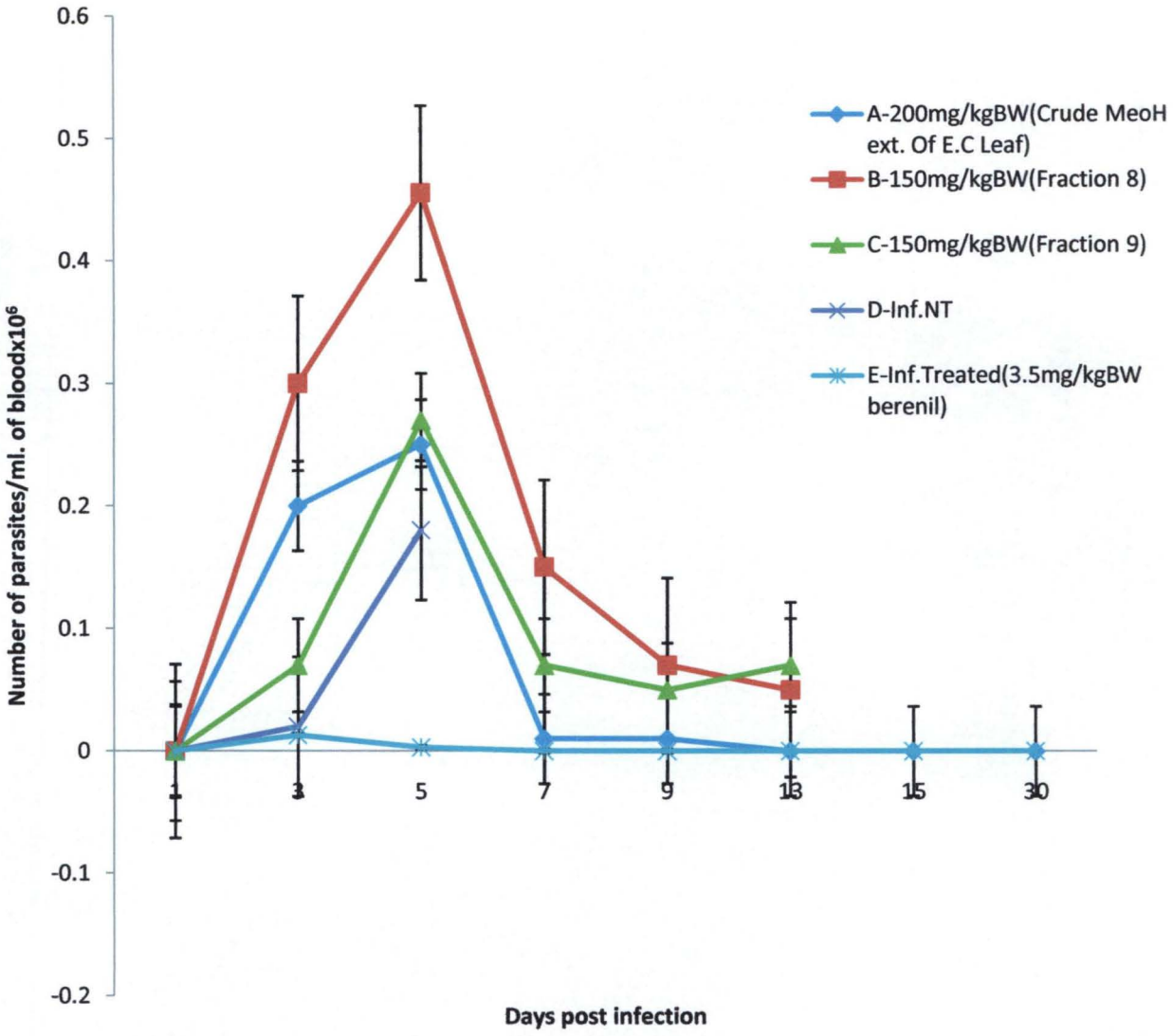


Fig.4.22: Course of parasitemia in infected mice treated with crude methanol extract of E.C, fractions 8, 9 and standard drug

4.16 Acute toxicity studies of the crude methanol extract of *E. camaldulensis* (leaf)

Table 4.16.1 shows the results of the effects of intraperitoneal administration of the crude methanol extract at concentrations ranging from 1000- 5000mg/kg bodyweight. As shown on the table, there was no death recorded in any of the dose groups, thus indicating the safety of the extract at this dose range. However there were visible signs of fatigue, shortness of breath, loss of appetite with consequent reduction in feeding (anorexia) and decreased activity in the first 24 hours. All these signs were reversed on the second day and the animals remained normal thereafter.

Table 4.16.1: Acute toxicity assay using mice

Groups	Dose (mg/kg bodyweight)	No. of dead/treated mice
Control	0.4ml of DMSO	0/3
1	1000	0/3
2	2000	0/3
3	3000	0/3
4	4000	0/3
5	5000	0/3

4.16.1 Changes in weights of animals in the acute toxicity assay groups

The initial and terminal weights of animals in the test and control groups were compared. The weights generally increased in all the groups after two weeks of monitoring, including the control group. There was a general increase in weights by about 8%. The initial weight range was between 25.55±5 to 30.50±4 and the terminal weight range was 27.43±2.5 to 32.23±5, an increase that was not significant ($P \leq 0.05$) as shown in Table 4.16.2.

4.16.2 Changes in percentage packed cell volume (%PCV) in the acute toxicity assay groups

Table 4.16.2 shows the changes in the percentage packed cell volume (%PCV) for animals administered acute doses of the crude methanol extract. The mean %PCV experienced a decrease in all groups except the one administered 5000mg/kg bodyweight and the control group that was given 0.4ml of physiological saline. The %PCV for the 5000mg/kg bodyweight group increased from an initial value of 35±20 to 37±25 while the control value increased from 40±10 to 42.3±10. There was no significant difference between the initial and final % PCV values ($P \leq 0.05$).

4.16.3 Changes in haemoglobin concentration (g/dl) in acute toxicity assay

The changes observed in the initial and terminal values of haemoglobin concentration are in the same proportion as observed for the %PCV. There was a decrease in haemoglobin concentration in groups A, B, C, and D, while E and F had an increase of about 8.8% from the initial to the terminal point (Table 4.16.2). There was no significant difference between the initial and final haemoglobin concentrations ($P \leq 0.05$).

4.16.4 Changes in Differential Leucocytes Counts

The changes observed in the values of basophils, eosinophils, and neutrophils are shown in Table 4.16.2. The mean percentages of the initial and terminal values experienced significant changes but the pattern was not consistent. The animals administered 1000mg/kg bodyweight had percentage basophils and eosinophils decreasing by 0.2% and 30% respectively, while that of neutrophils increased by about 30%. The groups administered with 5000mg/kg bodyweight also had the terminal percentages of basophils and eosinophils decreasing by about 2% and 30% respectively while the value for neutrophils increased by about 35%. The control group on the other hand experienced an increase in the percentage of basophils by about 37%, while the values for eosinophils and neutrophils fell by about 16% and 35% respectively.

Table 4.16.2: Changes observed in animals administered acute doses of crude methanol extract of *E. camaldulensis* (leaf)

GROUP		Weight (g)	%PCV	Hb Conc.	Basophils	Eosinophils	Neutrophils
A(1000mg/kgBW)	Initial	26.50±2.0	46.33±5.0	15.66±1.75	30.20±0.1	43.50±0.1	26.30±0.1
	Terminal	27.60±5.0	45.33±2.50	13.66±0.75	30.40±0.1	30.00±0.1	39.60±0.1
B(2000mg/kgBW)	Initial	27.80±5.0	43.66±12.0	14.86±4.0	40.30±0.1	32.30±0.1	28.40±0.1
	Terminal	30.25±5.0	43.33±8.0	14.20±2.70	43.80±0.1	31.20±0.1	25.00±0.1
C(3000mg/kgBW)	Initial	30.32±2.0	43.33±15.0	15.66±5.0	44.30±0.1	36.50±0.1	20.20±0.1
	Terminal	31.46±3.0	42.33±12.0	13.43±4.0	25.00±0.1	37.50±0.1	37.50±0.1
D(4000mg/kgBW)	Initial	29.40±6.0	44.33±10.0	15.76±3.50	33.30±0.1	43.40±0.1	23.30±0.1
	Terminal	31.85±6.0	43.33±15.0	14.10±5.0	38.00±0.1	26.00±0.1	36.00±0.1
E(5000mg/kgBW)	Initial	25.55±5.0	35.00±15.0	12.00±5.0	27.60±0.1	48.30±0.1	24.10±0.1
	Terminal	30.50±4.0	36.66±15	13.03±5.0	27.30±0.1	33.30±0.1	39.40±0.1
F(0.4ml saline)	Initial	27.43±2.50	40.00±5.0	13.33±1.75	24.10±0.1	48.30±0.1	27.60±0.1
	Terminal	32.23±5.0	42.33±5.0	14.10±1.5	40.50±0.1	38.00±0.1	20.50±0.1

Values are means of three (3) determinations ± SD

4.17 Sub chronic toxicity studies of crude methanol extract of *E. camaldulensis* (leaf)

The minimum curative dose of the extract (200mg/kg bodyweight) was administered to healthy Wister rats for three weeks, and thereafter monitored for another three weeks and then sacrificed to determine some parameters related to sub chronic toxicity in animals. The results obtained for the different parameters used are presented as follows:

4.17.1 Changes in the weights of animals

There was substantial increase in the mean weights of both the test and control groups as shown in Table 4.17.1. The mean weight of the test group increased from 206.06 ± 120 to 214.33 ± 125, while that of the control increased from 184.66 ± 23 to 188.66 ± 25.

4.17.2 Changes in the percentage packed cell volume (% PCV)

The percentage packed cell volume (% PCV) for both the test and control groups increased by about 2.5%. For the test group, the mean % PCV increased from 35.66 ± 10% to 36.66 ± 9.0% after four weeks. The control group had an increase from 39 ± 12 to 40 ± 7.5. This is presented in Table 4.17.1.

4.17.3 Changes in the haemoglobin concentration (g/dl)

The changes observed in the haemoglobin concentration of the test and control groups after four weeks of monitoring are shown in Table 4.17.1. The mean value for the test group increased from 11.88 ± 3.5 to 12.22 ± 4.0 while that of the control group increased from 12.99 ± 4.0 to 13.33 ± 2.5. The percentage increase was about 2.5%.

4.17.4 Changes in the differential leucocytes counts (%)

The differential leucocytes counts (%) (Basophils, eosinophils, and neutrophils) for the test and control groups were determined before the initiation of sub chronic dosage and at the point of termination after five weeks. The results are in Table 4.17.1. The mean % basophils for the test and control groups decreased from 49.64 ± 6.34 to 42.22 ± 3.13 and from 43.48 ± 1.5 to 41.45 ± 5.4. The mean % eosinophils also increased from 16.73 ± 2.55 to 23.89 ± 6.35(test group) and from 19.7 ± 2.94 to 22.62 ± 3.84 (control group). The mean % neutrophils decreased from 33.58 ± 3.95 to 32.19 ± 6.02 for the test group and from 36.8 ± 7.38 to 35.92 ± 6.23 for the control group.

4.17.5 Terminal organ bodyweight ratio

Table 4.17.1 contains values for the organ bodyweight ratios for both test and control animals in the sub chronic toxicity screening. The mean values for the control group are: 0.021 ± 0.002 (liver); .0053 ± 0.001 (kidney); and 0.0027 ± 0.00023 (heart), while the mean values for the test group are: 0.0243 ± 3.13 (liver); 0.0063 ± 0.0008 (kidney); and 0.0027 ± 0.0005 (heart). Only the organ bodyweight ratio for the kidney showed significant difference between the test and control groups.

4.17.6 Determination of liver enzymes activities

The activities of Alanine Amino Transferase (ALT) or Serum Glutamate-Pyruvate Transaminase (SGPT) and Aspartate AminoTransferase (AST) or Serum Glutamate – Oxaloacetate Transaminase (SGOT) are shown in Table 4.17.1 for both the control and test groups. There was no significant difference between the SGOT activities of animals in the control (24.89 ± 1.25) and test (24.22 ± 0.67) groups. The SGPT activities was however significantly different between the control (19.36 ± 2.12) and test (16.36 ± 2.44) groups. The activity was lower for the test group.

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4.17.7 Determination of kidney function parameters

The concentrations of electrolytes, creatinine, and urea for the control and test groups at the termination of six weeks of monitoring are presented in Table 4.17.1. The mean concentration of chloride (Cl^{-}) for the test group (101 ± 1.0 mmol/L) is not significantly different from the value for the control (101.33 ± 0.57 mmol/L). The mean values for potassium (K^{+}) for the test (5.36 ± 0.21 mmol/L) was not significantly different from that of the control (5.13 ± 0.12 mmol/L), while the concentration of sodium (Na^{+}) for the test (126 ± 5.29 mmol/L) was significantly different from the mean value for the control (133.67 ± 3.21 mmol/L). The bicarbonate concentration for the control (23.33 ± 0.58 mmol/L) and test (21.33 ± 2.08 mmol/L) groups were not significantly different ($p \leq 0.5$).

The levels of urea for the test (3.6 ± 0.6 mmol/L) and the control (3.7 ± 0.66 mmol/L); the creatinine concentrations for the test (92.33 ± 6.43 mmol/L) and control (99 ± 2.65 mmol/L) are not significantly different ($p \leq 0.5$).

Table 4.17.1: Changes observed in animals administered sub chronic dose of crude methanol extract of *E. camaldulensis* (leaf)

Parameter		Test group		Control group	
Weight (g)	Initial	206 ± 12.0	(167.0 – 256.0)	184.66 ± 23	(176.0 – 190.0
	Terminal	214.33 ± 12.0	(178.0 – 270.0)	188.66 ± 25	(178.0 – 190.0
%PCV	Initial	35.66 ± 10	(32.0 – 40.0)	39.0 ± 12	(35.0 – 44.0)
	Terminal	36.66 ± 9.0	(34.0 – 41.0)	40 ± 7.5	(37.0 – 43.0)
Hb conc.	Initial	11.80 ± 3.5	(10.0 – 13.0)	12.99 ± 4.0	(12.0 – 14.66)
	Terminal	12.22 ± 4.0	(11.0 – 13.0)	13.33 ± 3.5	(12.0 – 14.33)
Basophils	Initial	49.64 ± 6.34	(43.13 – 55.80)	43.48 ± 4.50	(38.77 – 47.82
	Terminal	42.32 ± 3.13	(38.63 – 44.40)	41.45 ± 5.40	(35.71 – 46.34
Eosinophils	Initial	16.73 ± 2.55	(14.70 – 19.60)	19.70 ± 2.94	(16.32 – 21.73
	Terminal	16.36 ± 2.44	(18.51 – 30.90)	22.62 ± 3.84	(19.51 – 26.92
Neutrophils	Initial	33.58 ± 3.95	(29.40 – 37.25)	36.80 ± 7.38	(30.43 – 44.89
	Terminal	32.19 ± 6.02	(25.45 – 37.04)	35.92 ± 6.23	(30.76 – 42.85
Organ BW ratio	Liver	0.0243 ± 0.03	(0.025 – 0.027)	0.021 ± 0.002	(0.019 – 0.022
	Kidney	0.0063 ± 0.0008	(0.0053–0.007)	0.0053 ± 0.001	(0.0046 - .006
	Heart	0.0027 ± 0.0005	(0.002 – 0.003)	0.0027±0.00023	(0.0026-0.00
Enzyme activity(IU/L)	ALT	16.36 ± 2.44	(13.50 - 17.80)	19.36 ± 2.12	(17.76 – 21.7
	AST	24.22 ± 0.67	(23.45 – 24.60)	24.89 ± 1.25	(23.45 – 25.7
Electrolyte Conc.	Na^+	126 ± 5.29	(120 -130)	133.67 ± 3.21	(130 – 136)
	K^+	5.36 ± 0.21	(5.20 – 5.60)	5.13 ± 0.12	(5.00 – 5.20)
	Cl^-	101 ± 1.0	(100 – 102)	101.33 ± 0.57	(101 – 102)
	HCO_3^-	21.33 ± 2.08	(19.00 – 23.00)	23.33 ± 0.58	(23.00 – 24.0
Creatinine Conc.		92.33 ±6.43	(85.00 – 97.00)	99.00 ± 2.65	(97.00 – 102)
Urea Conc.		3.60 ± 0.6	(3.00 – 4.20)	3.70 ± 0.66	(3.00 – 4.30)

Values are means of three determinations ± SD. Ranges of values are in parenthesis

4.18 Histopathological Studies of animal organs in the sub chronic toxicity, combined therapy and methanol extract treated groups

Plate I shows the micrograph of a normal rat liver from the control group. It shows a normal hepatic architecture, exhibiting maintenance of central vein and portal tract interphase. It also shows normal hepatic plates separated by sinusoids.

Plate II is a micrograph of the liver of *T. b. brucei* - infected mouse after treatment with 200mg/kg bodyweight of methanol extract of *E. camaldulensis* (leaf) for three weeks and surviving for three months. There were signs of hepatocytes' autolysis.

Plate III represents the micrograph of liver of a mouse treated with sub - chronic dose, 200mg/kg bodyweight) of crude methanol extract of *E. camaldulensis* (leaf) for three weeks. The hepatic architecture was normal, showing central vein and portal tract interphase with hepatic plates separated by sinusoids.

Plate IV represents the micrograph of liver of a *T. b. brucei*-infected mouse after treating with 200mg/kg bodyweight of combined therapy (methanol extracts of the leaves of *A. senegalensis* and *E. camaldulensis*) for three weeks and surviving for three months. There appeared to be oedema of the hepatocytes.

