SCREENING OF SELECTED MEDICINAL PLANTS FOR ACTIVITY AGAINST *Trypanosoma brucei rhodesiense*

PETER OGOTI MOSE

MASTER OF SCIENCE

(BIOCHEMISTRY)

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY

2008

Screening of selected medicinal plants for activity against *Trypanosoma brucei rhodesiense*.

Peter Ogoti Mose

A thesis submitted in partial fulfillment for the Degree of Master of Science in Biochemistry in the Jomo Kenyatta University of Agriculture and Technology.

2008

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

Signed…….. Date……

Peter Ogoti Mose

This thesis has been submitted for examination with our approval as University supervisors.

Signed…….. Date……..

Dr. Esther Magiri.

JKUAT, Kenya.

Signed…….. Date……

Prof. Gabriel Magoma.

JKUAT, Kenya.

Signed…….. Date……..

Prof. Mabel Imbuga.

JKUAT, Kenya.

Signed…….. Date……..

Dr Grace Murilla.

Trypanosomiasis Research Centre (TRC), Kenya.

DEDICATION

This thesis is dedicated to the almighty father for his providence, to my beloved father Mose, mother Esther and my paternal uncle Wilfred for their support and prayers.

ACKNOWLEDGEMENT

I am grateful to the almighty God for giving me life and strength to overcome challenges. I appreciate the cordial assistance rendered by my supervisors, Dr. E. Magiri, Professor .G .Magoma, and Professor M. Imbuga, all from the department of Biochemistry, JKUAT, and Dr. G. Murilla, TRC-KARI for their kind and keen guidance, supervision and persistent support throughout the course of research and writing up of the thesis. I would like to express my sincere gratitude to Dr. Muniru Tsanuo, Chairman, deparment of Chemistry and Mr Mawili Mapusa, Chief Technician for allowing me to use their laboratory facilities. I am grateful to Ms Joanna Auma and Jane Hanya, Biochemistry division, TRC-KARI for providing technical assistance throughout the research program. I am most grateful to JKUAT for funding much of my work.

I would also like to thank Mr. J. Kamau, (Botany department of JKUAT) for collection and identification of plant specimens. I appreciate the cooperation of my fellow postgraduate students; John Mining, Polycarp Lutta and Mirie.

I am greatly indebted to my Fiancée, Edner for the social and psychological support she accorded me throughout the entire period of the research work.

Finally, but not lastly, I am grateful to my brothers and sisters in the Lord who have unceasingly been praying for me as I struggled with the research work.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF CHARTS

LIST OF APPENDICES

ABBREVIATIONS

AdotMetDC: S-adenosylmethionine decarboxylase

AIDS: Acquired Immuno Deffiency Syndrome

ATP: Adenosine triphosphate

BBIQ: Bisbenzylisoquinoline

b.w: Body weight

CNS: Central nervous system

CO_2: Carbon dioxide

DALYs: Disability adjusted life years

dCAdoMet: decarboxylated Adomet

DE 52: Diethyl amino ethyl cellulose

DNA: Deoxyribonucleic acid

DFMO: Difluromethylornithine

DRC: Democratic republic of Congo

HAT: Human African Trypanosomiasis

HCl: Hydrochloric acid

HCT: Hemotocrit

HEPES: (N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid)

HI-FCS: Heat-inactivated fetal calf serum

HL: Human myeloids cell line

HMI9: Thymidine

IC_{50}: The drug concentration which inhibited growth of Trypanosome Populations by 50%

