THERAPEUTIC POTENTIAL OF STEM BARK EXTRACTS OF *ANNONA SENEGALENSIS* IN EXPERIMENTAL *TRYPANOSOMA BRUCEI BRUCEI* INFECTION IN MICE

BY

UGWU, UCHENNA BLESSING
M.TECH/SSSE/2007/1619

DEPARTMENT OF BIOCHEMISTRY

FEDERAL UNIVERSITY OF TECHNOLOGY, MINNA, NIGERIA

DECEMBER, 2010

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A THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL, FEDERAL UNIVERSITY OF TECHNOLOGY, MINNA, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF TECHNOLOGY (M.TECH) IN BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY
FEDERAL UNIVERSITY OF TECHNOLOGY, MINNA, NIGERIA

DECEMBER, 2010

DECLARATION

I hereby declare that this is the report of the original research carried out by me under the supervision of Dr. E.O. Ogbadoyi. Any part or the whole of this work has never been presented in whatever form to any institution apart from Federal University of Technology, Minna, Nigeria, for the award of any degree other than this. The works of others are dully acknowledged by means of references.

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(M.Tech/SSSE/2007/1619

28/01/11
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Date

CERTIFICATION

This thesis titled: Therapeutic potential of stem bark extracts of *Annona senegalensis* in experimental *Trypanosoma brucei brucei* infection in mice by Ugwu, Uchenna Blessing (M.Tech/SSSE/2007/1619), meets the regulations governing the award of the degree of Master of Technology in Biochemistry, Federal University of Technology, Minna and is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

This research project is dedicated to the Almighty God and my beloved husband, Mr. Alozieuwa Innocent.

ACKNOWLEDGEMENTS

My appreciation goes to my supervisor, Dr. E.O. Ogbadoyi for his suggestions and corrections in the course of this work. I acknowledge the head of the Department, Dr. A.A. Jigam, for his efforts and suggestions. I also wish to acknowledge all my lecturers for their unceasing efforts in nurturing me to a greater height. I appreciate Dr. Kabiru for all his efforts in making this work come through.

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I sincerely appreciate my lovely son, Master Uchechukwu Kyrian Alozieuwa, for his gentleness and calmness throughout this study. May Almighty God bless, protect and favour you abundantly.

Above all, I sincerely appreciate Almighty God for his immense grace and inspiration throughout this research project.

ABSTRACT

Chemotherapy of African trypanosomiasis is beset with problems of resistance to current drugs, toxicity, protracted treatment protocols and high cost of treatment. Moreover, there is no available trypanocidal drug that can be used routinely for the prevention of the disease during blood transfusion. These inherent problems associated with current trypanocides makes the sourcing of cheap, effective, and readily accessible trypanocides mandatory. Hence, *Annona senegalensis*, a shrub used in traditional medicine for treatment of sleeping sickness and cancer was investigated for potential in the treatment of trypanosomiasis. Hexane and methanol extracts of stem bark of *Annona senegalensis* were investigated for *in-vivo* anti-trypanosomal activity in albino mice infected with *Trypanosoma brucei brucei* and treated intraperitoneally with the extracts 24hours post infection, at doses ranging from 50 – 500mg/kg, for 14 days. The parasitaemia, packed cell volume and body weight in each mouse was monitored for 43 days. This was done in parallel with control mice, which had been given the standard drug; berenil. Hexane extract at a dose of 400 mg/kg showed significant ($P < 0.01$) trypanocidal activity; clearing the parasites completely from the circulation with 67% of the experimental animal surviving and no relapse was recorded over 43 days. Sub inoculation of blood and cerebrospinal fluid drawn from the cured mice into healthy mice failed to produce any infection within 2 months of post inoculation. Both the PCV and weight significantly improved when subjected to ANOVA after treatment with the crude hexane extracts. Although, the fractions obtained from column chromatography of the crude extracts failed to clear parasites from infected mice within the period of the study, significant reduction ($p<0.01$) of parasite level in infected mice that received 200mg/kg and 400mg/kg of the partially purified fractions was observed. Methanol extract did not show significant anti - trypanosomal activity. The treated control (berenil at dose of 3.5mg/kg), showed 100% survival and cleared parasites. The LD_{50} value of the crude hexane extract in mice was estimated to be 4355mg/kg. Phytochemical screening of the crude hexane extract of the plant indicated the presence of alkaloids, saponins, tannins and flavonoids. These results show that *Annona senegalensis* has great potential as anti trypanosomiasis agent, which could be developed into an alternative drug to complement treatment of trypanosomiasis.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 African Trypanosomiasis

Human African trypanosomiasis (HAT) or sleeping sickness is the result of a blood-borne protozoan infection caused by two subspecies of *Trypanosoma brucei, T. brucei gambiense* and *T. brucei rhodesiense*, while the third subspecies, *T. brucei brucei*, is only infectious to animals (Smith *et al.*, 1998). *T. b. gambiense* is responsible for the chronic form of sleeping sickness in West and Central Africa, whereas *T. b. rhodesiense* gives rise to the acute form of the disease in East and Southern Africa. Trypanosoma belongs to the family Trypanosomatidae of the order Kinetoplastida (Molyneux and Ashford, 1983). Both of these parasites have similar pathogenic features, including the presentation of indistinguishable clinical manifestations in infected humans. However, their epidemiological features differ greatly. Several species of hematophagous glossina, commonly known as tsetse flies, are the vectors of these related diseases, and are responsible for cyclical transmission of the parasitic protozoan between numerous vertebrate hosts. Wild and domestic animals may play a major role as parasite reservoirs for human infections with trypanosomes (WHO, 2006., Njiokou *et al.*, 2006. and Simo, 2006). Both forms of sleeping sickness affect the central nervous system.

The history of human African trypanosomiasis is closely linked to the slave trade. First accounts of sleeping sickness came from ship doctors and medical officers who worked for slave-trade companies. As sleeping sickness caused increasing losses, ship-owners and slave-traders pressed their ship doctors to investigate this disease (Cox, 2004).

Although epidemics of sleeping sickness were more rampant in the past, the most recent WHO estimates put 60 million people at risk of HAT today with approximately 500,000 people currently with infections. The disease is discontinuously spread over 9 million square kilometers and affects populations across 36 sub-Saharan African countries. However, only a small fraction of them are under surveillance with regular examination, have access to a health centre that can provide diagnostic facilities, or are protected by vector control interventions. Throughout history, African trypanosomiasis has severely repressed the economic and cultural development of Africa (Molyneux, 2006; WHO, 2006).

Because of animal trypanosomiasis, stock farming is very difficult within the tsetse belt. The disease in domestic animals and particularly cattle is a major obstacle to the economic development of the rural areas affected.

Today, most efforts to reduce transmission of the disease to humans and other vertebrate reservoirs focus on the control of the vector. Clinical treatment of both early and late onset sleeping sickness is limited and far from up-to-date, and thus cannot be relied upon for controlling the spread of the infection during times of epidemics.

In 2001, the Organisation of African Unity (OAU) launched a new initiative, the Pan African Tsetse and Trypanosomiasis Eradication Campaign (PATTEC) to eliminate the tsetse fly from Africa. It was planned to employ an area-wide approach using odour-baited traps, insecticide treated targets and ultra-low volume aerial spraying of insecticides to reduce the tsetse fly population, and finally the sterile male technique to ensure total elimination of the target Glossina species (Kabayo, 2002; Hide, 1999). The huge costs associated with the eradication

project are also a concern as most of the countries involved belong to the most heavily indebted poor countries in the world (Bhalla, 2002; WHO, 2008).

Disease management is by screening for potential infection, diagnosis to show whether the parasite is present and staging to determine the state of progression of the disease. Diagnosis must be made as early as possible and before the neurological stage in order to avoid complicated, difficult and risky treatment procedures. Exhaustive screening of exposed populations requires a major investment in human and material resources. In Africa such resources are often scarce, particularly in remote areas where the disease is mostly found. As a result, many infected individuals may die before they can ever be diagnosed.

1.2 Research Aims

The main objective of this research work is to obtain phytochemical capable of treating African trypanosomiasis and to possibly obtain lead compounds with the aims to:

(i) evaluate *Annona senegalensis* for trypanocidal activity.

(ii) isolate and purify the active ingredients.

(iii) identify the active ingredients responsible for trypanocidal effect.

(iv) establish the toxicological profile of the active extracts of *Annona senegalensis*

1.3 Justification for this Research

Trypanosomiasis, a disease of the rural poor prevailing in both humans and animals, is of considerable economic importance in many parts of Africa. The estimated loss in income from milk and meat alone caused by cattle trypanosomiasis is US$1000 million annually (Maurice, 1992). Currently, there is no vaccine for trypanosomiasis due to antigenic variation expressed by trypanosomes (Cross, 1975, 1990). Prevention is largely by vector control. Various measures of controlling tsetse fly are difficult to sustain and have collapsed (Esawi and srour, 2005; Onyeili and Egwu, 1995; kuzoe, 1993). The most practical control measure is chemotherapy. Chemotherapy of African trypanosomiasis is beset with problems of toxicity, high cost of treatment, growing resistant to current drugs and long period of treatment. Moreover, there is no available trypanocidal drug that can be used routinely for the prevention of the disease during blood transfusion. These inherent problems associated with current trypanocides make the sourcing of cheap, effective, and readily accessible trypanocides mandatory.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Trypanosome Parasite

Trypanosomes are of the Order kinetoplastida, a monophyletic (Hamilton *et al.*, 2004) group of unicellular parasitic protozoa. The name is derived from the Greek *trypano* (borer) and *soma* (body) because of their corkscrew-like motion. They are so-called because of the large DNA-containing structure, the kinetoplast, found at the base of the flagellum.

Characteristic of this order is the mitochondrial genome, known as the kinetoplast. It is made up of a highly complex series of catenatated circles and minicircles and requires a cohort of proteins for organisation during cell division.

The genus, Trypanosoma, is characterized by its parasitic mode of life. The characteristic morphological forms are trypomastigotes (Fig 2.3a), typically found in the blood in the invertebrate vector. However, other configurations of morphological forms are found, such as amastigotes (Fig 2.3b) and, more rarely, promastigotes. The infective forms which are produced in the vector are known as metacyclic and they are trypomastigote in configuration. Trypanosomes are transmitted to the vertebrate hosts by the bite of a vector or by contamination of the skin or mucous membranes by faecal material containing infective metacyclic trypanosomes. Trypanosomes infect a variety of hosts and cause various diseases, including the fatal disease sleeping sickness in humans.

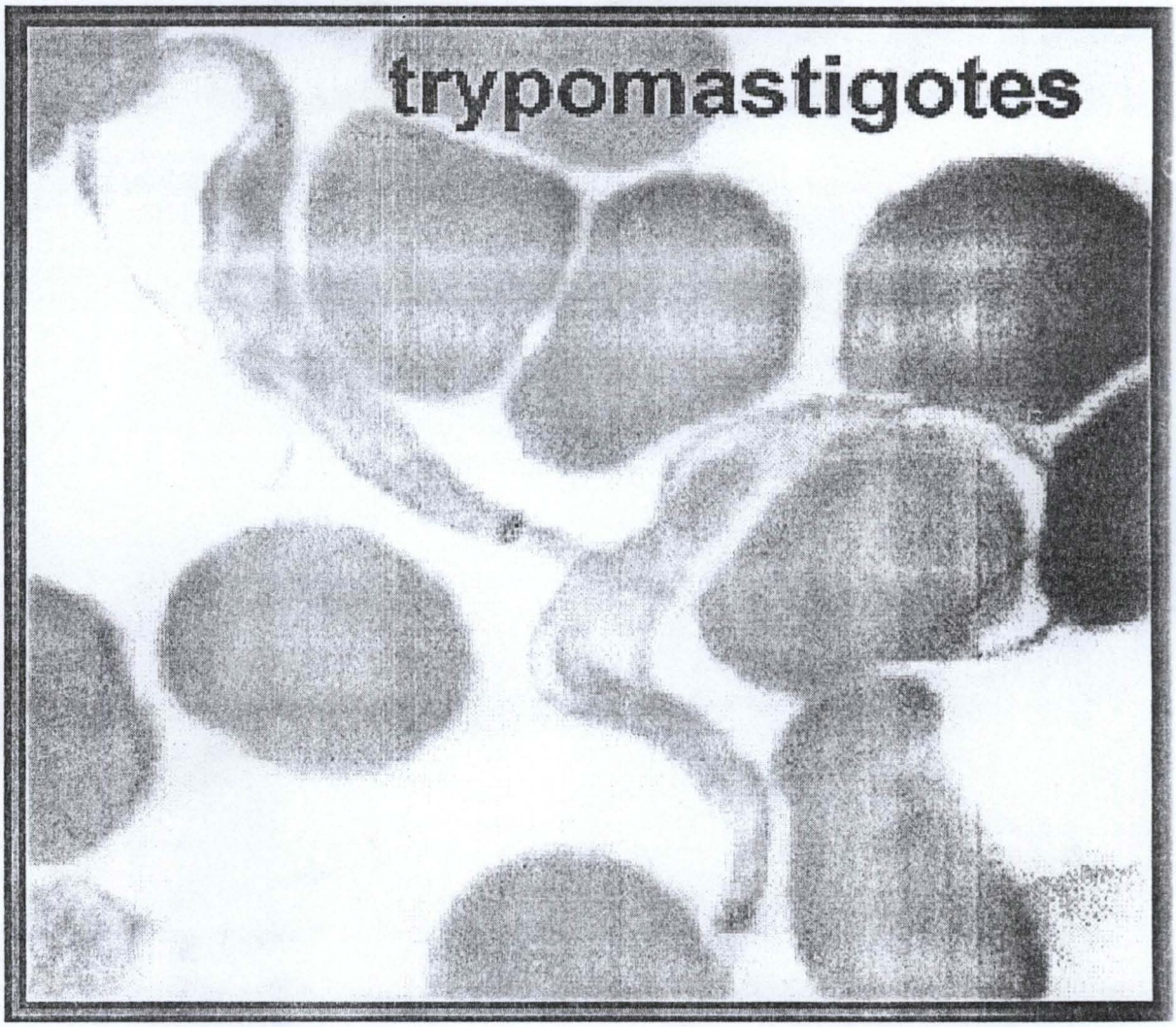

Fig 2.1a Trypomastigotes in blood (Hunt, 2007)

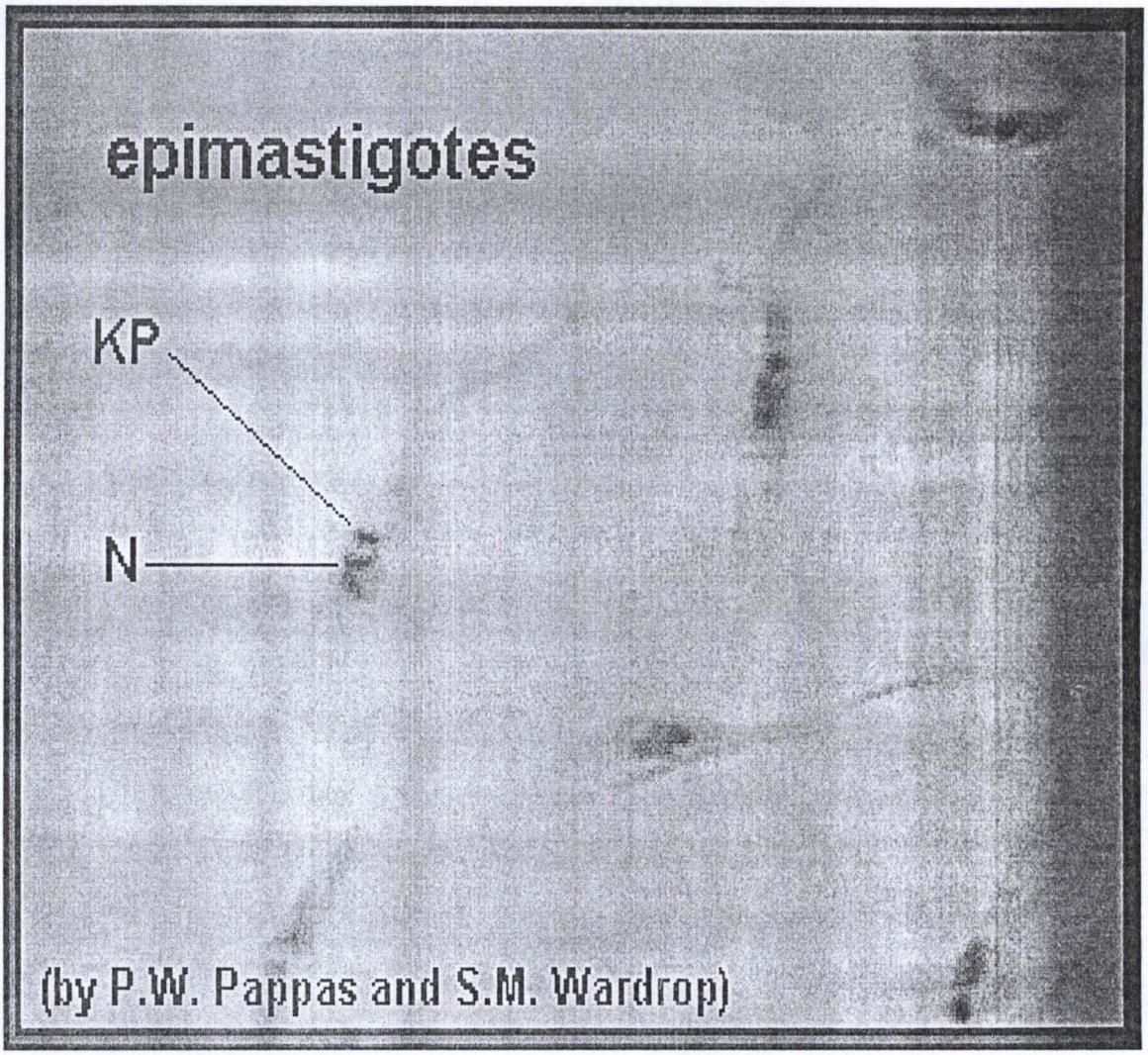

Fig 2.1b Epimastigotes grown in culture (Hunt, 2007).

2.1.1 Classification of Trypanosomes

Classification of pathogenic trypanosomes is based on their development in the vector and transmission by either the saliva or by fecal contamination of the wound caused by bite of vector. The genus *Trypanosoma* are divided into two sections, salivaria and stercoraria. Each section is subdivided into subgenera and species.

Trypanosoma vivax, and *T. uniforme,* salivarian, are species of the subgenus *Duttonella. Trypanosoma congolense,* and *T. smiae* are species of the subgenus *Nannomonas. Trypanosoma brucei, T. rhodesiense, T. evansi, T. equiperdum and T. gambiense* belongs to the subgenus *Trypanozoon. Trypanosoma suis* is also a salivarian belonging to the subgenus *Pycnomonas*. The Stercoraria are *Schizotrypanum (*species: *Trypanosoma cruzi)*, *Megatrypanum* (species: *Trypanosoma theileri*) and *Herpetosoma* (species: *Trypanosoma lewisi, T. musculi,* and *T. rangeli*. (Molyneux and Ashford, 1983)

2.1.2 Morphology and Life Cycle

2.1.2.1 Structure of an African Trypanosome

Trypanosomes are unicellular protozoans with a single flagellum that contains microtubules in the 9+2 arrangement typical of other flagella. At the base of the flagellum is the kinetoplast (figure 4a) 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 life cycle (Berriman *et al.*, 2005).

Most other organelles are those typical of any eucaryotic cell. At surface of the cell are sub-membranous pellicular microtubules which give the trypanosome its shape. These underlie a typical plasma membrane which is often covered by an electron-dense surface coat.

The two species of *T. brucei* are morphologically indistinguishable, but the differential diagnosis of the two infections can be made based on exposure history and serodiagnostic testing. The parasites range in size from 25 to 40 μm.

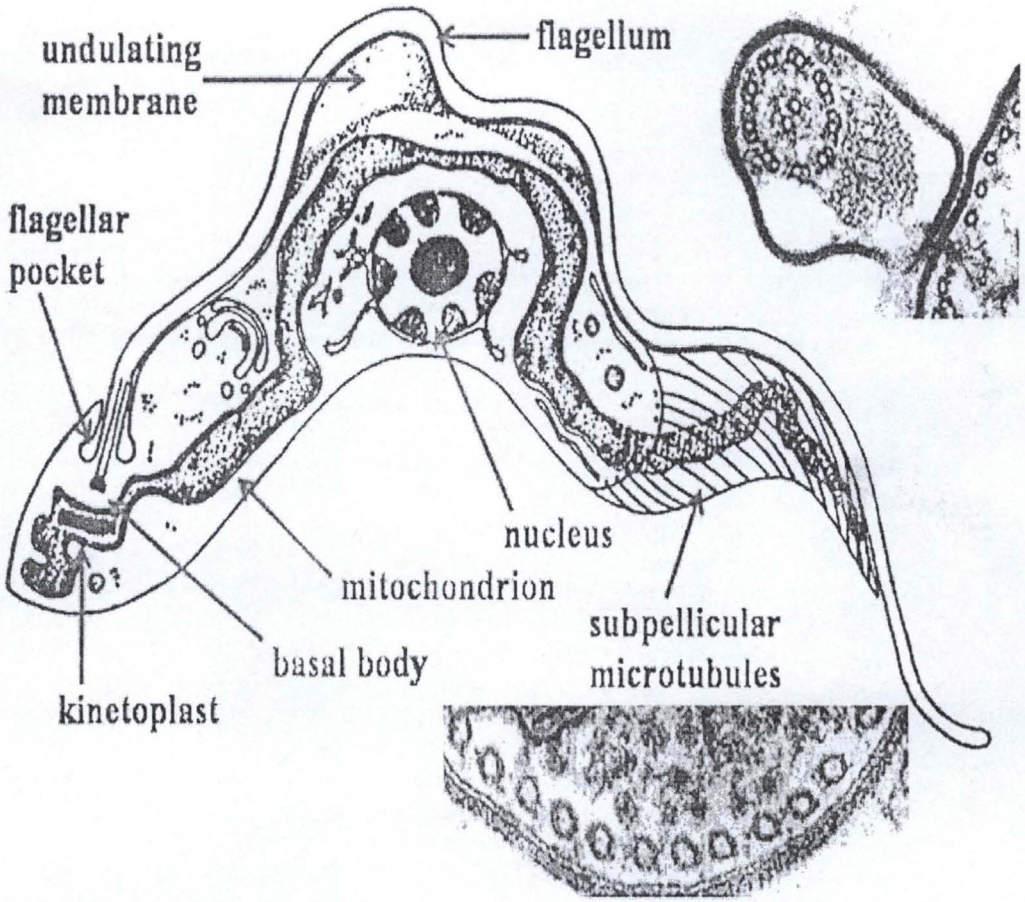

Fig 2.2 Diagram to show principal structures revealed by the electron microscope in the bloodstream trypomastigote form of the salivarian trypanosome. (Vickerman, 1969).

Several different morphological forms of trypanosomes are associated with different life cycle stages in the various species. The different forms are distinguished by the position of the kinetoplastid in relation to the nucleus and the presence or absence of an undulating membrane.

In the trypomastigote, the kinetoplast is located on the posterior end of the parasite. The flagellum emerges from the posterior end and folds back along the parasite's body. This attachment of the flagellum to the body forms an undulating membrane that spans the entire length of the parasite and the free flagellum emerges from the anterior end. This is considered the anterior end since the flagellum pulls the organism and the end with the free flagellum is the front in reference to the direction of movement. The undulating membrane functions like a fin and increases the motility of the organism.

The kinetoplast, in the epimastigote, is more centrally located usually just anterior to the nucleus. The flagellum emerges from the middle of the parasite and forms a shorter undulating membrane than observed in trypomastigotes. Epimastigotes are noticeably less motile than trypomastigotes.

The kinetoplast of promastigote is towards the anterior end and a free flagellum with no undulating membrane emerges. The end that the free flagellum emerges from in all three motile forms is designated as the anterior end because they swim in that direction. In other words, the flagellum pulls the organism.

In the Amastigote, the parasite is more spherical in shape and has no free flagellum. The structures associated with the point of origin of the flagellum kinetoplast and basal body may still be seen in the amastigote and are of diagnostic importance. This form is a non-motile intracellular stage.

2.1.2.2 Life Cycle of an African Trypanosome

Infection in the human host begins when the infective stage, known as the metacyclic trypomastigotes stage, is injected intradermally by the tsetse fly. The parasite exhibits a trypomastigote morphology in the bloodstream and is extracellular. These extracellular forms undergo an antigenic variation to evade the host immune system. Within the bloodstream the trypanosome undergoes asexual replication by longitudinal binary fission. The buildup of metabolic wastes and cell debris leads to the formation of a chancre. These replicating forms are generally long slender (LS) parasites. In addition to the long slender forms, intermediate and short stumpy (SS) forms are also found within the bloodstream of the mammalian host. The short stumpy forms are thought to be preadapted for the tsetse. However, *in vitro* experiments suggest that all bloodstream forms are infective for the tsetse.