Plate V is a micrograph of a normal rat kidney from the control group. Normal glomerular apparatus and tubules were clearly visible

Plate VI shows the micrograph of rat kidney after administering a sub chronic dose of 200mg/kg bodyweight for three weeks. There were no visible signs of negative effect of the extract on the kidney because the glomerular apparatus and tubules were intact after six weeks of monitoring.

Plate VII represents the micrograph of kidney of *T. b. brucei* - infected mouse after treatment with 200mg/kg bodyweight of combined therapy (methanol extracts of the leaves of *A. senegalensis* and *E. camaldulensis*) for three weeks and surviving for 3

months. It showed widespread intra renal tubular necrosis with micro thrombi formation.

Plate VIII Micrograph of the kidney of a *T. b. brucei - infected* mouse treated with 200mg/kg bodyweight of methanol extract of *E. camaldulensis* (leaf) for 3 weeks. The glomeruli were intact but there appeared to be necrosis of the cortex about 3 months later.

Plate I: Micrograph of normal liver at X 100 (control group). There is normal hepatic architecture

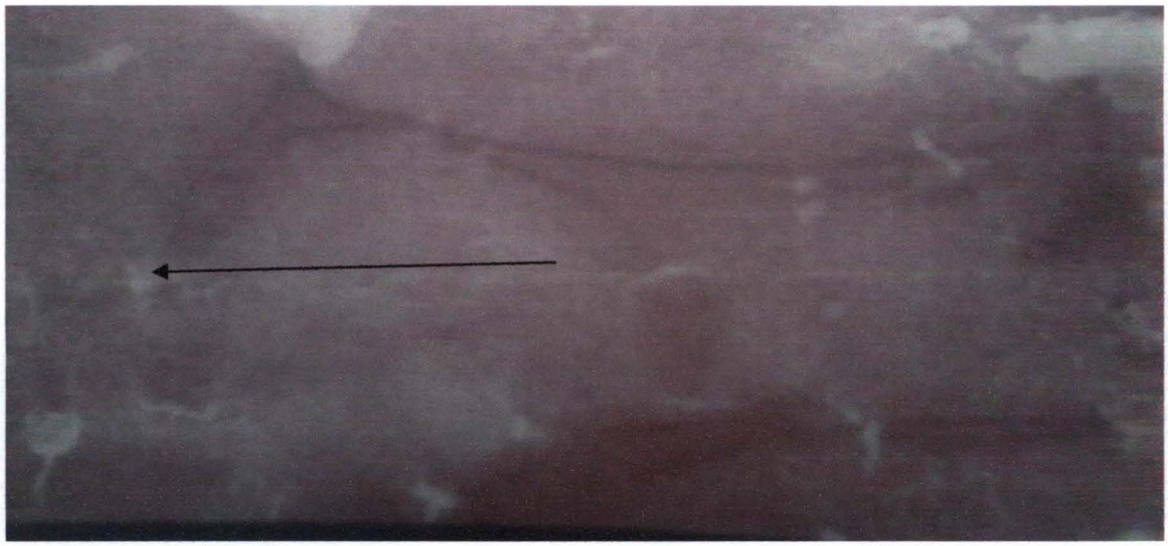


Plate II: Micrograph of Liver of *T. b. brucei* - infected mouse at X 100 treated with (200mg/kg bodyweight of methanol extract of *E. camaldulensis* leaf). There is autolysis of hepatocytes and cellular degeneration (arrow) (3 months later)

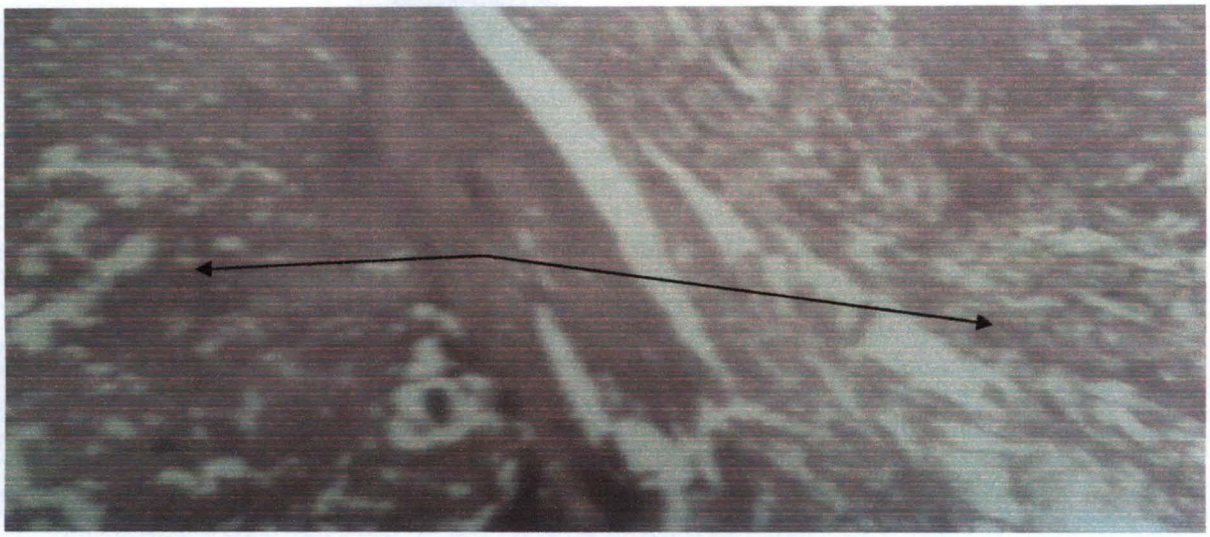


Plate V: Micrograph of normal kidney at X 100 from the control group. Normal glomerular apparatus and tubules are visible (arrows)

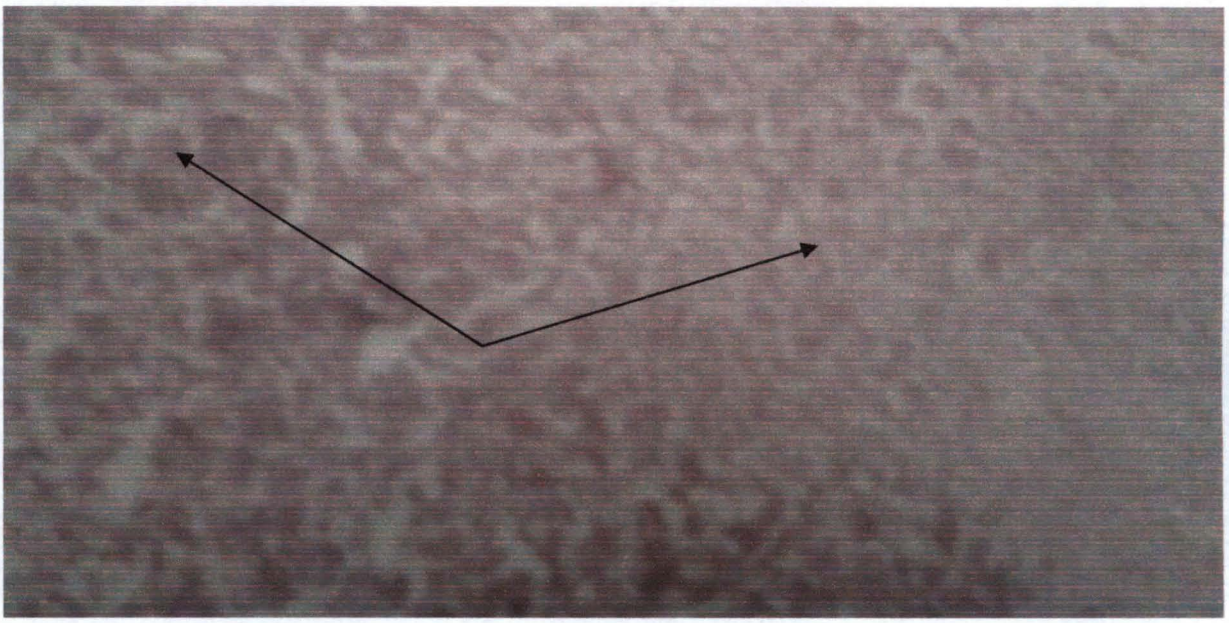


Plate VI: Micrograph of kidney at X 100 of mouse treated with sub chronic dose of 200mg/kg bodyweight of crude methanol extract of *E. camaldulensis* (leaf) for 3 weeks. Normal glomerular apparatus and tubules are intact (arrows).

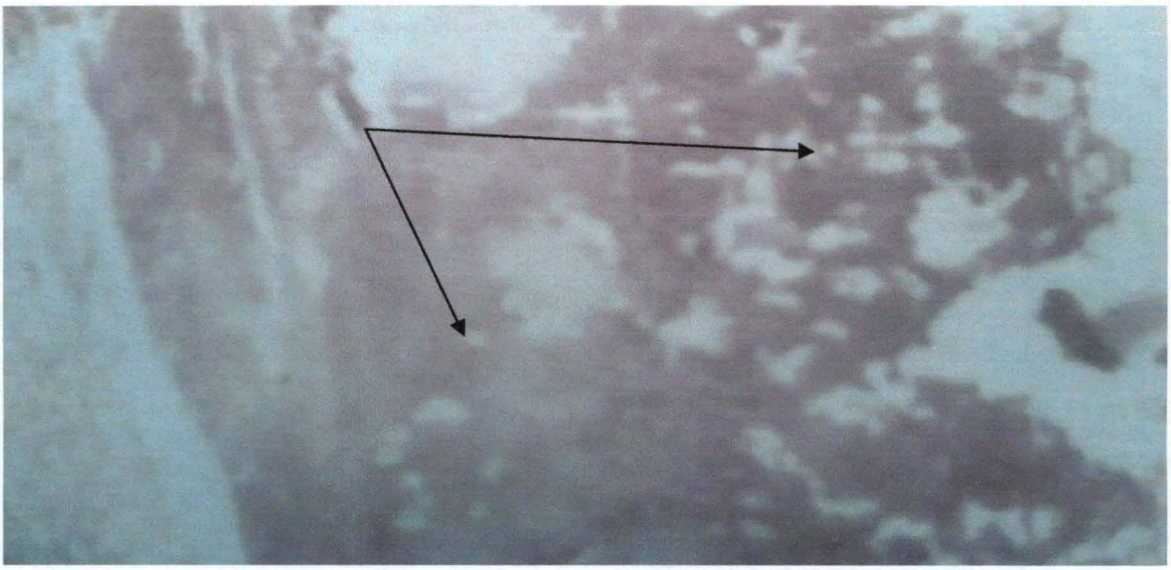


Plate VII: Micrograph of kidney of *T. b. brucei* - infected mouse at X 100 treated with combined therapy. There is intra renal tubular necrosis with micro thrombi formation (arrows), three (3) months later.

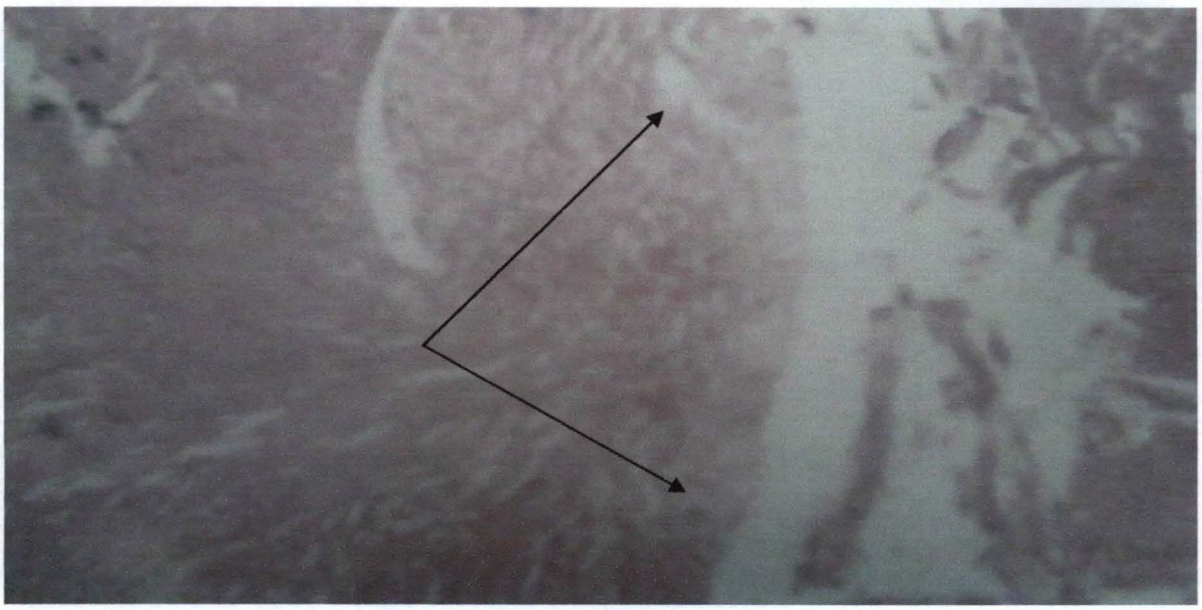


Plate VIII: Micrograph of the kidney of a *T. b. brucei* - infected mouse at X 100 treated with 200mg/kg bodyweight of methanol extract of *E. camaldulensis* (leaf) for 3 weeks). The glomeruli are intact but there appears to be necrosis of the cortex (arrow). Three (3) months later.

4.19 *In vitro* antibacterial activity of crude extracts of *E. camaldulensis* (leaf)

The antibacterial activities of the crude hexane, ethyl acetate, methanol and aqueous extracts of *E.camaldulensis* (leaf) were determined against three bacterial strains using a uniform concentration of 0.2mg/ml. It was only the methanol extract at this concentration that was most effective against *Staphylococcus aureus*, producing a zone of inhibition of 18mm (Plate IX), compared with its activity against *Klebsiella pneumoniae* (14mm) (Plate X), while there was no activity against *Salmonella typhi*. None of the other extracts inhibited the growth of any of the tested bacterial strains.

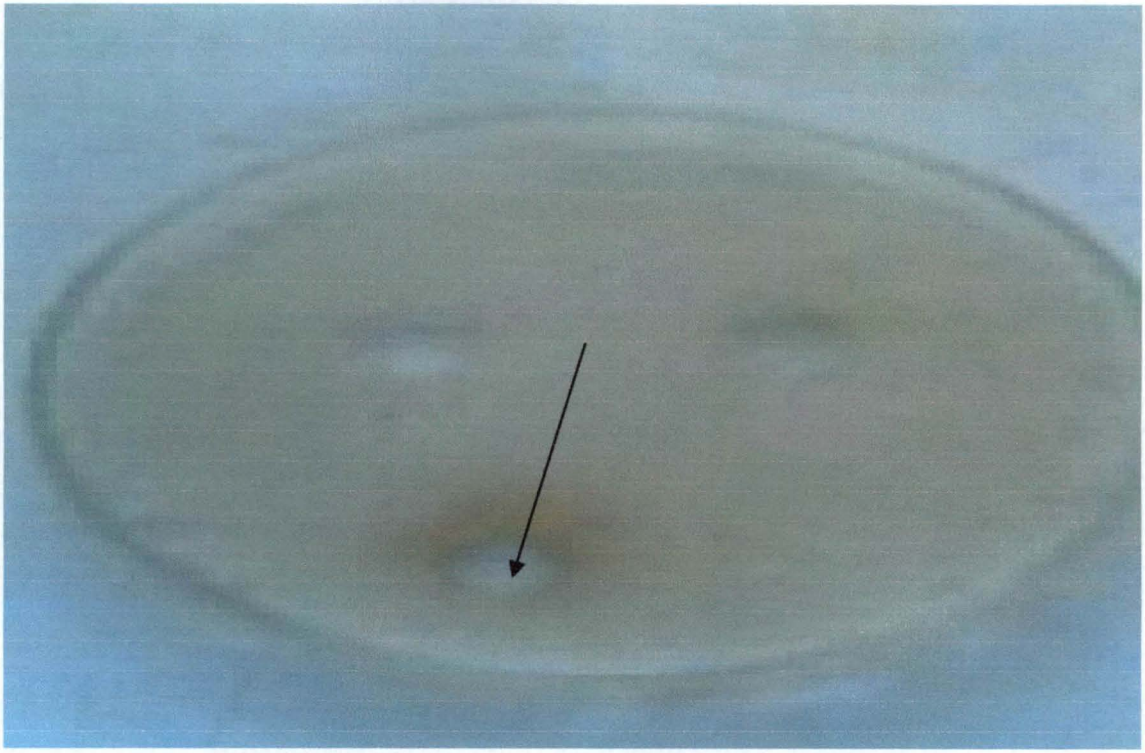


Plate IX: Inhibitory activity of methanol extract of *E. camaldulensis* (leaf) against *Staphylococcus aureus,* arrow indicates zone of inhibition (18mm)