i.p: intraperitoneal

kDNA: Kinetoplast deoxyribonucleic Acid

KETRI: Kenya Trypanosomiasis Research Institute

L: Leishimania

LDL: Low density lipoprotein

LILIT: Low inoculation long term incubation test

Melb: Melarsoprol

MEM: Minimum essential medium

MEMNEAA: Minimum essential medium-Non essential amino acid

MIC: Minimum inhibitory concentration

MRPA: Multidrug resistance protein

Mw: Molecular weight

NaOH: Sodium hydroxide

$NaHCO_3$: Sodium hydrogen carbonate

NIQ: Naphthylisoqunolin

NGO: Non governmental organization

NH_4Cl: Ammonium chloride

ODC: Ornithine decarboxylate

PCV: Packed cell volume

PSG: Phosphoseline glucose

RBC: Red blood cell

RNAi: Ribonucleic acid interference

Sp: Species

T: *Trypanosoma*

T.b.b: *Trypanosoma brucei brucei*

T.b.g: *Trypanosoma brucei gambiense*

T.b.r: *Trypanosoma brucei rhodesiense*

WHO: World Health Organization

ABSTRACT

The *in vitro* and *in vivo* antitrypanosomal activities of five plants species which are *Kigelia africana*, *Artemesia annnua*, *Bidens pilosa*, *Azadirachta indica* and *Senna didymobotyra* traditionally used in Kenya for treatment of parasitic diseases were evaluated. Dichloromethane, Methanol, Hexane, Ethyl acetate and Aqueous extracts of stem bark, fruits, leaves and pods of the five plants were evaluated for *in vitro* activity against *Trypanosoma brucei rhodesiense* KETRI 3798 isolate. Nineteen plant extracts were tested and six were active with MIC< 100µg/ml while four extracts afforded MIC values ranging from 3.91 to 62.6µg/ml respectively. Two of plant extracts, however, had low MIC values of between 3.91 and 11.42µg/ml.

All the extracts tested *in vitro* were also tested for *in vivo* antitrypanosomal activity in mice, experimentally infected with *T.b. rhodesiense* KETRI 3798. The results of *in vivo* studies showed that only one of the extracts, *Kigelia africana* was able to stop trypanosomes from establishing and multiplying in the animals. In the acute toxicity test, administration of 250mg/kg, 500mg/kg and 1000mg/kg of plant extracts, produced neither mortality nor significant reduction in body weight and packed cell volume between controls and the treated animals. Moreover, no gross lesions and histopathological changes were detectable between controls and the treated animals indicating that the extracts were safe and effective for the management of *T.b. rhodesiense*. Bio-guided fractionation, isolation and

characterization studies of compounds from the extracts will yield information on the active compounds and their mechanism of action.

CHAPTER ONE

1.0 INTRODUCTION

1.1 General introduction

1.1.1 Human African Trypanosomosis (HAT)

African trypanosomosis is caused by protozoan parasites, trypanosomes which are transmitted by tsetse flies (of genus *Glossina*). The disease occurs in two forms; chronic form caused by *Trypanosoma brucei gambiense*, which occurs in west and central Africa and an acute form caused by *Trypanosoma brucei rhodesiense*; which occurs in eastern and southern Africa. The chronic infection lasts for years, whilst the acute disease may last for only weeks before death occurs, if treatment is not administered (Kirchhoff, 2000).

Sleeping sickness is a re-emergent disease, but has not been given due attention, probably because its impact is regional. The disease occurs in 36-sub-Saharan countries, within the area of distribution of tsetse fly and over 60 million people living in some 250 foci within regions are at risk of contracting the disease (Scientific working group, 2001).

The number of cases reported annually is over 40,000, but this is highly underestimated, due to difficulties in diagnosis and remoteness of affected areas. It has been estimated that the actual number of cases is at least 300,000, the vast majority of whom are not diagnosed or treated (WHO 1998). These figures are relatively small compared to other tropical diseases. However, Africa trypanosomosis, without intervention, has the propensity to develop into epidemics

making it a major public health problem. Furthermore, fatality rate in untreated patients is 100%. This fact, combined with the focal nature of the disease, means that the disability adjusted life years (DALYs) averted per infection cured or prevented are very high. As a result, control of this disease in areas of risk is highly expensive; falling well below the accepted threshold value for money of US$25 per DALY averted (Shaw and Cattand, 1985).

At the beginning of the last century, huge sleeping sickness epidemics devastated large areas of the continent. During the early 1960s, the prevalence of the disease had been successfully reduced to less than 0.1% in all the endemic countries. This has been achieved through historical campaigns by the former colonial powers. Soon after independence, however, national governments failed to sustain such programmes due to lack or diversion of resources to other more pressing health problems. Breakdown of specialized mobile teams and health facilities in several countries, as a consequence of war and civil strife or change in health policy, resulted in dramatic resurgence of the disease. The distribution corresponds closely with that of major conflicts in sub-Saharan Africa (Scientific working group, 2001).