The tsetse fly becomes infected by ingesting a blood meal from an infected host. These short, stumpy forms are pre-adapted to the vector, having a well developed mitochondrion with a partial TCA cycle. In the insect vector, the trypanosomes develop into procyclic trypomastigotes in the midgut of the fly, and continue to divide for approximately 10 days. Accompanying this differentiation is a loss of the VSG surface coat and changes in the mitochondria and metabolism. The environment within the gut of fly is quite different than that of the mammalian bloodstream. The mammalian bloodstream is rich in glucose and parasite exhibits a high rate of

glycolysis which is carried out in a special organelle known as the glycosome. Because of this abundance of glucose the parasite does not carry out oxidative phosphorylation within the mitochondria and consequently the mitochondria are acristate and have minimal electron transport activity. Within the vector, mitochondrial functions associated with aerobic metabolism return and cristae develop within the mitochondria. Here they gain a fully functional cytochrome system and TCA cycle. When the division cycles are completed, the organisms migrate to the salivary glands, and transform into epimastigotes.

After reaching the salivary glands the procyclic trypomastigotes transform into epimastigotes and attach to epithelial cells via their flagella. The epimastigotes probably undergo further replication within the salivary gland. These forms, in turn, divide and transform further into metacyclic trypanosomes, the infective stage for humans and reservoir hosts (Fig 2.5a & 2.5b). The cycle in the insect takes 25-50 days, depending upon the species of the fly, the strain of the trypanosome, and the ambient temperature. The epimastigotes are non-infective for the mammalian host and they must first mature into metacyclic trypomastigotes (MT). During this maturation the surface coat is reformed, the mitochondria lose their cristae and the parasite detaches. These trypomastigotes are free within the lumen of the salivary gland waiting to be transferred to a vertebrate host when the tsetse feeds again, thus completing the life cycle (Vickerman, 1985).

Flies can remain infected for life (2-3 months). Tsetse flies inject over 40,000 metacyclic trypanosomes when they take a blood meal. The minimum infective dose for most hosts is 300-500 organisms, although experimental animals have been infected with a single organism.

Infection can also be acquired by eating raw meat from an infected animal. In East Africa, this mode of transmission may be important in maintaining the cycle in some reservoir hosts.

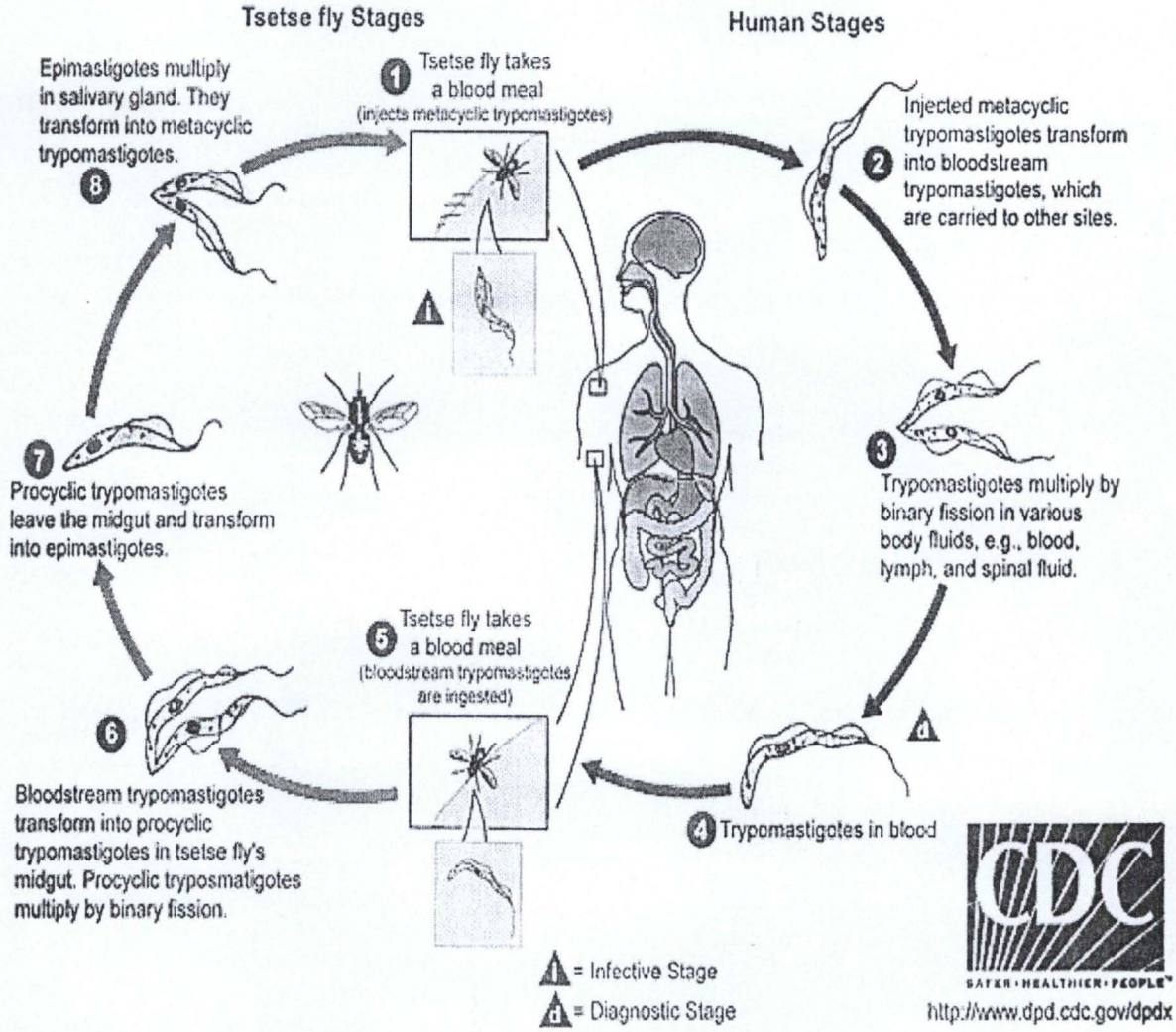

Fig 2.3a During a blood meal on the mammalian host, an infected tsetse fly (genus *Glossina*) injects metacyclic trypomastigotes into skin tissue (Vickerman, 1969).

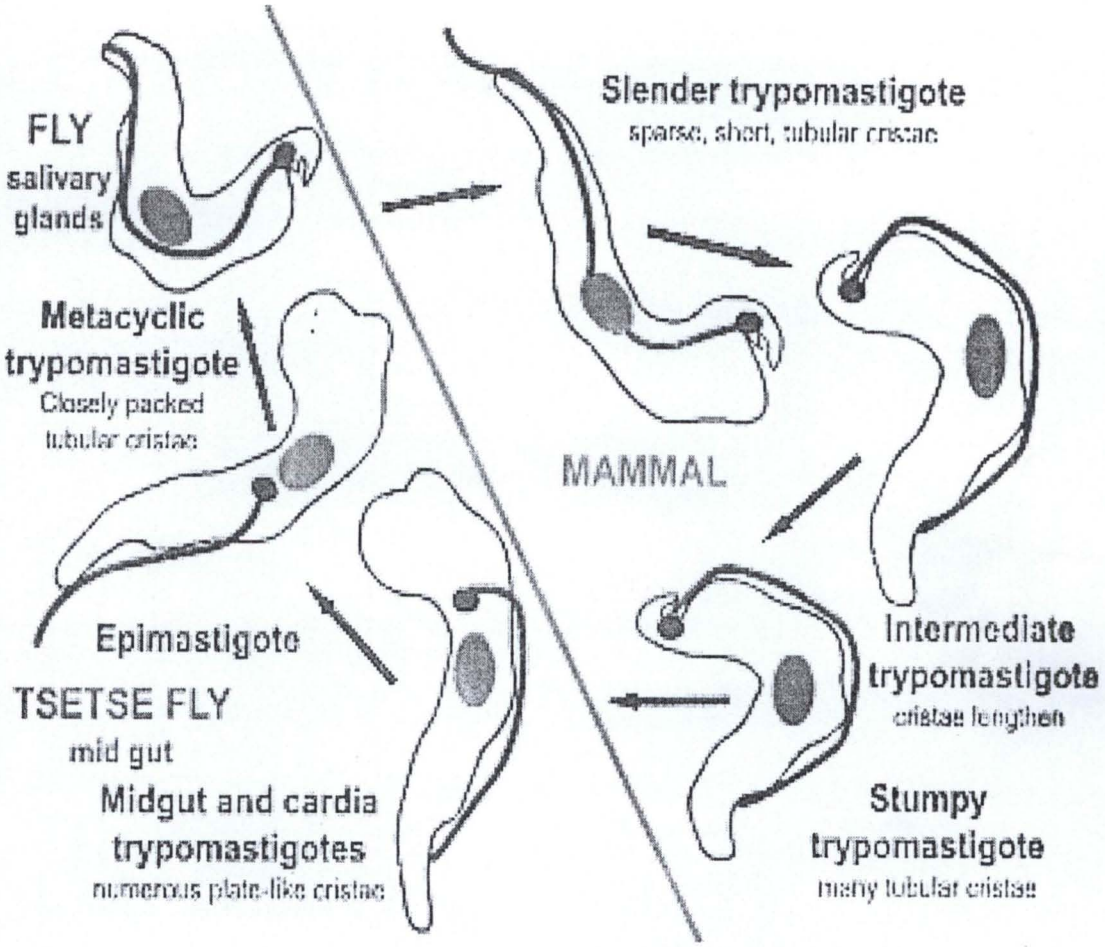

Fig 2.3b Life cycle of *Trypanosoma brucei brucei*. (Vickerman, 1969).

2.1.2.3 Morphology of Kinetoplast

The kinetoplast is a dark Giemsa-staining DNA containing structure which is distinct from the nucleus (Fig 2.4). The size of the kinetoplast varies according to species. The kinetoplast is found near the basal body which is located at the base of the flagellum. Because of this location near the flagellum, it was previously believed that the kinetoplast was somehow associated with cell movement--hence the name. However, the kinetoplast is actually a distinct region of the mitochondria and is not involved in motility. The staining of the kinetoplast is due to mitochondrial DNA

The kinetoplast is a DNA-rich structure which lies at the base of the flagellum and is at one end of the single long mitochondrion of the flagellate. 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 circle mitochondrial DNA of other cells. although our 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 mitochondrial transfer RNAs, the kinetoplast DNA does not code for any more RNAs or proteins than other mitochondrial DNAs. Indeed, some of the tRNAs of the kinetoplast are not encoded in this DNA and have to be imported from the cytoplasm which is not the case with mammalian mitochondria.The reason that kinetoplast DNA makes up such a high proportion of the total DNA of the cells is it complexity.

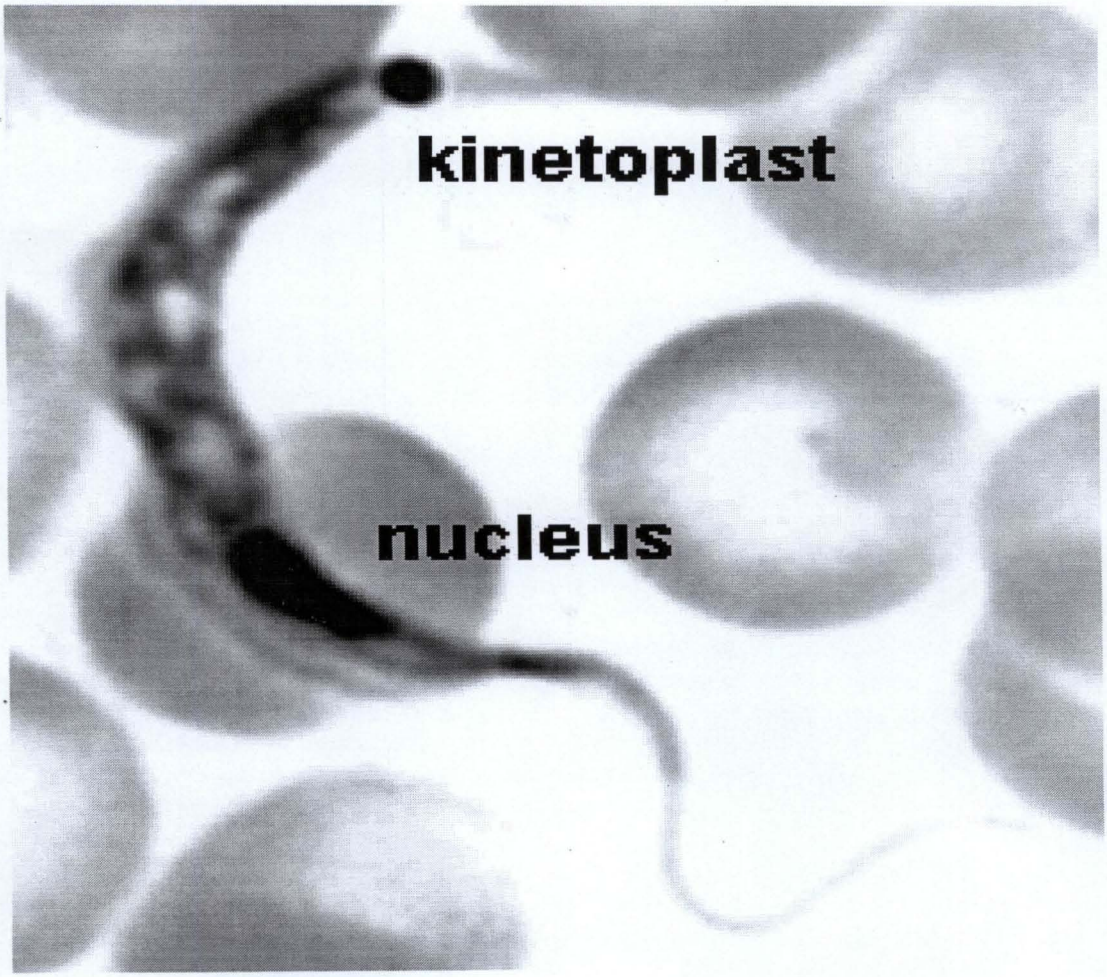

Fig 2.4 Dark Giemsa-stained kinetoplast (Hunt, 2010)

2.1.3 Biochemistry of Trypanosomes

2.1.3.1 Glycolysis in Trypanosomes

All living organisms make ATP as an energy carrier. This is produced mainly by the oxidation of carbohydrates using glycolysis and the tricarboxylic acid (TCA) cycle (Fig 2.6). Because free living organisms (like us) do not have an abundance of food, we rely on the much more efficient TCA cycle for most of our ATP production.

The trypanosome meets very different environments at different stages of its life cycle. In the mammalian blood stream there is an abundance of oxygen and glucose. The opposite is true in the insect gut or hemolymph. This is reflected in the number of reactions of glycolysis and TCA cycle that can be carried out and in the elaboration of the kinetoplast/mitochondrion.

The forms of *T. brucei* in the insect gut have a full complement of TCA and glycolysis enzymes since nutrients are not abundant. In most organisms that use oxidative phosphorylation, ATP production is sensitive to cyanide because cytochromes a/a_3 react with cyanide and can no longer transfer an electron to oxygen to form water; however, oxidative phosphorylation in the insect gut forms of Trypanosomes is only partially cyanide sensitive. Cytochrome a/a_3 system is still CN^--sensitive but in Trypanosomes there is an additional cytochrome O which is CN^- - insensitive. Little is known about the cytochrome O system. In the insect, partial aerobic fermentation produces succinate, pyruvate, acetate as well as carbon dioxide. Proline is a major fuel source (Hunt, 2007).

The forms of *T. brucei* in the mammalian bloodstream use only inefficient glycolysis because there is so much nutrient available; since glycolysis produces much less ATP than the TCA

cycle, respiration is 50 times that of normal mammalian cell and in the bloodstream, *T. brucei* uses 10 times the amount of fuel as in insect gut.

When only glycolysis is used to make ATP as is done in anaerobic respiration in our muscles or in yeast, pyruvate cannot just be excreted, which is what the trypanosome does in fact do, since all the NAD^+ will quickly be reduced to NADH and the whole pathway will stop for lack of oxidized substrate. We convert pyruvate to lactate while yeast converts it to ethanol to oxidize our NADH back to NAD^+ and keep glycolysis going. Trypanosomes excrete pyruvate and have another way of converting NADH back to NAD^+.

In trypanosomes, dihydroxyacetone phosphate metabolism is necessary for reoxidation of NADH. This is an aerobic system; however, requiring oxygen but oxygen consumption is CN^- - insensitive so it does not use the usual cytochrome chain. An FAD-containing dehydrogenase linked to copper containing oxidase. This complex is the glycerophosphate oxidase system. Trypanosomes in the bloodstream depend on this mechanism of keeping NAD oxidized. This system is the target of two trypanocidal drugs: SURAMIN and SHAM (salicylhydroxamic acid) which is a chelating agent. Terminal oxidase contains copper. These drugs have their specificity because this is a metabolic pathway that mammals do not use (Hunt, 2010).

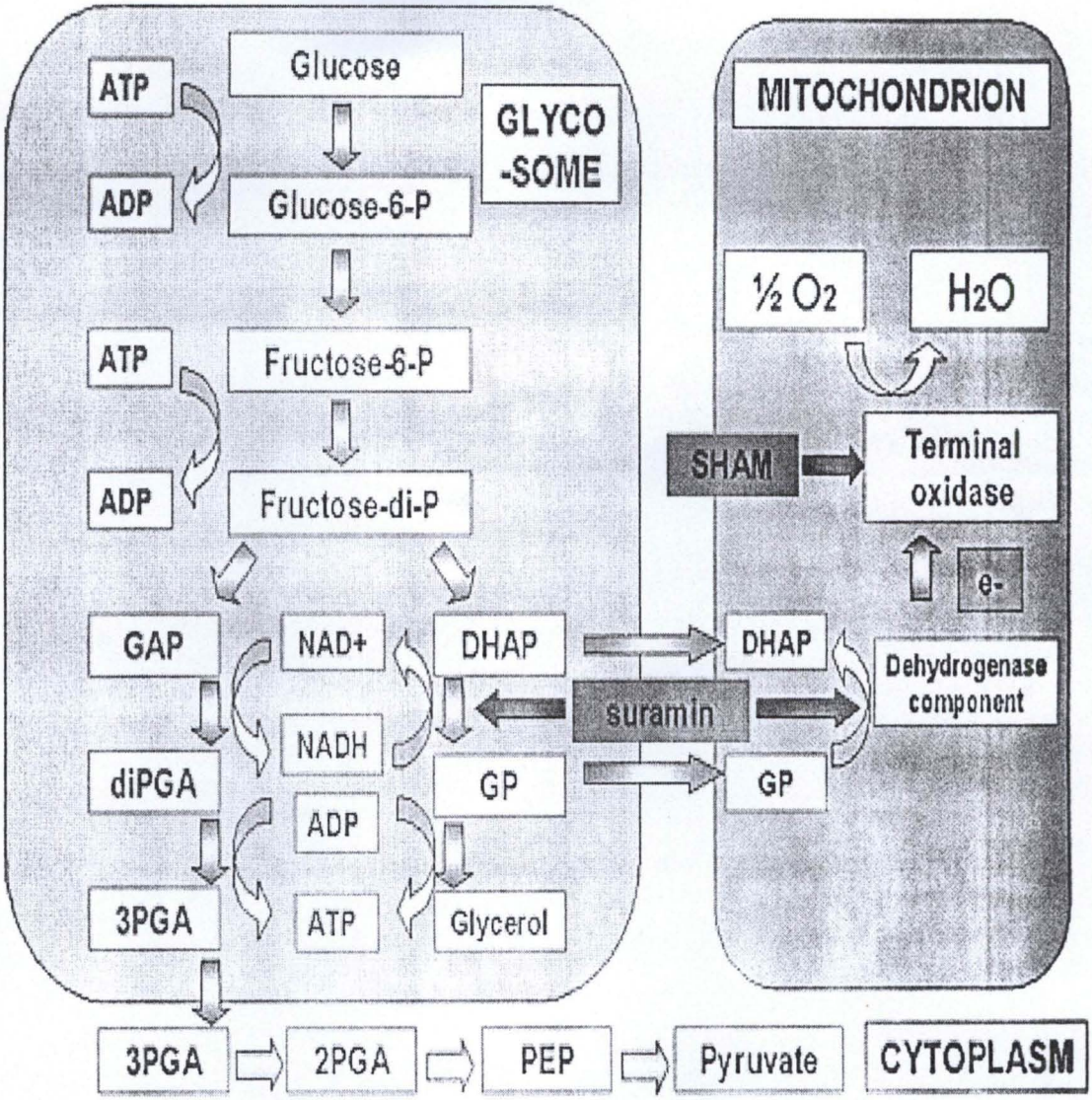

Fig 2.5 Glycolysis pathway in long – slender bloodstream form of *T. brucei* (Hunt, 2010).

2.1.3.2 Metabolism of Polyamine in African Trypanosomes

Polyamine metabolism in African trypanosomes is similar to mammalian polyamine metabolism, in that the organism synthesizes putrescine and spermidine from ornithine, and trypanosomal forms of ornithine decarboxylase and spermidine synthase have been identified. However, these organisms do not produce spermine, but instead convert two molecules of host-derived glutathione (1) and spermidine (2) into reduced trypanothione (4), which is used to protect the organism against oxidative stress. The formation of reduced trypanothione is mediated by two ATP-dependent enzymes, glutathionylspermidine synthetase, which produces glutathionylspermidine (3), and trypanothione synthetase (TS), which produces reduced trypanothione (4). In the presence of oxidative stress, oxidized trypanothione (5) is formed, and must be recycled to the reduced form by a third enzyme unique to the parasite, trypanothione reductase (TR).

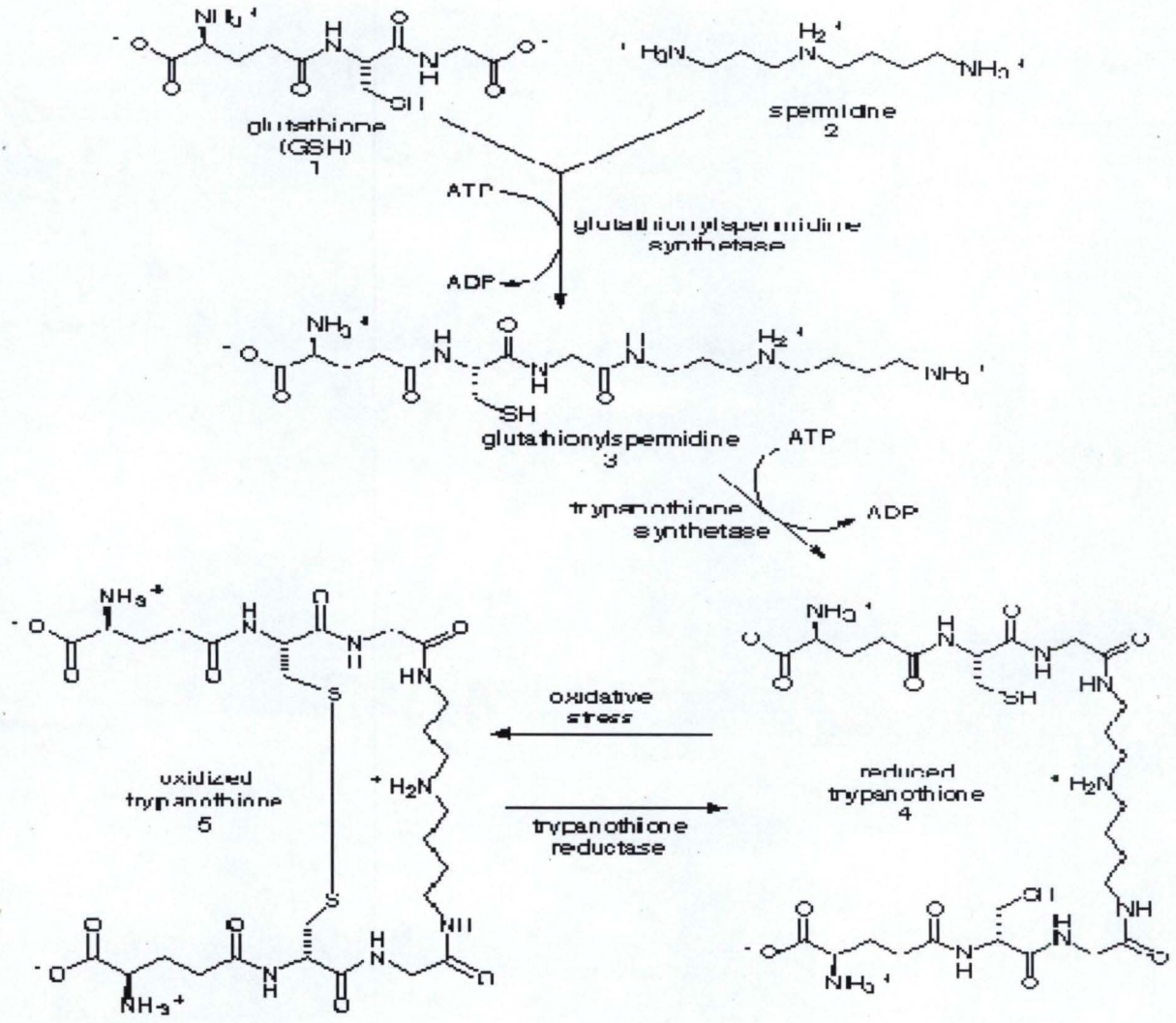

Fig 2.6 Metabolism of polyamine in African trypanosomes

2.1.4 Molecular Biology of African Trypanosome

2.1.4.1 Host Reactions to the Parasite

Usually, when parasite takes up residence within another organism, the host organism mounts an immune response to the parasite which may destroy it. Some parasites hide behind surfaces that disguise them so that they appear as normal cells to the immune system. Other parasites (including *Trypanosoma cruzi*) enter cells to get away from the immune system. *T. brucei* does neither. Instead, the parasite is very antigenic which is one reason for the symptoms shown by the infected patient. But the number of parasites in the bloodstream does not go on increasing and increasing until the patient dies. The patient undergoes waves of fever and cycles in parasite infestation. The waves of parasitemia correlate with the fever observed. Number of parasites in blood shows waves as the immune system partially overcomes the infection. Cyclic nature of parasitemia is very characteristic.