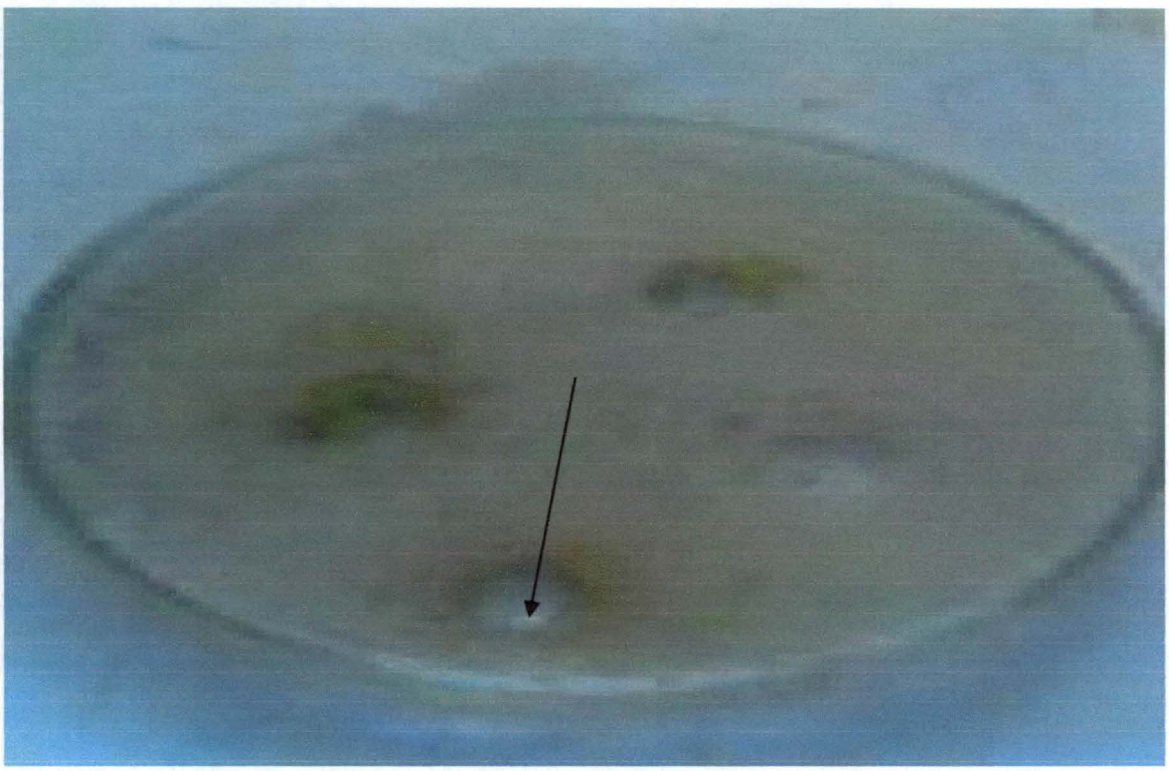


Plate X: Inhibitory activity of methanol extract of *E. camaldulensis* (leaf) against *Klebsiella pneumoniae* , arrow indicates zone of inhibition (14mm)

4.20 Phytochemical composition of crude methanol extract of *E. camaldulensis* (leaf)

Phytochemical analysis of the crude methanol extract of *E. camaldulensis* (leaf) revealed the presence of terpenes, steroids, saponins, tannins, alkaloids, and fatty acids while flavonoids and anthraquinones were absent as indicated in table 4.20.1 below:

Table 4.20.1: Phytochemical composition of crude methanol extract of *E. camaldulensis* (leaf)

Phytochemical	Indication
Terpenes	+ +
Steroids	+ +
Saponins	+ +
Flavonoids	–
Tannins	+
Alkaloids	+
Anthraquinones	–
Fatty acids	+ +

Notations:

++ Highly present

+ Fairly present

– Absent

4.21 Weight of fractions

Column chromatography of the crude methanol extract of E.camaldulensis (leaf) gave rise to 10 fractions of variable weights as shown in table 4.21.1 below.

Table 4.21.1: Weight of fractions obtained from column chromatography of crude methanol extract

Fraction	Weight (g)
1	0.218
2	0.288
3	0.528
4	0.628
5	0.718
6	0.378
7	0.348
8	0.638
9	3.588
10	18.078

4.22 Phytochemical composition of fractions 8 and 9

Phytochemical analysis of the fractions revealed the presence of terpenes, steroids and fatty acids in fraction 8, while fraction 9 contains terpenes, steroids, saponins, flavonoids, tannins and fatty acids. Alkaloids were absent in both fractions. The result is shown in table 4.22.1 below:

Table 4.22.1: Phytochemical constituents of fractions 8 and 9

Phytochemical	Fraction 8	Fraction 9
Terpenes	+	+ +
Steroids	+	+ +
Saponins	–	+
Flavonoids	–	+
Tannins	–	+
Alkaloids	–	–
Anthraquinones	–	–
Fatty acids	+ +	+ +

Notations:

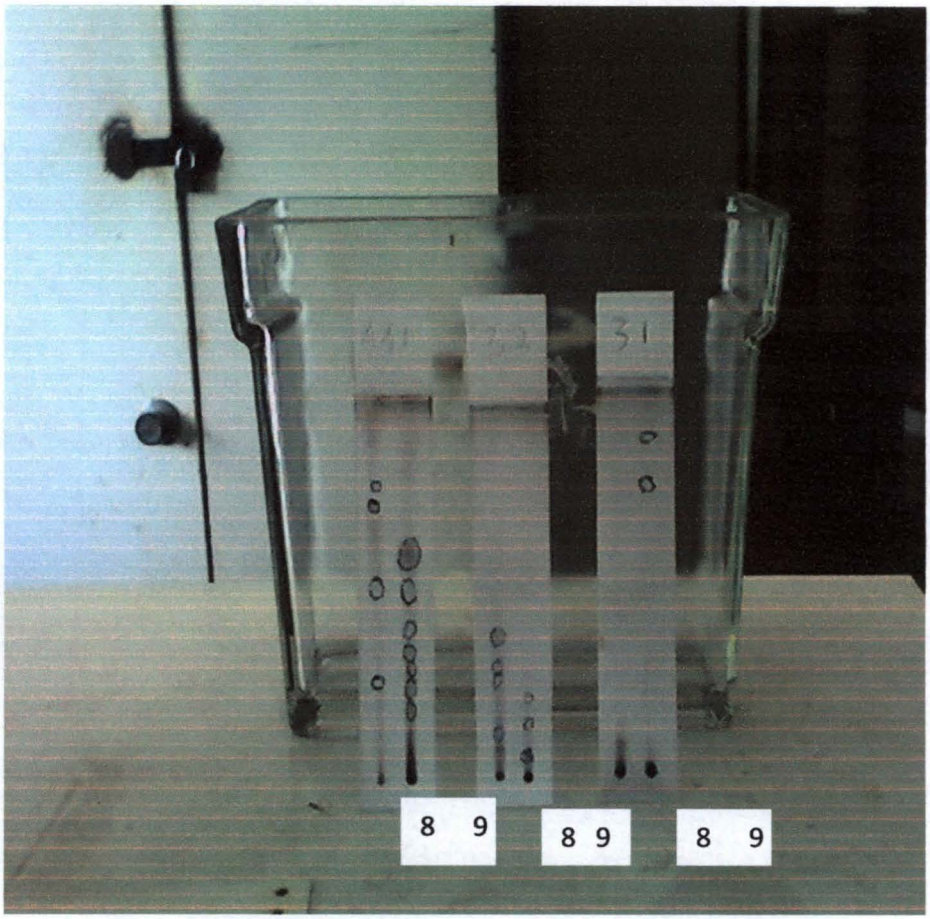
++ Highly present

+ Fairly present

– Absent

4.23 Thin layer chromatography of fractions 8 and 9

The best visualization of the components of the fractions was obtained with the 4:4:1 solvent system; the spots were located in iodine tank and sprayed with vanillin in Sulphuric acid. Fig. 4.23 shows that fraction 8 contains about 5 - 6 components while fraction 9 contains 6 – 7 components.



Solvent systems used

Hex:	Et.Ac	:	Met.
4	: 4	:	1
3	: 2	:	0
3	: 1	:	0

Hex. -------Hexane

Et.Ac.------Ethyl acetate

Met.--------Methanol

Fig.4.23: Thin layer plates in different systems, spots located in iodine tank and sprayed with vanillin in sulphuric acid, showing separation of fractions 8 and 9

4.24 Gas Chromatography – Mass Spectroscopy (GC-MS) Analysis of fraction 8

Analysis of fraction 8 using GC-MS gave rise to six peaks that were representative of six compounds as shown on fig. 4.24. Based on the spectroscopic data obtained from GC-MS analysis, the relative intensities, selectivity index and the peak values of components, and deductions made in accordance with the database of the National Institute of Science and Technology (NIST) library, the following compounds/structures were identified for fraction 8: 2-Chloro-N-(1, 3-thiozol-2-yl) acetamide (an amide) (fig.4.25) , 9-Octadecenamide (amide) (fig. 4.26), 1-Nonadecene (long chain alkene) (fig.4.27), (Z)-9-Eicosene (long chain alkene) (fig.4.28), Hexadecanol (alcoholl) (fig.4.29), and 1-Pentadecanol (alcohol) (fig.4.30).

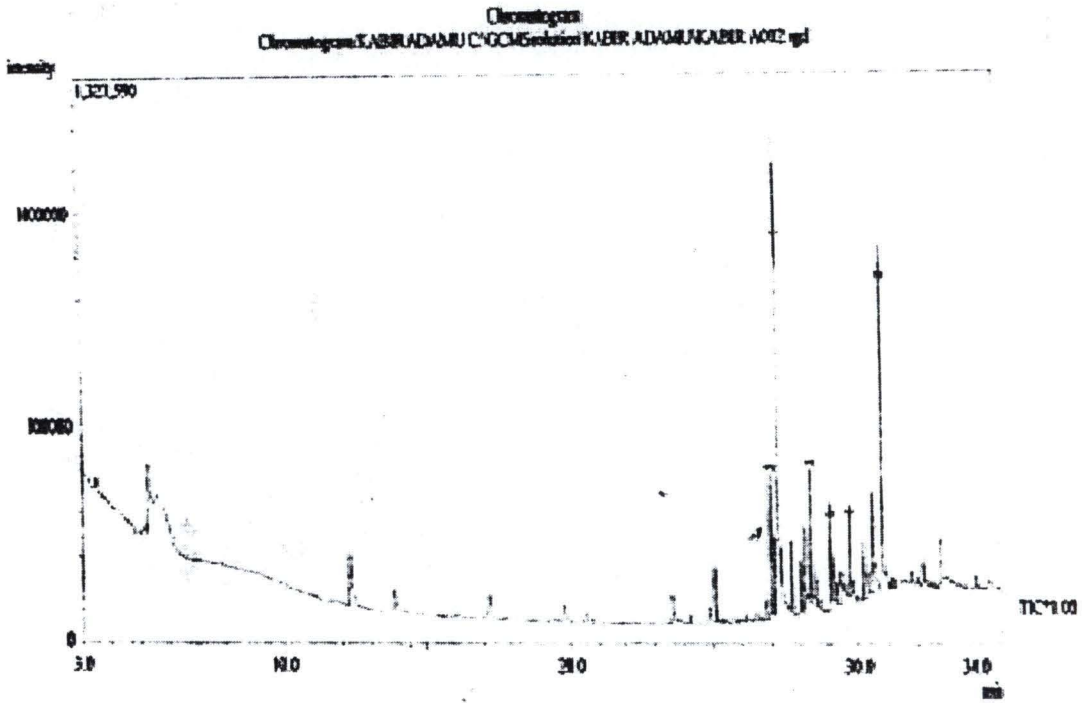


Fig . 4.24: Chromatogram of fraction 8

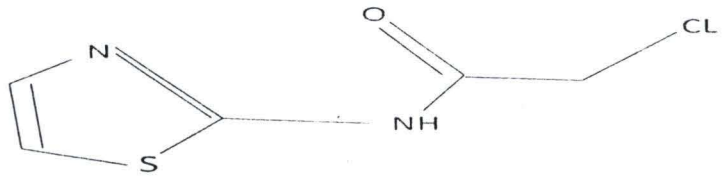


Fig. 4.25 2-Chloro-N-(1, 3-thiozol-2-yl) acetamide (Line 1)
CAS No.5448-49-7
Molecular Formula: $C_5H_5ClN_2OS$
Formula Weight: 176
Ret. Index: 1557

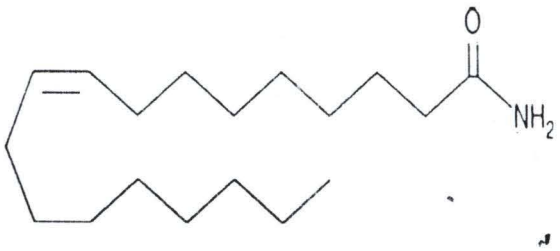


Fig. 4.26 9 -Octadecenamide (Line 2)

Common name: Oleamide (CAS.No: 301-02-0)
Molecular formula: $C_{18}H_{35}NO$
Molecular weight : 281
Ret. Index: 2228

Fig. 4.27: 1-NONADECENE (Line 3)

CAS No.	18435-45-5
Molecular Formula:	$C_{19}H_{38}$
Molecular Weight:	266
Ret. Index :	1900

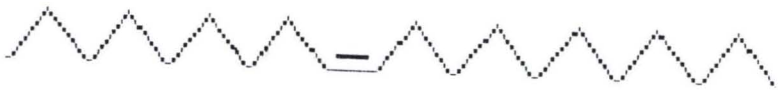


Fig. 4.28: (Z)-9-Eicosene (Line 4)
CAS.No. :74685-29-3
Molecular formula: $C_{20}H_{40}$
Molecular weight: 280
Ret. Index :2017

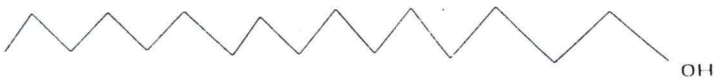


Fig.4.29 : Hexadecanol (Line 5)

CAS . No.	36653-82-4
Molecular formula :	$C_{16}H_{34}O$
Molar formula :	242
Ret. Index:	1854

Fig. 4.30 : 1-PENTADECANOL (Line 6)

4.25 Gas Chromatography – Mass Spectroscopy (GC-MS) Analysis of fraction 9

Analysis of fraction 9 using GC-MS gave rise to six peaks that were representative of six compounds as shown on fig. 4.31. Based on the spectroscopic data from GC-MS, the relative intensities, selectivity index and the peak values of components, and deductions made from the National Institute of Science and Technology (NIST) library, the following compounds/structures were identified for fraction 9: Methyl hexadecanoate (ester) (fig.4.32), Methyl-cis-9- octadecenoate (ester) (fig.4.33), Methyl-n-octadecanoate (ester) (fig.4.34) and 1-Heptadecanol (alcohol) (fig.4.35). The fifth component made an unusual presentation and therefore could not be identified.

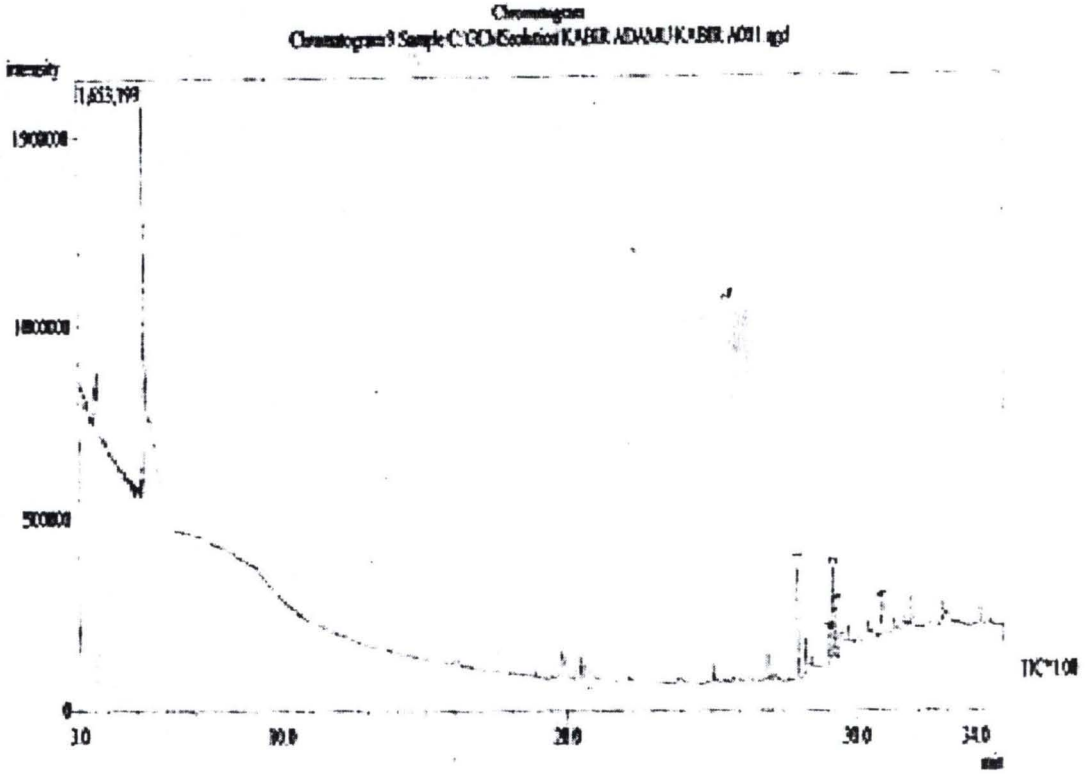


Fig .4.31 : Chromatogram of fraction 9

Fig.4.32: Methyl hexadecanoate (Line 1)

CAS. No.	112-39-0
Molecular formula:	$C_{17}H_{34}O_2$
Molecular Weight:	270
Ret. Index:	1878

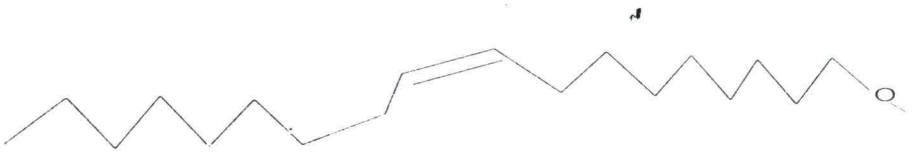


Fig. 4.33: Methyl cis-9- octadecenoate (Line 2)

CAS. No.	112-62-9
Molecular formula:	$C_{19}H_{36}O_2$
Molecular weight :	296
Ret. Index :	2085

CHAPTER FIVE

5.0 DISCUSSION OF RESULTS, SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION OF RESULTS

In this study with *E. camaldulensis*, a plant that have been identified with several several folkloric values, the crude methanol extract of the leaf exhibited very promising activity against *Trypanosoma brucei brucei*. The extract when administered consecutively for three weeks completely cleared parasites from circulation and all animals in the different dose groups survived thereafter for more than three months until they were used for further studies (fig.4.3). The remaining eleven extracts; consisting of hexane , ethyl acetate and aqueous extracts of the leaf (fig.4.1, 4.2 and 4.4); hexane, ethyl acetate, methanol extracts of the stem and root barks (fig.4.5 – 4.10) exhibited varying degrees of antitrypanosomal activities that were not significant ($P \leq 0.05$) when weighed against the performance of the methanol extract of the leaf. However, it is interesting to note that the hexane and ethyl acetate extracts of the leaf in the preliminary screening for antitrypanosomal activity produced results that are worth re-visiting because both extracts at some dose levels cleared parasites from circulation but the survival rate was less than 50%.