The social and economic impact of sleeping sickness is often under estimated. During epidemics, large proportions of communities are affected, with loss of life and untold suffering. These are serious social and economical consequences, which far outweigh the cost of maintaining surveillance. The disease has been a major cause of depopulation of large tracts of Africa. The fear it causes has led to

abandonment of fertile lands and is an impediment to development. Some affected countries have agriculture-based economies, and workers in cocoa and coffee plantations are always at risk of contracting the disease, consequently decreasing the labor force. This is reinforced by the fact that the disease mainly strikes the active adult population (Scientific working group, 2001).

Regular medical surveillance, involving accurate case detection and appropriate treatment, and tsetse control where applicable, is the backbone of strategy for the control of sleeping sickness (WHO 1998).

1.1.2 Life cycle of *Trypanosoma brucei* parasites

African trypanosomes undergo life cycles (fig. 1) which alternates between a vertebrate host (blood and other body fluids, and tissues) and the tsetse fly (gut, salivary glands and mouth parts). To survive in these different environments, various metabolic and morphological changes are necessary which involve the mitochondrial system and the surface membrane. During a blood meal, the vector ingests bloodstream trypomastigotes from an infected animal. The parasites multiply in the fly and go through several developmental stages (procyclic, epimastigotes and metacyclic trypanosomes) in a period of three to four weeks. When the blood sucking fly bites another mammal, it injects metacyclic trypanosomes which transform into bloodstream, trypomastigotes in the skin and subsequently disseminate into the bloodstream *via* the lymphatic system. The parasites proliferate in waves, evading the host's immune system by continuously

changing their antigenic coat of variant surface glycoproteins (Kirchhoff, 2000, Vickerman and Cox, 1967, and Barret *et al*., 2003).