Massive immune response with strikingly high levels of Ig (especially IgM) and profound B lymphocyte proliferation mounted by the infected individual does not clear the entire parasite from the body. In the chronic phase of the infection, lymphoid organs are depleted of lymphocytes, they shrink and patchy fibrosis replaces the lymphocytes. Immunodepression sets in and the parasitemia is uncontrolled leading to death. The cells coat from different infected animals or patients has been shown to be biochemically so different. Moreover cells from a defined wave of parasitemia in the same patient shows that all of the trypanosomes in that wave of organisms are expressing the same single surface antigen whereas in other waves, all of the parasites are expressing a single but completely different antigen (Fig 2.8a). In other words, a different surface antigen gene is being expressed. The surface coat is therefore made of

VARIABLE SURFACE ANTIGENS or VARIABLE SURFACE GLYCOPROTEINS (VSGs). Thus escape from the immune response depends upon 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) and no antigen is repeated, there must be an equal number of VSG genes. In fact, there are probably 1000-2000 such genes C10% of the cells genome devoted to genes that express these surface molecules that allow the organism to be one step ahead of the host's immune response (LaCount, 2003).

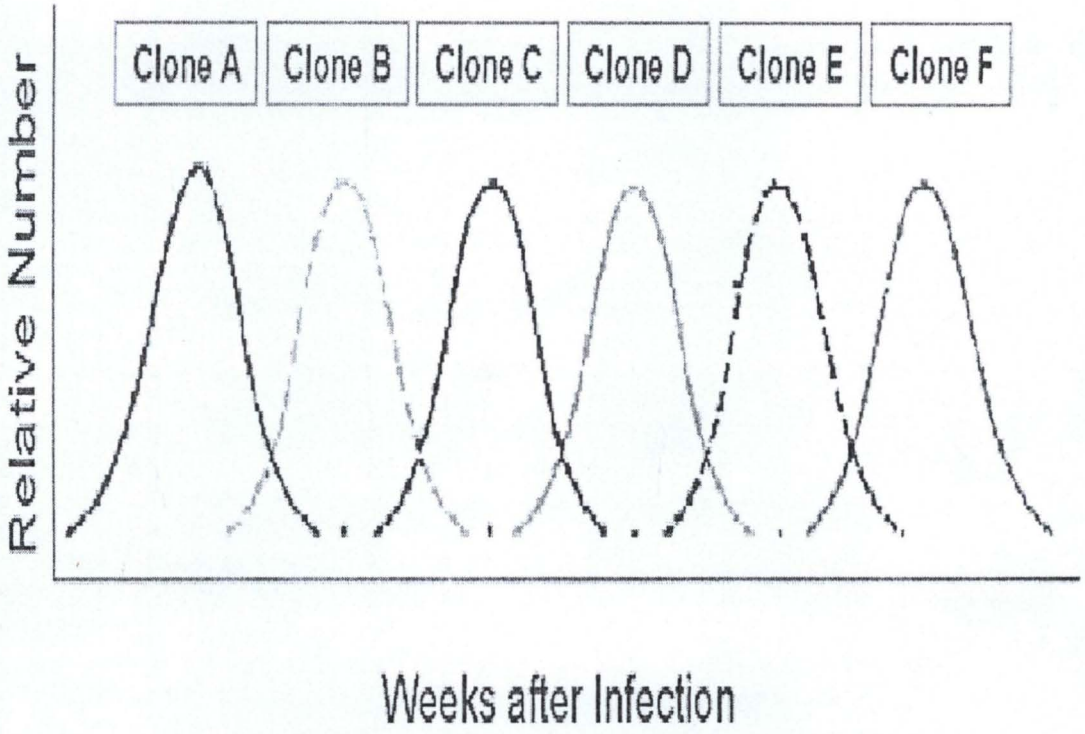

Fig 2.7 Successive waves of parasites in the blood are characteristic of sleeping.

2.1.4.2 Antigenic Variation in African Trypanosomes

Normally the host immune system is quite efficient at generating antibodies against infectious organisms and eliminating them from the circulation. In this regard, a characteristic of African trypanosomiasis is a fluctuating parasitemia. In other words the number of parasites in the circulation dramatically rises and falls. Generally, fever and other clinical symptoms are associated with the peaks in parasitemia. Further examination of parasites obtained from successive peaks reveals that they are antigenically distinct, or exhibit variant antigenic types (VAT).

The VAT are determined by a protein known as the variant surface glycoprotein (VSG). VSG is an abundunt protein (10^7 copies per cell) and is a major component of the 12-15 nm thick electron dense 'surface coat' covering bloodstream trypomastigotes. The parasite is estimated to have more than 1000 distinct VSG genes (occupying ~10% of the trypanosome's genome). Periodically the parasite will express a different VSG gene which is antigenically distinct from the previously expressed VSG with a switch rate of approximately 10^{-2} per cell per generation.

VSG is immunogenic and antibodies against VSG do lead to parasite elimination. Switching expression to another VSG results in a new surface coat which is now not recognized by the host antibodies. These parasites will then rapidly increase in number until the host mounts an immune response against the new VSG. The large repertoire of antigenically distinct VSG proteins means that the parasite stays one step ahead of the host and avoids complete elimination by the immune system (Turner, 1999).

There are two types of expression sites corresponding to the two life cycle stages in which VSG is expressed on the parasite surface: metacyclic expression sites and blood-stream form expression sites. The blood-stream form expression sites produce a polycistronic RNA containing several genes in addition to VSG that is processed to the mature mRNA and the metacyclic expression sites only have a VSG gene (Donelson, 2003., Rocha *et al.*, 2004).

'In situ' gene activation (i.e., transcriptional control) involves turning off the active expression site and initiating expression from another expression site. This in situ switching appears to predominate early in the infection and may represent a 'preprograming' of antigenic variation. Similarly, transcriptional regulation is also associated with metacyclogenesis. A limited subset of VSG genes can be expressed by metacyclic trypomastigotes found within the salivary glands of the tsetse. The expression of VSG and the surface coat is a preadaptation of the parasite for the vertebrate host. Another mechanism of VSG switching involves telomere exchange between the telomere with the active expression site and a telomere of a silent expression site or the telomere of a minichromosome. The exchange of the telomeres results in the expression of a previously silent gene and the silencing of the previously expressed gene. The third mechanism of VSG switching involves a duplicative transposition of these VSG genes into the active expression site. The archive copy of the VSG gene is copied and the template remains intact. The copied gene then replaces the gene in the active expression site by a gene conversion process (Mervyn, 2010).

The cDNA (gives sequence encoded by gene) 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, phospho-inositol 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. Therefore this site is of no use in vaccine development (Hunt, 2007).

2.1.4.4 Sequential Expression of Variable Surface Antigens (VSGs)

Analyses have shown that when African trypanosomes express a new coat, a new copy of the gene for that coat, called expression-linked copy (ELC) is expressed. It is only the gene that is being expressed that occurs in an extra copy. Thus there is gene duplication but it is only temporary as when the trypanosome switches to a new VSG the extra copy usually disappears and is replaced by another expression-linked copy (ELC). It is the extra copy that is being transcribed into mRNA for translation into protein, not the copy that is permanently in genome. The new copy is in an EXPRESSION SITE which is always close to a telomere that is near the end of a chromosome. An ELC and a permanent gene may be on different chromosome. This suggests a copy/translocate mechanism for a cassette of information. Sometimes, an expression linked copy is not produced but the permanent copy is transcribed. These transcribed non-ELC genes are always at telomeres. The temporary copying of a gene and using the copy is very unusual.

Indeed, the order in which these genes are expressed is not absolute. At the beginning of the infective phase, VSGs are produced by parasites in the insect salivary glands. A subset of the repertoire (about 12) of the VSGs are produced here. This remains during the first wave of parasitemia in the mammal. 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. Hence the fact that there is an initial subset of VSGs that are expressed gives hope for a vaccine.

mRNAs in trypanosome are also very unusual. Almost every mRNA that has been looked at starts with the same 35 nucleotides. This is coded by an exon far away from the rest of the gene (Hunt, 2010).

2.1.4.5 Shedding of the Variable Surface Antigens (VSG) Coat

The trypanosome parasite sheds all of its coat at a given time in order to survive the developing immune response against its original VSG. The form of the VSG at the cell surface is attached through a glycolipid and not via a transmembrane protein sequence. This give a clue to how shedding occurs. A trypanosome- and VSG-specific phospholipase C is found in bloodstream forms and since all VSGs have the same attachment structure only one enzyme is needed for rapid and complete cleavage (Hunt, 2010).

2.1.4.6 Extraordinary RNA Editing by Trypanosomes

The trypanosomes have a very odd nuclear genome. The chromosomes do not condense in nuclear division and so the exact number chromosomes are not known. The nuclear genes include 1000-2000 genes that encode the variable surface antigens that allow the coast of the organisms to be changed regularly so that it can avoid the host's immune response. The way in which this is done is extraordinary and 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 (Rocha *et al.*, 2004).

2.1.4.7 The Enigma of the Cytochrome Oxidase of *Trypanosoma Brucei*

Researchers have shown that in all the trypanosomes studied, the cytochrome oxidase subunit III (CO III) is encoded in the kinetoplast DNA, and are all in the same position within that DNA. *T. brucei* kDNA lacks COIII gene which differentiates them from two other trypanosomes, *Crithidia fasciculata* and *Leishmania tarentolae.* In the other two species, this gene is upstream of the apocytochrome b gene. Evidence was found for an mRNA transcript in *T. brucei* that had a sequence similar to the gene for COIII in the other trypanosomes. The similarity of the sequences was exploited in the determination of the correct open reading frame for the *T. brucei* transcript and the following were established: Of the 181 amino acids predicted by the sequence, 135 conserved amongst all three. Taking conservative replacements and those conserved in one other species, 160 out of 181 (88%) are conserved.

Heterologous hybridization using *L. tarentolae* and *C. fasciculata* COIII probes could not detect COIII sequences in *T. brucei* genes. This was confirmed by Southern blot analyses using probes that were predicted form the sequence that was obtained for the transcript. The probes do not hybridize to kDNA or to total DNA. Two possibilities were put forward to explain this: (1) the transcript may be made by splicing together small fragments of RNA transcribed from multiple sites. There is after all precedent for mini-exons in the trypanosome system. There are data that would tend to exclude this possibility. (2) 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.

Researchers have shown that the sequence in the upstream of apo-cytochrome b gene in the kinetoplast where the COIII gene is located in so many other trypanosomes *matches* the transcript sequence exactly except for the presence of uridines in the transcript that are not in the

DNA of the gene. In spite of the differences between the RNA and DNA sequences, only the number and positions of Us are affected; Cs, Gs, and As occur in the same sequence in the mRNA and the genomic DNA. Of the 626 nucleotides sequenced initially in the *T. brucei* COIII 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 COIII genes. In addition, 16 uridines, predicted by the genomic sequence at 7 sites appear to be deleted. The protein coding portion of the transcript contains 315 additions and 15 deletions in 546 nucleotides. Thus 58% of the coding part of the mRNA results from editing and is not in the original gene. The editing is the reason why cDNA probes do no hybridize to the genomic DNA (Hunt, 2007).

2.2 African Trypanosomiasis

African trypanosomiasis is restricted to equatorial Africa with a patchy distribution depending upon specific topographical features. An estimated 60 million people in 36 nations are at risk with an estimated 500,000 cases occuring annually. However, only 45-50,000 annual cases are reported. Since 1970 the incidence of the disease has been increasing. The control of African trypanosomiasis is complicated by poverty, political instability, and civil wars often found in areas endemic for the parasite.

The parasites responsible for causing African sleeping sickness belong to a group of closely related trypanosomes in the *Trypanosoma brucei* species complex. The three morphologically indistinguishable species are *T. brucei, T. rhodesiense, T. gambiense.*

2.2.1 Human African Trypanosomiasis (HAT)

Human African Trypanosomiasis, also known as sleeping sickness, is a vector-borne parasitic disease. The parasites concerned are protozoa belonging to the *Trypanosoma* Genus. They are transmitted to humans by tsetse fly (*Glossina* Genus) bites which have acquired their infection from human beings or from animals harbouring the human pathogenic parasites.

Tsetse flies are found in Sub-Saharan Africa. Only certain species transmit the disease. Different species have different habitats. They are mainly found in vegetation by rivers and lakes, in gallery-forests and in vast stretches of wooded savannah. Sleeping sickness occurs only in sub-Saharan Africa in regions where there are tsetse flies that can transmit the disease. For reasons that are so far unexplained, there are many regions where tsetse flies are found, but sleeping sickness is not. The rural populations living in regions where transmission occurs and which depend on agriculture, fishing, animal husbandry or hunting are the most exposed to the bite of the tsetse fly and therefore to the disease.

Sleeping sickness generally occurs in remote rural areas where health systems are weak or non-existent. The disease spreads in poor settings. Displacement of populations, war and poverty are important factors leading to increased transmission. The disease develops in areas whose size can range from a village to an entire region. Within a given area, the intensity of the disease can vary from one village to the next (WHO, 2006).

Human African Trypanosomiasis takes two forms, depending on the parasite involved:

- *Trypanosoma brucei gambiense (T.b.g.)* is found in west and central Africa. This form represents more than 90% of reported cases of sleeping sickness and causes a chronic

infection. A person can be infected for months or even years without major signs or symptoms of the disease. When symptoms do emerge, the patient is often already in an advanced disease stage when the central nervous system is affected.

- *Trypanosoma brucei rhodesiense (T.b.r.)* is found in eastern and southern Africa. This form represents less than 10% of reported cases and causes an acute infection. First signs and symptoms are observed after a few months or weeks. The disease develops rapidly and invades the central nervous system.

However, in both cases without proper diagnosis and treatment the outcome is death.

Major Differences Between African Trypanosome Species

Attribute	*T. rhodesiense*	*T. gambiense*
tsetse vector	*G. morsitans* group	*G. palpalis* group
ecology	dry bush, woodland	rainforest, riverine, lakes
transmission cycle	ungulate-fly-human	human-fly-human
non-human reservoir	wild animals	domestic animals
epidemiology	sporadic, safaris	endemic, some epidemics
disease progression	rapid, often fatal	slow (~1 yr)acute ⇒ chronic
Parasitemia	High	Low
asymptomatic carriers	Rare	common

(Barrett, 2003)

2.2.2 Geographical Distribution of the Disease

All cases of African trypanosomiasis are imported from Africa by travelers to endemic areas. Infections among travelers are rare, with less than 1 case per year reported among US travelers. Most of these infections are caused by *T brucei rhodesiense* and are acquired in East African game parks (Simo, 2006). 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 African trypanosomiasis varies by country and region. In 2005, major outbreaks were observed in Angola, the Democratic Republic of Congo, and Sudan. 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. Fewer than 50 new cases per year are reported in countries such as Burkina Faso, Cameroon, Equatorial Guinea, Gabon, Kenya, Mozambique, Nigeria, Rwanda, Zambia, and Zimbabwe.

T. brucei transmission seems to have stopped and no new cases of African trypanosomiasis 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.

Sleeping sickness threatens millions of people in 36 countries of sub-Saharan Africa. The current situation is difficult to assess in numerous endemic countries because of a lack of surveillance and diagnostic expertise.

In 1986, a panel of experts convened by the World Health Organization (WHO) estimated that 70 million people lived in areas where transmission of African trypanosomiasis is possible. In 1998, almost 40,000 cases of the disease were reported, but this number did not reflect the true situation given the remoteness of affected regions and the focal nature of the disease. Between 300,000 and 500,000 more cases were estimated as remaining undiagnosed and therefore untreated.

During recent epidemic, the prevalence of sleeping sickness reached 50% in several villages in the Democratic Republic of Congo, Angola, and Southern Sudan. Sleeping sickness was considered the first or second greatest cause of mortality in those communities, even ahead of HIV infection and AIDS. By 2005, surveillance had been reinforced and the number of new cases reported throughout the continent had substantially reduced; between 1998 and 2004, the figures for both forms of African trypanosomiasis together fell from 37,991 to 17,616.

The estimated number of cases is currently between 50,000 and 70,000. The current epidemic, which began in 1970, is thought to have been facilitated by factors such as the halting of screening programs, population migration, civil war, economic decline, and reduced health care financing (WHO, 2006).

2.2.3 Origin of *Trypanosoma rhodesiense*

The genetic and evolutionary relationships between *T. gambiense*, *T. rhodesiense*, and *T. brucei* provide insight into the evolution of human disease. Molecular techniques show that *T. gambiense* is relatively homogeneous throughout its wide distribution in central and western Africa. In contrast, distinguishing *T. rhodesiense* and *T. brucei* has been much more problematic. Molecular

analyses indicate that both of these species exhibit a wide range of sequence heterogeneity and in some cases more homology is observed between *T. rhodesiense* and *T. brucei* than between isolates of the same species obtained from different geographical regions. The discovery of a single gene, called SRA, that can confer resistance to trypanosome lytic factor has provided some insight into the evolutionary relationships between *T. rhodesiense* and *T. brucei*.

SRA is a truncated VSG and is only found in *T. rhodesiense*. Presumably this mutation in the ancestral *T. brucei* conferred human infectivity and thus represented the origin of *T. rhodesiense*. Transfer of this gene to other *T. brucei* isolates with different genetic backgrounds via sexual recombination accounts for the genetic heterogeneity of *T. rhodesiense* and its spread in eastern Africa. In this regard, one might also consider *T. rhodesiense* a host-range variant of *T. brucei* (Gibson, 2002., Tait *et al*, 1985. and Gibson, 1989).

2.2.4 Epidemiology of Human African Trypanosomiasis

2.2.4.1 *Trypanosoma brucei gambiense*

West African sleeping sickness is typically a chronic disease, making it a difficult disease to diagnose in the field. Low levels of trypanosomes in circulating blood make it difficult to detect the presence of parasites in blood smears, requiring more sophisticated means of detecting trypanosomes such as with the use of miniature anion-exchange / centrifugation (mAEC) technique. In comparison to the East African form, *T. b. gambiense* has a longer evolutionary history with humans, having successfully adapted to establishing infections in human hosts without manifesting severe symptoms. Astonishingly, infection rates of *T. b. gambiense* in wild

glossina populations are as low 0.1%, even in areas with an epidemic of sleeping sickness (Jordan, 1986).

Vectors of the West African sleeping sickness are species of the palpalis group, most of which are in close contact with humans. Several different reservoirs for *T. b. gambiense* have been identified, strongly suggesting that the persistence of sleeping sickness in human populations may be maintained by other animals, such as the African domestic pig (Watson, 1962; Gibson *et al.*, 1978; Mehlitz *et al.*, 1982). However, *T. b. gambiense* has not been observed or proven experimentally to reach significantly infectious levels of parasitemia in other reservoir hosts. Although it is widely accepted that the human-to-fly contact is the main route of transmission, some suggest a minor cycle involving an animal reservoir may help explain the re-emergence and persistence of the disease in West Africa (Noireau *et al.*, 1989).

The epidemiology of *T. b. gambiense* sleeping sickness is far from being fully understood. Despite the low levels of parasitemia in humans, the disease has successfully established endemicity in many regions of West Africa. It has also long been observed that the incidence of disease is not related to the density of the glossina populations and that epidemics often occur in areas where the density of the vector is low (Jordan, 1986). In Nigeria, sleeping sickness occured in the north where the distribution of *G. p. palpalis* and *G. tachinoides* were scarce and restricted to vegetation close to watercourses during the dry season (Edeghere *et al.*, 1989). In Southern Nigeria, the same species of tsetse flies are found in abundance due to favorable climatic conditions, yet cases of sleeping sickness have never been observed. It is thought that the nature of the human-fly contact is of particular importance in the transmission of *T. b. gambiense* and the distribution of the disease, and that human-fly contacts can be classified as "personal" or

"impersonal" depending on the ecological circumstances of the interaction (Nash and Page, 1953). "Personal" contact refers to situations where fly movements are restricted to areas where exposure to humans are frequent, such as a watering hole or a stream, and single tsetse fly can have multiple opportunities to feed on humans. "Impersonal" contact occurs when fly movements are less restricted, and where repeated contacts are not likely. In general, ecological isolation of tsetse flies in the vicinity of human populations lead to increased "personal" contact. Climatic stress, lack of natural hosts where humans have destroyed wild animals close to villages, or clearing vegetation for cultivation are all examples of restrict movements of palpalis group vectors.

2.2.4.2 *Trypanosoma brucei rhodensiense*

East African sleeping sickness differs from West African sleeping sickness in both its epidemiology as well as its clinical manifestations in mammalian hosts (Baker, 1974). The clinical symptoms of East African sleeping sickness are more severe, and the onset of the disease is rapid. In contrast to *T. b. gambiense, T. b. rhodensiense* occurs with higher levels of parasitemia in ungulates, and humans are the adventitious hosts. The vectors of *T. b. rhodensiense* are the *G. mositans* subspecies, *G. pallidipes* and *G. swnnertoni* species from the morsitans group, and on lesser occasions the peridomestic vectors from the palpalis group, *G. fuscipes* and *G. tachinoides.* Sporadic cases usually arise from among those in the population whose activities bring them into contact with the savannah woodland habitats of the morsitans group. Although the vectors normally feed on game animals, under extreme situations where "personal" contact is increased due to social and/or environmental factors, a human-fly-human transmission cycle may ensue resulting in an outbreak. Droughts and political turmoil are known

to increase the number of cases when entire communities relocate to hitherto unoccupied areas in search of safety or fertile lands and water (Molyneaux and Ashford, 1983).

2.2.5 Transmission of African Trypanosomiasis

The disease is transmitted through the bite of an infected tsetse fly. At first the trypanosomes multiply in subcutaneous tissues, blood and lymph. In time, the parasites cross the blood-brain barrier to infect the central nervous system. The process can take years with *T.b. gambiense*. In addition to the bite of the tsetse fly, the disease is contractible in the following ways:

- Mother-to-child infection: the trypanosome can cross the placenta and infect the fetus. Laboratories: accidental infections, for example, through the handling of blood of an infected person, pricks from contaminated needles and organ transplantation can occur although this is uncommon.
- Blood transfusion
- Sexual contact (may be possible, but appears rare). (Rocha *et al.,* 2004).

2.2.6 Vectors of Salivarian Trypanosomes

Glossina species, tsetse flies, are the insect vectors of the salivarian trypanosomes of Africa and are thus among the most economically important vectors. They are commonly known as tsetse flies. *Glossina* belong to the family glossinidae and order Diptera. Glossina are nondescript in colour, being of brownish grey hue occasionally with darker bands on the abdomen (Fig. 2.1). Currently, this species of flies are restricted to sub-Saharan Africa north of the Kalahari Dessert, which currently restricts the transmission of the disease to within this region. However, with

rapid and frequent intercontinental travel, the introduction of this species to naïve regions poses a threat.

Tsetse flies are all haematophagous, totally dependent on blood sucking to derive nutrients. Different species of *Glossina* have different preferences for the source of their blood meal with some specifically preferring human blood and are therefore important vectors of the disease in human populations. These host preferences are important in the epidemiology of the diseases transmitted by *Glossina*. Both male and female flies feed on blood and are both vectors of the parasites (Molyneux and Ashford, 1983).