The crude methanol extract of *E. camaldulensis* (leaf) cleared parasites completely from circulation only when it was administered 24 hours after infection, but did not clear parasites completely when treatment was delayed beyond 24 hours although some activity was recorded (fig.4.14). The partially purified fractions (fractions 8 and 9) that exhibited minimal antitrypanosomal activities did not completely clear parasites from circulation using the same treatment regime (fig.4.22). There are three possible inferences that can be deduced from these observations. First,

the crude extract is only active at the initial phase of infection; secondly, fractionation of the crude extract must have resulted in a partial loss of activity, and thirdly there is the possibility of synergistic action between the bioactive components of the crude extract since the fractions, when administered individually, were not as active as the crude extract. It has been observed before now that the active components of herbal remedies have the advantage of being combined with many other substances that appear to be inactive. However, these complementary components give the plant as a whole a safety and efficiency much superior to that of its isolated and pure active components (Shariff, 2001). The importance of these observations are that: first, the crude extract can be standardised and packaged as phytomedicine, just the way it is done in traditional medical practice. Secondly, the partially purified fractions or their components, though not as active as the crude extract could be subjected to chemical modification to potentiate their antitrypanosomal activities.

An overview of the identified components in fraction 8 shows that 1-Nonadecene and (Z) - 9- Eicosene (fig.4.27 and 4.28) are sex pheromones in some insects, where they serve as attractants for males (El-Sayed, 2009); hexadecanol (fig.4.29), as a commercial product is used as a cosmetic ingredient, while excessive exposure to 1- pentadecanol (fig.4.30) produces some central nervous system depression and prolonged contact produces skin irritation. It is not clear what role they could be playing in the antitrypanosomal activity of the crude methanol extract of *E. camaldulensis*. The case of the other two components, 2-Chloro-N-(1, 3-thiozol-2-yl) acetamide (fig.4.25) and 9-Octadecenamide (fig.4.26) is different because evidence from literature shows that some acetanamides are potent inhibitors of histone deacetylase (HDAC), an enzyme responsible for the deacetylation of N-acetyl lysine residues of histone and non-histone proteins (James and Henry, 2006). And there is

growing evidence that the acetylation state of proteins and thus the HDAC enzyme plays a crucial role in the modulation of a number of biological processes, including transcription and cell cycle, which is the basis for their use in the treatment of cancer (Marks *et al.*, 2000). On the basis of this, we may infer that the amides in fraction 8 could be playing the role of inhibitors of certain enzyme (s) unknown to us, but because their concentration in the fraction was small, they elicited a minimal effect on the trypanosomes.

Another aspect of the present study that could be exploited is the treatment of trypanosomiasis by administration of methylating agents. Some methylating agents have been found to be effective against *T. rhodesiense* and *T. gambiense*, which cause fatal diseases in man, and also against *T. brucei*, *T. evansi* and *T. equiperdum*, which are of veterinary importance (Hoare, 1967). That study concerned a method of treating trypanosomiasis in patients, e.g., warm-blooded animals, such as humans, horses, sheep, goats, swine, camels or cattle, by administering to such patients a trypanocidal effective amount of a methylating agent of the formula CH_3 N=NX', wherein X' is a leaving group, e.g., OH or SO_2 R''' and R''' an alkyl or an aryl, more particularly an unsubstituted or substituted alkyl having 1 to 10, preferably 1 to 6, carbon atoms or an unsubstituted or substituted aryl, including other species capable of generating methyl radicals ($CH_3^{\bullet}$), diazomethane (CH_2 N_2) or methyldiazonium (CH_3 N_2^{+}) (Shyam *et al.*, 1987). Some methylating agents for use as trypanocidals have been described by Shyam *et al.*, (1987).

Interestingly, fraction 9 contains compounds like Methyl hexadecanoate (fig.4.32), Methyl-cis-9-octadecanoate (fig.4.33) and Methyl-n-octadecanoate (fig.4.34), which might be likely methylating agents that could play positive roles in the therapy of African trypanosomiasis. Methyl-cis-9-octadecenoate was first identified in the

cerebrospinal fluid of sleep-deprived cats, rats and humans. It induced physiological sleep when injected into rats intraperitoneally at 5-50mg doses (Cravatt *et al.*, 1995). Methylating agents appear to have two major effects on trypanosomes, depending upon the dose level. At high levels, cytokinesis appears to be inhibited almost immediately and the cells are transformed into transitional forms containing multiple nuclei and kinetoplasts. These cells disappear from the bloodstream in 48 to 72 hours. When administered at repetitive low doses, methylating agents induce the entire population to differentiate into short-stumpy forms (short-stumpy forms cannot differentiate further unless they are taken up by a feeding tsetse fly or placed in appropriate culture conditions), as judged by morphology, NADH diaphorase positivity and other biochemical and physiological criteria. Short-stumpy forms are non-dividing differentiated cells and are not infective to the mammalian host. The latter property may make these agents useful biochemical tools in the study of differentiation in trypanosomes, since, with these compounds, it is possible to induce the entire population of trypanosomes to differentiate in a moderately synchronous manner and through this approach early events in the differentiation process can be studied. Both single high dose regiments and repetitive low doses can result in cures of trypanosomiasis using a number of the methylating agents (Shyam *et al.*, 1987).

Difluoro Methyl Ornithine (DFMO) or eflornithine has also been shown to induce differentiation in *T. brucei* (Giffin *et al.*, 1986). This effect is generally attributed to the depletion of polyamines. DFMO, however, also causes a 1000-fold increase in decarboxylated S-adenosylmethionine (DSAM) and S-adenosylmethionine (SAM) (Fairlamb *et al.*, 1983). These latter metabolites are weak chemical methylating agents and, therefore, may be in part responsible for the differentiating action of DFMO. The depletion of polyamines and trypanothione as a result of the DFMO treatment may

potentiate the actions of SAM and DSAM as methylating agents by decreasing the levels of competing nucleophiles. Depletion of polyamines may also make the nucleic acids more susceptible to methylation (Fairlamb *et al.*, 1983). SAM is also the methyl donor used by many methylases; therefore, enzymatically – mediated – methylation reactions may also be affected.

Although methylating agents in general are mutagenic, in cases of multi-drug resistant trypanosomiasis which have failed to respond to existing therapies, these compounds may be extremely effective. The distinct advantages of methylating agents over existing trypanocides include (a) high therapeutic indices, (b) oral activity, (c) novel mechanism of action, (d) broad spectrum antitrypanosomal activity, and (e) favourable pharmacokinetics which make these compounds candidates for both agricultural and clinical development. The fourth component of fraction 9; 1-heptadecanol is used commercially as an additive to feed and food products. From the foregoing, it may be deduced that the minimal antitrypanosomal activity exhibited by fraction 9 is due to the presence of three potential methyl donors and the relatively low concentration of these agents prevented complete clearance of parasites from circulation.

There has been no previous report on the *in vivo* antitrypanosomal activity of *E. camaldulensis* except that of Ogbadoyi *et al.*, (2007b), in which the aqueous extract of the leaf, was found to prolong the lifespan of *T. b. brucei* - infected mice by 40 days when treatment was effected for three weeks consecutively. That study did not go beyond the preliminary stage, but our study has demonstrated the ability of the methanol extract to produce a better and more promising result.

The efficacy of `methanol extracts of several medicinal plants obtained from some African and Asian countries against *T.b.brucei* and other strains, have been

demonstrated *in vitro* (Kiuchi *et al.*, 1998; Atawodi *et al.*, 2003; 2005; Kamanzi *et al.*, 2004; Van Baren et *al.*, 2006; Mesia *et al.*, 2007; Essam *et al.*, 2009; Nibret *et al.*, 2009). The results obtained in these studies tend to lay credence to the fact that methanol as an extracting solvent, has the ability to extract phytochemicals that exhibit strong antitrypanosomal profile, thus making methanol a good extracting solvent for plants that are reported to have antitrypanosomal activity.

The antitrypanosomal activity exhibited by the methanol extract of *E. camaldulensis* was very obvious because mice in the control group that were infected but not treated presented with massive parasitemia culminating in death less than three weeks post infection (fig.4.3). And for the cured mice, the initial fall observed in the mean weights and % PCV was reversed two weeks into treatment (fig.4.21), thus indicating the ability of the extract to halt anaemia and weight loss associated with trypanosomiasis. However, the fact that histopathological studies of the livers and kidneys of treated animals' organs indicated that there was autolysis of hepatocytes (plate II), while the glomeruli of the kidney remained intact (plate VIII), virtually unaffected by the extracts. However the appearance of necrosis of the cortex is a cause for concern. The efficacy of any therapeutic regimen has to be weighed against the side effects observed over time, but we are convinced that the advantages derivable from using this extract to treat African Trypanosomiasis far outweigh the disadvantages. Some of these advantages include the cheapness and availability of the plant source, and the fact that the extract was not fatal even at an dose of 5000mg/kg bodyweight (Table 4.16.1), although there were visible signs of fatigue, shortness of breath, loss of appetite with consequent reduction in feeding (anorexia) and decreased activity in the first 24 hours; however these signs were reversed on the second day and the animals remained normal thereafter. The extract had no adverse effect on the weights of the animals

because they resumed normal feeding after 24 hours and the weight increased with time but the % PCV and haemoglobin concentration of the animals except for those administered the highest dose of 5000mg/kg bodyweight experienced a decrease. The differential leucocytes counts, expressed as % basophils, % neutrophils and % eosinophils for all animals experienced changes that were consistent with the normal pattern when animals are exposed to external agents like drugs and medicinal plant extracts (Table 4.16.2).

Neutrophils, also known as Granulocytes or, segmented neutrophils, are the main defender of the body against infection and antigens. High levels may indicate an active infection; a low count may indicate a compromised immune system or depressed bone marrow (low neutrophil production). The normal adult range is between 48 – 73%. Eosinophils are used by the body to protect against allergic reactions and parasites. Therefore, elevated levels may indicate an allergic response. A low count is normal. The normal adult range is 0 – 5%. Basophilic activity is not fully understood but it is known to carry histamine, heparin and serotonin. High levels are found in allergic reactions, low levels are normal. The normal adult range is 0 – 2%.

One of the problems associated with some trypanocidal drugs presently in use is long treatment protocol that is excruciating to patients. An attempt to reduce the treatment period in this study to two weeks did not produce a result that was similar to the three weeks treatment protocol (fig.4.12), because only the animals treated with the highest dose of 600mg/kg bodyweight/per day survived for 30 days with significant number of parasites in circulation, while animals in the lower dose groups died earlier. This observation indicates that, for complete clearance of parasites and lifespan prolongation to be achieved, treatment of infected animals with the crude methanol extract of *E.camaldulensis* (leaf) has to be sustained for three weeks. But the fact that

the group treated with the highest survived for thirty days is encouraging because the possibility exists that a refinement of the crude could result in obtaining lower effective doses. It is clear from the result of this study that the treatment protocol for medicinal plants' extracts depend on the plant type, extracting solvent, route of administration, phytochemical composition and degree of efficacy because reports of previous *in vivo* studies have demonstrated different treatment protocols for attaining the desired antitrypanosomal activity from different plants (Igwe and Onabanjo, 1989, Nok *et al.*, 1993, Ogbadoyi *et al.*, 2007a).

Conventionally, any product with therapeutic value should have a minimum curative dose (MCD) in order to enable the establishment of a therapeutic index for such product. The minimum curative dose for the crude methanol extract of *E. camaldulensis* (leaf) was found to be 200mg/kg bodyweight/day. At this dose level the extract was well tolerated and produced rapid clearance of parasitemia (fig.4.13). Dosages below this value did not clear parasites completely from the circulation of infected animals. A similar curative dose was reported for the aqueous extract of *A. senegalensis* (leaf) by Ogbadoyi *et al.*, (2007a). However, lower minimum curative doses have been reported (Igwe and Onabanjo, 1989; Nok *et al.*, 1993). A low minimum curative dose for any drug or therapeutic regimen is most desirable in order to avoid cases of drug overload especially when we are dealing with medicinal plant extracts. In Nigeria, the practice among traditional medical practitioners is to decoct the plant parts at boiling temperature and administer orally to patients without much regard to the quantity. Therefore, the effort in this study to establish a therapeutic index was worthwhile, but there is room for improvement.

The antibacterial activity of the methanol extract of *E. camaldulensis* (leaf) has been demonstrated (Babayi *et al.*, 2004; Ayepola *et al.*, 2008), but in our own study, we

widened the horizon of the screening by including hexane and ethyl acetate extracts. Previous reports of antibacterial activity were further verified, because only the crude methanol extract inhibited the growth of *Staphylococcus aureus* (gram positive bacteria) (Plate IX) and *Klebsiella pneumoniae* (gram negative bacteria) (Plate X), while *Salmonella typhi* was resistant. It is noteworthy that the extract exhibited an appreciable measure of inhibitory activity against *Klebsiella pneumoniae,* an organism that causes pneumonia. Gram-negative organisms are considered to be more resistant to treatment due to the lipoprotein content of their outer membrane which acts as a barrier to many environmental substances, including antibiotics. Though the level of activity recorded for the methanol extract of *E. camaldulensis* against some of the test organisms may appear to be low, this can be attributed to the fact that the extract being in crude form, contains very small amounts of bioactive compounds. At the same time, the values are rational because several workers have reported bioactivity of crude extracts of medicinal plants within such ranges of zones of inhibitions (Olukoya *et al.*, 1993; Ilori *et al.,* 1996; Ogbeche *et al.*, 1997; Akinyemi *et al.*, 2005). The hexane and ethyl acetate extracts were resisted by the test organisms.

Contrary to the synthetic drugs, antimicrobials of plant origin are not associated with many side effects and have an enormous therapeutic potential to heal many infectious diseases and natural products of higher plants may give a new source of antimicrobial agents with possibly novel mechanisms of action (Hamil *et al.*, 2003; Barbour *et al.*, 2004).

A major problem besetting the chemotherapy of African Trypanosomiasis is parasite resistance to the few available drugs (De Koning, 2001). Drug combinations can potentially avert or delay the emergence of drug-resistant organisms. Dosage reductions of each drug combined may reduce the overall toxicity while maintaining

good efficacy (Gerardo *et al.*, 2007). An attempt was made in this study to explore the potentials of combination therapy using extracts of *Annona senegalensis* (leaf) and *Eucalyptus camaldulensis* (leaf) in different combinations to treat *T.b.brucei*-infected mice. The only combination that cleared parasites from the circulation of infected mice was the combination of the methanol extracts of the leaves of the two plants (fig.4.17). Though the survival rate was not very high, the mere fact that one animal in the treated group survived after treatment is an interesting development because this is the first reported screening for antitrypanosomal activity using combination of extracts from these two plants or in fact any other combination of plant extracts.

We strongly believe that this combination therapy project can be carried forward bearing in mind the fact that the practice among traditional medicine practitioners in Nigeria is to combine an array of medicinal plants for the treatment of single or multiple diseases and in most cases these combinations have produced positive results. What this screening has succeeded in doing is to establish the possibility of synergistic action in the treatment of diseases using animal models and this will provide a basis for further studies using combinations of extracts from different plants. Preliminary studies using the two extracts separately showed that the methanol extract of *A. senegalensis* (leaf) considerably halted the severe anaemia associated with the African Trypanosomiasis as demonstrated by a relatively stable %PCV throughout the course of infection, while the methanol extract of *E. camaldulensis* (leaf) cleared parasites from the circulation of infected mice. The attempt made in this screening to explore synergy between the two extracts and the result obtained gave an indication of this possibility. It was also observed in this study that treatment with this combination resulted in a tremendous increase in the weight and an appreciable rise in percentage packed cell volume (%PCV) of the animal (fig.4.20). These observations give credence

to the ability of the combination to reverse the two principal symptoms of African trypanosomiasis namely, loss of weight and anaemia. Although the histopathological profile of the liver and kidney of the animal showed widespread intra renal tubular necrosis with micro thrombi formation (plate VII) and oedema of the hepatocytes (plate IV), there is room for improvement and possible perfection of the protocol with a view to preventing these undesirable side effects.

The liver is the principal organ that is capable of converting drugs into forms that can be readily eliminated from the body. A broad spectrum of adverse drug's effects on liver functions and structures has been documented. The reactions range from mild and transient changes in the results of liver function tests to complete liver failure with death of the host. Many drugs may affect the liver adversely in more than one way.