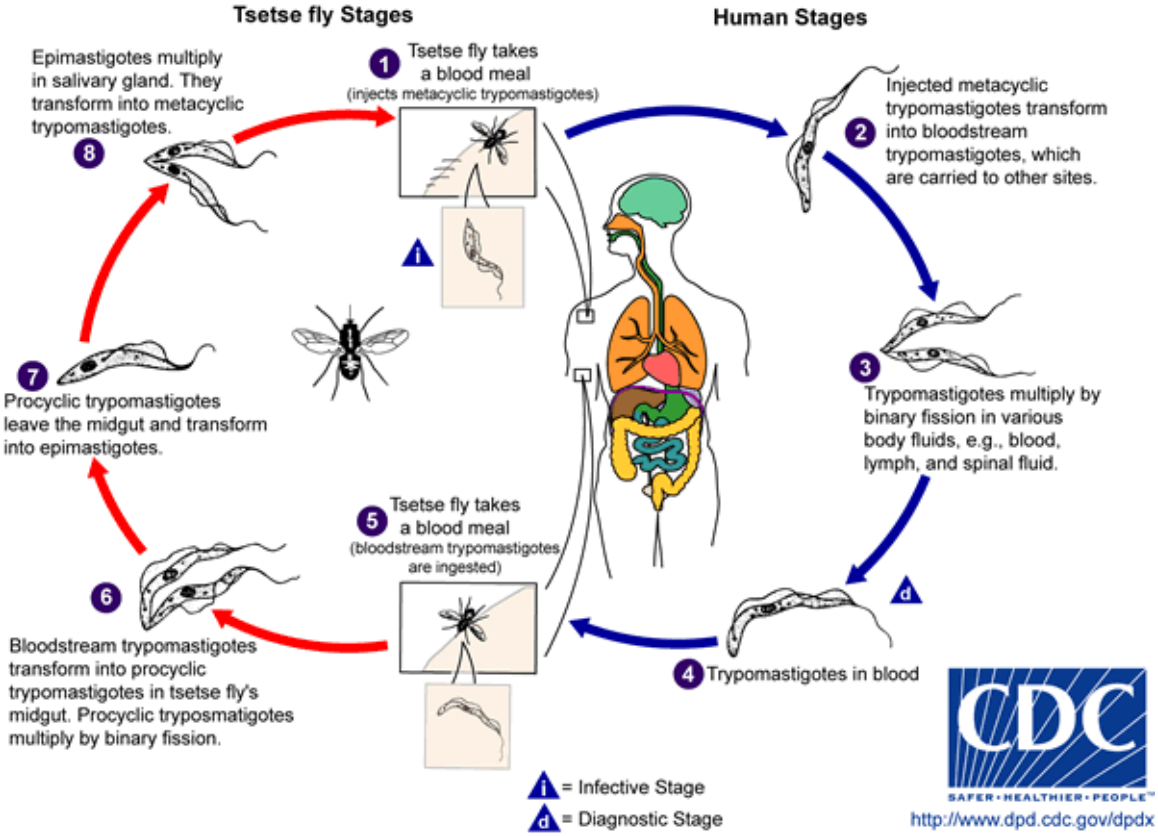


Fig. 1: Life cycle of *T. brucei* parasites. Source: CDC

This first haemolyphatic stage of the disease, which appears one to three weeks after inoculation, is characterized by non specific symptoms such as irregular bouts of fever, headaches, joint pains and itching which are very often misinterpreted as influenza or malaria. After weeks or months depending on the parasite involved, trypanosomes cross the blood –brain-barrier to invade the central nervous system resulting in a chronic meningo-encephalitis eventually leading to encephalopathy. During this second stage of HAT, characteristic symptoms appear; headaches, neurological symptoms, personality and behavior

alterations, poor coordination, changes in sleep cycles (giving the disease its name) and body wasting, leading eventually to a terminal somnolent state and finally to death if untreated. An early diagnosis as well as early treatment are thus important for better management of the second stage of the disease.

T. brucei species (sp) are extra-cellular parasites living in blood and lymph as elongated trypamastigotes. Here, they obtain their energy by glycolysis, whereas in their insect vector they switch to oxidative metabolism using mainly proline. In contrast to intracellular parasites such as *T. cruzi* and *Leishmania* sp., *T. brucei* are vulnerable to humoral defense mechanisms. Complement-activating antibodies may bind to their surface, facilitating recognition, phagocytosis and destruction by the monocytic phagocyte system. *T.brucei* evades eradication by antigenic variation, sequentially producing new clones differing in their surface glycoproteins which are not susceptible to the prevailing antibody population. This strategy of antigen variation effectively neutralizes the host's immune defense mechanism and has confounded all efforts of vaccine development. Further, *T.brucei* organs have been found to activate immunosuppressive microphage function (Hoet *et al.*, 2004).

1.1.3 Current approaches of treating Human African Trypanosomosis (HAT)

Current chemotherapeutics options are very limited and far from ideal. There are only four approved drugs for HAT, three of which were developed more than half a century ago: Suramin, Pentamidine, Melarsoprol and Eflornithine. Other molecules such as Homodium, Isometamidium and Diminazene aceturate are used in animal infections. Only Melarsoprol and Eflorthine, which are able to cross the

blood-brain barrier, can be used for the second stage of HAT, with Eflorthine only effective against *T.b gambiense* infection. The mechanisms of action of these molecules remain poorly understood except for Eflorthine, which inhibits the polyamine biosynthesis pathway (Hoet *et al.*, 2004).

The frequency and extend of the use of standard drugs against Africa trypanosomosis, melarsoprol and pentamidine, has led to development of resistance. Indeed, there has been an increase in late stage cases refractory to melarsoprol treatment in the past decade (Legros, 1999). The market availability of Malarsoprol and Difluoromethylornithin (DFMO) was not assured until in 2001. However, Aventis, in association with Médecins Sans Frontières and the WHO, signed a long-term agreement to manufacture and donate the drug. An international research teams working in the Democratic Republic of the Congo, new Sudan and Angola involving, Immtech international and University of north Carolina at Chapel Hill completed Phase II clinical trial and commenced Phase III trial in 2005 testing the efficacy of the first oral treatment for Sleeping Sickness, known as "DB289" (Williamson *et al.*, 2005 and Staff clinical trial up dates, 2005).

1.1.4 Mechanisms of action of current chemotherapeutics of HAT

Three of the four currently approved drugs for the treatment of African trypanosomosis (sleeping sickness) were developed over 50 years ago. All of the current therapies are unsatisfactory for various reasons, including unacceptable toxicity, poor efficacy, undesirable route of administration, and drug resistance.