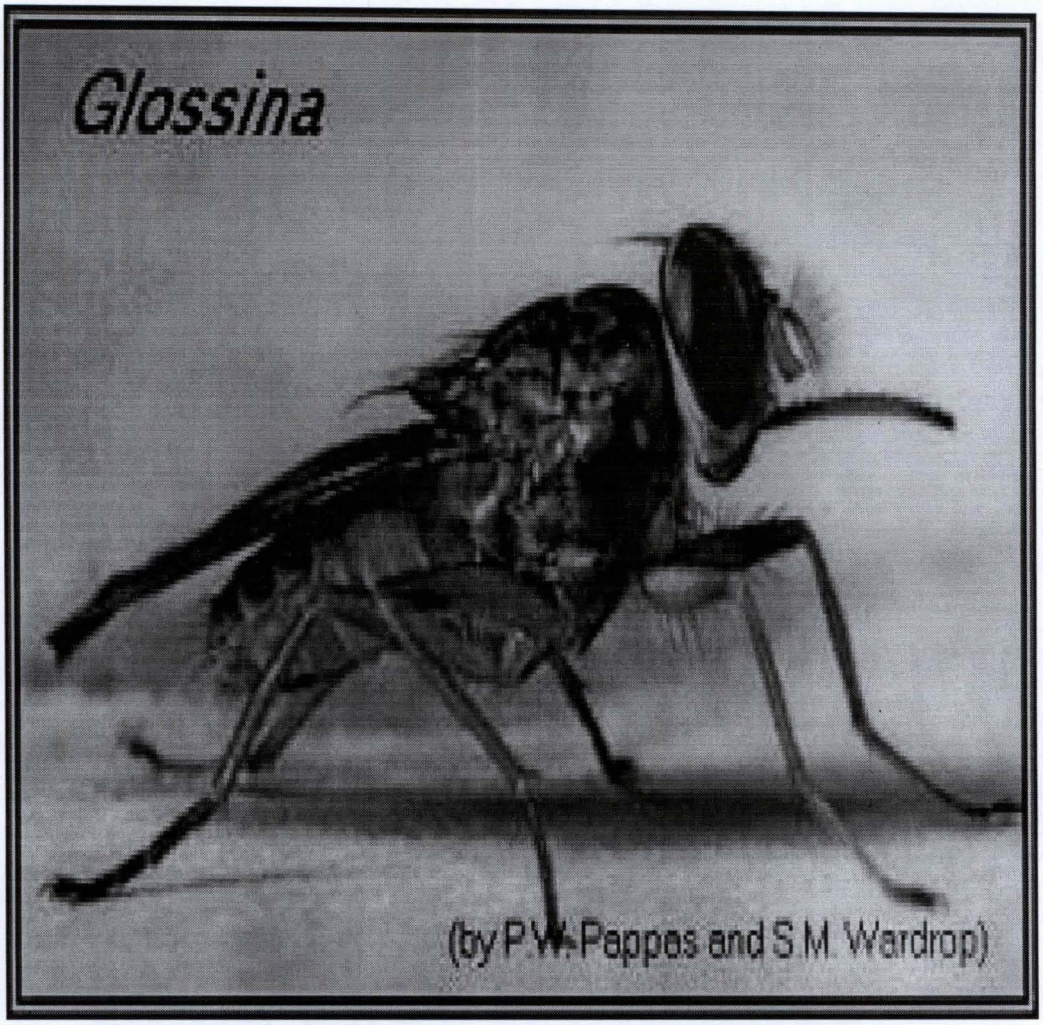

Fig 2.8 Picture of Glossina, the tsetse fly and vector of African trypanosomiasis

2.2.6.1 Biology and Ecology of Tsetse Fly

2.2.6.2 Classification of Tsetse Fly

There are 31 species and subspecies of tsetse flies under the genus *Glossina*, family *Glossinidae*, and order *Diptera*. Tsetse flies are largely classified into three subgenera based on morphological differences in the structure of the genitalia: *Morsitans* (Savannah species), *Palpalis* (Riverine species), and *Fusca* (Forest species) groups. All members of a particular species will breed successfully with each other and give fertile offsprings. Although the tsetse flies can be found over some 9 million squared kilometers of the African continent, presence of *Glossina* populations throughout the continent are far from continuous. Although tsetse fly habitats may vary considerably, climate and altitude - through their direct effects on vegetation, rainfall, and temperature are still the primary determinants for proliferation. Unlike other insects, there are no seasonal interruptions in the life cycles of tsetse flies. However both adult longevity and puparial duration are related to temperature and a significant seasonal decline in tsetse populations is normal, particularly in savannah habitats during the dry season. The 3 groups of tsetse flies are generally adapted to different habitats and ecozones (Leak, 1999).

2.2.6.3 Distribution and Habitat of Tsetse Fly

The southern limits of the Glossina distribution in Africa lies north of a line drawn from Benguela in Angola to Durban in the Republic of South Africa, while the northern limits are roughly a line from Dakar in Senegal across to Ethiopia and Mogadishu in Somalia on the East coast (Ford, 1960).

The Morsitans group is not found in the wet areas but is present throughout much of the savannah (grassy woodland) of Africa. Their distribution appears to be limited by cold winter conditions in the north of west and central Africa. The Palpalis group is called the riverine flies, which are associated with local patches of dense vegetation along the banks of rivers and lakes in the arid areas and are also limited to the very humid areas of Africa. On the other hand, the Fusca group is limited to the more thickly forested areas of Africa (Moloo, 1993).

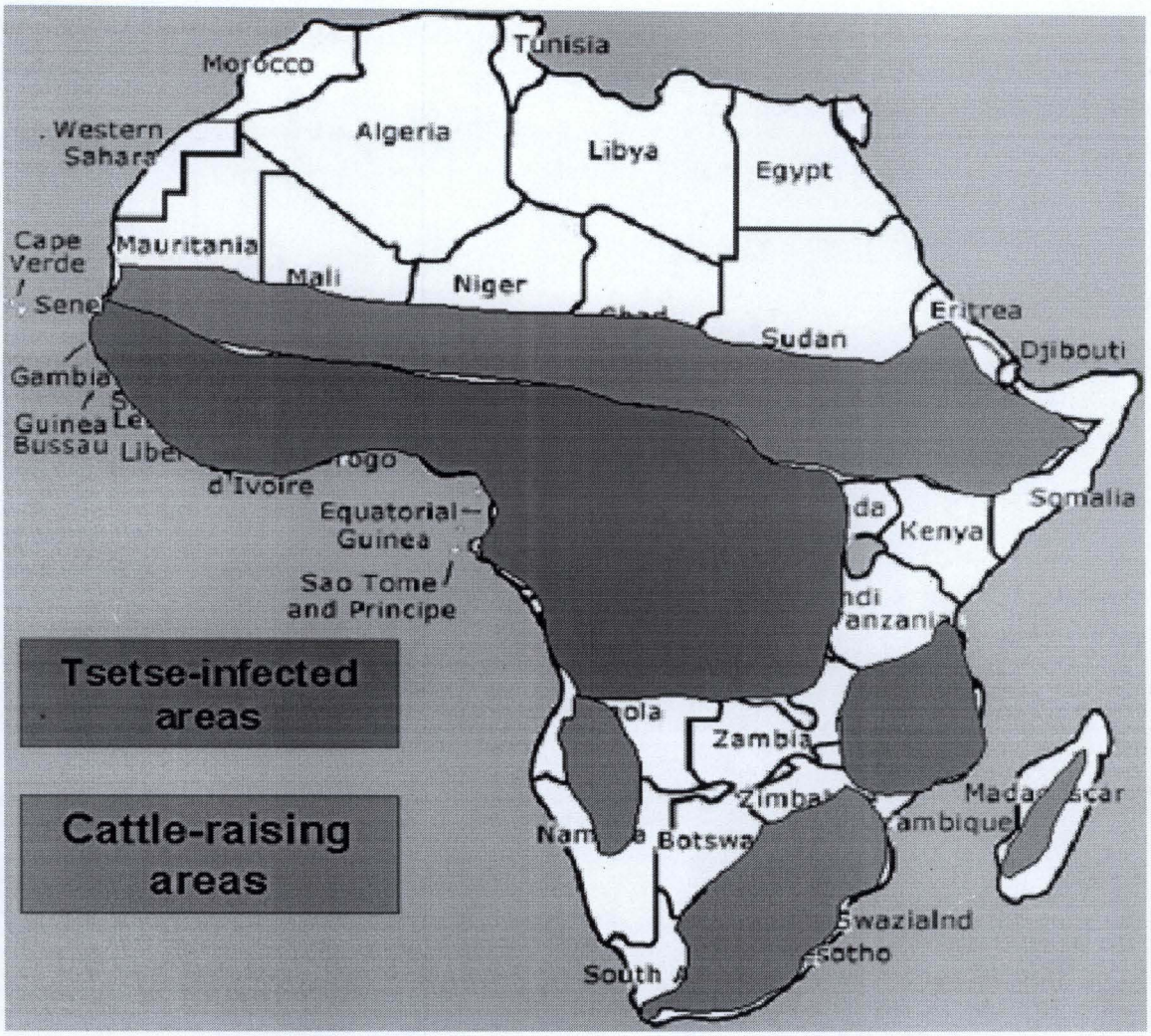

Fig 2.9 Map of the distribution of tsetse fly and cattle raising areas in Africa (Hunt, 2007).

2.2.6.4 Life Cycle of Tsetse Fly

Unlike most insect species (r-strategists) that produce large quantities of eggs, fertilized female tsetse flies (k-strategists) "give birth" to one larva. A typical female tsetse fly will produce one full grown larva approximately every 9-10 days depending on temperature and humidity. A single egg will hatch and develop to a third-stage larva in the uterus of the female fly, where it is nurtured and supplied with nutrients. This reproductive process is known as adenotrophic viviparity. This form of reproduction ensures the higher degree of survival of each offspring, but is also the reason why reproductive rates are considerably low in tsetse fly populations. In laboratory colonies, a single adult female can produce up to 12 offspring this way, but in the wild the number is speculated to be lower (Leak, 1999).

Larviposition takes place when the third-instar larva is deposited onto a suitable site, usually soil or sand depending on the species, and the larva burrows down to its optimum depth to become a pupa. Usually an adult fly will emerge after the puparial period which varies according to temperature but on average is around 30 days at 24°C (Leak, 1999). Longevity of the adult fly varies greatly according to seasonal factors. For the general tsetse population to increase, it is critical that the average female lifespan exceed 36 days. During optimal conditions, female flies can live as long as 3 months, producing as much 10 offspring during her lifetime (Jordan, 1986).

Many population control methods in the past have been successful because tsetse populations are far more vulnerable to disruptions in life cycles than insects that are r-strategists, such as mosquitoes. When both larval and adult mortality rates are artificially increased through control methods such as fly traps, insecticide spraying, and sterile insect techniques, the reduction in reproductive rate is profound.

2.2.7 Clinical Presentation of African Trypanosomiasis

Generally, there are two stages to sleeping sickness; the early stage refers to the hemolymphatic infection, and the late stage refers to the neurological phase (infection of the CNS). The progress of the disease is as follows (Fig 2.9):

Incubation Period: The clinical manifestations of both forms of sleeping sickness are usually quite different, but can be easily confused because of the variability of symptoms and length of time until onset depends heavily on host characteristics (Molyneaux and Ashford, 1983). The first clinical manifestation of African trypanosomiasis occurs a few days after infection as a chancre at the site of tsetse fly inoculation, primarily for *T. b. rhodensiense*. This is due to the localized proliferation of the pathogens within the subcutaneous tissue. Incubation period for *T.b. rhodesiense* may be two to three weeks, while the incubation period for the Gambian species may last several weeks to months.

Dissemination: With the conclusion of the incubation period, the organisms have already disseminated into the bloodstream, leading to the emergence of a characteristic intermittent fever pattern that correlates directly with high versus low levels of parasitemia. This occurs within weeks and is characteristic of *T. b. rhodensiense* infections. The reason for the oscillating levels of parasite load is linked to the ability of the organisms to change their variable surface glycoproteins (VSGs) and evade the host's immune system. Lymphadenopathy, the swelling of lymph nodes, especially in the posterior cervical nodes (on the back of the neck) is characteristic sign of African sleeping sickness and is termed Winterbottom's sign. For *T. b. gambiense*,

lymphoadenopathy occurs more frequently. Oedema of the face is another frequent sign of infection, and anemia may be present, particularly in *T. b. rhodensiense*.

Invasion of the Central Nervous System: Invasion of the central nervous system (CNS) occurs within several weeks in the Rhodesian species and months to even years in the Gambian species of trypanosomiasis. Symptoms include headache, weight loss, weakness, rash, itching, stiff neck, sleep disturbance, and depression, followed by progressive mental deterioration, focal seizures, tremors, and palsies. This progresses to coma and the ultimate death of the patient often secondary to pneumonia or sepsis. Without treatment, African trypanosomiasis is a universally fatal illness (WHO, 2006., Dumas and Bisser, 1999).

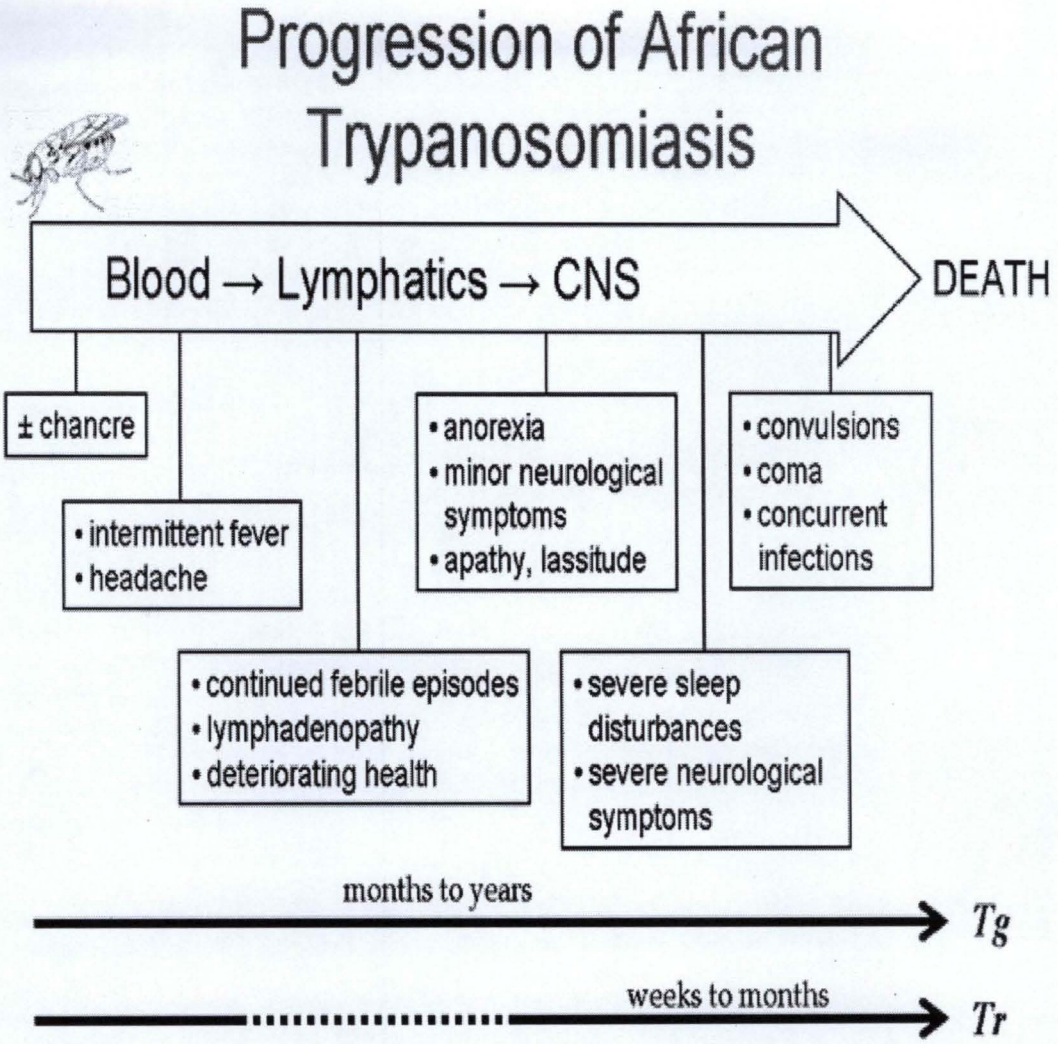

Fig 2.10 Progression of African Trypanosomiasis

2.2.8 Diagnosis of the disease

Confirmed diagnosis depends upon the detection of trypanosomes in the blood, lymph node aspirations, or spinal fluid. Typically few trypanosomes are detected in the blood or other bodily fluids during the *T. gambiense* infections. The trypanosomes are more likely to be detected during symptomatic periods (eg., during febrile episodes). In the absence of detectable parasites, travel or residence in an endemic area combined with the symptoms discussed above can be used as a presumptive diagnosis. Because of the low parasitemias exhibited during *T. gambiense* infections it is often not possible to detect parasites by standard thin and thick blood smears. Techniques to increase the sensitivity of detection are often needed (Box). For example, looking at fresh whole blood mounts may increase the sensitivity due to the distinctive movement of the trypomastigote. Another method to increase sensitivity is to centrifuge the blood in a microhematocrit tube. The parasites are enriched in the 'buffy coat' which is the band of white cells directly about the packed erythrocytes. The tube is then broken at the buffy coat and Giemsa-stained blood smears are prepared from these cells. Inoculation of mice or rats with patient blood and looking for the development of parasitemia is also possible. This generally works much better for *T. rhodesiense* infections than *T. gambiense* infections.

The miniature anion-exchange chromatography technique (mAECT) is another method used in the diagnosis of human sleeping sickness. Blood is passed through an anion exchange column. Blood cells are more negatively charged than the trypanosomes and they are retained on the column. The trypanosomes pass through the column and are collected at the bottom of a sealed glass tube by low-speed centrifugation. The tip of the glass tube is then examined in a special holder under the microscope for the presence of trypanosomes. The large blood volume (300 µl)

enables the detection of less than 100 trypanosomes/ml. Although highly sensitive, the manipulations are somewhat tedious and time-consuming.

One important issue in diagnosis of African trypanosomiasis is to distinguish the late encephalitic stage of the disease from the early stage. This is important since the treatment is different depending on whether there is CNS involvement. Criteria for CNS involvement include detection of parasites in the cerebral spinal fluid (CSF) or elevated white blood cells in the CSF. There is some controversy in regards to the exact level of white blood cells in the CSF should constitute a classification of CNS involvement.

2.2.9 Disease Management

Disease management is performed in three steps:

Screening for potential infection: This involves the use of serological tests and/or checking for clinical signs - generally swollen cervical glands.

Diagnosis of the parasite: Diagnosis of HAT requires confirming the presence of the parasite. The disease is difficult to diagnose early by both, lack of specific signs and symptoms in the first stage of the disease and lack of sensitivity of the parasitological methods available. Serological tests available today are only useful for screening and establishing suspicion of infection. Confirmation of infection will require the performance of parasitological tests to demonstrate the presence of trypanosomes in the patient. The parasites can be present in any body fluids. However, the number of parasite can be so low (mainly in the *gambiense* form of the disease) that available parasitological methods may not be sensitive enough to find them. Thus a negative

parasitological result in the presence of a positive serological test does not necessarily indicate absence of infection, and tests may have to be repeated over time to achieve diagnosis.

Staging to determine the state of progression of the disease: This entails examination of cerebro-spinal fluid obtained by lumbar puncture and is used to determine the course of treatment.

Diagnosis must be made as early as possible and before the neurological stage in order to avoid complicated, difficult and risky treatment procedures.

The long, asymptomatic first stage of *T.b. gambiense* sleeping sickness is one of the factors that require the use of exhaustive active screening of the population at risk in order to identify patients at an early stage and reduce transmission. Exhaustive screening of exposed populations requires a major investment in human and material resources. In Africa such resources are often scarce, particularly in remote areas where the disease is mostly found. As a result, many infected individuals may die before they can ever be diagnosed and treated.

2.3 Animal Trypanosomiasis

Other parasite species and sub-species of the *Trypanosoma* Genus are pathogenic to animals and cause animal Trypanosomiasis in many wild and domestic animal species (in cattle the disease is called *Nagana*, a Zulu word meaning "to be depressed"). The trypanosomes infect the blood of the vertebrate host, causing fever, weakness, and lethargy which leads to weight loss and anemia; in some animals the disease is fatal unless treated. The trypanosomes are transmitted by tsetse flies. Animals can host the human pathogen parasites, especially *T.b. rhodesiense*; thus domestic and wild animals are an important parasite reservoir. Animals can also be infected with *T.b.*

gambiense, however the precise epidemiological role of this reservoir is not yet well known. This disease kills animals.

The disease in domestic animals and particularly cattle is a major obstacle to the economic development of the rural areas affected.

T. brucei is a natural parasite of wild game in Africa and are non-infective to humans. This inability to infect humans is due to a 'trypanosome lytic factor' found in human sera. *T. brucei* and two morphologically distinct trypanosmes, *T. vivax* and *T. congolense*, are major pathogens for wild and domestic animals and have far reaching effects on raising livestock. In fact, although trypanosomiasis can be a devastating human disease, the greatest impact of trypanosomiasis on human health is at the agricultural level. Large areas of Africa are unsuitable for raising cattle and other livestock due to the presence of the tsetse vector and the transmission of trypanosomes. This contributes to protein deficient diets among the indigenous population.

2.3.1 Trypanosome Lytic Factor

Humans and some primates are naturally resistant to the cattle parasite *T. brucei* because of a serum factor that lyses the parasite. This trypanolytic activity has been demonstrated to be associated with high density lipoprotein (HDL) particles (Pays *et al.*, 2006). These particles consist of lipids and several proteins collectively known as apolipoproteins. African trypanosomes take up these particles via receptor-mediated endocytosis and this uptake is important in regards to the toxicity (Hager and Hajduk, 1997). Two proteins found in HDL particles have been proposed to be the trypanolytic factor: haptoglobin-related protein (HRP) and apolipoprotein L1 (ApoL1).

HRP has been demonstrated to have trypanolytic activity (Drain *et al.*, 2001). Furthermore, characterization of sera from primates which do and do not lyse trypanosomes shows a correlation between lytic activity and the presence of HRP (Lugli *et al.*, 2004). HRP results from a gene duplication of haptoglobin which is only found in humans and some other primates. Haptoglobin is a serum protein which functions to bind hemoglobin. One hypothesis is that the HRP-hemoglobin complex is taken up by the trypanosome via receptor-medicated endocytosis. Fusion of these coated vesicles with the lysosome would result in a lower pH which could stimulate the production of free radicals by the HRP-hemoglobin complex. The free radicals would then damage the membranes of the lysosome and release hydrolytic enzymes into the parasite cytoplasm leading to parasite lysis and death. However, questions about whether HRP actually binds hemoglobin have been raised. In addition, no direct evidence for a role of peroxidative mechanisms could be shown for TLF-mediated lysis (Portela *et al.*, 2000).

Because of these problems others have proposed that ApoL1 may by the trypanosome lytic factor. ApoL1 has also been demonstrated to be taken up by trypanosomes and targeted to the lysosomes (Vanhamme *et al.*, 2003). ApoL1 contains a pore-forming domain and is capable of forming pores on the lysosomal membrane (Perez *et al.*, 2005). This results in a depolarization of the lysosome due to an influx of chloride ions and the accompanying water and osmotic swelling of the lysosomes. The continual enlargement of the lysosome and osmotic changes may lead to parasite lysis. A synergy between HRP, ApoL1 and other HDL components has also been suggested (Shiflett *et al.*, 2005). Subsequently, it was shown that HRP facilitates the uptake of HDL particles and does not directly participate in the lysis of the trypanosomes (Vanhollebeke *et al.*, 2007).

T. gambiense and *T. rhodesiense* are resistant to the trypanosome lytic factor found in human serum. One possible mechanism of this resistance to human serum is a decrease receptor-mediated endocytosis of HDL by the trypanosome (Hager and Hajduk, 1997). In addition, a serum resistance associate (SRA) gene has been described in *T. rhodesiense* (Xong *et al.*, 1998; Milner and Hajduk, 1999). SRA is a truncated variant surface glycoprotein (VSG) and is localized to the lysosome. Furthermore, SRA binds to ApoL1 in the lysosome and this interaction is believed to neutralize the toxic effect of apoL-1 (Vanhamme *et al.*, 2003), thus suggesting that ApoL1 is TLF. However, the SRA gene is only found in *T. rhodesiense*, and not *T. gambiense*, suggesting that there are multiple mechanisms for human infectivity.

2.4 Prevention and Control of African Trypanosomiasis

Measures currently used to control trypanosomiasis are diagnosis and treatment, chemoprophylaxis, chemotherapy, tsetse fly control or eradication of tsetse flies, and the utilization of trypanotolerant breeds (Leak, 1999).. This most challenging task in Africa is complicated and hampered by several specific factors. The number of tsetse flies (and thus the occurrence of disease) fluctuates greatly over periods of several years and makes assessment of the actual risk to which livestock are exposed difficult. In addition, control of trypanosomiasis is hindered considerably by the fact that African trypanosomes are able to establish chronic infections in their mammalian hosts because of their highly developed system of antigenic variation. Individual members of the parasite population change the composition of their surface coat so that variations in the composition of these variant surface glycoproteins (VSGs) allow the parasite to escape the host's immune system. Thus fluctuating parasitemias produced by *T. brucei* are associated not only with the phenomena of variable antigen type (VAT) but certainly also the

potential for regulation of trypanosome growth by environmental factors such as epidermal growth factor (EGF), transferrin and low-density lipoprotein. This makes effective immunoprophylaxis unlikely (Vanhamme *et al.*, 2003).