Elevated liver enzymes indicate inflammation or damage to cells in the liver. Inflamed or injured liver cells may leak higher than normal amounts of certain chemicals, including liver enzymes, into the bloodstream, resulting in elevated liver enzymes on blood tests. The specific elevated liver enzymes most commonly found are: Alanine transaminase (ALT) and Aspartate transaminase (AST). In most cases, liver enzyme levels are elevated mildly and temporarily. Most of the time, elevated liver enzymes don't signal a chronic, serious liver problem (Green, 2002).

Results from sub chronic toxicity studies of the crude methanol extract of *E.camaldulensis* (leaf) indicate that the liver and kidney of test animals were spared from damage as could be inferred from the measured activities of Alanine Transaminase and Aspartate Amino Transaminase; and the concentrations of urea, creatinine, chlorine, sodium, potassium and bicarbonate (Table 4.17). We observed that there was a significant difference between ALT activities in the control and test groups. The enzyme activity was lower for the test group that was administered the crude extract.

This observation apparently indicates a likely damage to the liver that ultimately affected the enzyme by lowering its activity but the results obtained from the histopathological studies of the liver did not substantiate this observation because there was no observable hepatocyte damage on the slides because the normal hepatic architecture showing central vein and portal tract interphase with hepatic plates separated by sinusoids was maintained (plate III). On the other hand, the activities of AST in the control and test groups were not significantly different.

The insignificant differences between the concentrations of Na+, Cl-, HCO3-, K+, Urea and Creatinine for the test and control groups indicates that the kidney was spared from any damage and so maintained its integrity as confirmed by the result of the histopathological studies which revealed that there were no visible signs of effect of the extract on the kidney because the glomerular apparatus and tubules were intact (plate VI). Uric acid is the end product of purine metabolism and is normally excreted through the urine. High levels are noted in gout, infections, kidney disease, alcoholism, high protein diets, and with toxaemia in pregnancy. Low levels may be indicative of kidney disease, malabsorption, poor diet, liver damage or an overly acid kidney.

It could be inferred from these observations that the liver and kidney damage seen in the infected and cured mice is not due to the extracts but rather due to parasite infiltration of the tissues and organs. On a general perspective, the organ bodyweight ratios of the test animals were consistent with the fluctuations observed in the initial and terminal weights of the animals in each group. It was observed that the mean weight, % packed cell volume, haemoglobin concentration generally increased with time, while the differential leucocytes counts fluctuated. The organ bodyweight ratio as indicated in Table 4.17.1 was only found to be significantly different for the kidney, the organ of excretion, indicating a likely negative effect of the extract on the kidneys of the test

animals, although this was not confirmed by the histopathological and enzymatic studies.

In the evaluation of *A. senegalensis*, the hexane and aqueous extracts of the stem bark were found to possess antitrypanosomal activities. Both preliminary and subsequent experiments with these extracts demonstrated their antitrypanosomal activities at 400mg/kg for hexane extract and 300mg/kg bodyweight/per day for the aqueous extract (fig.4.15 and 4.16). This was evidenced by the ability of the extracts to prolong the lifespan of the infected animals beyond two months and the inability of their blood and CSF inoculums to produce infection in healthy mice which demonstrates the likelihood of the extracts crossing the blood brain barrier in case of a chronic infection although these was not established. Different parts of *A. senegalensis* have been reported to possess trypanocidal activities (Ogbadoyi *et al.*, 2007a; Igwe and Onabanjo, 1987; Freiburghaus *et.al.*, 1997). It is interesting to note that we have demonstrated the antitrypanosomal activity of the hexane and aqueous extracts of the stem bark, contrary to the result obtained by Ogbadoyi *et al.*, (2007a), in which the stem bark extracts were found to be inactive against *T.b.brucei*. This is not surprising because the efficacy of medicinal plants in part depends on factors like season and time or period of harvest, and the location of the plant (Atawodi *et al.*, 2005).

Furthermore, what we have demonstrated in terms of the efficacy of the stem bark extracts that different solvent extracts of the same plant may exhibit different antitrypanosomal activity, just as extracts of different parts of the same plant (Freiburghaus *et.al.*, 1997). Therefore, the statement that a plant extract is trypanocidal or not should be taken in the context of the solvent used, the part investigated, the season of harvest and the geographical location of the plant (Atawodi *et al.*, 2003).

Pre-treatment of healthy mice with some potentially active crude extracts of both *A.senegalensis* and *E.camaldulensis* did not confer protection on infected mice (fig.4.19). All animals in the test groups experienced a continuous rise in parasitemia to the point of death; a trend that was not different from that of the control group. It was evident from the result obtained that the extracts used do not possess prophylactic activity. Similar observations were made by Igwe and Onabanjo, 1989; Ogbadoyi *et al.*, 2007a when the curative doses of *A.senegalensis* (root) and *A.senegalensis* (leaf) respectively were administered on healthy mice before infection. The mice were not protected by pre-treatment with the extracts.

The results obtained in this study give credence to the acclaimed therapeutic potency of *E. camaldulensis* (leaf) as an anaesthetic, antiseptic, astringent, and as a folk remedy for colds, colic, coughs, diarrhoea, dysentery, haemorrhage, laryngalgia, laryngitis, pharyngitis, sore throat, spasm, trachalgia, and wounds. The leaves are used in some local communities for the treatment of feverish conditions (undocumented).

In Nigeria, different parts of the *Annona* plant are used locally to treat ailments ranging from "fever" and sexually transmitted diseases (Ogbadoyi *et al.*, 2007a), to cancer (Gbile and Adesina,1985). The ripe fruit is a delicacy among the Hausas of Northern Nigeria. The demonstration of the antitrypanosomal activity of the stem bark is therefore encouraging and the need to exploit the potentials in the two plants, to the fullest, cannot be over emphasized bearing in mind the global interest in the sourcing of trypanocides from natural products (Nok *et.al.*, 1993).

In addition; these results form a good basis for selection of the plants for further phytochemical and pharmacological investigation and possibly the formulation of new drugs for the therapy of parasitic diseases.

5.2 SUMMARY AND CONCLUSIONS

Five medicinal plants, namely *Annona senegalensis* (Gwandan daji), *Eucalyptus camaldulensis* (Itchen turare), *Enanthia chloranta* (Dokita agbo), *Allium sativum* (Tafarnuwa), *and Adansonia digitata* (Kuka); that are widely used locally for the treatments of various illnesses were evaluated for antitrypanosomal activities. The leaves; stem and root bark; whole root; and the bulbs of these plants were extracted with hexane, ethyl acetate, methanol, ethanol, and water to obtain a total of twenty seven (27) extracts. The extracts were screened for activity against trypanosomes using *T.b.brucei*-infected mice and their efficacy was judged on their ability to clear parasites from the blood of infected and treated mice and prolong their life beyond that of the untreated mice.

Only three (3) of the extracts screened exhibited profound antitrypanosomal activities. These were: the crude methanol extract of *E.camaldulensis* (leaf); hexane and aqueous extracts of the *A.senegalensis* (stem bark).

Acute and sub chronic toxicity studies showed that the crude methanol extract was safe because no death was recorded even at an acute dose of 5000mg/kg bodyweight and a sub chronic dose of 200mg/kg bodyweight did not affect the normal architecture of the liver and kidney cells.

Bioassay-guided fractionation of the crude methanol extract showed that only fractions 8 and 9 out of the 10 fractions obtained exhibited minimal antitrypanosomal activities that were not as effective as the crude extract in terms of time taken to clear parasites from circulation and the extent to which they prolonged the life of treated mice.

Gas chromatography-Mass spectroscopy analysis of the two minimally active fractions revealed the presence of a mixture of methyl esters, esters, long-chain alkenes

and alcohols. Based on the results obtained in this study, it can be concluded that the work has succeeded in:

(i) obtaining extracts in the forms of crude methanol extract of *E. camaldulensis* (leaf), hexane and aqueous extracts of *A. senegalensis* (stem bark) capable of managing experimental African animal trypanosomiasis

(ii) Identifying compounds which are potentially useful lead compounds that could form the basis for further work towards the synthesis of modern pharmaceuticals.

5.3 RECOMMENDATIONS

We recommend that further studies be carried out on the identified active extracts with a view to screening them for activity against other sub species of *T. brucei* and *T. congolense* and other protozoans e.g. *plasmodium.* It is also worthwhile to recommend the exploration of the potentials in combination therapy using medicinal plants whose efficacies have been verified experimentally. This will go a long way in helping us understand the wisdom behind the practice among Traditional Medicine practitioners who combine different plants to treat various ailments.

The prevalence of a variety of climatic conditions puts Nigeria in a comfortable position with respect to richness of medicinal flora. As such, Nigeria should occupy a significant position in the world trade of botanical drugs. Nigeria should focus on agro technology, process technology, standardization, quality control, research and development of herbal drugs. It is time come to compile, document and coordinate available knowledge on our valuable plant resources and to prove their utility scientifically through detailed phytochemical, biological and pharmacological investigations.

REFERENCES

Agranoff, D., Stich, A., Abel, P., Krishna, S. (2005). Proteomic Fingerprinting for the Diagnosis of Human African Trypanosomiasis. *Trends in Parasitology.*, **21:**154-157.

Akinyemi, K. O., . Oluwa, O. K ., Omomigbehin, E. O.(2006). Antimicrobial activity of crude extracts of three medicinal plants used in South-West Nigerian folk medicine on some food borne bacterial pathogens. *African Journal of Traditional, Complementary and Alternative Medicines.*, **3** (4): 13-22

Akinyemi, K. O., Bayagbon, C., Oyefolu, A. O. B., Akinside, K. A., Omonigbeyin, E. A., Coker, A. O. (2000). Antibacterial screening of five indigenous Nigerian medicinal plants against *S. typhi* and *S. paratyphi*. *Journal of Nigerian Infection Control Association.*, **3** (1): 30-33.

Atawodi, S.E., Bulus, T., Ibrahim, S., Ameh, D.A., Nok, A.J., Mamman, M. and Galadima, M. (2003). *In vitro* trypanocidal effect of methanolic extract of some Nigerian savannah plants. *African Journal of Biotechnology.*, **2** (9): 317-321

Atawodi, S.E. (2005). Comparative *in vitro* trypanocidal activities of petroleum ether, chloroform, methanol and aqueous extracts of some Nigerian savannah plants. *African Journal of Biotechnology.*, **4** (2): 171-182

Apak, L. and Olila, D. (2006). The *in-vitro* antibacterial activity of *Annona senegalensis, Securidacca longipendiculata* and *Steganotaenia araliacea* - Ugandan medicinal plants. *African Health Science.*, **6** (1): 31–35.

Augustyns , K., Amssoms, K., Yamani, A., Rajan, P. K and Haemers, A.(2001). Trypanothione as a target in the design of antitrypanosomal and antileishmanial agent *Current Pharmarceutical Des.*, **7** (12):1117-41.

Ayepola, O.O and Adeniyi, B.A. (2008). The antibacterial activity of leaf extracts of *Eucalyptus camaldulensis* (Myrtaceae). *Journal of Applied Sciences Research.*, **4** (11): 1410-1413.

Babayi, H., Kolo, I., Okogun, J. I. and Ijah, U. J. J. (2004). The antimicrobial activities of methanolic extracts of *Eucalyptus camaldulensis* and *Terminalia catappa* against some pathogenic microorganisms. *Biokemistri.*, **16** (2):106-111

Bacchi, C. J. (1989). Inhibition of polyamine metabolism. *Proceedings of a workshop* held at ILARAD, Nairobi, Kenya. p. 17-24.

Bacchi, C. J., Garofolo, J., Mockenhaupt, D., McCann, P. P., Diekema, K., Pegg, A. E., Nathan, H.C., Mullaney, E. A., Chunosoff, L., Sjoerdsm, A and Hunter, S. H (1983). *Molecular and Biochemical Parasitology.*, **7:** 209-225.

Bacchi, C. J and McCann, P. P (1987). Inhibition of polyamine metabolism. New York: Academic Press, p .317-344.

Baker, B. M., Westerhoff, H.V., and Michels, P. A.M. (1995). Regulation and Control of compartmentalized glycolysis in bloodstream form of *Trypanosoma brucei. Journal of Bioenergetics and Bio membranes.*, **27**: 513-525.

Barbour, A.G and Restrepo, B.I (2000). "Antigenic variation in vector-borne pathogens". *Emerging Infect Diseases.*, **6** (5): 449–57.

Barret, M. P. (1999). The fall and rise of sleeping sickness. *Lancet* **353**: 1113- 4.

Barrett, M.P, Burchmore, R.J, and Stich, A (2003). "The trypanosomiasis". *Lancet.*, **362** (9394): 1469–80.

Barry, J.D, and McCulloch, R (2001). "Antigenic variation in trypanosomes: enhanced phenotypic variation in a eukaryotic parasite". *Advanced Parasitology.*, **49**: 1–70.

Batish, D. R; Singh, H. P; Kohli, R. K and Kaur, S. (2008). Eucalyptus essential oil as a natural pesticide http//www.eucalypt.oil.

Berriman, M., Ghedin, E and Hertz-Fowler, C. (2005). 'The genome of the African Trypanosome, *Trypanosoma brucei. Science.*, **309** (5733):416-22

Bhattacharya, S.K., Sur, D., Sinha, P.K., Karbwang, J. (2006). Elimination of leishmaniasis (kala-azar) from the Indian subcontinent is technically feasible and operationally achievable. *Indian Journal Medical Research.*, **123:**195-196.

Bitonti, A. J., Dumont, J. A. and McCann, P. P. (1986). Recent developments in the chemotheraphy of African trypanosomiasis. *Biochemical Journal.*, **237**: 685-689.

Bodeker, G. (1998). The therapeutic potential of plants used as traditionally as antimalarials: New directions of research. In: Health in the Common Wealth, Kinsington press. p.120-125.

Bodeker, G. and Willcox, M. (2000). New research initiative of plant-based antimalarials. *Lancet* ., **355**: 761.

Borst, P., Vanderploeg, L., Van hock, J. F. M., Tas, J. and James, J. (1982). On the DNA content and ploidy of trypanosomes. *Molecular and Biochemical Parasitology.*, **6**: 13-23.

Brun, R., Schumacher, R., Schmid, C., Kunz, C and Burri, C. (2001). The phenomenon of treatment failures in Human African Trypanosomiasis. *Tropical Medicine and International Health.*, **6** (11): 906-914.

Butcher, P.A., Otero, A., McDonald, M. W. and Moran, G. F. (2001) Nuclear RFLP variation in *Eucalyptus camaldulensis* Dehnh. from northern Australia. *Heredity.*, **88**: 402-412.

Buckner, F.S., Wilson, A.J., White, T.C. and Van Voorhis, W.C. (1998). Introduction of resistance to azole drugs in *Trypanosoma cruzi*. *Antimicrobial Agents and Chemotherapy.*, **42**, 3245-3250.

Canadian Council on Animal Care (CCAC) (1997) Guidelines on: Animal use and Protocol Review.

Callen, M., Kuntz, D. A and Opperdoes, F. R. (1991). Characterization of pyruvate kinase from Trypanosoma brucei and its role in the regulation of carbohydrate metabolism. *Molecular and Biochemical Parasitology.*, **47**: 19-30.

Cazzulo, J. J. (1992). Aerobic fermentation of glucose by trypanosomatids. *FASEB Journal.*, **6**: 3153-3161.

CDC (2005). Life cycle of *Trypanosoma brucei*. Centre for Disease Control and Prevention http//www.dpc.cdc.gov/dpdx

CDC (2005). Cell structure of *Trypanosoma*. Centre for Disease Control and Prevention CDC :http://www.dpd.cdc.gov/dpdx

Cravatt, B.F., Prospero-Garcia, O and Siuzdah, G. (1995). Chemical characterization of a family of brain lipids that induce sleep. *Science.*, **268**, 1506-1509

Croft, S. L., Neal, R. A., Thorton, E. A and Herrmann, D. B. J. (1993). Antileshmanial activity of the ether phospholipid, ilmofosine. *Transactions of the Royal Society of Tropical Medicine and Hygien.*,. **87**: 217-219.

Croft, S. L. (1997). The current status of antiparasitic chemotherapy. *Parasitology.*, **114**: 83-96.

Cross, G. A. M. (2001). African trypanosomes in the 21st century: What is their future in science and in health? *International Journal of parasitology.*, **31**: 427-433.

Das, B., Padmarao, S. and Kashinatham, A. (1998). Taxol content in the storage samples of the needles of Himalayan *Taxus baccata* and their extracts. *Planta Medicine.*, **64**: 96.

De Koning, H.P. (2001). Transporters in African trypanosomes: role in drug action and resistance. *International Journal Parasitology.*, **31**: 512-522.

Donelson, J. E. (1995). Mechanisms of antigenic variation in Borrehia hermsii and African trypanosomes. *Journal of Biological Chemistry.*, **270**: 7783-7786.

Doran, J. and Brophy, J. J. (1990) Tropical gums – a source of 1,8-cineole-rich *Eucalyptus* oil. *New Forests.*, **4:** 157-178.

Dubey, N.K., Rajesh, K. and Pramilla, T. (2004). Global promotion of herbal medicine. India's opportunity. *Current Science.*, **86** (1): 41

Eloff, J. N. (2004). Quantification of the bioactivity of plant extracts during screening and bioassay guided fractionation. Phytomedicine*: International Journal of* Phytotherapy and Phytopharmacology., **4**:23-26

El-Sayed, A. M. (2009). The pherobase: Database of Insect pheromones and semichemicals. <http://www.pherobase.com>

El sayed, N. M., Alarcon, C. M., Beck, J. C., Sheffield, V. C. and Donelson, J. E. (1995). cDNA Expressed sequence of *Trypanosoma brucei rhodesiense* provides new insight into the biology of the parasite. *Molecular and Biochemical Parasitology.*, **73**: 75-90.