The possible modes of action of these drugs are briefly reviewed, as are the possible mechanisms of resistance. The inter-mediate and long-term prospects for the development of safer, effective drugs are discussed. The chemotherapy of HAT has historical roots that date back to the pioneering work by Paul Ehrlich on dyes and arsenicals. Yet research over the past century has yielded only four clinically approved drugs, three of which were introduced more than 50 years ago. Set against the spectacular advances in other areas of chemotherapy over the past half-century, parasitic diseases in general, and trypanosomes in particular, have fared extremely badly. As pharmaceutical companies have merged and evolved integer-larger multinational conglomerates, so has investment declined in drug development for tropical diseases and other less profitable 'orphan diseases?' There are some exceptions to this deplorable situation, but the fact still remains that only a handful of new drugs (, 1% of all new chemical entities) has been launched for the treatment of all tropical parasitic diseases over the past 25 years. With the exception of antimalarials, these drugs arose as result of research and development for veterinary or anticancer indications, and only one of these (eflornithine).

1.1.4.1 Suramin

Suramin (Germanine; manufactured by Bayer: http://www.bayer.com/) was introduced in the early 1920s and to this day remains the drug of choice for treatment of the early phase of *T. b. rhodesiense* infections. Suramin is a colourless, polyanionic sulfonated naphthylamine that is chemically related to Paul Ehrlich's trypan red and toother dyes with *in vivo* trypanocidal activity. Suramin

is highly soluble in water and has to be given intravenously by injection. Immediate life threatening side-effects include collapse, with nausea, vomiting and shock. Severe delayed reactions include kidney damage (particularly in malnourished patients), exfoliative dermatitis, agranulo-cytosis, haemolytic anaemia, jaundice and severe diarrhea, all of which can be fatal. Owing to its highly ionic nature, the drug does not penetrate well into the Central nervous system (CNS) (1% of serum levels) and is therefore only effective in the primary stage of the disease. Most of the drug is tightly bound in serum to albumin and low-density lipoprotein (LDL), accounting for its long terminal half-life (90 days) and prophylactic properties. No significant clinical resistance to suramin has emerged despite 80years of use. Suramin has also been used in the treatment of onchocerciasis (Awadzi, 1995) and as an experimental antitumour agent (Walther, 1996 and Kaur, 2002). The mode of action of suramin in killing trypanosomesis is still a complete mystery. The drug is taken up by endocytosis within the flagella pocket into the endocytic compartment of the cell. Simple fluid-phase endocytosis is too slow to account for uptake (Fairlamb, 1980) and uptake is thought to involve receptor-mediated endocytosis of suramin bound to LDL (Vansterkburg, 1983). LDL has been proposed as the only carrier for suramin uptake (Vansterkburg, 1983) but this has recently been disputed (Pal, 2002). Whether suramin remains trapped within the endo-cytic compartment of the cell or escapes into the cytosol has never been clearly established. The trypanocidal action of suramin is slow. Diminished growth rate *in vivo* is associated with a decrease in respiration before parasite clearance from the blood (Fairlamb, 1980). Because rates of respiration

and aerobic glycolysis are tightly coupled in bloodstream forms, it has been suggested that the action of suramin involves inhibition of variours glycolytic enzymes (Fairlamb, 1980 and Opperdoes, 1989). However, the rate of Adenosine triphosphate (ATP) turn over is tightly coupled to the glycolytic rate and therefore these effects could simply be secondary to a generalized decrease in macromolecular synthesis owing to cessation of cell growth. Another clue to the action of suramin is its pronounced synergism with DFMO (eflornithine) (Clarkson, 1984), implicating polyamine metabolism. Unfortunately, inhibition of poly-amine biosynthesis by DFMO itself produces pleiotropic downstream effects, so the reason for synergism between these two drugs might be difficult to establish. A survey of the literature indicates that suramin is a promiscuous inhibitor of many enzymes and receptors, and therefore could inhibit multiple targets (Fries, 2003). Certainly, this would explain the fact that no significant resistance has ever been observed in clinical isolates and that high-level resistance has not been obtained by laboratory selectionuous.