2.4.1 Vector Control

In areas with low tsetse fly density the method of choice for controlling African trypanosomiasis seems to be the eradication of the vector. Although complete eradication of the vector is impossible, the most successful attempts at controlling tsetse flies are likely to be at the extreme limits for survival of the fly, where both the density of the fly is low and "personal" contact with humans may be highest (Rogers, 1979). For the time being the spraying of insecticides dominates in tsetse fly eradication. In regions with very low levels of infestation, e.g., by riverine tsetse species (Glossina palpalis group, e.g., *G. palpalis, G. fuscipes*), trypanosomiasis can be controlled by surveillance and treatment only. Nevertheless, flies of the savanna group (*G. morsitans* group, e.g., G. morsitans, G. pallidipes, G. austeni) may give rise to severe trypanosomiasis in susceptible stock even if their numbers are low. In these areas commercial cattle ranching may be possible under chemoprophylactic protection. However, tsetse fly density and thus contact between cattle and vector must be reduced by additional spraying of insecticides with residual effects (e.g., synthetic pyrethroids) and by setting up impregnated traps and screens. In areas with medium tsetse fly density the further exploration and logical exploitation of trypanotolerant cattle, including crossbreeding trials with European breeds to increase milk and meat productivity of indigenous trypanotolerant cattle, may offer a realistic alternative to not yet available vaccination. At least in areas with high tsetse fly density even trypanotolerant animals may not survive unless they are treated prophylactically against trypanosomiasis. The

control of the disease in fully susceptible stock even under chemoprophylaxis seems to be impossible in regions heavily infected with tsetse.

Tsetse flies can detect odors by means of receptors on their antennae. Experience with insect pheromones was used to identify the chemical components of the ox odor, which might attract tsetse flies and led to the discovery of 1-octen-3-ol. It proved highly attractive to flies of the savanna (G. pallidipes and *G. m. morsitans*). Thus live bait (e.g. cattle treated with insecticides: spot-on, pour-on), fly traps, and screens impregnated with "essence of ox" and pyrethroid insecticides (e.g., deltamethrin, aplphametrin, or cyfluthrin), and sophisticated ground spraying technology may markedly reduce tsetse infestation in limited areas of riverine woodland or transitional forest-savanna zones. Traps baited with acetone and 1-octen-3-ol have been used in Zimbabwe, Zambia and Malawi to detect the presence and distribution of tsetse flies (Vreysen *et al.*, 2000; (Steverding, 2006; Steverding, 2008; El-Sayed and Donelson, 1997. It has been shown that isometamidium is capable of eliminating the insect vector form of *T. v. vivax*. This experimental finding may be of potential significance in the control of trypanosomiasis in the field, particularly in the operation of the sterile insect technique (e.g. in Nigeria) (Dransfield *et al.,* 1990; Wall and Langely, 1991; Weidhaas and Haile, 1978; Hargrove, 1981).

Exploiting the knowledge that tsetse flies concentrated in certain areas lead to numerous bush-clearing projects all over West and East Africa to drastically alter and maintain the area unsuitable for tsetse fly habitation. Discriminative bush-clearing was used in Uganda to control for *G.m. centralis* by clearing taller Acacia trees in the Ankole district (Harley and Pilson, 1961). (Morris, 1949) in 1958(Rogers and Randolph, 1985) In Tanzania, between 1923 and 1930, bush-clearing methods were also widely employed to stop the spread of sleeping sickness epidemic in

Maswa district, where *G. swynnertoni* was prevalent (Leak, 1999). Similar tactics were used in Ghana to control sleeping sickness around villages were human-fly contacts were high (Morris, 1949) in 1958(Rogers and Randolph, 1985). Despite the apparent success of these methods, it is widely accepted that bush-clearing is unsuitable as a long term control measure due to the expense and speed of reinvasion, as well as the environmental damage it causes through soil erosion, decreased soil fertility, and its adverse effects on water supplies.

One of the more modern methods of non-insecticidal control is the Sterile Insect Technique (SIT) which was first considered as a means to control tsetse by Simpson in 1958(Rogers and Randolph, 1985).. This technique relies on the mating of wild females with sterile male flies. Physiologically, female tsetse flies are only required to mate once to store sperm in its spermathecae in sufficient quantity such that fertilization can occur over its entire reproductive life. Mating with a sterile male would thus result in no offspring. However, SIT was considered to be impractical for control of high-density tsetse populations above 1000 males per square mile due to the large number of sterilized males that would be required. For SIT to be effective, it has been estimated that 10% of the females in the population need to be inseminated, and in order to achieve that, the number of sterile males released must constitute 80% of the male population (Rogers and Randolph, 1985).

Today, there is neither a breakthrough in biological control of tsetse flies nor are there promising solutions for a vaccine against African trypanosomes (Barrett, 1997; Harley and Pilson, 1946; Morris, 1949; Simpson, 1958; Rogers and Randolph, 1985).

2.4.2 Chemotherapy and Chemoprophylaxis of Animal Trypanosomiasis

Presently, no vaccine is available for African animal trypanosomiasis (Murray *et al.*, 1979). Chemotherapeutic and chemoprophylaxis have been of great importance in the prevention and control of animal trypanosomiasis but trypanosomes have developed resistant to each drug introduced. Although 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 cattles for up to three months. The drug pyrithidium bromade (Prothidium and AD2801) is useful in the prophylasis of *T.vivax* and *T. congolense* infections in cattle, sheep, and goats and can give protection for up to 6 months.

Isometamidium chloride is the most widely used of the newer chemoprophylactic drugs and also the least expensive. It gives protection for 3 – 6 months for all the three African animal trypanosomes. In both East and West Africa, development of resitance to this drug has been reported (Kuzoe, 1991). Diminazine aceturate (berenil) is widely used in the treatment of all three African animal trypanosomes. Homidium bromide is also effective chemoprophylactic drug in Kenya, and the newly introduced arsenical Clymelarsan is effective in treatment of *T.b. brucei* infection (Kuzoe, 1991).

2.4.2.1 Trypanotolerance of Indigenous African Breeds

The term `trypanotolerance' means reduced susceptibility to trypanosomiasis and denotes an inherited biological property allowing animals to live, breed, grow and survive in a naturally infected environment without exhibiting clinical signs of trypanosomiasis after harboring pathogenic trypanosomes (Murray *et al.*, 1979).

Attention has recently focused on genetic resistance and various selection programs are being discussed to select trypanotolerant animals. Such programs could involve selection of trypanotolerant animals under natural challenge or selection of marker traits (e.g., aspects of the immune response). Selection could also act on polymorphic loci that may affect trypanotolerance, and may be closely linked to genes acting upon tolerance via marker loci. Trypanotolerance is found not only in cattle (all dwarf semiachondroplastic West and Central African types) but also in sheep, goats, and in some rare pony types, such as the Kotokoli of the Ivory Coast. The N'Dama (Hamitic Longhorn of the Bos taurus type as well as those breeds of the West African Shorthorn) is a West African breed (e.g., Gambian cattle) noted for its small size and its trypanotolerance. This humpless breed responds very well to improved management and can attain levels of productivity comparable to that of many African beef breeds of the Bos indicus type, such as the West African Zebu, the Orma Boran, the Ankole, or the Afrikander. In addition the N'Dama can maintain reasonable production levels under conditions of poor management, climate, nutrition and high tsetse fly densities. Trypanotolerant breeds of Zebus, sheep and goats may also exist in East Africa. Field studies on two types of large East African Zebu (Bos indicus) Boran cattle on a beef ranch in Kenyo have demonstrated that a boran type bred by the Orma tribe had a superior response to tsetse fly challenge compared to an improved Boran when introduced to a new locality. Superior resistance to tsetse fly challenge was evident by lower trypanosome infection rate, and when this was untreated, by lower anemia and decreased mortality (Murray *et al.*, 1979; Moulton and Sollod, 1979).

2.4.3 Chemotherapy and Chemoprophylaxis of Human African Trypanosomiasis

Since vaccine against trypanosomiasis remains elusive, chemotherapy and chemoprophylaxis is the most important and major aspect of the control and prevention of this disease. The first line drug for the treatment of African Trypanosomiasis were developed over 30 years ago (WHO, 2006). Current chemotherapy of trypanosomiasis is beset with problem of toxicity, growing resistant to current drugs, long period of treatment and high cost of treatment. The type of treatment depends on the species of trypanosome causing infection, stage of the disease, the species of the animal and occurrence of resistant strains. The drugs used in the first stage of the disease are less toxic, easier to administer and more effective. The earlier the identification of the disease, the better the prospect of a cure. Treatment success in the second stage depends on a drug that can cross the blood-brain barrier to reach the parasite. Such drugs are quite toxic and complicated to administer. Four drugs are registered for the treatment of trypanosomiasis.

First stage treatments

Pentamidine was discovered in 1941. It is used for the treatment of the first stage of *T.b. gambiense* sleeping sickness. Despite a few undesirable effects, it is tolerated by patients.

Suramin was discovered in 1921. It is used for the treatment of the first stage of *T.b. rhodesiense*. It provokes certain undesirable effects, in the urinary tract and allergic reactions.

Second stage treatments

Melarsoprol was discovered in 1949. It is used in both forms of infection. It derives from arsenic and has many undesired side effects. The most dramatic being a reactive encephalopathy (encephalopathic syndrome) which can be fatal (3% to 10%). An increase of resistance to the drug has been observed in several foci particularly in central Africa.

Eflornithine is less toxic than melarsoprol and was registered in 1990. It is only effective against *T.b. gambiense*. It is an alternative to melarsoprol treatment. The regimen is strict and difficult to apply (WHO, 200; Bouteille *et al.*, 2003).

2.5 American Trypanosomiasis

American trypanosomiasis, known as Chagas' disease, was first characterized by Carlos Chagas in Brazil in 1909, and continues to be among the most important diseases in tropical and subtropical Mexico, Central America and South America. Chagas Disease is caused by the trypanosomatid species, *Trypanosoma cruzi*, which is similar but distinct from the species that cause HAT. According to the World Health Organization (2006), 25 % of the total population in Central and South America is at risk. Currently, there are between 16-18 million people infected, with 6 million cases advancing to clinically significant disease and more than 45,000 deaths annually. The disease is transmitted through the bite of several species of triatomine bugs (also referred to as reduviid bugs, assassin bugs or "kissing bugs"), including *Triatoma infestans, Triatoma dimidiata* and *Rhodnius prolixa*, all of which live in dry, forested areas. These insects hide during the day in dark crevices or behind objects that are abundant in the type of housing used in endemic areas, as well as in animal nests and thatched roofs. At night, the insects emerge and feed on the blood of a variety of mammals, including humans. During a blood meal, the disease is not transmitted by the bite of the insect, but rather through its feces, which contains the organism. The parasite enters the host when the wound is scratched, or through the conjunctiva of the eye or the mucosa of the nose or mouth, and invades a variety of cell types including macrophages, smooth and striated muscle and fibroblasts. When the site of infection is near the eye, acute swelling of one eyelid is observed, a phenomenon known as Romaña's sign.

After a 1-2 week incubation period, the disease progresses through three phases, termed acute, indeterminate and chronic. In the acute phase patients have high levels of the parasite in blood and tissues, and symptoms are generally mild (high fever and edema). Immunosuppressed patients and children can develop a more severe form of infection, with cardiac involvement and encephalomyelitis. Following the acute phase, the parasite burden in blood and tissues decreases dramatically, although low levels of the parasite are still detectable in certain tissues. Patients then experience an asymptomatic lag period of anywhere from 10 to 30 years that is known as the indeterminate form of Chagas disease. A percentage of these patients advance to chronic Chagas disease, and develop moderate to severe clinical symptoms including cardiomyopathy, heart failure and digestive tract abnormalities such as megacolon and megaoesophagus. If severe enough, these manifestations of the disease are the main causes of death.

Trypanosoma cruzi is much more difficult to treat than Human African Trypanosomiasis, since this trypanosomatid parasite is intracellular, and drugs used for the disease must pass through mammalian and parasite cell membranes to be effective. Laboratory and clinical studies conducted since 1969 have demonstrated that nifurtimox and benznidazole are the best agents for treating human *T. cruzi* infection, although they are far from being ideal drugs. Nifurtimox and benznidazole are indicated in the acute phase of the infection, the congenital form, reactivation of disease associated with immunosuppression, and in transfusions and organ transplants involving infected individuals. Both drugs are taken orally and must be given divided into 2-3 fractions after meals. They are generally well tolerated by children, particularly in the acute phase of the disease, but relatively frequent and severe gastrointestinal or dermatological adverse reactions may be observed. Recurrence of the disease is a significant problem, and as such these

drugs are considered generally ineffective. The main limitations of both drugs are their long courses of administration and the occurrence of adverse side effects. The related compound megazol has also been used for Chagas disease, but its use was discontinued because of severe mutagenic and cytotoxic effects (Hunt, 2007).

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 (*B*erriman *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.

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.

2.6.1 Vaccine Strategies against *Trypanosoma brucei*

2.6.2 The Challenge of the Variable Surface Glycoproteins (VSG)

Variable Surface Glycoproteins (VSG) cannot be used as vaccine targets due to the high degree of their variability. At the time of infection almost all the Trypanosomes will express only one

VSG. The host clears all of the parasites bearing the first particular VSG but does not generate a response to those few that have switched. On the genomic level VSG switching occurs by two mechanisms. VSG expression sites are located at the telomeric ends of chromosomes. Individual genes can be copied and then displace the currently expressed copy of the VSG, or alternatively the active telomeric site can be silenced, and another activated. Only one expression site, and thus only one VSG is expressed on a trypanosome at a time. On the parasite surface, the VSGs are physically switched both spontaneously and by immune pressure. The switch is mediated by phospholipase C, which rapidly cleaves the glycophosphoinositol (GPI) anchor that binds the VSG to the parasite and allows another VSG to take the former's place. The rapid switching of VSGs and the large number of different VSGs, postulated to be over a 1000 unique types, plus variants that result from recombination of genes, allow the trypanosome to constantly evade the immune response. The antigenic switching found in African trypanosomiasis makes vaccine development very challenging. However, there are a few other hopeful options.

2.6.3 Glycolysis an Antiparasitic Drug

Glycolysis in the bloodstream form of *Trypanosoma brucei* provides a convenient context for studying the prospects for using enzyme inhibitors as antiparasitic drugs. A recent supplement to Nature entitled "Intelligent Drug Design" has determined that metabolism, and metabolic effects of drugs, should be considered as one of the steps when designing a drug for vaccines. The glycolytic pathway is an attractive target because the predominant bloodstream form of T. brucei has no energy resources. Once *T. brucei* enters the bloodstream the parasite relies entirely on rapid glycolysis for its energy supply. So the ability to kill trypanosomes by halting glycolysis

may prove to be important in the development of a drug against African sleeping sickness (Einsenthal, 1997).

There are two basic metabolic methods of killing an organism. Either the flux through an essential metabolic pathway can be decreased to the point where life is no longer possible, or the metabolic concentration can be increased to toxic levels. The model for trypanosomal glycolysis developed by Bakker *et al* (2000) provides an excellent starting point for assessing effects of inhibition in whole pathways, as not only is a large amount of detailed kinetic information embedded in it, but also because the model gives an excellent account of the known properties of intact trypanosomes. Bakker *et al* determined there are two possible ways of altering glycolysis of trypanosomes in the bloodstream. The first, inhibition of glucose transport was found to have the highest degree of control over typanosome growth. The second, inhibition of pyruvate transport is an alternate strategy to decrease the carbohydrate flux and increase metabolite concentration to catastrophic levels. When the stoichiometric constraints are taken into account, the glycerol transport inhibition can be eliminated due to the fact that under aerobic condition, it has such a low rate of efflux that some authors have doubted whether it occurs at all. Fortunately, the remaining candidate, pyruvate transport has proved to respond to inhibition well. It is expected to have powerful anti-trypanosomal activity. If the metabolic pathway of blood stream tryoanpsomes can be targeted it may prove to be more successful than inhibition during transcription or translation (Einsenthal, 1997)

2.6.4 Phenothiazine Inhibitors as Antitrypanosomal Drugs

Present chemotherapies are inadequate, toxic, or both with current drugs including the arsenicals, nifurtimox, and pentamidine. The trivalent arsenical drug melarsoprol is still widely used against the second stage of African trypanosomiasis in which the parasitic pathogen has invaded the central nervous system, a stage refractory to the drugs suramin and pentamidine. As an arsenical it is no surprise that melarsoprol is toxic, the drug itself leading to the deaths of 4-8% of patients treated with it. Nifurtimox and benxnidazole are the two major drugs available for Chagas' disease, and there is continuing debate about their effectiveness and safety. Glutathione is responsible for many cellular protection activities including those against free radicals and oxygen-derived species. In the course of this action glutathione disulfide is formed from glutathione reductase. Trypanosomes do not contain glutathione reductase but rather an analogous enzyme, trypanothione reductase. The mutual substrate exclusivity indicated that selective ligand design should be possible, making trypanothione reductase an important potential target for drug design against parasitic diseases involving trypanosomes. Effective inhibition of TR would compromise the parasites' redox defenses, making them more sensitive to redox-damage drugs, such as nifurtimox. A trypanothione reductase inhibitor might be an effective antitrypanosomal drug in its own right, or coadministration with the redox-active drug such as nifurtimox (Chan, 1998).

2.6.5 Flagellar pocket antigens

The flagellar pocket of the trypanosome is an area where receptors are used in specific host macromolecule uptake, and thus it is conjectured that these antigens are highly conserved

amongst flagellated protozoans. For this reason a vaccine against the flagellar proteins of *T. brucei* may also confer protection against many other parasites. In one study a flagellar pocket antigen from *T. brucei rhodesiense*, with bovine serum albumin as the carrier and alum as the adjuvant, was used to inoculate 90 cattle in Kenya. The rate of infection was reduced from 13 % to 0.9 % (Mkunza *et al.*, 1995). This experiment is of particular note because it was carried out in an environment of natural exposure with a naturally infectable species. Most studies have used a murine model which is highly effected by disease-based immunosupression.

One specific example of a flagellar pocket protein is Trypanosomal transferrin-binding protein (TFBP), a flagellar pocket protein whose gene is present in multiple versions, and to which antibodies are produced in chronic infection. Borst (1991) proposes that those antibodies hinder the multiplication of trypanosomes, and proposes that a vaccine stimulating antibody production against the repertoire of TFBPs may hinder trypanosome proliferation. He also notes that several other proteins are present in the flagellar pocket, and they may also serve as targets for vaccines.

2.6.6 Congopain

A second focus of vaccine research has been on attempting to eliminate the pathogenicity of the parasite, not the infection itself. Cysteine proteases of microorganisms can degrade host proteins such as immunoglobulins and complement factors. They can also modulate cytokine activities, and are suspected of interfering with antigen presentation and processing. Some cattle species in Africa are more resistant to infection than others. Studies have shown that a *Trypanosoma congolense* cysteine protease (congopain) may play a role in the different levels of tolerance. The more resistant cattle generate a stronger IgG response to congopain than the less resistant cattle.

Attempts are being made to use congopain antigens in a vaccine which would generate antibodies capable of neutralizing the enzyme activity (Authie, 1994).

2.6.7 Intracellular Antigens

A third strategy involves intracellular antigens. Members of the Trypanosomatidae family have subpeullicular microtubules cross-linked to each other and to the plasma membrane by unique trypanosomal microtubule-associated proteins (MAPs). The trypnaosomal MAP (p52) has been used in an antigenic preparation with the enzymes aldolase and GAPDH (which the protein copurifies with) as a vaccine in mice and rats. The p52 was isolated from T. brucei brucei, and when the animals were immunized three times over a period of three weeks, 100% protection was achieved. The serum from immunized animals also cross reacted with *T. b. rhodesiense* and *T. b. evansi*, which gives hope for cross protection at least among trypanosomes species, and possibly for the whole family (Balaban *et al.*, 1995).

2.7 Medicinal Plants in the Treatment of Diseases

Medicinal plants include plants or plants part in which one or more of its organs, contains substance that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs (WHO, 1977). A number of medicinal plants have been used in traditional medicine for many years. Some do seem to work although there may not be sufficient scientific data to confirm their efficacy (Sofowora, 1993). Undoubtedly, the plant kingdom still holds many species of plants containing substances of medicinal value, which are yet to be discovered. Large numbers of plants are constantly been screened for their possible pharmacological value.

Today there are at least 120 distinct chemical substances derived from plants that are considered as important drugs currently in use in one or more countries in the world (Leslie, 2000). Several of the drugs sold today are simple synthetic modifications or copies of the naturally obtained substances. For example, many years ago a plant chemical was discovered in a tropical plant, Cephaelis ipecacuanha, and the chemical was named *emetine*. A drug was developed from this plant chemical called *Ipecac* which was used for many years to induce vomiting mostly if someone accidently swallowed a poisonous or harmful substance. Another example of this is the plant chemical named *taxol.* The name taxol is the name of the plant chemical orginally discovered in the plant. A pharmaceutical company copied this chemical and patented a drug named *Paclitaxel*™ which is used in various types of tumors today in the U.S. and many other countries (Leslie, 2000).

Some of the drug/chemicals shown in table 1.1 are still sold as plant based drugs requiring the processing of the actual plant material. Others have been chemically copied or synthesized by laboratories and no plant materials are used in the manufacture of the drug. A good example of this is the plant chemical quinine, which was discovered in a rainforest tree (*Cinchona ledgeriana*) over 100 years ago. For many years the quinine chemical was extracted from the bark of this tree and processed into pills to treat malaria. Then a scientist was able to synthesize or copy this plant alkaloid into a chemical drug without using the original tree bark for manufacturing the drug. Today, all quinine drugs sold are manufactured chemically without the use of any tree bark. However, another chemical in the tree called *quinidine* which was found to be useful for various heart conditions couldn't be completely copied in the laboratory and the tree bark is still harvested and used to extract this plant chemical from it (Leslie, 2000). Quinidine extracted from the bark is still used today to produce quinidine-based drugs.

Table 2.1 Drugs made from plants (Leslie, 2000).

Drug/Chemical	Action/Clinical Use	Plant Source
Acetyldigoxin	Cardiotonic	*Digitalis lanata*
Adoniside	Cardiotonic	*Adonis venalis*
Aescin	Anti-inflammatory	*Aesculus hippocastanum*
Aesculetin	Anti-dysentery	*Frazinus rhychophylla*
Agrimophol	Anthelmintic	*Agrimonia supatoria*
Ajmalicine	Circulatory Disorders	*Rauvolfia sepentina*
Allyl isothiocyanate	Rubefacient	*Brassica nigra*
Anabesine	Skeletal muscle relaxant	*Anabasis sphylla*
Andrographolide	Baccillary dysentery	*Andrographis paniculata*
Anisodamine	Anticholinergic	*Anisodus tanguticus*
Anisodine	Anticholinergic	*Anisodus tanguticus*
Arecoline	Anthelmintic	*Areca catechu*
Asiaticoside	Vulnerary	*Centella asiatica*
Atropine	Anticholinergic	*Atropa belladonna*
Berberine	Bacillary dysentery	*Berberis vulgaris*
Bergenin	Antitussive	*Ardisia japonica*

Drug/Chemical	Action/Clinical Use	Plant Source
Betulinic acid	Anticancerous	*Betula alba*
Bromelain	Anti-inflammatory,	proteolytic *Ananas comosus*
Caffeine	CNS stimulant	*Camellia sinensis*
Camphor	Rubefacient	*Cinnamomum camphora*
Camptothecin	Anticancerous	*Camptotheca acuminata*
(+)-Catechin	Haemostatic	*Potentilla fragarioides*
Chymopapain	Proteolytic, mucolytic	*Carica papaya*
Cissampeline	Skeletal muscle relaxant	*Cissampelos pareira*
Cocaine	Local anaesthetic	*Erythroxylum coca*
Codeine	Analgesic, antitussive	*Papaver somniferum*
Colchiceine amide	Antitumor agent	*Colchicum autumnale*
Colchicine	Antitumor agent, anti-gout	*Colchicum autumnale*
Convallatoxin	Cardiotonic	*Convallaria majalis*
Curcumin	Choleretic	*Curcuma longa*
Cynarin	Choleretic	*Cynara scolymus*
Danthron	Laxative	*Cassia species*
Demecolcine	Antitumor agent	*Colchicum autumnale*
Deserpidine	Antihypertensive, tranquillizer	*Rauvolfia canescens*

Drug/Chemical	Action/Clinical Use	Plant Source
Deslanoside	Cardiotonic	*Digitalis lanata*
L-Dopa	Anti-parkinsonism	*Mucuna sp*
Digitalin	Cardiotonic	*Digitalis purpurea*
Digitoxin	Cardiotonic	*Digitalis purpurea*
Digoxin	Cardiotonic	*Digitalis purpurea*
Emetine	Amoebicide, emetic	*Cephaelis ipecacuanha*

2.8 Trypanocidal Potential of Some Medicinal Plants

Kigelia africana

Kigelia Africana is commonly used in Africa as traditional medicine for wide spectrum of other medical indications. The ethanolic stem extract have been shown to have antibacterial activity (Grace *et al.*, 2002). Other reports have demonstrated that the ethanolic extracts of the *K. africana* have analgesic and anti-inflammatory activities (Owolabi and Omogbai, 2007), as well as having a potential central nervous system stimulant effect that can be explored for therapeutic purposes (Owolabi *et al.*, 2008). The dichloromethane extracts of *K. africana* fruits was administered intraperitoneally to Swiss white mice that had previously been inoculated with *Trypanosoma brucei rhodesiense* KETRI 3798. This extract was effective at 2000 mg/kg, curing 60% of the animals treated (Ogoti *et al.,* 2009).