Enserink, M. (2007). Entomology. Welcome to Ethiopia's fly factory. *Science.*, **317:**310-313.

Essam, A., Fathalla, M. H., Soliman, M. A. A., Sahar, E., Chikara, I., Hiroaki, K., Kazuhiko, O., Satoshi, O., and Haruki, Y. (2009). Antiplasmodial and

antitrypanosomal activity of plants from the Kingdom of Saudi Arabia. *Journal of Natural Medicines.*, **63** (2): 232-239.

Evans, W. C. (2002) *Trease and Evans Pharmacognosy*, W.B. Saunders, Edinburgh, London, p.72.

Fairlamb, A.H., Henderson, V C., Bacchi, J and Cerami, A. (1983) *Molecular Biochememistry and Parasitology.*, 7: 209-225

Fairlamb, A. H. (1989). Interaction of trypanocidal drugs with the metabolism and functions of trypanothione. *Proceedings of a Workshop held at ILRAD*. Nairobi, Kenya. p.25.

Feldman, M. (2006). *Sleisenger & Fordtran's Gastrointestinal and Liver Disease: Pathophysiology, Diagnosis, Management*. 8th ed. Philadelphia, Pa.: Saunders Elsevier; p.1575.

Ferri, F.F. (2009). *Ferri's Clinical Advisor*. Philadelphia, Pa.: Mosby Elsevier; p.1434.

Finelle, P. (1973). African animal trypanosomiasis. *World Animal Review.*, **7:** 1-6.

Fournet, A and Muñoz, V (2002). Natural products as trypanocidal, antileishmanial and antimalarial drugs. *Current Topics in Medicinal Chemistry.*, **2**(11):1215-37.

Freiburghaus, F., Jonker, S.A., Nkuna, M.H.N., Mwasumbi, L.B. and Brun, R. (1997). Evaluation of African Medicinal Plants for their *in vitro* trypanocidal activity. *Journal of Ethnopharmacology.*, **55:**1-11

Gamaliel, K. S. (2000). Toxicity from medicinal plants and their products. *Nigerian Journal of Natural Products and Medicine.*, **4:** 4- 5.

Gbile, Z. O and Adesina, S. K. (1985). *Nigerian Flora and their Pharmacological Potential.* University Press Ltd., Ibadan, p.15.

Gerardo, P., Serena, K., Daniel, N., Sara, G., Ute, A., Salah, G and Unni, K (2007). Nifurtimox-Eflornithine Combination Therapy for Second-Stage *Trypanosoma*

brucei gambiense Sleeping Sickness: A Randomized Clinical Trial in Congo. *Clinical Infectious Diseases*., **45:**1435–42.

Giffin, B. F., McCann, P. P., Bitanti, A. J. and Bacchi, C. J (1986) *Journal of Protozoology*., **33**:238-243

Gijtenbeek, J. M. M., Vanden Bent, M. J. and Vecht, C. J.(1999). Cyclosporin neurotoxicity. *Journal of Neurology*., **246**, 339–346.

Gotaskei, G. E. and Andreassi, B. F. (1994). Paclitaxel: a new antimitotic chemotherapeutic agent. *Cancer practice; A Multidisciplinary Journal of Cancer Care.*, **2:** 27- 33.

Gottesdiener, K., Garcia-Anoveros, J., Lee, M. G and Vander Ploeg, L. H. (1990). Chromosome organization of the protozoan *Trypanosoma brucei. Molecular and Cell Biology*., **10:** 6079-6083.

Goto, H., Gomes, C.M., Corbett, C.E., Monteiro, H.P. and Gidlund, M. (1998). Insulin-like growth factor I is a growth-promoting factor for Leishmania promastigotes and amastigotes. *Proceedings of the National Academy of Sciences of the USA* **95**: 13211-13216.

Gottsberger, G. (1988). "Comments on flower evolution and beetle pollination in the genera *Annona* and *Rollinia* (Annonaceae)". *Plant Systematics and Evolution* (Springer Science.Business Media)., **167** (3-4): 189–194.

Green, R. M. (2002). AGA technical review on the evaluation of liver chemistry tests. *Gastroenterology*., **123**:1367.

Greenwood, D. (1992). The quinine connection. *Journal of Antimicrobial Chemotherapy.*, **30**: 417- 427.

Groves, M. J. and Bisset, N. G. (1991). A note on the use of topical digitalis prior to William Withering, *Journal of Ethnopharmacology.*, **35**: 99- 103.

Heby, O., Persson, L and Rentala; M. (2007). Targeting the polyamine biosynthetic enzymes: a promising approach to therapy of African sleeping sickness, Chagas' disease, and Ieishmaniasis. *Amino Acids*., **33**(2): 359-66

Herbert, W.J and Lumsden, W.H. (1976). *Trypanosoma brucei*: a rapid 'matching' method for estimating the host's parasitemia. *Experimental Parasitology*., **40**:427-31

Hide, G. (1999). History of Sleeping Sickness in East Africa. *Clinical Microbiology Review*., **12**: 112- 125

Hoare, C.A. (1967). The use of methylating agents for the treatment of trypanosomiasis. *Advanced Parasitology*.,**5:**47-91

Horonok, l. (1992). *Cultivation and Processing of Medicinal Plants*, Wiley and Sons, Chickester, UK, p. 221–235.

Hunt, R. C. (2004). "Trypanosomiasis page, "Microbiology and Immunology On-line"". University of South Carolina http//www.sanger.ac.uk/projects/T.brucei/

Huston, C. D. and Petri, W. A., jr. (1998). Host- pathogen interaction in amoebiasis and progress in vaccine development. *European Journal of Clinical Microbiology and Infectious Diseases.*, **17**: 601- 614.

International Federation of Clinical Chemists (IFCC). (1980). *In vitro* determination of SGPT (ALT) and SGOT (AST) in serum or plasma. *Clinical Chemistry Acta*., **105:** 147-172.

Igwe, A. C. and Onabanjo, A. O. (1989). Chemotherapeutic effects of *Annona senegalensis* in *Trypanosoma brucei brucei. Annals of Tropical Medicine and Parasitology.*, **83** (5): 527- 534.

James, R. C and Henry, W. K. (2006). Synthesis and Biological Characterization of 10, 11- Methylene-9-Octadecene, an analogue of sterculic acid. *Lipids.*, **7**(12): 769-773

Janice, R. S., Arthur, J.S.,Canio, J. M., Donna, R and Bacchi, C. J. (2008). Novel Trypanocidal Analogs of 5^1-(Methylthio)-Adenisine. *Antimicrobial Agents Chemotherapy.*, **52**(1): 211-219.

Johnson, W. C. and William, O. W. (2002). Warfarin toxicity. *Journal of Vascular Surgery.*, 413– 421.

Kamanzi, A. K., Schmid, C., Brun, R., Kone, M. W and Traore, D. (2004). Antitrypanosomal and antiplasmodial activity of medicinal plants from Cote d Ivoire. *Journal of Ethnopharmacology.*, **90**:221-227.

Kiuchi, F., Takemura, M., Ohta, T., Nakajima-Shimada, J and Aoki, T (1998) Anti-trypanosomal activities of natural medicines. IX International Congress of Parasitology (ICOPA IX). p. 97-101.

Leak, S. (1998) *Tsetse Biology and Ecology: Their role in the Epidemiology and Control of Trypanosomiasis.* New York, NY, USA: CABI Publishing., p.50-62.

Legros, D., Olliver, G., Gastellu-Etchegory, M., Paquet, C., Burri, C., Jannin, J and Buscher, P. (2002). Treatment of human African trypanosomiasis—present situation and needs for research and development. *The Lancet Infectious Diseases.*, **2:** 437 – 440.

Losos, G. J. and Chuinard, A. (1979). *Pathogenicity in trypanosomes*. Ottawa: IDRC press. p. 448 - 500.

Losos, G. J. (1986). *Infectious Tropical Diseases of Domestic Animals*. Longman Scientific and Technical. England. p. 183 - 194.

Mair, G., Shi, H. F., Li, H. J., Djikeng, A., Aviles, H. O., Bishop, J. R., Falcone, F . H., Gavrilescu, C., Montgomery. J. L., Santori, M. I., Stern, L. S., Wang, Z. F., Ullu, F. and Tschudi, C. (2000). A new twist in trypanosome RNA metabolism. Cis-splicing of Pre- mRNA. *RNA.*, **6**: 163- 9.

Marks, P.A., Ellis, A and Peters, S.A. (2000). Structural classes of HDAC inhibitors (a review). *Journal of National Cancer Institute.*, **92**: 1210-1215

Marton, L. J. and Morris, D. R. (1987). *Inhibition of polyamine metabolism.* New York Academic Press, p. 79- 106.

Masake, R. A. and Nantulya, V. M. (1991). Sensitivity of an antigen- detecting enzyme immunoassay for diagnosis of *Trypanosoma congolense* infections in goats and cattle. *Journal of Parasitology.*, **77**: 231- 236.

Mbagwe, B. O. (2003). *In vivo* antitrypanosomal activity of a novel pyrimidinedione derivative of ellipticine, an antibiotic analogue. *Paper delivered at African Conference of Toxicological Sciences.* Held at ILRAD, Nairobi, Kenya.p. 12 - 15.

Marcello, L and Barry, J. D (2007). "Analysis of the VSG gene silent archive in Trypanosoma brucei reveals that mosaic gene expression is prominent in antigenic variation and is favoured by archive substructure". *Genome Research.*, **17** (9): 1344–52.

Merlin, L. W., Cosentino, M. J., Richard, P., Gerard, B. and Steven, W. (2001). Natural Products for the Treatment of Tropical Diseases. *Trends in Parasitology.*, **17** (2) p.59.

Mesia, G. K., Tona, G. L., Nanga, T. H., Cimanga, R. K., Apers, S., Cos, P., Maes, L., Pieters, L and Vlietinck, A. J. (2008). Antiprotozoal and cytotoxic

screening of 45 plant extracts from Democratic Republic of Congo. *Journal of Ethnopharmacology.*, **115** (3):409-15.

Michels, P. A. M. and Hannert, V. (1994). The evolution of Kinetoplastida glycosomes. *Journal of Bioenergetics and Biomembranes.*, **26**: 213- 219.

Michels, P. A. M. and Hannert, V. and Bringaurd, F. (2000). Metabolic aspects of glycosomes in trypanosomatidae- new data and vies. *Parasitology Today.*, **16:** 482-9.

Moulton, J. E. and Sollod, A. E. (1976). Clinical, serological and pathological changes in calves with experimentally- induced *Trypanosoma brucei* infection. *Annual Journal of Veterinary Research.*, **37** : 791.

Murray, M., Trail, J. C. M., Davies, C. E. and Black, S. J. (1984). Genetic resistance to African Trypanosomiasis. *Journal of Infectious Diseases.*, **149:** 311- 319.

Nadia, L., Loïc, R., Virginie, C., Graham, H. C., Michael, P. B. and Frédéric B. (2005). Proline Metabolism in Procyclic *Trypanosoma brucei* Is Down-regulated in the Presence of Glucose. *Journal of Biology and Chemistry.*, **280** (12):11902-11910

Nigerian Society of Parasitology.(2002). NSP Annual Report. *Proceedings of the National Conference of Parasitology* held in Abuja, Nigeria.

Natural Resources Conservation Service (NRC). (2008). "Plants Profile, *Annona* L." *The PLANTS Database*. United States Department of Agriculture.p.234-247.

Nibret, E., Sporer, F., Asres, K., and Wink, M. (2009). Antitrypanosomal and Cytotoxic activities of pyrrolizidine alkaloid-producing plants of Ethiopia. *Journal of pharmacy and pharmacology.*, **66** (6): 801-808.

Niessen, W. M. A. (2001). *Current practice of gas chromatography—mass spectrometry*. New York, N.Y: Marcel Dekker, p. 40-45.

Noble, R. L. (1990). The discovery of the vinca alkaloids- chemotherapeutic agent against cancer. *Biochemistry and Cell Biology.*, **68**: 1344- 1351.

Nok, A. J., Esievo, K. A. N., Longdet, I., Arowosafe, S., Onyenekwe, P. C., Gimba, C. E. and Kagbu, J. A. (1993). Trypanocidal potentials of Azadirachta Indica: In vivo activity of leaf extract against *Trypanosoma brucei brucei. Journal of Clinical Biochemistry and Nutrition.*, **15:** 113- 118.

Nwaka, S and Hudson, A. (2006) Innovative lead discovery strategies for tropical diseases. *National Review of Drug Discovery.*, **5:**941-955.

Ogbadoyi, E, Ersfeld, K, Robinson, D, Sherwin, T, and Gull, K (2000). "Architecture of the *Trypanosoma brucei* nucleus during interphase and mitosis". *Chromosoma.*, **108** (8): 501–13.

Ogbadoyi, E. O., Akinsunbo, O. A., Theophilus, Z. A and Okogun, J. I. (2007)a. *In vivo* trypanocidal activity of *Annona senegalensis* Pers. Leaf against *Trypanosoma brucei brucei. Journal of Ethnopharmacology.*, **112**: 85-89.

Ogbadoyi, E.O., Hafsatu, B., Adamu. Y. K., Chinenye. E. N., Solomon, I., Rasheedat, B.A., Emmanuella, I.O., Paul, B.M., Akinsunbo, A.O and Theophilus, Z.A (2007)b. Preliminary Studies of Antitrypanosomal Properties of Selected Nigerian Medicinal Plants. *Journal of Research in Bioscience.*, **3** (3):38-43.

Ogbeche, A. K., Ajayi, G. O. and Onyeneta, P. (1997). Antibacterial activities of the leaf extract of *Ageratum conyzoides Nigerian Quarterly Journal of Hospital Medicine.*, **7:** 397-399.

Olukoya, D. K., Idika, N and Odugbemi, T. (1993). Antimicrobial activity of some medicinal plants from Nigeria. *Journal of Ethnopharmacology.*, **39**: 69-72.

Okpanyi, S.N. and Ezekwu, G.C. (1973). Anti-inflammatory and anti-pyretic activities of *Azadirachta indica, Planta Medica.*, **4**: 44-45

Opperdoes, F.R. (1987). Compartmentation of Carbohydrate metabolism in trypanosomes. *Annual Review of Microbiology.*, **41**:127-151

Overath, P., Chaudhri, M., Steverding, D., and Ziegelbauer, K. (1994). *"Invariant surface proteins in bloodstream forms of Trypanosoma brucei".Parasitology Today.*, **10** (2): 53–8.

Pays, E.(2005). "Regulation of antigen gene expression in Trypanosoma brucei". *Trends in Parasitology.*, **21** (11): 517–20.

Pilar Rauter, A., Dos Santos, A. F and. Santana, A. E. G. (2002). "Toxicity of Some species of *Annona* toward *Artemia Salina* Leach and *Biomphalaria Glabrata* Say". *Natural Products in the New Millennium: Prospects and Industrial Application*. Springer Science.Business Media. p. 540.

Phillipson, J.D. and O'Neil, M.J. (1986). Novel antimalarial drugs from plants? *Parasitology Today.*, **2**, 355-358.

Phillipson, J. D. and Wright, C. W. (1991). Medicinal plants in tropical medicine. Medicinal plants against protozoal diseases. *Transactions of the Royal society of Tropical Medicine and Hygiene.*, **85**, 18-21

Robert , V and Gardener, M. (2008). "Cherimoya" . *Minor subtropicals*. Ventura CountyCooperativeExtension.http://ucce.ucdavis.edu/counties/ceventura/Agriculture265/Cherimoya.htm

Sara, H., Frederik, O., Reto, B., Victor, A and Joëlle, Q.(2004). In vitro antitrypanosomal activity of ethnopharmacologically selected Beninese plants. *Journal of Ethnopharmacology.*, **91(1):** 37-42 *-42*

Shale, T. L., Stirk, W. A., van Staden, J.(1999). Screening of medicinal plants used in Lesotho for anti-bacterial and anti-inflammatory activity. *Journal of Ethnopharmacology.*, **67:** 347-354

Shariff, Z. U. (2001). *Modern Herbal Therapy for Common Ailments. Nature Pharmacy Series* (**1**), Spectrum Books Limited, Ibadan, Nigeria in Association with Safari Books (Export) Limited, United Kingdom, p. 9 – 84.

Shyam, K., Hrubiec, R. T., Furubayashi, R., Cosby, L. A and Sartorelli, A. C. (1987). Methylating agents for use in the treatment of trypanosomiasis. *Journal of Medical Chemistry.*, **30**, 2157-2161

Smith, A. B., Esko, J. D. and Hajduk, S. L. (1995). Killing of trypanosomes by the human haptaglobin- related protein. *Science.*, **268:** 284- 286.

Sommer, J.M. and Wang. C. C. (1994). Targeting proteins to the glycosomes of African trypanosomes. *Annual Review of Microbiology.*, **48**: 105-38.

Spriggs, D. R. (1985). Antigenic variation in trypanosomes. Genomes in flux. *Journal of Infectious Diseases.*, **152**: 855- 856.

Stevens, J. R. and Gibson, W. (1999). The molecular evolution of trypanosomes. *parasitology Today.*, **15**: 432- 7.

Steiger, R.F. (1973). On the ultra structure of trypanosome (Trypanozoon) brucei in the course of its life cycle and some related aspects. *Acta Tropica.*, **30** (1-2): 65-168

Still, W.C., Kahn, M. and Mitra, A. (1978). Column chromatography. *Journal of organic chemistry.*, **43**(14): 29923-2925.

Swerdlow, J.L. (2000). Medicines in nature. *National Geographic.*, **4**, 98-117.