1.1.4.2 Pentamidine

Pentamidine (Lomidinee; manufactured by Aventis: http://www.aventis.com/), an aromatic diamidine, was first introduced in the 1940s and remains the drug of the choice for early *T. b. gambiense* infections. Treatment of *T. b. rhodesiense* infections is reported to be less reliable and thus pentamidine is only used as the second-line drug when therapy with suramin is contraindicated (Pepin, 1994). Pentamidine can also be used in the treatment of antimony resistant leishmaniasis and for *Pneumocystis carinii* infections in patients who cannot tolerate

trimethoprim-sulphamethoxazole. Pentamidine is highly protonated at physiological pH and thus has poor oral bioavailability, requiring parenteral administration by injection. Efficacy is restricted to early-phase disease because the compound does not readily penetrate the CNS. Severe hypotensive reactions can occur following intravenous administration, so the intramuscular route is preferred. Pentamidine can also cause damage to the liver, kidneys and the pancreas. Damage to the pancreas can lead to diabetes. Mass chemoprophylaxis campaigns were carried out in the 1950s and 1960s in endemic areas of West and Equatorial Africa in an attempt to eradicate Gambian sleeping sickness (Waddy, 1970). However, although the incidence of the disease was significantly reduced, mass chemoprophylaxis was finally abandoned as impractical.

The mechanism of action of pentamidine is not well understood. The drug is taken up by at least three transporters (Carter, 1995 and Dekonning, 2001) and accumulates to mill molar concentrations in cells. Pentamidine binds to negative charged cellular components, such as phospholipids and nucleic acids, and disrupts the structure of kinetoplast Deoxyribonucleic acid (kDNA), possibly by inhibiting topoisomerase II (Shipiro and Lynd, 1990). Recent observations suggesting that silencing expression of mitochondrial topoisomerase II by Ribonucleic acid interference (RNAi) causes shrinking and loss of the kDNA network would support this hypothesis (Wang, 2000. However, dyskinetoplasty alone does not confer drug resistance, and inhibition of kDNA function could not account for the broad antiprotozoal and antimicrobial spectrum of the drug (Fries, 2003).

Pentamidine also binds to RNA and has been reported to inhibit group I intron catalytic activity in *Candida albicans* (Zhang, 2002) and *P. carinii* (Liu, 1994. Possible effects of pentamidine on trans-splicing and RNA editing in trypanosomes deserve investigation. Another possible mode of action involves perturbation of polyamine biosynthesis because pentamidine inhibits the enzyme S-adenosylmethionine decarboxylase (AdoMetDC) in vitro. AdoMetDC catalyses formation of decarboxylated AdoMet (dcAdoMet) required for the conversion of putrescine into spermidine. However, no changes in putrescine or spermidine levels were observed in *T. b. brucei* exposed to pentamidine *In vivo* (Berger, 1993). Moreover, null mutants and overexpression of AdoMetDC in Leishmania donovani showed no alterations in sensitivity to the drug (Roberts, 2002). Thus, inhibition of AdoMetDC cannot be involved in the mode of action in the intact cell.

Other proposed targets for pentamidine include inhibition of a plasma-membrane Ca_2þATPase in *T. brucei* (Benaim, 1993) and disruption of mitochondrial membrane potential in L. donovani (Vercesi and Decampo, 1992). Despite the massive use of pentamidine prophylaxis in the 1950s and 1960s for control of Gambian sleeping sickness, widespread resistance has not emerged in the field Pepin, 1994). The reason for clinical failures with *T. b. rhodesiense* infections has never been established.

Laboratory strains of *T. brucei* selected for resistance to melaminophenyl arsenicals often show cross-resistance to diamidines, and vice versa (Borst and Fairlamb, 1998, Bray, 2003). In part, this is because of the activity of the P2

purine transporter (Carter and Fairlamb, 1993) , which not only transports melamino-phenyl arsenicals such as melarsoprol, but also contributes to transport of diamidines, including pentamidine (Carter, 1995). However, the fact that pentamidine has multiple modes of uptake could account for the failure to observe pronounced resistance in field isolates (Dekonning, 2001 and Jarvis, 2001). One laboratory strain selected for resistance to pentamidine showed no alterations in transport activity, indicating that other mechanisms of resistance can be involved (Berger, 1995). An understanding of non-transport-resistance mechanisms might shed light on the mode of action of this drug.