Azadirachta indica (Neem)

The Neem tree, *Azadirachta indica,* is a common plant that grows wild and thrives well even in harsh environments. It has been exploited for a long time as a potential source of traditional drugs and several parts of the plant are known to have extensive medicine application (Okpanyi and Ezenkwu, 1981). An aqueous extract of defatted leaves of this plant was found to possess trypanocidal activity against *Trypanosome brucei brucei.* One of the fractions obtained from column chromatography of the crude extract retained trypanocidal properties and cured mice chronically infected with trypanosome brucei brucei. Histopathological studies of the brains, livers, hearts, and spleens of the treated mice showed no cellular infiltrations (Nok *et al.*, 1992).

Annona senegalensis

Annona senegalensis, commonly known as "Gwandar daji" in Hausa, Northern part of Nigeria is a medicinal plant of the Annonaceae family commonly found in the savannah area and near stream. Mice infected with trypanosome brucei brucei 8/18 strain were treated orally and intramuscularly (im). With aqueous root extracts of A*nnona senegalensis*, in doses of 27.8mg/kg and 9.5 mg/kg respectively, for four consecutive days commencing 72 hours after the mice were infected. Although, Igwe and Onabanjo (1989) reported that at this dosage the mice were cleared from the circulation and no relapse was recorded over 60 days, Ogbadoyi *et al.,* (2007) showed that the whole root extract of the same plant is only trypanostatic.

Crude and partially purified aqueous extract of the leaves of *Annona senegalensis*, at a dose of 200mg/kg body weight per day completely cured experimental *Trypanosoma brucei brucei* infection in mice. Sub- inoculation of blood and cerebrospinal fluid drawn from the cured mice into healthy mice failed to produce any infection within 60 days of post-inoculation. The crude extract had no prophylactic action when given prior to infection. Administration of 5000 mg/kg body weight of the crude extract did not lead to fatality in mice (Ogbadoyi *et al*., 2007).

Morinda lucida (leaf), Combretum dolicopetalum (root bark), Alstania boonei (bark)

Udem *et al*., (2006) in their research macerated dried, powered plant samples with 95% ethanol overnight. The ethanolic extracts were concentrated to dryness under reduced pressure and subjected to exhaustive gradient solvent extraction in a soxhlet, and concentrated with a rotary evaporator. Albino cats infected with a known strain of *T. b brucei* (CT/70/NITR) were treated with the crude extracts and packed cell volume (% PCV) and, parasitaemia were monitored.

Udem *et al.*, (2006) reported that the extracts of *Morinda lucida, Combretum dolicopetalum* showed significant ($P < 0.05$) trypanocidal activity at high dosage levels. The extract was most effective when given simultaneously with infection. Both the PCV and clinical symptoms improved after treatment with the extracts. *Alstonia boonei* extracts appeared to reduce the level of parasitaemia although this was not significant when subjected to ANOVA.

2.8.1 Other Medicinal Plants that have been tested for Trypanocidal Activity

Ochrosia elliptica

Elliptieine 1, a plant extract, is a naturally occurring DNA reactive antiecoplasmic and antibiotic indole alkaloid that was first isolated in 1959 by Goodwin and Co-workers from the leaves of Chrosia elliptica. Some elliptieine derivatives are potent and specific inhibitors of DNA topoisomerases II (TOPO 2) are cytotoxic to African trypanosomes (Mbagu, 2003).

Aqueous and methanol extracts of seventeen natural medicines purchased in markets in South. East Asian countries were tested for anti- trypanosomal activity using an invitro assay system which enabled quantitative determination of infection of *T. cruzi* to Hela cells and its propagation. All of these natural medicines have been shown to have invitro nematocidal activity against second-stage larvae of dog roundworm, *Toxocara canis* (Kiuchei *et al.*, 1998).

The activity of the aqueos extracts were generally weak and only Ritha showed appreciable activity. The methanol extracts of Devdaru, Bakurchi, Defence yapragi and Asilbent showed strong inhibition against the infection of *T. cruzi* to Hela cells.

Among the Natural medicine which showed strong activity, the active principle of Bakuchi was investigated. Bakuchi is used as antihelmintic, laxative, stomachic, diuretic and diaphoretic in Ayurvedic medicine (Arya, 1995), it is also said to be a good hair tonic,

2.9 General Profile of *Annona senegalensis*

Annona senegalensis is a species of plant in the Annonaceae family, found in the savannah area and near stream. This little-known fruit has the potential to improve nutrition, boost food security, foster rural development and support sustainable landcare (NRC, 2008). *Annona senegalensis* is commonly known as a wild custard-apple and locally called "Gwandar daji" in Hausa, "Abo" in Yoruba, and "Uburu ocha" in Ibo

2.9.1 Taxonomy

Kingdom:	Plantae
Class:	Angiosperms
(unranked):	Magnoliids
Order:	Magnoliales
Family:	Annonaceae
Genus:	*Annona*
Species:	*A. senegalensis*

(NRC, 2008).

2.9.2 Botanic Description

Annona senegalensis is a shrub or small tree 2-6 m tall but may reach 11 m under favourable conditions; bark smooth to roughish, silvery grey or grey-brown, with leaf scars and roughly circular flakes exposing paler patches of under bark.

Young branches with dense, brown, yellow or grey hairs that are lost later. Leaves alternate, simple, oblong, ovate or elliptic, 6-18.5 x 2.5-11.5 cm, green to bluish-green, almost without hairs on top, but often with brownish hairs on underside, net veining green to reddish on both surfaces; apex rounded or slightly notched; base square to slightly lobed; margin entire; petiole short, 0.5-2.5 cm, thickset. Flowers up to 3 cm in diameter, on stalks 2 cm long, solitary or in groups of 2-4, arising above the leaf axils; 6 fleshy cream to yellow petals in 2 whorls, greenish outside, creamy or crimson, 0.8-1.5 x 0.9-1.1 cm, glabrous or minutely papillose within; inner whorl of the petals curving over the stamens and ovary; sepals ovate, 3 in number, free, smaller than the petals, 3-4 x 4-5 mm; stamens 1.7-2.5 mm long. Fruit formed from many fused carpels, fleshy, lumpy, egg shaped, 2.5-5 x 2.5-4 cm, ovoid or globose; unripe fruit green, turning yellow to orange on ripening; stalk 1.5-5 cm long; seeds numerous, cylindrical, oblong, orange-brown. The genus name, 'Annona', is from the Latin word 'anon', meaning 'yearly produce', referring to the production habits of fruits of the various species in this genus. The specific name means 'of Senegal', which is where the type specimen was collected (Beentje, 1994; Mbuya *et al.*, 1994; Vogt, 1995; Katende *et al.*, 1995;Bekele, 1993; ICRAF, 1992 and Coates, 1988).

2.9.3 Ecology and Distribution

Wild fruit trees of this species are found in semi-arid to subhumid all over regions of Africa. The species occurs along riverbanks, fallow land, swamp forests and at the coast. Commonly grows as a single plant in the understorey of savannah woodlands.

Native : Botswana, Cameroon, Congo, Cote d'Ivoire, Democratic Republic of Congo, Ethiopia, Gambia, Guinea, Kenya, Lesotho, Mali, Mozambique, Senegal, Sierra Leone, South Africa, Sudan, Swaziland, Tanzania, Nigeria, Uganda Exotic : India.

Altitude: 0-2400 m, Mean annual temperature: 17-30 deg. C, Mean annual rainfall: 700-2500 mm. Soil type: Although A. senegalensis grows on various soil types, it does well on coral rocks dominated by sandy loam soils.

2.9.4 Reproduction

The flowers of Annona genus have both male and female parts, but the stigmas are generally not receptive at the time the pollen is shed. Beetles of several species are important in carrying out natural pollination. But complete pollination seldom occurs, explaining the frequency of misshapened fruits. Hand pollination may improve both yield and quality of the fruit.

2.9.5 Propagation and Management

Regeneration through seed, root suckers and coppice is possible. On disintegration of the fruit, seeds fall to the ground, where they germinate if conditions are favourable. Germination is good on recently cultivated or burnt areas. For plants that are to be raised in the nursery, scarification improves germination rates. Root suckers are produced on wounding of roots by fire and trampling by cultivators and animals.

A. senegalensis should be protected from fire and browsing. The planting site should be cleared, and the site should be weeded, as the young plants are not hardy enough to compete with weeds. A light shade should also be provided. Fruit cracking is common.

Seed storage behaviour is orthodox. Seeds are susceptible to insect damage and lose viability within 6 months. However, viability can be maintained for more than 2 years in air-dry storage at 5 deg. C.

2.9.6. Functional Uses

Products

Food: The leaves are sometimes used as vegetables, while the edible white pulp of the ripe fruit has a pleasant, pineapple-like taste. Flowers serve as a spice for various meals.

Fodder: Livestock browse the leaves.

Fibre: Fibre from young sucker shoots is used in binding.

Timber: Wood is soft and white or light brown in colour; it is used for poles and tool handles.
Tannin or dyestuff: A yellow or brown dye is obtained from the bark.

Essential oil: The major constituents are car-3-ene in the fruit and linalool in the leaves.

Poison: An effective insecticide is obtained from the bark.

Medicine: It is used in the folk loric medicine in the treatment of cancer (Gbile and Adesina, 1985). The bark is used for treating guinea worms and other worms, diarrhoea, gastroenteritis, snakebite, toothache and respiratory infections. Gum from the bark is used in sealing cuts and wounds. The leaves are used for treating pneumonia and as a tonic to promote general well being. The roots are used for stomach-ache, venereal diseases, chest colds and dizziness. Various plant parts are combined for treating dermatological diseases and ophthalmic disorders.

Other products: Ash from the wood is added to chewing or snuff tobacco and also is a solvent in soap production. Leaves are sometimes used in filling mattresses and pillows, and in Sudan a perfume is made from boiled leaves. In South Africa, roots are said to cure madness, and in Mozambique, they are fed to small children to induce them to forget the breast and thus hasten weaning. It has also been claimed that leaves picked on a Thursday morning and thrown over the right shoulder bring good luck.

2.9.7 Pests and Diseases

The primary disease that affects the genus is anthracnose, caused by *Colletotrichum gloesporioides*. It induces small, light green spots on leaves and dark spots on flowers, causing them to drop prematurely and leading to mummification of the fruit. It is controlled by spraying with fermate, phygon and fungicides.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant Samples

Fresh stem bark of *Annona Senegalensis* (Annonaceae) was collected in March from Beji - Minna, Niger State, Nigeria. The plant was identified in the Department of Crop Production, Federal University of Technology, Minna.

3.1.2 Trypanosomes

Trypanosoma brucei brucei was obtained from the Nigerian Institute for Trypanosomiasis Research, Vom, Plateau State, Nigeria, and maintained in the laboratory by serial blood passage in mice. Passage was by sacrificing heavily infected mice.

3.1.3 Animals

Albino mice, male and female, weighing between 19.32 - 28.82 were purchased from the Nigerian Institute for Trypanosomiasis Research, Vom, Plateau State, Nigeria. They were adequately fed on mouse pellets and given water *ad libidum* throughout the study. The experiment was conducted in compliance with the internationally accepted principles for laboratory animal use and care as contained in the Canadian Council on Animal Care (CCAC) guidelines on animal use protocol review (1997).

3.2 METHODS

3.2.1 Preparation of Crude Extracts

Stem bark of *Annona Senegalensis* was washed with tap water; air dried to constant weight and kept in polythene bags until required for use. Dried sample was cut into pieces and then pounded to powder form using mortar and pestle. Fifty grams (50g) of the powdered form was extracted under reflux in 400ml each of hexane, and then methanol for 2h with Medline Extraction Mantle. The extracts were filtered hot through muslin cloth, and then concentrated with steam bath (Gallen kamp, Britain, App.No. 954005E). The extracts were transferred into sterile universal tubes, and kept in the refrigerator until required for use. The stock solution was prepared just before use by dissolving the extracts in 10% dimethylsulfoxide (DMSO).

3.2.2 Partial Purification of Plant Extracts

3.2.2.1 Column Chromatography

The crude extract was partially purified using column chromatography. A slurry was prepared by shaking 80g of silica gel (60-120) with 100ml of hexane and packed in a column. The column was then loaded with 10g of the extract that had been previously adsorbed from hexane on 4g of the silica gel, and then eluted with 100ml hexane/ethylacetate in the ratio as follows

Table 3.1 Column chromatography

S/no	Hexane (ml)	Ethylacetate (ml)	Volume (ml)
1.	100	-	100
2.	95	5	100
3.	90	10	100
4.	80	20	100
5.	70	30	100
6.	60	40	100
7.	50	50	100
8.	40	60	100
9.	30	70	100
10.	20	80	100
11.	10	90	100
12	-	100	100

Twelve different fractions were collected under pressure using mechanical pump, and then evaporated to dryness.

3.2.2.2 Thin Layer Chromatography (TLC)

The twelve fractions were each spotted on a thin layer plate and developed in chromatographic tank of hexane/ethylacetate solvent system (4:1). After removal of the plate from the solvent, it

was sprayed with iodine vapour and kept in an ovum for 3mins in order to make the spots conspicuous. From the spots on the thin layer plate, the twelve fractions were pulled together to obtain six fractions (fractions 1, 2&3, 4, 5-8, 9, 10-12)

3.2.3 Infection of Animals

Blood was collected by cardiac puncture with an EDTA coated syringe from a heavily infected mouse and immediately diluted with physiological saline to serve as inoculum. Healthy mice were infected intraperitoneally with 0.02ml of the inoculum containing about 10^3 trypanosome cells. Infection was monitored at two days interval throughout the study by microscopic examination of blood samples taken from tails of infected animals.

3.2.4 Acute Toxicity Testing

3.2.4.1 Pilot Study

A pilot test was carried out to determine a range of doses which is estimated to cause 10 -90% deaths in the final test. Varying doses of the hexane extract of the stem bark was given to five different groups (A- 1000, B- 2000, C- 3000, D- 4000, E- 5000mg/kg body weight) of mice having average weight of 25g, each containing three mice. The sixth group was the control, administered 0.4ml of physiological saline. They were observed for 24h, and thereafter for one week.

3.2.4.2 Median Lethal Dose (LD_{50})

Median lethal dose (LD_{50}) was carried out by administering varying doses of the extract to five different groups (A- 4500, B- 4000, C- 3600, D- 3300, E- 3000mg/kg body weight) of mice having average weight of 25g, each containing 10 mice. The sixth group was the control,

administered 0.4ml of physiological saline. The doses were chosen based on the result obtained from the pilot test. They were observed for 24h, and thereafter for two weeks.

Table 3.2 LD_{50} determination by arithmetic method of Karber

Group	Dose (mg/kg)	Dose Difference	Dead	Mean Death	Dose Diff. x Mean Dead
A	4500		4		
		500		2.5	1250
B	4000		1		
		400		0.5	200
C	3600		0		
		300			
D	3300		0		
		300			
E	3000		0		
Total					1450

The sum of the products of the mean dead and the dose difference was divided by the number of animals in each group. The resulting quotient was subtracted from the apparent least lethal dose in order to obtain the LD_{50} as follows:

$$LD_{50} = 4500 - \frac{(1450)}{10} = 4355 mg/kg$$

3.2.5 Screening of Extracts for Antitrypanosomal Activity

3.2.5.1 In Vivo Antitrypanosomal Activity of the Crude Hexane Extract

Seven groups of albino mice of both sexes, each group containing three mice, were infected with *Trypanosoma brucei brucei* intraperitoneally as described above. These infected mice were administered intraperitoneally hexane extract at doses of 50 (A), 100 (B), 300 (C), 400 (D), 500mg/kg body weight (E) per day. The sixth group (F) was administered with 3.5mg/kg body weight of berenil, a standard drug used in the treatment of animal trypanosomiasis and the infected but not treated, seventh group (G), served as the control. Administration of extract commenced 24 hours post-infection. Infection was monitored at two days interval by microscopic examination of blood samples taken from tails of infected animals.

3.2.5.2 In Vivo Antitrypanosomal Activity of the Methanol Extract

Twenty one albino mice were infected with *Trypanosoma brucei brucei* intraperitoneally as described above. These were divided into seven groups of three mice each and intraperitoneally administered methanol extract at doses of 50 (group A), 100 (B), 300(C), 400 (D), 500mg/kg body weight (E) per day. The sixth group (F) was administered with 3.5mg/kg body weight of berenil, standard drug and the infected but not treated, seventh group (G), served as the control.

Mice treated with 100mg/kg body weight were cleared of parasites on day 9 but reappeared later. Hence, further screening was carried on Group B (100mg/kg body weight) using three groups of mice, each group containing three mice each, to ascertain its trypanocidal activity. Group A- 100mg/kg body weight, B- 3.5mg/kg body weight, C- infected not treated. Administration of extract commenced 24 hours post-infection. Blood was taking from tails of infected mice into a

glass slide and viewed under the microscope for parasites. This was done at two days interval throughout the study.

3.2.5.3 In Vivo Antitrypanosomal Activity of Partially Purified Hexane Extract

Eleven groups of mice, each group containing three mice, were infected with *Trypanosoma brucei brucei* intraperitoneally as described above. Groups A – D and E – H were each treated respectively with 200mg/kg and 400mg/kg of the fractions I – IV. Group I was treated with 400mg/kg of the crude extract, group J - standard drug (3.5mg/kg berenil), group K- control (infected not treated). Administration of extract commenced 24 hours post-infection. Blood was taking from tails of infected mice into a glass slide and viewed under the microscope for parasites. This was done at two days interval throughout the study.

3.2.6 Blood and Cerebrospinal Fluid (CSF) Infectivity Tests

3.2.6.1 Blood Infectivity Test

One of the mice that survived after treatment with the crude hexane extract was sacrificed 6 weeks post- treatment and 0.02ml of blood sample was drawn from the tail and sub-inoculated intraperitoneally into three parasite-free mice. Infection was monitored at two days interval by microscopic examination of blood samples taken from tails of infected animals for two 2 months.

3.2.6.2 Cerebrospinal Fluid (CSF) Infectivity Test

Inoculation of mice with CSF obtained from the second surviving mouse was done as described by Nok *et al.*, (1993). The hair of the mice was shaved and positioned such that the head touched the limbs to make the vertebrae conspicuous. The lumber was then punctured by the insertion of the clean needle. A clean syringe was then fixed to the needle to obtain the clean, clear, and

transparent fluid (Cerebrospinal fluid) that gushed into the needle. Three clean, parasite-free mice were each sub-inoculated with 0.02ml of Cerebrospinal fluid, and parasitemia was monitored at two days interval by microscopic examination of blood samples taken from tails of infected animals for two 2 months.

3.2.7 Haematocrit Determination

A small volume of blood was collected from the tail of the mice into a heparinized capillary tube, one end of which was sealed with plasticine and then spun for 5 minutes in a micro-haematocrit centrifuge. The packed cell volume (PCV) was determined with the aid of micro- haematocrit reader, which gave the value as percentage.

3.2.8 Weight Determination

The weights of the mice were determined twice a week on a weighing balance. This was done by zeroing an empty beaker in a weighing balance and then, placing each mice in the beaker to determine the weight in grams.

3.2.9 Phytochemical Analysis for Hexane Extract

This analysis was carried out to determine the active phytochemical constituents of crude hexane extract of stem bark of *Annona Senegalensis.*

3.2.9.1 Test for Carbohydrate (Molisch's Test)

The powdered sample (0.1g) was boiled in 2ml of distilled water on a hot plate for 3mins and filtered while hot. A few drops of molisch reagent was added to 2ml of the water extract. Then, a small quantity of concentrated sulphuric acid was added and allowed to form a lower layer. A purple ring in the interface of the liquid indicates the presence of carbohydrates. This was then

shaken and allowed to stand for 2mins and diluted with 5ml of water. A purple precipitate also indicates the presence of carbohydrates (Evans, 1989).

3.2.9.2 Test for Tannins

A quantity of 1g of the powdered sample was boiled in 20ml of distilled water for 3mins on a hot plate. The mixture was filtered and the resulting filtrate was used to carry out the following test for tannins.

Ferric chloride test- a portion of the filtrate was diluted with distilled water in a ration 1:4 and a few drops of 10% ferric chloride solution was added and observed for a blue –black colour which indicates the presence of tannins (Evans, 1989).

Lead acetate test- to a little of the filtrate was added lead acetate solution and observed for a reddish colour.

3.2.9.3 Test for Saponins

i) *Froth Test*: to a small quantity of each of the powdered samples was added 95% ethanol and boiled. The mixture was filtered and 2.5ml of the filterate was added to 10ml of distilled water in a test tube. The test tube was stoppered and shaken vigorously for about 30secs and allowed to stand for over 30mins. A honey comb froth indicates the presence of saponins. (Sofowora, 1993).

ii) *Emulsion test*: to the frothing solution was added 2drops of olive oil and the contents shaking vigorously and observed for emulsion formation.

iii) *Fehling's Test*: to 5ml of the filtrate was added 5ml of Fehling's solution and the content was heated in a water bath. Then, observed for a reddish precipitate which turns brick red on further heating with sulphuric acid.

3.2.9.4 Test for Terpenes and Sterols

A quantity of 5g of the powdered sample was extracted by maceration with 50ml of ethylalcohol (95%), filtered and the filtrate was evaporated to dryness. The residue was dissolved in 10ml of anhydrous chlorofoam and then filtered. The filterate was divided into equal portions and the following test carried out.

i) *Liebermann-Burchard test*: The first portion of the chlorofoam solution from above was mixed with 1ml of acetic anhydride, followed by the addition of 1ml of concentrated sulphuric acid down the wall of the test to foam a layer underneath. This was observed for the formation of a reddish violet colour at the junction of the two liquid and a green colour at the layer of the chlorofoam indicates the presence of terpenes (Sofowora, 1993).

ii) *Salkowski's Test*: The second potion of the solution was mixed with 2ml of conc. Sulphuric acid carefully so that the sulphuric acid formed a lower layer. A reddish brown colour at the interface indicates the presence of steroidal ring (sofowora, 1993).

3.2.9.5 Test for Resins (Precipitation Test)

The powdered sample (0.1g) was extracted with 15ml of petroleum ether and filtered into a test tube. An equal volume of copper acetate solution was added and shaken vigorously, then allowed to separate. A green colour indicates the presence of resins.

3.2.9.6 Test for Balsams

About 2 drops of alcoholic ferric chloride solution was added to 5ml of 90% ethanol extract of the powdered sample. A dark green colour indicates the presence of balsams.

3.2.9.7 Test for Flavonoids

A quantity of 1g of the powdered sample was boiled in 20ml of distilled water for 3mins on a hot plate. The mixture was filtered and the resulting filtrate was used to carry out the following test for tannins.

Ferric chloride test- a portion of the filtrate was diluted with distilled water in a ration 1:4 and a few drops of 10% ferric chloride solution was added and observed for a blue –black colour which indicates the presence of flavonoids (Evans, 1989).

3.2.9.8 Test for Alkaloids (General Test)

A quantity of 20ml of 3% sulphuric acid in 50% ethanol was added to 2g of the sample in a set of five test tubes and heated on a boiling water bath for 10 minutes and cooled. A drop wise of a few drops of the following reagents were added

Meyer's reagent - (potassium mercuric iodine solution)

Dragner Dorff's reagent-(potassium bismuth iodine solution)

Wagner's reagent (solution of iodine I potassium iodine)

Hager's reagents – (a saturated solution of picric acid 1%)

10% tannic acid solution

The presence of precipitate in at least 3 or all of the above reagents indicates the presence of alkaloids.

3.2.9.9 Test for Glycosides

A small amount of the powdered sample was placed in a test tube and sufficient distilled water was added to cover the sample. A prepared moist sodium picrate paper was suspended in the neck of the tube by means of a cork. The tube was placed in water bath for one hour. A brick red colour in the paper indicates the presence of cyanogenic glycosides (Evans, 1989).

3.2.9.10 Test for Phenols

To a small amount of the sample in a test tube was added methanol. Ferric chloride was added to the methanolic extract. A green colour appearing in the test tube indicates the presence of phenols.

CHAPTER FOUR

4.0 RESULTS

4.1 Screening for Antitrypanosomal Activity of Hexane Extract of Stem Bark of *Annona senegalensis*

4.1.1 Antitrypanosomal activity of crude hexane extract of stem bark of *Annona senegalensis*

Although varying doses of hexane extract were administered to mice infected with *trypanosoma brucei brucei*, only 400mg/kg of the crude extract showed trypanocidal activity (fig 4.1). Comparing the parasitemia of the infected mice administered 400mg/kg of the crude extract with the control (infected but not treated), there was no significant difference ($p>0.05$) observed on day 1 and 3 after administration of the extract. On days 5 and 7, a significant difference ($p<0.01$) was observed. The control died on day 9 while the infected mice treated with 400mg/kg of the crude extract completely cleared the parasites from circulation on day 11 with 2/3 of the mice surviving without parasite up to 43 days before they were sacrificed for blood and cerebrospinal fluid (CSF) infectivity tests.