Trails, J. C. M., Dieteren, G. D. M., Maille, J. C., Yangari, G. and Nantulya, V.M. (1991). Use of antigen-detection enzyme immunoassays in assessment of trypano-tolerance in N'Dama cattle. *Acta Tropica.*, **50**: 11- 18.

Tropical Disease Research (TDR). (1998). Progress Report. Fourteenth Programme Report. p. 8- 12.

Turner, C.M (1997). "The rate of antigenic variation in fly-transmitted and syringe-passaged infections of Trypanosoma brucei". *FEMS Microbiology Letters.*, **153** (1): 227–31.

Van Baren , C., Anao, I., Leo Di Lira, P., Debenedetti , S., Houghton, P., Croft, S and Martino, V. (2006). Triterpenic acids and flavonoids from Satureja parvifolia. Evaluation of their antiprotozoal activity. *Naturforsch C.*, **61** (3-4):189-92.

Vanhamme, L, Paturiaux-Hanocq, F, and Poelvoorde, P (2003). "Apo lipoprotein L-I is the trypanosome lytic factor of human serum". *Nature.*, **422** (6927): 83–7.

Van Nieuwenhove, S., Schechter, P.J., Declercq, J. (1985). "Treatment of gambiense sleeping sickness in the Sudan with oral DFMO (DL-alfa-difluoromethyl ornithine) an inhibitor of ornithine decarboxylase: first field trial". *Transaction of Royal Society of Tropical Medical Hygiene.*, **79** (5): 692–8.

Van Schaftingen, E., Opperdoes, F.R. and Hers, H. G. (1987). Effects of various metabolic conditions and of trivalent arsenical melarsen oxide on the intracellular levels of fructose 2, 6- bisphosphate and of glycolytic intermediates in *Trypanosoma brucei. European Journal of Biochemistry.*, **166**: 653- 661.

Vickerman, K. (1962). The mechanism of cyclic development in Trypanosoma brucei sub group: a hypothesis based on ultra structural observations. *Transactions of the Royal Society of Tropical Medicine and Hygiene.*, **56**:487-495

Vickerman, K. (1965). Polymorphism and mitochondrial activity in sleeping sickness trypanosomes. *Nature.*, **208**: 762-766

Vickerman, K. and Preston, T.M. (1970). Spindle microtubules in the dividing nuclei of trypanosomes. *Journal of Cell Science.*, **6**: 365-383

Vickerman, K. and Preston, T. M.(1976). *Comparative cell biology of the Kinetoplastida flagellates.* In: 'Biology of the Kinetoplastida, Vol.1 (ed. W.C.A. Lumsden and D.A. Evans), New York, Academic Press. p.35-130

Vickerman, K. (1985). Developmental Cycles and Biology of Pathogenic Trypanosomes. *British Medical Bulletin.,* **41**: 105- 114.

Wallace, H. A. (2001). *Principles and Methods of Toxicology.* Raven Press, New York. p. 45-345.

Warrington, I and Warrington, J. (2008) "Annonaceae" . *Apples: Botany, Production and Uses*. CABI Publishing. ISBN 0851995926.

.Weiden, M., Osheim, Y. N., Beyer, A. L. and Vanderploeg, L. H. T. (1991). Chromosome structure- DNA nucleotide sequence elements of a subset of minichromosomes of the protozoan *Trypanosoma brucei. Molecular and Cell Biology.,* **11**: 3823 – 3834.

Wilcox, M. L., W, M., Cosentino, M. J, Pink, R., Bodeker, G and Wayling, S (2001). Natural products for the treatment of tropical diseases. *Trends in Parasitology.*, **17** (2): 59.

Wilcox, M.L and Bodeker, G. (2000). Plant-based malaria control: Research initiative on traditional anti-malaria methods. *Parasitology. Today.,* **16**: 220-221

Williams, B. I. (1996). *African trypanosomiasis*. In: The Wellcome Trust. Illustrated History of Tropical Diseases. Wellcome Trust London. Pp. 178-191

World Health Organization (1990). Control and Surveillance of African Trypanosomes. *WHO Technical Report Series,* No. 113.

World Health Organization (1998). Control and Surveillance of African Trypanosomes. *WHO Technical Report Series,* No. 881.

World Health Organization (2003). Expert Committee on Control and Surveillance of African trypanosomiasis (Geneva), "*WHO Technical Report Series,No.881*".

World Health Organization/TDR. (2004). African Trypanosomiasis. World Health Report. Expert group meeting (14-15 April).

World Health Organization/TDR. (2009). African Trypanosomiasis. World Health Report No. 10. HAT epidemic in Uganda.

World Health Organization. (2006). African trypanosomiasis or sleeping sickness "Fact sheet No.259: ".

WHO (2008) "Uganda: Sleeping Sickness Reaching Alarming Levels," World Health Report. 'Fact sheet No.270'.

Wurochekke, A.U and Nok, A. J. (2004) Invitro antitrypanosomal activity of some medicinal plants used in the treatment of trypanosomiasis in Northern Nigeria. *African Journal of Biotechnology.*, **3** (9): 481-483

APPENDICES

Appendix I: Statistical analysis for screening with hexane extract of *E.camaldulensis* (Leaf)

Report

OBSERVATION NUMBEROFPARASITES/ ml of blood	Mean	N	Std. Deviation	Std. Error of Mean
A-200mg/Kg Bw	.1320	10	.22685	.07174
B-400mg/Kg Bw	.5250	10	.98918	.31281
C-600 mg/Kg Bw	.2800	10	.51758	.16367
D-600 mg/Kg Bw (Not infected)	.0000	10	.00000	.00000
E-Infected,not treated	1.3850	10	2.49813	.78998
F-Infected, treated with 3.5 mg/Kg Bw of Berenil	.0220	10	.06957	.02200
Total	.3907	60	1.17628	.15186

ANOVA Table

			Sum of Squares	df	Mean Square	F	Sig
OBSERVATION * Number of parasites/ml of blood	Between Groups	(Combined)	13.744	5	2.749	2.186	.069
		Linearity	.438	1	.438	.348	.558
		Deviation from Linearity	13.307	4	3.327	2.646	.043
	Within Groups		67.890	54	1.257		
	Total		81.634	59			

Measures of Association

	R	R Squared	Eta	Eta Squared
OBSERVATION * NUMBEROFPARASITES	.073	.005	.410	.168

Appendix II: Statistical analysis for screening with ethyl acetate extract of *E.camaldulensis* (Leaf)

Report

OBSERVATION NUMBEROFPARASITES, /ml of blood	Mean	N	Std. Deviation	Std. Error of Mean
A-200mg/Kg Bw	.3130	10	.64852	.20508
B-400mg/Kg Bw	.7050	10	1.30223	.41180
C-600mg/Kg Bw	.4100	10	.87108	.27546
D-600mg/Kg Bw (Not Infected)	.0000	10	.00000	.00000
E-Infected,Not Treated	1.3170	10	2.21540	.70057
F-Infected,treated with 3.5mg/kg Bw of Berenil	.0250	10	.07906	.02500
Total	.4617	60	1.18106	.15247

ANOVA Table

			Sum of Squares	df	Mean Square	F	Sig
OBSERVATION * Numberofpara SITEs/ml of blood	Between Groups	(Combined)	12.194	5	2.439	1.879	.113
		Linearity	.000	1	.000	.000	.996
		Deviation from Linearity	12.194	4	3.048	2.348	.066
	Within Groups		70.105	54	1.298		
	Total		82.299	59			

Measures of Association

	R	R Squared	Eta	Eta Squared
OBSERVATION * Number of para sites/ml of blood	-.001	.000	.385	.148

Appendix III: Statistical analysis for screening with methanol extract of *E.camaldulensis* (Leaf)

Case Processing Summary

	Cases					
	Included		Excluded		Total	
	N	Percent	N	Percent	N	Percent
OBSERVATION * Number of para sites/ml of blood	60	98.4%	1	1.6%	61	100.0%

Report

OBSERVATION Number of parasites/ ml of blood	Mean	N	Std. Deviation	Std. Error of Mean
A-200mg/Kg BW	.0460	10	.09513	.03008
B-400mg/Kg Bw	.0420	10	.08677	.02744
C-600mg/Kg BW	.0515	10	.10734	.03394
D-600mg/Kg BW(NOT INFECTED)	.0000	10	.00000	.00000
E-INFECTED,NOT TREATED	.8040	10	1.52224	.48137
F-INFECTED,TREATED WITH 3.5mg/Kg Bw OF BERENIL	.0280	10	.07857	.02485
Total	.1619	60	.66546	.08591

ANOVA Table

			Sum of Squares	df	Mean Square	F	Sig
OBSERVATION * Number of para sites/ml of blood	Between Groups	(Combined)	4.964	5	993	2 533	039
		Linearity	657	1	657	1 676	201
		Deviation from Linearity	4 307	4	1 077	2 748	037
	Within Groups		21.163	54	392		
	Total		26.128	59			

Appendix IV: Statistical analysis for screening with aqueous extract of *E.camaldulensis* (Leaf)

Case Processing Summary

	Cases					
	Included		Excluded		Total	
	N	Percent	N	Percent	N	Percent
OBSERVATION * Number of par sites of blood	48	100.0%	0	.0%	48	100.0%

Report

OBSERVATION Number of parasites /ml of blood	Mean	N	Std. Deviation	Std. Error of Mean
A-200mg/Kg Bw	.5250	8	1.40458	.49660
B-400mg/Kg Bw	.4275	8	1.14164	.40363
C-600mg/Kg Bw	.3538	8	.76564	.27069
D-600mg/kg Bw(NOT INFECTED)	.0000	8	.00000	.00000
E-INFECTED,NOT TREATED	.0163	8	.03852	.01362
F-INFECTED,TREATED WITH 3.5mg/Kg Bw OF BERENIL.	.0063	8	.01188	.00420
Total	.2215	48	.79043	.11409

Appendix IV cont'd

ANOVA Table

		Sum of Squares	Df	Mean Square	F	Sig.
OBSERVATION * NUMBEROFPARSITEOFBLOOD	Between Groups	(Combined)	2.316	5	.463	.719	.613
		Linearity	1.998	1	1.998	3.103	.085
		Deviation from Linearity	.318	4	.080	.124	.973
	Within Groups		27.048	42	.644		
	Total		29.365	47			

Measures of Association

	R	R Squared	Eta	Eta Squared
OBSERVATION * Number of par sites/ml of blood	-.261	.068	.281	.079

Appendix V: Statistical analysis for screening with hexane extract of *E.camaldulensis* (Stem bark)

Case Processing Summary

	Cases					
	Included		Excluded		Total	
	N	Percent	N	Percent	N	Percent
OBSERVATION * Number of para Sites/ml of blood	36	100.0%	0	.0%	36	100.0%

Report

OBSERVATION NUMBER OF PARASITES ml ofblood	Mean	N	Std. Deviation	Std. Error of Mean
A-200mg/KgBw	.9700	6	1.62327	.66270
B-400mg/KgBw	.5917	6	1.01509	.41441
C-600mg/KgBw	.7867	6	1.24397	.50785
D-600mg/KgBw(NOT INFECTED)	.0000	6	.00000	.00000
E-INFECTED.NOT TREATED	.1700	6	.40669	.16603
F-INFECTED,TREATED WITH 3.5mg/KgBW OF BERENIL.	.0050	6	.00837	.00342
Total	.4206	36	.95853	.15975

ANOVA Table

			Sum of Squares	df	Mean Squar	F	Sig.
OBSERVATION * Number of para Sites/mlof blood	Between Groups	(Combined)	5.265	5	1.053	1.175	.345
		Linearity	4.053	1	4.053	4.522	.042
		Deviation from Linearity	1.212	4	.303	.338	.850
	Within Groups		26.892	30	.896		
	Total		32.157	35			

Measures of Association

	R	R Squared	Eta	Eta Squared
OBSERVATION * Number of para Sites/ml of blood	-.355	.126	.405	.164

Appendix VI: Statistical analysis for screening with ethyl acetate extract of *E.camaldulensis* (Stem bark)

Case Processing Summary

	Cases					
	Included		Excluded		Total	
	N	Percent	N	Percent	N	Percent
OBSERVATION * Number of para Sites/ml of blood	42	100.0%	0	.0%	42	100.0%

Report

OBSERVATION Number of parasites/ ml of blood	Mean	N	Std. Deviation	Std. Error of Mean
A200mg/KgBw	.0129	7	.01890	.00714
B-400mg/KgBw	.2157	7	.39251	.14835
C-600mg/KGBw	.2543	7	.38087	.14396
D-600mg/KgBw (NOT INFECTED)	.0000	7	.00000	.00000
E-INFECTED,NOT TREATED	.0757	7	.18743	.07084
F-INFECTED, TREATED WITH 3.5mg/KgBw OF BERENIL	.0051	7	.01119	.00423
Total	.0940	42	.24482	.03778

ANOVA Table

			Sum of Squares	df	Mean Square	F	Sig
OBSERVATION * Number of para sites/ml of blood	Between Groups	(Combined)	.449	5	.090	1.610	.182
		Linearity	.051	1	.051	.911	.346
		Deviation from Linearity	.398	4	.100	1.785	.153
	Within Groups		2.008	36	.056		
	Total		2.457	41			

Appendix VII: Statistical analysis for screening with methanol extract of *E.camaldulensis* (Stem bark)

Case Processing Summary

	Cases					
	Included		Excluded		Total	
	N	Percent	N	Percent	N	Percent
OBSERVATION * Number of parasites/ml of blood	48	100.0%	0	.0%	48	100.0%

Report

OBSERVATION

NUMBEROFPARASITES	Mean	N	Std. Deviation	Std. Error of Mean
A-200mg/KgBw	.5375	8	.67449	.23847
B-400mg/KgBw	.1900	8	.37064	.13104
C-600mg/KgBw	.2475	8	.68799	.24324
D-600mg/KgBw (NOT INFECTED)	.0000	8	.00000	.00000
E- INFECTED, NOT TREATED	.0813	8	.21034	.07436
F- INFECTED, TREATED WITH 3.5mg/KgBw OF BERENIL	.0038	8	.00744	.00263
Total	.1767	48	.44756	.06460

ANOVA Table

			Sum of Squares	df	Mean Square	F	Sig
OBSERVATION * NUMBEROFPARASITES	Between Groups	(Combined)	1.645	5	329	1 778	138
		Linearity	1.202	1	1 202	6 495	015
		Deviation from Linearity	.443	4	.111	599	665
	Within Groups		7.770	42	185		
	Total		9.414	47			

Measures of Association

	R	R Squared	Eta	Eta Squared
OBSERVATION * Number of parasites/ml of blood	-.357	.128	.418	.175

Appendix VIII: Statistical analysis for screening with hexane extract of *E.camaldulensis* (Root bark)

Case Processing Summary

	Cases					
	Included		Excluded		Total	
	N	Percent	N	Percent	N	Percent
OBSERVATION * Number of par asites/ml of blood	42	100.0%	0	.0%	42	100.0%

Report

OBSERVATION NUMBEROFPARASITES/ ml of blood	Mean	N	Std. Deviation	Std. Error of Mean
A-200mg/Kg Bw	.4243	7	.74753	.28254
B-400mg/Kg Bw	.3629	7	.54070	.20437
C-600mg/Kg Bw	.3314	7	.49522	.18718
D-600mg/Kg Bw (NOT INFECTED)	.0000	7	.00000	.00000
E-INFECTED, NOT TREATED	.0786	7	.18676	.07059
F- INFECTED, TREATED WITH 3. 5mg/KgBw OF BRENIL.	.0043	7	.00787	.00297
Total	.2002	42	.44443	.06858

ANOVA Table

			Sum of Squares	df	Mean Square	F	Sig
OBSERVATION * Number of para sites/ml of blood	Between Groups	(Combined)	1.310	5	.262	1.390	.251
		Linearity	1.079	1	1.079	5.721	.022
		Deviation from Linearity	.231	4	.058	.307	.872
	Within Groups		6.788	36	.189		
	Total		8.098	41			

Measures of Association

	R	R Squared	Eta	Eta Squared
OBSERVATION * NUMBEROFPAR ASITESml	-.365	.133	.402	.162

Appendix IX: Statistical analysis for screening with ethyl acetate extract of *E.camaldulensis* (Root bark)

Case Processing Summary

	Cases					
	Included		Excluded		Total	
	N	Percent	N	Percent	N	Percent
OBSERVATION * Number of par asites/ml of blood	48	100.0%	0	.0%	48	100.0%

Report

OBSERVATION NUMBEROFPARASITES/ ml of blood	Mean	N	Std. Deviation	Std. Error of Mean
A-200mg/Kg BW	.1863	8	.35367	.12504
B-400mg/Kg BW	.0100	8	.01773	.00627
C-600mg/Kg BW	.0363	8	.08700	.03076
D-600mg/Kg BW (NOT INFECTED)	.0000	8	.00000	.00000
E-INFECTED, NOT TREATED	.0325	8	.08795	.03110
F- INFECTED, TREATED WITH 3. 5mg/Kg BW OF BRENIL.	.0113	8	.02800	.00990
Total	.0460	48	.15892	.02294

ANOVA Table

			Sum of Squares	df	Mean Square	F	Sig
OBSERVATION * Number of par asites/ml of blood	Between Groups	(Combined)	.197	5	039	1.667	164
		Linearity	081	1	081	3.450	070
		Deviation from Linearity	115	4	029	1.221	316
	Within Groups		990	42	024		
	Total		1.187	47			

Measures of Association

	R	R Squared	Eta	Eta Squared
OBSERVATION * Number of par asites/mlof blood	-.262	.069	.407	.166

Appendix X: Statistical analysis for screening with methanol extract of *E.camaldulensis* (Root bark)