Continuous increase in packed cell volume (PCV) and Weight of the treated mice were also observed except for a slight decrease in the PCV on days 4 and 7 but not a significant decrease ($p>0.05$) (table4.1, 4.2)

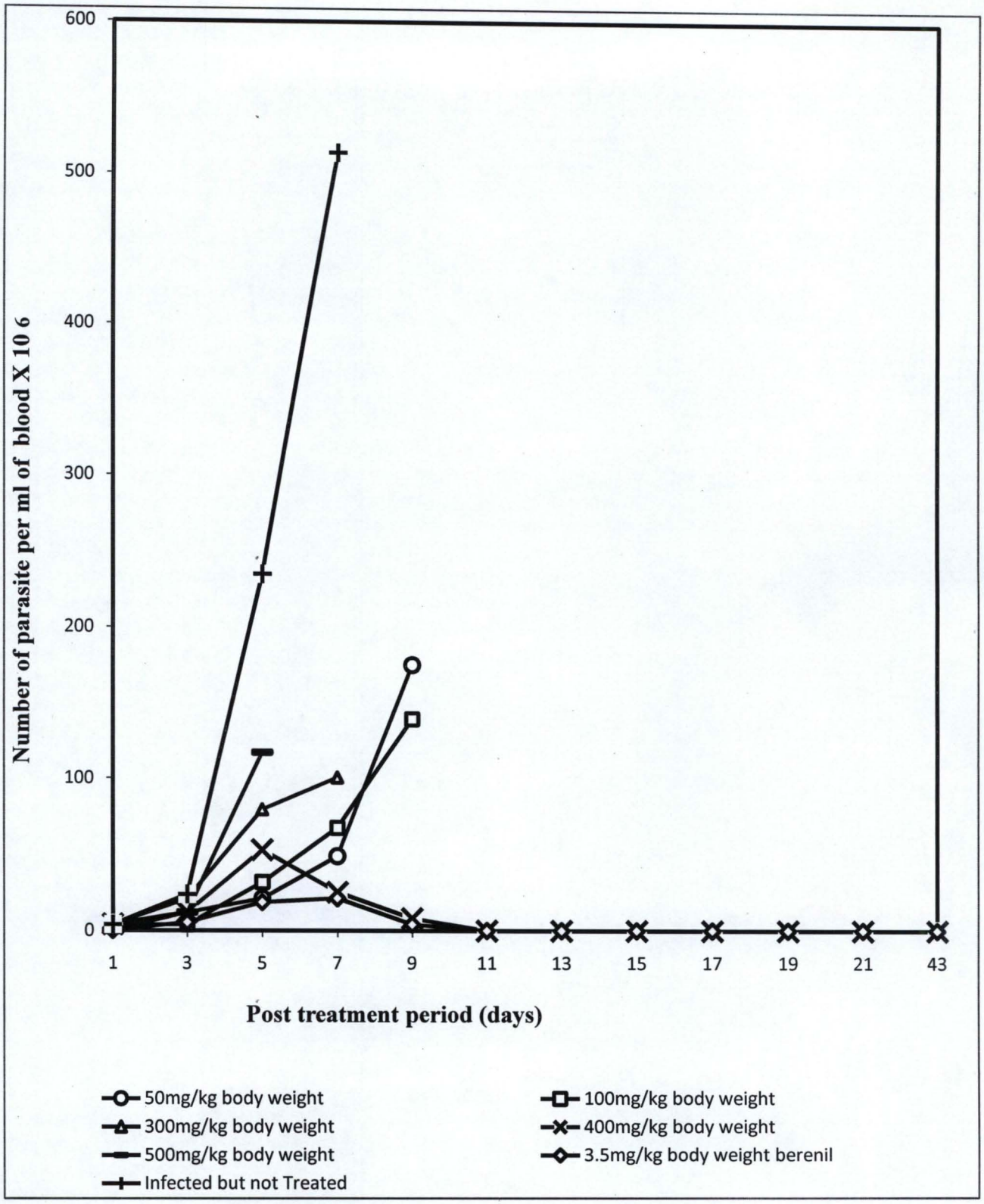

Fig 4.1 Antitrypanosomal activity of crude hexane extract of stem bark of *Annona senegalensis* administered intraperitoneally at different dose levels.

Table 4.1 Weight (g) of infected mice treated with crude hexane extract of stem bark of *Annona senegalensis*.

Dose (mg/kg body weight)	Days after infection						
	Before infection	3	5	16	22	33	43
50	20.000 ± 0.7200	21.050 ± 0.9300	19.520 ± 0.6310	-	-	-	-
100	21.040 ± 0.8407	21.000 ± 0.1100*	21.010 ± 0.1217	-	-	-	-
300	22.440 ± 0.6286	21.900 ± 0.4877	20.420 ± 0.2427**	-	-	-	-
400	19.783 ± 0.1258	20.253 ± 0.5749	21.047 ± 0.9451	21.690* ±0.6126	22.900** ±0.8660	25.530 ** ± 0.5233	26.595 ±0.5728
500	21.133 ± 0.9607 **	22.717 ±1.277	19.725 ± 0.5445	-	-	-	-
3.5 berenil	19.380 ± 0.2254**	19.070 ±0.0818*	20.115 ± 0.6152	-	-	-	-
Infected but not Treated mice	25.173 ± 0.3535	24.407 ±0.3955	19.835 ± 1.181	-	-	-	-

- dead

** = P<0.01 means that the difference in weight before infection when compared with the weight after infection is extremely significant.

* = P<0.05 means that the difference in weight before infection when compared with the weight after infection is significant.

= p>0.05 means that the difference in weight before infection when compared with the weight after infection is not significant.

Table 4.2 Packed cell volume (PCV) of infected mice treated with crude hexane extract of *Annona senegalensis*

Dose (mg/kg body weight)	Days after infection					
	Before infection	4	7	11	40	43
50	52.000 ± 2.000	48.333 ± 1.528*	45.000 ± 2.000 **	-	-	-
100	50.333 ± 1.528	43.333 ± 4.619	39.000 ± 3.000**	-	-	-
300	48.667 ± 4.041	45.500 ± 3.536	44.000 ± 5.657	-	-	-
400	52.333 ± 3.215	49.667 ± 3.055	51.333 ± 4.163	52.000 ±3.464	57.000 ±4.243	57.000 ± 4.243
500	49.667 ± 8.021	46.667 ± 8.327	39.000 ± 2.820	-	-	-
3.5 berenil	49.333 ± 0.577	49.333 ± 0.577	49.333 ± 1.528	-	-	-
Infected but not Treated mice	53.000 ± 1.732	49.000 ± 1.000	42.000 ± 1.000**	-	-	-

- Dead

** = P<0.01 means that the difference in PCV before infection when compared with the PCV after infection is extremely significant.

* = P<0.05 means that the difference in PCV before infection when compared with the PCV after infection is significant.

= p>0.05 means that the difference in PCV before infection when compared with the PCV after infection is not significant.

4.1.2 Antitrypanosomal activity of partially purified hexane extract of stem bark of *Annona senegalensis*

Four fractions were obtained from partial purification of crude hexane extract using thin layer chromatography. All the fractions failed to clear parasites from infected mice within the period of the study. Although, comparing the control with the fractions, significant reduction ($p<0.01$) of parasite level in infected mice that received 200mg/kg and 400mg/kg of the partially purified fractions was observed (Fig 4.2). Mice treated with fraction A and B survived for 13 and 11days respectively while the control died on the ninth day. Fraction C (200mg/kg) showed a significant reduction ($p<0.01$) in parasitemias on the fifth and seventh day when compared with the control. Fraction D (200mg/kg) survived for 13 days with a significant decrease ($p<0.01$) in the parasitemia level observed on the fifth and seventh day. There was also a decrease in the PCV and Weight of all the mice treated with various fractions (table 4.3 and 4.4). Infected mice treated with the crude extract to ascertain its antitrypanosomal activity completely cleared the parasites from the circulation within 11 days of continued treatment at a dose level of 400mg/kg body weight per day.

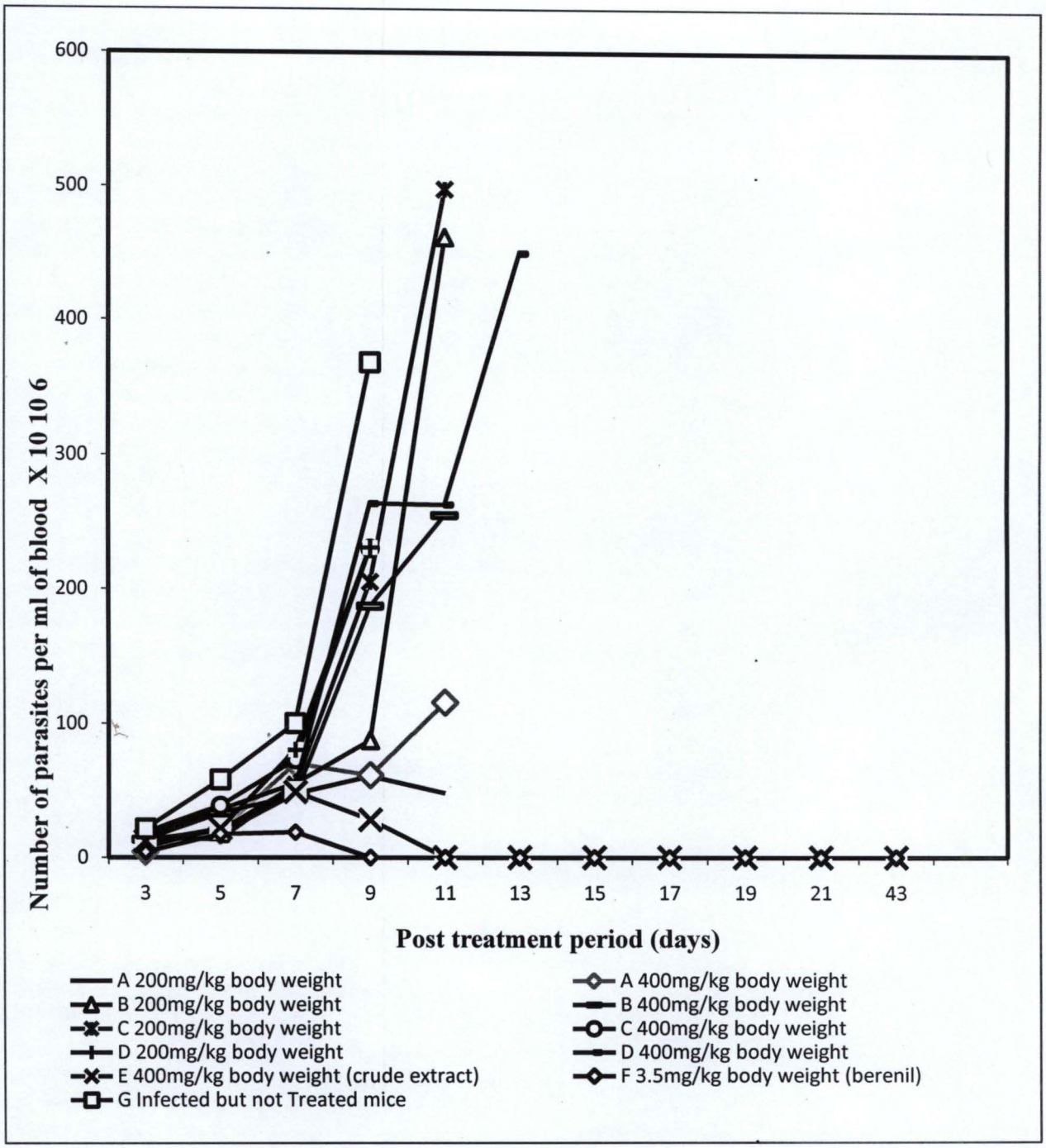

Fig 4.2 Antitrypanosomal activity of partially purified hexane extract of stem bark of *Annona senegalensis* administered intraperitoneally at different dose levels.

Table 4.3 Weight (g) of infected mice treated with partially purified hexane extract of stem bark of *Annona senegalensis*

Animals	Dose(mg/kg body weight)	Weeks after infection		
		before infection	1	2
A	200	25.64 ± 0.360	25.00 ± 0.700	25.00 ± 1.000
A	400	23.99 ± 1.315	22.00 ± 1.000	21.12 ± 0.1664
B	200	25.71 ± 0.6092	24.80 ± 1.136	21.00 ± 0.200**
B	400	24.31 ± 1.815	23.00 ± 0.100	20.00 ± 1.000
C	200	22.37 ± 1.383	21.680 ± 0.5456	19.43 ± 0.04359*
C	400	21.62 ± 0.3704	20.970 ± 0.9254	18.01 ± 0.01732**
D	200	24.80 ± 0.3464	26.040 ± 0.2425 **	27.90 ± 0.4583**
D	400	23.10 ± 0.1732	20.113 ± 0.02309**	20.00 ± 1.000**
E	400 crude extract	24.8 ± 0.3464	26.040 ± 0.2425**	27.90 ± 0.4583**
F	3.5 berenil	21.07 ± 0.1212	21.500 ± 0.3000	23.04 ± 0.1229**
G	Infected but not Treated mice	25.17 ± 0.1868	24.400 ± 0.2646	20.00 ± 0.5292**

** = $P<0.01$ means that the difference in weight before infection when compared with the weight after infection is extremely significant.

* = $P<0.05$ means that the difference in weight before infection when compared with the weight after infection is significant.

= $p>0.05$ means that the difference in weight before infection when compared with the weight after infection is not significant.

Table 4.4 Packed cell volume (PCV) of infected mice treated with partially purified hexane extract of *Annona senegalensis* stem bark

Animals	Dosage (mg/kg body weight)	weeks after infection		
		Before infection	1	2
A	200	52.000 ± 2.000	52.000 ± 1.732	50.000 ± 0.000
A	400	50.000 ± 2.000	49.000 ± 2.646	49.000 ± 2.646
B	200	56.000 ± 1.000	53.000 ± 1.000	50.000 ± 2.000 **
B	400	47.000 ± 3.464	44.000 ± 2.000	42.000 ± 1.000
C	200	22.370 ± 1.383	21.680 ± 0.5456 **	19.430 ± 0.04359 **
C	400	48.000 ± 2.000	47.000 ± 3.606	43.000 ± 3.000
D	200	47.000 ± 1.000	45.000 ± 1.000	42.000 ± 2.000 **
D	400	49.000 ± 0.000	46.000 ± 1.732 *	45.000 ± 1.000 **
E	400 crude extract	51.000 ± 1.000	49.000 ± 3.000	50.000 ± 2.000 **
F	3.5 berenil	51.000 ± 0.000	52.000 ± 1.732	54.000 ± 1.000 *
G	Infected but not Treated mice	55.000 ± 1.000	51.000 ± 1.000 **	48.000 ± 0.000 **

** = $P<0.01$ means that the difference in PCV before infection when compared with the PCV after infection is extremely significant.

* = $P<0.05$ means that the difference in PCV before infection when compared with the PCV after infection is significant.

= $p>0.05$ means that the difference in PCV before infection when compared with the PCV after infection is not significant.

4.2 Antitrypanosomal Activity of Methanol Extract of Stem Bark of *Annona senegalensis*

Varying doses of methanol extract of *Annona senegalensis* stem bark were screened for antitrypanosomal activity. Administration of 100mg/kg of the crude methanol extract to mice infected with *Trypanosoma brucei brucei* cleared the parasites from circulation within 7 days of continued administration of the extract but reappeared 2 days later. Continuous significant reduction ($p<0.01$) in parasitemias in all infected mice treated with the extract when compared with the control was observed throughout the study (Fig 4.3). Decrease in the PCV and weight of all the mice treated with the extract was also observed (table 4.5 and 4.6). In the final screening of the crude methanol extract, a significant reduction ($p<0.01$) in the parasite level was observed as compared with the infected but not treated mice that showed significant increase ($p<0.01$) in the parasitemia level in the circulation (Fig 4.4). No significant difference was observed in the PCV of the mice treated with the extract and the control but a significant decrease ($p<0.01$) was observed in the weight of the control group (table 4.7 and 4.8). Infected mice treated with the extract survived for 9 days while infected but not treated group survived for 5 days.

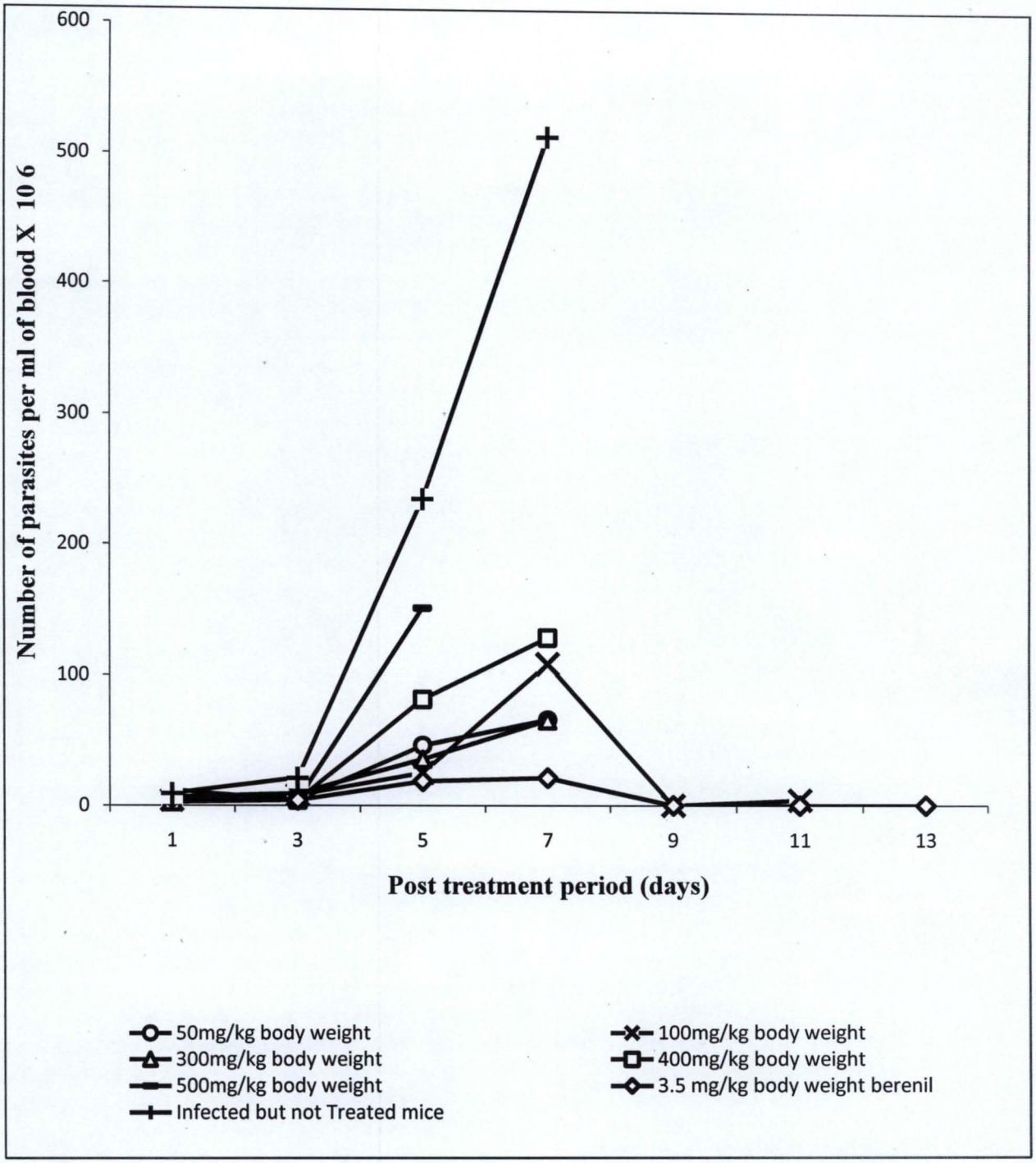

Fig 4.3 Antitrypanosomal activity of methanol extract of stem bark of *Annona senegalensis* administered intraperitoneally at different dose levels.

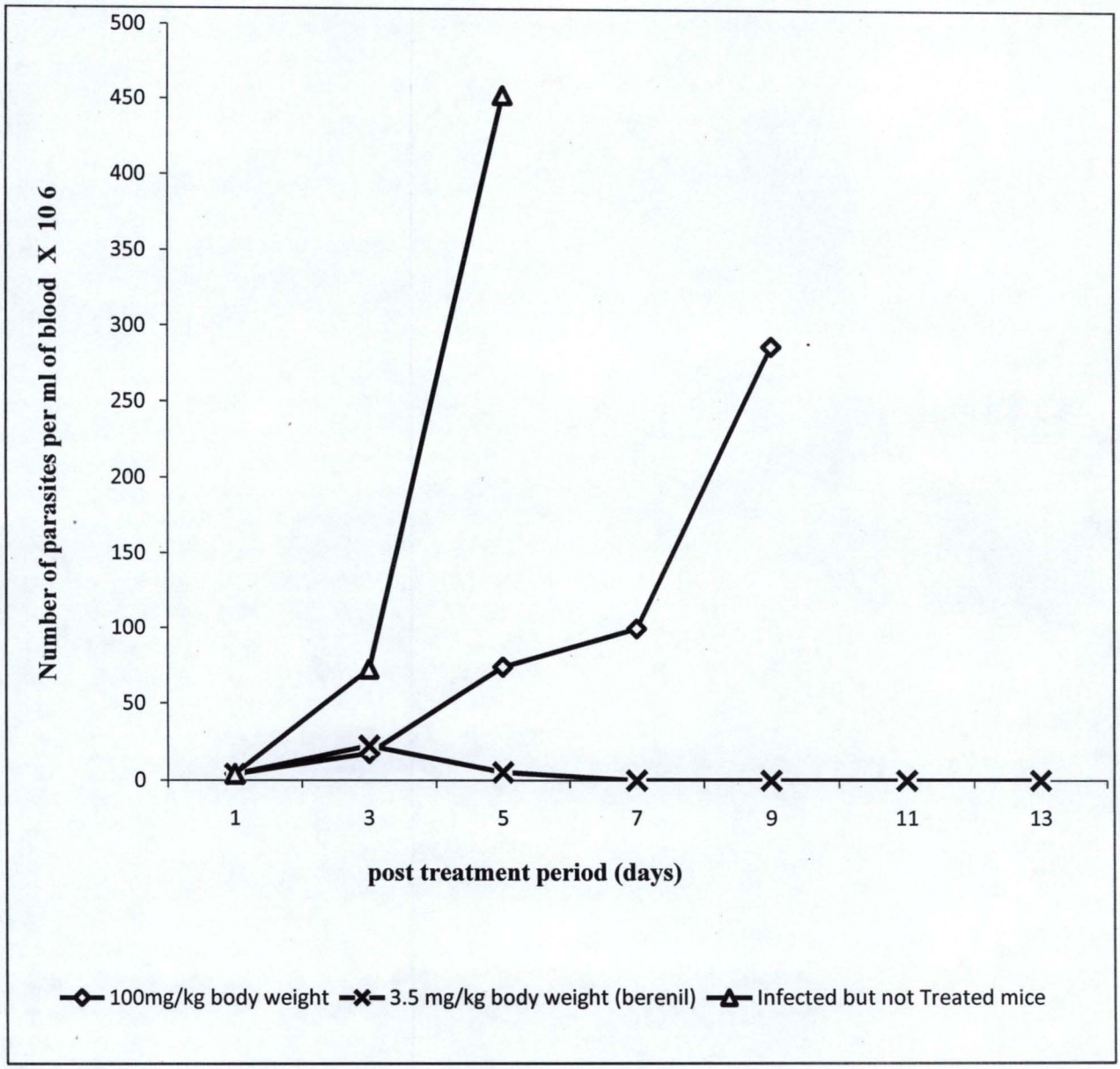

Fig 4.4 Final screening for antitrypanosomal activity of methanol extract of stem bark of *Annona senegalensis* administered intraperitoneally at different dose levels.

Table 4.5 Weight (g) of infected mice treated with methanol extract of stem bark of *Annona senegalensis* (initial screening)

Dose (mg/kg body weight)	weeks after infection		
	Before infection	4	7
50	21.350 ± 0.5635	22.310 ± 0.4293	19.400 ± 0.5292 **
100	21.610 ± 0.7299	21.720 ± 0.7219	20.770 ± 0.7750
300	23.290 ± 0.2524	23.910 ± 0.6894	22.260 ± 0.5575
400	21.330 ± 0.3300	22.050 ± 1.026	20.300 ± 0.2784
500	28.820 ± 0.5620	26.230 ± 0.3984	20.300 ± 0.2784 **
3.5 berenil	19.380 ± 0.2524	19.077 ± 0.07095	20.120 ± 0.8266
Infected but not Treated mice	20.710 ± 0.8982	21.930 ± 2.395	17.200 ± 1.114

** = $P<0.01$ means that the difference in weight before infection when compared with the weight after infection is extremely significant.

= $p>0.05$ means that the difference in weight before infection when compared with the weight after infection is not significant.

Table 4.6 Packed cell volume (PCV) of infected mice treated with methanol extract of stem bark of *Annona senegalensis* (initial Screening)

Dose (mg/kg body weight)	(days)	
	Before infection	after infection (4)
50	52.00 ± 3.00	49.00 ± 3.00
100	52.00 ± 2.00	49.00 ± 3.00
300	48.00 ± 3.00	45.00 ± 1.00
400	52.00 ± 1.732	48.00 ± 1.732
500	50.00 ± 1.00	42.00 ± 2.00
3.5 berenil	44.00 ± 1.732	44.00 ± 1.00
Infected but not Treated mice	53.00 ± 1.732	43.00 ± 1.155

= $p>0.05$ means that the difference in PCV before infection when compared with the PCV after infection is not significant.

Table 4.7 Weight (g) of infected mice treated with methanol extract of stem bark of *Annona senegalensis* (final screening)

Dose (mg/kg body weight)	Days after infection		
	Before infection	3	7
100	24.897 ± 0.6215	24.803 ± 0.7009	23.697 ± 1.055
3.5 berenil	22.413 ± 0.3951	22.417 ± 0.3785	23.043 ± 0.1692
Infected but not Treated mice	25.253 ± 0.3361	24.137 ± 0.1795	20.557 ± 0.8707 **

** = $P<0.05$ means that the difference in weight before infection when compared with the weight after infection is extremely significant.

= $p>0.05$ means that the difference in weight before infection when compared with the weight after infection is not significant.

Table 4.8 Packed cell volume (PCV) of infected mice treated with methanol extract of stem bark of *Annona senegalensis* (final screening)

Dose (mg/kg body weight	Days after infection		
	Before infection	3	7
100	50.667 ± 2.517	50.000 ± 3.000	48.667 ± 1.528
3.5 berenil	51.000 ± 1.000	49.333 ± 0.5774	51.333 ± 0.5774
Infected but not Treated mice	55.333 ± 7.572	54.333 ± 7.506	45.667 ± 3.055

= $p>0.05$ means that the difference in PCV before infection when compared with the PCV after infection is not significant.

4.3 Blood and Cerebrospinal Fluid (CSF) Infectivity Test

Sub- inoculation of blood and cerebrospinal fluid (CSF) drawn from the cured mice into three parasite free mice was to ascertain the complete clearance of the parasites from the infected animals. Two months after the sub-inoculation, both the blood and cerebrospinal fluid failed to develop any infection.

4.4 Acute Toxicity Studies

In the initial study (Table 4.9), when the hexane extract was administered intraperitoneally to mice at doses between 1000 and 3000mg/kg to non-infected mice, no death was recorded but some mice given extract at dose level of 4000 and 5000mg/kg were less active and one mouse died from each group within the observation period. Hence, it was considered necessary to determine the median lethal dose (LD_{50}) which was estimated to be 4355mg/kg body weight (table 4.10).

Table 4.9 Acute toxicity studies (pilot testing)

Group	Dose	Number of mice	Dead
A	5000	3	1
B	4000	3	1
C	3000	3	0
D	2000	3	0
E	1000	3	0

Table 4.10 Median lethal dose (LD_{50})

Group	Dose (mg/kg)	Dose Difference	Dead	Mean Death	Dose Diff. x Mean Dead
A	4500		4		
		500		2.5	1250
B	4000		1		
		400		0.5	200
C	3600		0		
		300			
D	3300		0		
		300			
E	3000		0		
Total					1450

The Median lethal dose (LD_{50}) of the hexane extract was estimated to be 4355mg/kg body weight.

4.5 Phytochemical Analysis

Phytochemical analysis of the crude hexane extract showed that it contained alkaloids, saponins, sterols, tannins, steroids, flavonoids and phenol (table 4.11).

Table 4.11 Phytochemical Analysis of crude hexane extract

Phytochemical Test	Inference
Carbohydrate	+
Tannin	+
Saponins	+
Terpenes	-
Sterols	+
Resins	-
Balsams	-
Flavonoids	+
Alkaloids	+
Glycosides	-
Phenols	+

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

The crude methanol extract and various fractions of hexane extract failed to clear parasites from circulation although a reduction of parasitemia was observed when compared with the control. The crude hexane extract of *Annona senegalensis* was found to possess trypanocidal activity against *Trypanosoma brucei brucei* in mice. These findings agree with the claims of traditional health practioners in Northern Nigeria, who use the herb, alone (decoction) or in combination with other plants (concoction) in the treatment of animals infected with *Trypanosoma brucei brucei.*

Although the parasitemia in the infected mice treated at 400mg/kg body weight per day of crude hexane extract was high on day 5, to about 52.652 x 10^6 of trypanosomes per ml, a significant difference ($p<0.01$) of parasite level was observed when compared with infected but not treated mice on the same day. Though a progressive increase in parasitemia was observed in all the infected mice treated at various dose level of crude hexane extract, a reduction in parasitemia was observed from day 7 in infected mice treated at 400mg/kg, and the infection was nil on the eleventh day post infection. No parasite was seen in the infected group treated at 400mg/kg 43 days after the commencement of treatment (Fig 4.1). It is obvious that the trypanocidal effect is attributable to the plant extract since the control group that were infected but not treated presented with massive parasitemia culminating into death 7 days post infection. Ogbadoyi *et al.*,

(2007) showed that the aqueous leaf extract of *Annona senegalensis* has trypanocidal activity while the whole root extract of the same plant is trypanostatic.

A significant increase ($p< 0.01$) in weight of infected mice treated at 400mg/kg body weight/ day was observed where as decrease in weight was observed in the infected but not treated mice throughout the study (Table 4.1). An increase in PCV though not significant ($p>0.05$) was observed in the group treated at 400mg/kg where as the PCV of infected but not treated mice showed progressive significant decrease ($p<0.01$) throughout the study (Table 4.2). The significant decrease in PCV of infected but not treated group could be a result of the enormous increase in numbers of circulating trypanosomes causing lysis of the erythrocytes by the lashing action of the parasites' flagella. The breakdown of plasma protein, and the enzymes neuraminidase produced by the trypanosomes, may also be involved in the process (Igwe and Onabanjo, 1988).

Crude hexane extract of *Annona senegalensis* below a dosage of 4000mg/kg did not cause death in experimental mice. The LD_{50} was estimated to be 4355mg/kg (Table 4.9 and 4.10).

Crude hexane extract of *Annona senegalensis* was also found to contain saponins and tannins (Table 4.11). This agrees with the findings of Ogbadoyi *et al.*, (2007) and Igwe and Onabanjo (1988). They showed that the aqueous leaf extract of *Annona senegalensis* contained saponins and tannins. The extract also showed the presence of alkaloids where as glycosides were completely absent (Table 4.11). This result also agrees with the findings of Igwe and Onabanjo, (1988) who showed that the same plant contains alkaloids while glycosides were absent.

In the subinoculation experiments, both the blood and the cerebrospinal fluid from the cured mice failed to develop any infection 2 months after sub-innoculation which signifies that parasites have been cleared from the systems. It also suggests that the drug probably crossed the blood brain barrier to kill the parasites if any escaped into the cental nervous system. Hence, this result is of great interest since a major problem militating against the effective chemotherapeutic control of late stage sleeping sickness is the inability of most existing drugs to cross the blood brain barrier (WHO, 2003). Therefore, only drugs that can cross the blood brain barrier and clear the parasites from the central nervous system (CNS) are useful in the treatment of late stage of the disease.

Although the crude extract cleared the parasites, the partially purified fractions of the extracts failed to clear the parasites from circulation but there was a reduction in parasitemia in infected mice treated with fraction A as compared with the control. The failure of the various fractions to clear parasites from circulation could be that the active phytochemical components are acting synergistically. Therefore, the crude extract which showed trypanocidal activity can be standardized and packaged to be used as phytomedicine.

Though, varying doses of methanol extract of *Annona senegalensis* stem bark when administered to infected mice failed to clear parasites from circulation, at 100mg/kg lives of infected mice treated with this extract were prolonged for further 4 days as compared with infected but not treated mice (Fig 4.3). Infected mice were presented with massive parasitemia which lead to decrease in PCV and weight of all the infected mice treated with the extract. This resulted to death, 9 days post infection (Fig. 4.3, Table 4.5 and 4.6).

As reported by Gbile and Adesina (1985), *Annona senegalensis* is used in the treatment of cancer. This is of considerable interest because all four drugs used clinically in the treatment of sleeping sickness are known to have significant cytotoxic effect on cancer cells (Barret and Barret, 2000). Since sleeping sickness is a disease of the rural poor in Africa where there is no profitable market, drug developers are reluctant in the development of such drugs but the anticancer activity of such plant is an added advantage as it will draw the attention of investor to invest in the development of this drug because of wider market potentials.

5.2 Conclusions

In conclusion, hexane-extractable phytochemicals from *Annona senegalensis* stem back possess *in vivo* antitrypanosomal activities. Hence, standardization and packaging of the crude extract will be very necessary for the proper utilization of the plant potentials.

5.3 Recommendations

In other to exploit the chemotherapeutic potential of this plant to the fullest, it is suggested that

further research be carried out on combination therapy involving the stem bark and other parts of the plant to find out if this will be more effective in the treatment of sleeping sickness is recommended. Standardization and packaging of the crude extract will be very necessary for the proper utilization of the plant potentials.

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APPENDIX

Appendix I

Preparation of the Stock Solution and Determination of Dose Level

Crude hexane and methanol extracts (1g each) were each dissolved in 5ml of dimethyl sulfoxide (DMSO) and made up to 10ml with physiological saline. Various fractions of partially purified hexane extract were each dissolved in the equivalent volume of Dimethylsulphoxide (DMSO) and stored in the refrigerator. The preparation is as follows:

1g of extract = 10ml of solvent

1000mg = 10ml of solvent

= 100mg/ml

Therefore, 1g of extract in 10ml of solvent is equivalent to 100mg/ml being the concentration of the stock solution.

For mice of average weight of 20g, 50mg of the extract was administered following this calculation as follows

50mg/kg body weight: 50mg/1000g

Xmg/20g

Xmg = 1.0mg

But 100mg/ml,

Therefore, 100mg/ml

1.0mg/y

Y= 0.01ml

Hence, the volume of extract administered is 0.01ml of the stock solution for 50mg/kg body weight.

The above calculation was followed for all the doses administered.

Appendix ii

a) Screening for antitrypanosomal activity of crude hexane extract of stem bark of *Annona senegalensis* (raw data)

Animals	Dose (mg/kg body weight)	Average number of parasite in the blood per field (days after infection)																		
		1	3	5	7	9	11	13	15	17	19	21	24	26	28	30	35	38	40	43
A	50	3	5	12	44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	100	1	1	8	17	35	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C	300	1	4	20	26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D	400	1	3	16	6	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E	500	0	3	30	52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F	3.5 berenil	0	1	5	5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G	Infected but not Treated mice	1	6	59	129	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

b) Screening for antitrypanosomal activity of hexane extract of stem bark of *Annona senegalensis* (statistical data)

Dose (mg/kg body weight)	Average number of parasite per ml of blood x 10^6 (days after infection)					
	1	3	5	7	11	43
50	3.981 ± 2.656E-19	11.905 ± 3.954	21.125 ± 9.912	48.742 ± 22.761	-	-
100	3.981 ± 2.656E-19	3.974 ± 3.971	31.665 ± 6.817	67.302 ± 41.708	-	-
300	3.981 ± 2.656E-19	21.125 ± 9.912	79.198 ± 16.223	100.46 ± 35.975	-	-
400	3.981 ± 2.656E-19	11.930 ± 3.991	52.652 ± 29.042	25.106 ± 9.904	0.000	0.000
500	0.000	11.912 ± 7.925	117.02 ± 45.563		-	-
3.5 berenil	0.000	5.301 ± 2.287	18.516 ± 6.043	21.107 ± 9.105	0.000	0.000
Infected but not Treated mice	3.981 ±2.656E-19	23.773 ± 11.839	234.73 ± 39.450	513.55 ± 0.000	-	-

- Dead

c) Weight(g) of mice treated with crude hexane extract of stem bark of *Annona senegalensis*

Animals	Dose (mg/kg body weight)	Days after infection									
		Before Infection	3	7	11	16	22	27	33	40	43
A	50	20.00	21.05	19.52	-	-	-	-	-	-	-
B	100	21.04	21.00	21.01	-	-	-	-	-	-	-
C	300	22.44	21.90	20.42	-	-	-	-	-	-	-
D	400	19.78	20.25	21.05	19.83	21.69	22.90	24.56	25.53	26.00	26.60
E	500	21.13	22.7 2	19.73	-	-	-	-	-	-	-
F	3.5 berenil	19.38	19.07	20.12	20.62	23.75	25.00	25.53	25.98	27.45	27.76
G	Infected but not Treated mice	20.71	19.93	16.61	-	-	-	-	-	-	-

d) Packed cell volume (PCV) of mice treated with crude hexane extract stem bark of *Annona senegalensis*

Animals	Dose (mg/kg Body weight)	days after infection									
		Before infection	4	7	11	16	22	27	33	40	43
A	50	52	48	45	-	-	-	-	-	-	-
B	100	50	43	39	-	-	-	-	-	-	-
C	300	49	46	44	-	-	-	-	-	-	-
D	400	52	50	51	52	52	52	56	56	57	57
E	500	50	47	39	-	-	-	-	-	-	-
F	3.5 berenil	49	49	49	51	51	51	52	52	55	56
G	Infected but not treated mice	53	49	42	-	-	-	-	-	-	-

- Dead

Appendix iii

a) Screening for antitrypanosomal activity of partially purified extract of stem bark of *Annona senegalensis* (raw data)

Animals	Dosage (mg/kg body weight)	Average number of parasite in the blood per field (Days after infection)																	
		1	3	5	7	9	11	13	15	17	19	21	24	26	28	30	35	38	40	43
A	200	1	2	4	12	1	12	-	-	-	-	-	-	-	-	-	-	-	-	-
A	400	1	1	5	18	16	29	-	-	-	-	-	-	-	-	-	-	-	-	-
B	200	0	4	9	14	28	116	-	-	-	-	-	-	-	-	-	-	-	-	-
B	400	1	9	8	12	47	64	-	-	-	-	-	-	-	-	-	-	-	-	-
C	200	1	3	5	13	52	125	-	-	-	-	-	-	-	-	-	-	-	-	-
C	400	1	6	10	18	58	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D	200	0	25	5	20	58	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D	400	1	5	3	14	10	66	113	-	-	-	-	-	-	-	-	-	-	-	-
E	400 crude Extract	1	3	5	12	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F	3.5 berenil	1	1	4	5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G	Infected but not treated mice	1	5	16	27	93	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- Dead

b) Screening for antitrypanosomal activity of partially purified extract of stem bark of *Annona senegalensis* (statistical data)

Animals	Dosage (mg/kg body weight)	Average number of parasite in the blood per field X 10^6(days after infection)							
		1	3	5	7	9	15	21	43
A	200	3.975 ± 3.972	7.961 ± 7.971	17.189 ± 8.249	47.415 ± 14.152	60.538 ± 19.818	-	-	-
A	400	2.654 ± 2.298	3.981± 2.656E-19	19.825 ± 6.884	70.126 ± 16.479	61.247 ± 19.484	-	-	-
B	200	0.000 ± 0.000	15.868 ± 10.484	35.621± 11.839	55.254 ± 13.589	87.348 ± 50.898	-	-	-
B	400	3.981 ± 2.656	13.238 ± 6.050	32.986 ± 21.777	46.137 ± 2.309	187.11 ± 22.520	-	-	-
C	200	3.981± 2.656	13.233 ± 2.267	19.825 ± 6.884	54.126 ± 19.818	205.02 ± 171.71	-	-	-
C	400	2.654 ± 2.298	18.504 ± 2.298	38.551 ± 6.484	72.780 ± 12.100	230.10 ± 16.852	-	-	-
D	200	0.000 ± 0.000	7.942 ± 0.000	21.125 ± 9.912	80.336 ± 27.960	230.90 ± 50.670	-	-	-
D	400	3.981 ± 0.000	14.541 ± 9.971	13.233 ± 6.051	54.325 ± 12.270	263.58 ± 120.57	-	-	-
E	400 crude extract	2.654 ± 2.298	11.912 ± 7.925	21.152 ± 2.287	48.736 ± 12.598	27.867 ± 8.267	0.00	0.00	0.00
F	3.5 berenil	2.654 ± 2.298	3.981 ± 2.656	17.189 ± 6.047	18.471 ± 16.468	0.00	0.00	0.00	0.00
G	Infected but not treated mice	2.654 ± 2.298	21.125 ± 9.912	58.082 ± 14.827	100.11 ± 22.626	368.24 ± 25.335	-	-	-

- Dead

c) Weight (g) of mice treated with partially purified hexane extract

Animals	Dose (mg/kg body weight)	weeks after infection						
		before Infection	1	2	3	4	5	6
A	200	25.64	25.00	25.00	-	-	-	-
A	400	23.99	22.00	21.12	-	-	-	-
B	200	25.71	24.80	21.00	-	-	-	-
B	400	24.31	23.00	20.00	-	-	-	-
C	200	22.37	21.68	19.43	-	-	-	-
C	400	21.62	20.97	18.01	-	-	-	-
D	200	21.07	18.70	17.00	-	-	-	-
D	400	23.10	20.12	20.00	-	-	-	-
E	400 crude extract	24.80	26.04	27.90	27.98	29.00	29.30	29.99
F	3.5 berenil	21.07	21.50	23.04	23.70	23.79	24.06	25.00
G	Infected but not Treated mice	25.17	24.40	20.00	-	-	-	-

- Dead

d) Packed cell volume of mice treated with partially purified hexane extract

Animals	Dose (mg/kg body weight)	Weeks after infection						
		Before Infection	1	2	3	4	5	6
A	200	52	52	50	-	-	-	-
A	400	50	49	49	-	-	-	-
B	200	56	53	50	-	-	-	-
B	400	47	44	42	-	-	-	-
C	200	47	45	45	-	-	-	-
C	400	48	47	43	-	-	-	-
D	200	47	45	42	-	-	-	-
D	400	49	46	45	-	-	-	-
E	400 crude extract	51	49	50	50	52	56	56
F	3.5 berenil	51	52	54	54	55	55	56
G	Infected but not Treated mice	55	51	48	-	-	-	-

- Dead

Appendix iv

a) Initial screening for antitrypanosomal activity of methanol extract of stem bark of *Annona senegalensis* (raw data)

Animals	Dose (mg/kg body weight)	Average number of parasite in the blood per field (days after infection)						
		1	3	5	7	9	11	13
A	50	1	1	12	17	-	-	-
B	100	1	2	6	29	0	01	-
C	300	2	2	9	17	-	-	-
D	400	1	3	23	33	-	-	-
E	500	3	1	38	-	-	-	-
F	3.5 berenil	1	1	5	5	1	0	0
G	Infected but not Treated mice	2	6	59	129	-	-	-

- Dead

b) Initial screening for antitrypanosomal activity of methanol extract of stem bark of *Annona senegalensis* (statistical data)

Dose (mg/kg body weight)	Average number of parasite per ml of blood x 10^6 (days after infection)				
	1	3	5	7	13
50	2.654 ± 2.298	3.981 ± 2.656E-19	46.115 ± 17.702	65.640 ± 53.551	-
100	3.981 ± 2.656E-19	7.949 ± 3.972	25.100 ± 12.063	108.37 ± 52.184	0.000
300	6.629 ± 4.586	9.258 ± 6.043	35.575 ± 23.920	65.219 ± 13.867	-
400	2.654 ± 2.298	3.981 ± 2.656E-19	81.299 ± 49.668	128.34 ± 85.788	-
500	11.924 ±1.062E-18	3.981 ± 2.656E-19	150.92 ± 33.513	-	-
3.5 berenil	5.301 ± 2.287	3.981 ± 2.656E-19	18.516 ± 6.043	21.107 ± 9.105	0.000
Infected but not Treated mice	9.269 ± 2.299	21.145 ± 4.586	234.73 ± 39.450	513.55 ± 0.000	-

- Dead

c) Weight (g) of mice treated with methanol extract of stem bark of *Annona senegalensis* (initial screening)

Animals	dose (mg/kg body weight)	days after infection			
		Before infection	4	7	11
A	50	21.35	22.31	19.40	-
B	100	21.61	21.72	20.77	20.03
C	300	23.29	23.91	22.26	-
D	400	21.33	22.05	20.30	-
E	500	28.82	26.23	-	-
F	3.5 berenil	19.38	19.07	20.12	20.62
G	Infected but not treated mice	20.71	21.93	17.20	-

d) Packed cell volume (PCV) of mice treated with methanol extract of stem bark of *Annona senegalensis* (initial screening)

Animals	Dose (mg/kg body weight)	days	
		before infection	after infection(4)
A	50	52	49
B	100	52	49
C	300	48	45
D	400	52	48
E	500	50	42
F	3.5 berenil	44	44
G	Infected but not treated mice	53	43

- Dead

Appendix v

a) Final screening for antitrypanosomal activity of methanol extract of stem bark of *Annona senegalensis* (raw data)

Animals	Dosage mg/kg body weight	Average number of parasite in the blood per field(Days after infection)						
		1	3	5	7	9	11	13
A	100	1	4	18	25	72	-	-
B	3.5 berenil	1	6	1	0	0	0	0
C	Infected but not Treated mice	1	18	114	-	-	-	-

b) Final screening for antitrypanosomal activity of methanol extract of stem bark of *Annona senegalensis* (statistical data)

Dose (mg/kg body weight)	Average number of parasite in the blood per field x 10^6 (Days)				
	1	3	5	11	13
100	3.975 ± 3.972	17.522 ± 6.595	73.908 ± 10.296	-	-
3.5 berenil	3.981 ± 0.000	22.446 ± 9.917	5.302 ± 6.071	0.000	0.000
Infected but not Treated mice	3.981 ± 2.656	72.358 ± 8.254	452.91 ± 69.461	-	-

- Dead

c) Weight (g) of mice treated with methanol extract of stem bark of *Annona senegalensis* (final screening)

Animals	Dose (mg/kg body weight)	days after infection		
		Before infection	3	7
A	100	24.90	24.80	23.70
B	3.5 berenil	22.41	22.42	23.10
C	Infected but not Treated mice	25.30	24.13	20.56

d) Packed cell volume (PCV) of mice treated with methanol extract of stem bark of *Annona senegalensis* (final screening)

Animals	Dose (mg/kg body weight)	days after infection		
		Before infection	3	7
A	100	51	50	49
B	3.5 berenil	51	50	51
C	Infected but not treated mice	55	54	46

- Dead

Appendix vi

Toxicity studies

a) Pilot study (Doses)

Group A 5000mg/kg body weidht = 0.125g/o.4ml

Group B 4000mg/kg body weight = 0.1g/0.4ml

C 3000mg/kg body weight = 0.075g/0.4ml

Group D 2000mg/kg body weight = 0.05g/0.4ml

Group E 1000mg/kg body weight = 0.025g/0.4ml

(Average weight of mice is 25g)

b) Median lethal dose (LD_{50}) determination

Group A 4500mg/kg body weight = 0.1125g/0.4ml

Group B 4000mg/kg body weight = 0.1g/0.4ml

Group C 3600mg/kg body weight = 0.09g/0.4ml

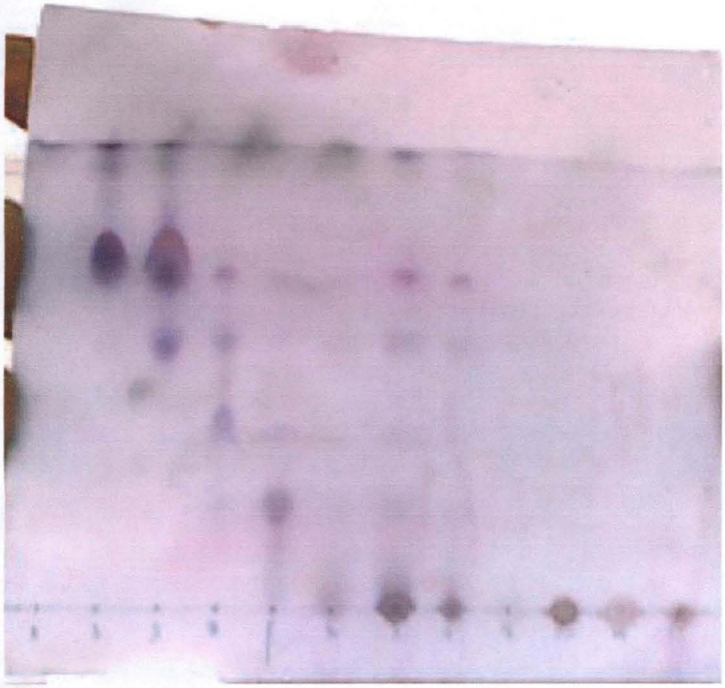
Group D 3300mg/kg body weight = 0.0825g/0.4ml

Group F 3000mg/kg body weight = 0.075/0.4ml

(Average weight of mice is 25g)

Appendix vii

Thin layer chromatograph of Crude Hexane extract



Twelve fractions of hexane extract spotted on a thin layer plate. Based on the spots on the plate, the twelve fractions were pulled together to obtain six fractions (fractions 1, 2&3, 4, 5-8, 9, 10-12).