Case Processing Summary

	Cases					
	Included		Excluded		Total	
	N	Percent	N	Percent	N	Percent
OBSERVATION * Number of parasites/ml of blood	36	100.0%	0	.0%	36	100.0%

Report

OBSERVATION

NUMBEROFPARASITES	Mean	N	Std. Deviation	Std. Error of Mean
A-200mg/KgBw	.0608	6	.10141	.04140
B-400mg/KgBw	.0867	6	.20265	.08273
C-600mg/KgBw	.0433	6	.10132	.04137
D-600mg/KgBw(NOT INFECTED)	.0000	6	.00000	.00000
E-INFECTED, NOT TREATED	.0450	6	.10075	.04113
F- INFECTED, TREATED WITH 3.5 mg/KgBw OF BERENIL.	.0200	6	.04000	.01633
Total	.0426	36	.10616	.01769

ANOVA Table

			Sum of Squares	df	Mean Squar	F	Sig.
OBSERVATION * Number of parasites/ml of blood	Between Groups	(Combined)	.028	5	.006	.452	.808
		Linearity	.012	1	.012	.973	.332
		Deviation from Linearity	.016	4	.004	.322	.861
	Within Groups		.367	30	.012		
	Total		.394	35			

Measures of Association

	R	R Squared	Eta	Eta Squared
OBSERVATION * NUMBEROFPARASITES	-.174	.030	.265	.070

Appendix XI: Statistical analysis for screening with Combination therapy (*A.senegalensis E.camaldulensis*)

Case Processing Summary

	Cases					
	Included		Excluded		Total	
	N	Percent	N	Percent	N	Percent
A_200mg_KgBW * DAYOFPOSTINFECTION	24	100.0%	0	.0%	24	100.0%
B_200mg_KgBW * DAYOFPOSTINFECTION	24	100.0%	0	.0%	24	100.0%
C_200mg_KgBW * DAYOFPOSTINFECTION	24	100.0%	0	.0%	24	100.0%
D_untreated_Control * DAYOFPOSTINFECTION	24	100.0%	0	.0%	24	100.0%
E_TreatedWithBerenil * DAYOFPOSTINFECTION	24	100.0%	0	.0%	24	100.0%

Report

DAYOFPOSTINFECTION		A_200mg_ KgBW	B_200mg_ KgBW	C_200mg_ KgBW	D_untreated_ Control	E_ TreatedWith Berenil
1	Mean	0000	.0000	0000	0000	00
	N	3	3	3	3	
	Std. Deviation	00000	00000	00000	00000	000
	Std. Error of Mean	00000	00000	00000	00000	000
3	Mean	0167	.0067	0200	0200	01
	N	3	3	3	3	
	Std. Deviation	00577	00577	01000	01000	005
	Std. Error of Mean	00333	00333	00577	00577	003
6	Mean	1767	.1333	1333	1800	00
	N	3	3	3	3	
	Std. Deviation	08737	05774	05774	08185	005
	Std. Error of Mean	05044	03333	03333	04726	003
9	Mean	0967	.0667	2067	0000	00
	N	3	3	3	3	
	Std. Deviation	15885	11547	17926	00000	000
	Std. Error of Mean	09171	06667	10349	00000	000
11	Mean	0000	0833	0833	0000	00
	N	3	3	3	3	
	Std. Deviation	00000	14434	14434	00000	000
	Std. Error of Mean	00000	08333	08333	00000	000
13	Mean	0000	0000	0667	0000	00
	N	3	3	3	3	
	Std. Deviation	00000	00000	11547	00000	000
	Std. Error of Mean	00000	00000	06667	00000	000
20	Mean	0000	0000	0333	0000	00
	N	3	3	3	3	
	Std. Deviation	00000	00000	05774	00000	000
	Std. Error of Mean	00000	00000	03333	00000	000
30	Mean	0000	0000	0000	0000	00
	N	3	3	3	3	
	Std. Deviation	00000	00000	00000	00000	000
	Std. Error of Mean	00000	00000	00000	00000	000
Total	Mean	0363	.0363	0679	0250	00
	N	24	24	24	24	
	Std. Deviation	08256	07552	10542	06494	005
	Std. Error of Mean	01685	01541	02152	01326	001

Appendix XII: Statistical analysis for determination of minimum curative dose for methanol extract of E.camaldulensis (leaf)

Report

DAYSPOSTINFECTION		GrpA50mg KgBodyweight	GrpB100m GKg Bodyweight	GrpC150mg KgBodyweight	GrpD200mg KgBodyweight	GrpE3 5mgBerenil Kgbodyweight	GrpFinfected NotTreated
1	Mean	00	00	00	00	00	00
	N	3	3	3	3	3	3
	Std. Deviation	000	000	000	000	000	000
	Std. Error of Mean	000	000	000	000	000	000
4	Mean	8.33	5.00	2.00	2.33	2.67	2.00
	N	3	3	3	3	3	3
	Std. Deviation	.577	1.000	1.000	.577	.577	000
	Std. Error of Mean	.333	.577	.577	.333	.333	000
6	Mean	4.67	4.67	8.33	6.67	3.00	7.00
	N	3	3	3	3	3	3
	Std. Deviation	.577	.577	.577	1.528	000	000
	Std. Error of Mean	.333	.333	.333	.882	000	000
8	Mean	9.67	8.67	5.67	5.67	2.00	4.00
	N	3	3	3	3	3	3
	Std. Deviation	1.528	3.786	2.517	3.215	000	000
	Std. Error of Mean	.882	2.186	1.453	1.856	000	000
10	Mean	7.67	8.00	10.67	8.33	10.33	8.67
	N	3	3	3	3	3	3
	Std. Deviation	.577	1.000	1.155	2.082	577	577
	Std. Error of Mean	.333	577	667	1.202	333	333
12	Mean	8.00	6.33	4.27	5.67	2.67	10.00
	N	3	3	3	3	3	3
	Std. Deviation	1.000	3.215	635	2.887	577	000
	Std. Error of Mean	577	1.856	367	1.667	333	000
14	Mean	20.33	21.00	14.33	9.67	2.00	26.67
	N	3	3	3	3	3	3
	Std. Deviation	3.215	1.000	5.033	.577	000	2.887
	Std. Error of Mean	1.856	577	2.906	333	000	1.667
16	Mean	26.00	39.33	45.33	25.00	00	96.67
	N	3	3	3	3	3	3
	Std. Deviation	22.539	34.196	19.009	3.000	000	83.865
	Std. Error of Mean	13.013	19.743	10.975	1.732	000	48.419
18	Mean	13.67	13.00	82.67	2.67	00	00
	N	3	3	3	3	3	3
	Std. Deviation	23.671	22.517	51.588	4.619	000	000
	Std. Error of Mean	13.667	13.000	29.784	2.667	000	000
20	Mean	33.33	1.67	2.67	1.00	00	00
	N	3	3	3	3	3	3
	Std. Deviation	57.735	2.887	2.517	1.732	000	000
	Std. Error of Mean	33.333	1.667	1.453	1.000	000	000
22	Mean	40.00	1.00	1.00	00	00	00
	N	3	3	3	3	3	3
	Std. Deviation	69.282	1.732	1.732	000	000	000
	Std. Error of Mean	40.000	1.000	1.000	000	000	000
26	Mean	166.67	100.00	26.67	00	00	00
	N	3	3	3	3	3	3
	Std. Deviation	288.675	173.205	25.166	000	000	000
	Std. Error of Mean	166.667	100.000	14.530	000	000	000
28	Mean	00	00	26.67	00	00	00
	N	3	3	3	3	3	3
	Std. Deviation	.000	.000	30.551	.000	000	000
	Std. Error of Mean	.000	.000	17.638	.000	.000	000
30	Mean	.00	.00	.00	.00	00	00
	N	3	3	3	3	3	3
	Std. Deviation	.000	.000	.000	000	000	000
	Std. Error of Mean	.000	.000	.000	000	000	000
Total	Mean	24.17	14.90	16.45	4.79	1.62	11.07
	N	42	42	42	42	42	42
	Std. Deviation	79.140	47.179	27.136	6.784	2.731	31.170
	Std. Error of Mean	12.212	7.280	4.187	1.047	421	4.810

Appendix XIII: Statistical analysis for screening for Time-dependent Therapy using 200mg/kg BW of Methanol extract of E.camaldulensis (leaf)

Report

DAYSPOSTINFECTION		GrpA0hrs	GrpB24hrs	GrpC48hrs	GrpD72hrs	GrpE3. 5mgBerenil	GrpFinfected NotTreated
1	Mean	.000	.00	.00	.00	.000	00
	N	3	3	3	3	3	3
	Std. Deviation	.0000	.000	.000	.000	0000	000
	Std. Error of Mean	.0000	.000	.000	.000	.0000	000
4	Mean	233	32.00	39.67	56.33	4.833	129.33
	N	3	3	3	3	3	3
	Std. Deviation	.0577	2.646	6.807	4.041	1.0408	25.482
	Std. Error of Mean	.0333	1.528	3.930	2.333	.6009	14.712
6	Mean	000	105.67	44.67	52.33	000	198.67
	N	3	3	3	3	3	3
	Std. Deviation	.0000	14.364	4.509	7.506	.0000	52.013
	Std. Error of Mean	.0000	8.293	2.603	4.333	.0000	30.030
8	Mean	4.333	39.67	165.33	118.33	.000	417.67
	N	3	3	3	3	3	3
	Std. Deviation	4.9329	17.039	56.616	71.122	.0000	128.165
	Std. Error of Mean	2.8480	9.838	32.687	41.062	.0000	73.996
10	Mean	14.000	24.33	87.00	88.33	.000	300.00
	N	3	3	3	3	3	3
	Std. Deviation	4.0000	9.018	37.643	29.023	.0000	264.575
	Std. Error of Mean	2.3094	5.207	21.733	16.756	.0000	152.753
12	Mean	21.333	11.67	51.67	340.67	.000	.00
	N	3	3	3	3	3	3
	Std. Deviation	24.0901	7.638	18.824	197.930	.0000	000
	Std. Error of Mean	13.9084	4.410	10.868	114.275	.0000	000
14	Mean	37.667	4.33	29.67	418.67	.000	.00
	N	3	3	3	3	3	3
	Std. Deviation	24.8261	4.933	22.368	59.543	0000	000
	Std. Error of Mean	14.3333	2.848	12.914	34.377	.0000	000
16	Mean	6.000	1.67	116.67	279.67	.000	00
	N	3	3	3	3	3	3
	Std. Deviation	3.6056	2.887	125.831	246.253	.0000	000
	Std. Error of Mean	2.0817	1.667	72.648	142.174	.0000	000
18	Mean	.333	.00	205.00	.00	.000	00
	N	3	3	3	3	3	3
	Std. Deviation	.5774	.000	181.452	.000	.0000	000
	Std. Error of Mean	.3333	.000	104.762	.000	.0000	000
20	Mean	.000	.00	331.67	.00	.000	00
	N	3	3	3	3	3	3
	Std. Deviation	.0000	.000	297.504	.000	.0000	000
	Std. Error of Mean	.0000	.000	171.764	.000	.0000	000
22	Mean	.333	.00	56.67	.00	.000	00
	N	3	3	3	3	3	3
	Std. Deviation	.5774	.000	98.150	.000	.0000	000
	Std. Error of Mean	.3333	.000	56.667	.000	.0000	000
26	Mean	.000	.00	00	.00	000	00
	N	3	3	3	3	3	3
	Std. Deviation	.0000	.000	.000	.000	.0000	.000
	Std. Error of Mean	.0000	.000	.000	.000	.0000	.000
Total	Mean	7.019	18.28	94.00	112.86	.403	87.14
	N	36	36	36	36	36	36
	Std. Deviation	14.2452	30.642	133.031	164.917	1.3775	157.492
	Std. Error of Mean	2.3742	5.107	22.172	27.486	.2296	26.249

Appendix XIV: Statistical analysis for bioassay of fractions at 150mg/kg BW

Report

DAYSPOSTINFECTION		Fraction1	Fraction2	Fraction3	Fraction4	Fraction5	Fraction6	Fraction7	Fraction8	Fraction9	Fraction10	UninfectedNot Treated	Uninfected Treatedwith3 5mgKgBw
1	Mean	00	00	00	00	00	00	00	00	00	00	00	0(
	N	3	3	3	3	3	3	3	3	3	3	3	3
	Std. Deviation	000	000	000	000	000	000	000	000	000	000	000	00(
	Std. Error of Mean	000	000	000	000	000	000	000	000	000	000	000	00(
3	Mean	116.67	250.00	66.67	60.00	150.00	60.00	90.00	30.00	67	220.00	4.00	1.33
	N	3	3	3	3	3	3	3	3	3	3	3	3
	Std. Deviation	28.868	50.000	28.868	10.000	50.000	10.000	36.056	17.321	1.155	72.111	2.646	.57
	Std. Error of Mean	16.667	28.868	16.667	5.774	28.868	5.774	20.817	10.000	.667	41.633	1.528	.333
5	Mean	333.33	00	350.00	00	00	236.67	193.33	45.00	2.67	00	250.00	00
	N	3	3	3	3	3	3	3	3	3	3	3	3
	Std. Deviation	152.753	000	132.288	000	000	77.675	40.415	30.414	2.082	000	50.000	000
	Std. Error of Mean	88.192	000	76.376	000	000	44.845	23.333	17.559	1.202	000	28.868	000
7	Mean	00	00	00	00	00	00	00	26.67	67	00	00	00
	N	3	3	3	3	3	3	3	3	3	3	3	3
	Std. Deviation	000	000	000	000	000	000	000	20.817	1.155	000	000	000
	Std. Error of Mean	000	000	000	000	000	000	000	12.019	.667	000	000	000
9	Mean	00	00	00	00	00	00	00	9.00	67	00	00	00
	N	3	3	3	3	3	3	3	3	3	3	3	3
	Std. Deviation	000	000	000	000	000	000	000	9.644	577	000	000	000
	Std. Error of Mean	000	000	000	000	000	000	000	5.568	333	000	000	000
11	Mean	00	00	00	00	00	00	00	1.67	67	00	00	00
	N	3	3	3	3	3	3	3	3	3	3	3	3
	Std. Deviation	000	000	000	000	000	000	000	1.528	577	000	000	000
	Std. Error of Mean	000	000	000	000	000	000	000	.882	333	000	000	000
13	Mean	00	00	00	00	00	00	00	00	00	00	00	00
	N	3	3	3	3	3	3	3	3	3	3	3	3
	Std. Deviation	000	000	000	000	000	000	000	000	000	000	000	000
	Std. Error of Mean	000	000	000	000	000	000	000	000	000	000	000	000
Total	Mean	64.29	35.71	59.52	8.57	21.43	42.38	40.48	16.05	76	31.43	36.29	1(
	N	21	21	21	21	21	21	21	21	21	21	21	2
	Std. Deviation	129.560	91.026	130.976	21.745	56.061	87.573	73.449	21.516	1.221	82.115	90.805	51.
	Std. Error of Mean	28.272	19.863	28.581	4.745	12.234	19.110	16.028	4.695	266	17.919	19.815	11.

Appendix XV: Statistical analysis for screening with crude methanol extract, fractions 8 and 9; and berenil

Case Processing Summary

	Cases					
	Included		Excluded		Total	
	N	Percent	N	Percent	N	Percent
A_200mg_KgBW * DAYSOFPOSTINFECTION	24	100.0%	0	.0%	24	100.0%
B_150mg_KgBW * DAYSOFPOSTINFECTION	24	100.0%	0	.0%	24	100.0%
C_150mg_KgBW * DAYSOFPOSTINFECTION	24	100.0%	0	0%	24	100.0%
D_3.5mg_KgBW * DAYSOFPOSTINFECTION	24	100.0%	0	0%	24	100.0%

Report

DAYSOFPOSTINFECTION		A_200mg_KgBW	B_150mg_KgBW	C_150mg_KgBW	D_3.5mg_KgBW
1	Mean	.0000	.0000	.0000	.0000
	N	3	3	3	3
	Std. Deviation	.00000	.00000	.00000	.00000
	Std. Error of Mean	.00000	.00000	.00000	.00000
3	Mean	.2000	.3000	.0667	.1333
	N	3	3	3	3
	Std. Deviation	.05000	.17321	.11547	.05774
	Std. Error of Mean	.02887	.10000	.06667	.03333
6	Mean	.2500	.4500	.2667	.0000
	N	3	3	3	3
	Std. Deviation	.00000	.30414	.20817	.00000
	Std. Error of Mean	.00000	.17559	.12019	.00000
9	Mean	.0100	.1500	.0667	.0000
	N	3	3	3	3
	Std. Deviation	.01732	.08660	.11547	.00000
	Std. Error of Mean	.01000	.05000	.06667	.00000
11	Mean	.0000	.0667	.0667	.0000
	N	3	3	3	3
	Std. Deviation	.00000	.05774	.05774	.00000
	Std. Error of Mean	.00000	.03333	.03333	.00000
13	Mean	.0000	.0000	.0000	.0000
	N	3	3	3	3
	Std. Deviation	.00000	.00000	.00000	.00000
	Std. Error of Mean	.00000	.00000	.00000	.00000
20	Mean	.0000	.0000	.0000	.0000
	N	3	3	3	3
	Std. Deviation	.00000	.00000	.00000	.00000
	Std. Error of Mean	.00000	.00000	.00000	.00000
30	Mean	.0000	.0000	.0000	.0000
	N	3	3	3	3
	Std. Deviation	.00000	.00000	.00000	.00000
	Std. Error of Mean	.00000	.00000	.00000	.00000
Total	Mean	.0575	.1208	.0583	.0167
	N	24	24	24	24
	Std. Deviation	.10088	.19500	.11765	.04815
	Std. Error of Mean	.02059	.03980	.02401	.00983