1.1.4.3 Eflornithine

Eflornithine (Ornidyle; manufactured by Aventis) is the drug of choice for treatment of late-stage HAT caused by *T. b. gambiense*. The drug is not recommended for *T. b. rhodesiense* infections because many cases respond poorly to eflornithine. Eflornithine is a mechanism based irreversible inhibitor of Ornithine decarboxylase (ODC), the first committed step in the polyamine biosynthetic pathway. The reason for its efficacy is not fully understood because it is an equally effective suicide inhibitor of mammalian ODC. Although the pronounced differences in turnover rates between host and parasite ODC have been invoked to explain selectivity (Heby, 2003), this might not be the only factor. Of crucial importance is the fact that mammalian cells are able to induce high-affinity polyamine transporters in response to polyamine starvation, whereas *T. brucei* appears to have a limited capacity to scavenge the trace amounts of polyamines present in serum (Fairlamb and Le Quesne, 1997). Inhibition of

polyamine biosynthesis triggers a wide range of downstream biochemical effects, but opinion is divided as to which of these is responsible for the trypanocidal effect (Heby, 2003 and Fairlamb and Le Quesne, 1997). The immediate consequences of inhibition of ODC are: (i) a loss of putrescine and a decrease in spermidine levels; (ii) a decrease in trypanothione levels, and (iii) a profound disturbance of AdoMet metabolism. Downstream of these metabolic changes are: (i) a generalized decrease in DNA, RNA and protein synthesis, including synthesis of variant surface glycoprotein; and (ii) morphological and biochemical changes resembling differentiation into non dividing short stumpy forms. *In vitro* eflornithine initially causes cytostasis, with cell death following after a few days. *In vivo*, rapid clearance of parasites from the bloodstream requires an intact immune humoral response, presumably because non dividing trypanosomes are unable to undergo antigenic variation.

1.1.4.4 Melarsoprol

Melarsoprol (Mel B, Arsobale; manufactured by Aventis) was introduced in 1949 for the treatment of late-stage HAT caused by either *T. b. gambiense* or *T. b. rhodesiense*. Melarsoprol is practically insoluble in water and must be given intravenously dissolved in propylene glycol, a solvent that is highly irritant to tissues. Melarsoprol causes a serious reactive encephalopathy in 5–10% of cases, half of which are fatal (Pepin, 1994). Other common side effects include vomiting, abdominal colic, peripheral neuropathy, arthralgia and thrombophlebitis (as a result of leakage of propylene glycol into tissues at the injection site). Acute haemolysis can occur in patients with glucose-6 phosphate dehydrogenase

deficiency. The currently used therapeutic regimen was developed on a trial and error basis and recent pharmacokinetic studies suggest that the current dose regimens are not optimal (Burri, 1993). However, the interim results of a clinical trial comparing a new 10-day schedule with the standard 26 day regimen have been disappointing because the incidence of reactive encephalopathy was the same in both treatments (Burri, 2000). The mechanism of this devastating side effect is unknown, but is probably not specific to HAT because patients receiving melarsoprol in clinical trials for the treatment of advanced leukaemia suffered a similar incidence of CNS toxicity (Soignet, 1999). If reactive encephalopathy is an intrinsic property of the drug and unrelated to the disease, then the sooner a safer alternative to melarsoprol is developed, the better.

Melarsoprol is a pro-drug in which the reactivity of the trivalent arsenic moiety is masked with 2, 3-dimercapto-propanol (British anti Lewisite, originally developed as an antidote to arsenical-based nerve gases). In patients, melarsoprol is rapidly converted into melarsen oxide, which binds rapidly and reversibly to serum proteins (Keiser, 2000). Review enzymes or substrates that contain vicinal (neighbouring) thiol groups and thus part of the selective toxic action of the drug could be a result of selective uptake into the parasite by the trypanosomal P2-purine transporter (Carter and Fairlamb, 1993).

Once within the cell, arsenical drugs could conceivably inhibit several important metabolic and transport functions. The extremely rapid loss of motility and cell lysis of bloodstream *T. brucei* was for a long time attributed to inhibition of

pyruvate kinase, disrupting glycolysis and energy metabolism. However, this is now thought to be an immediate consequence of loss of cell integrity, rather than a direct cause of cell lysis.

Melarsen oxide reacts with trypanothione to form a reversible, but stable, adduct a competitive inhibitor of trypanothione reductase (Fairlamb, 1989), an enzyme essential for maintaining the correct intracellular thiol-redox balance for other cellular biochemical and biological processes (Fairlamb and Cerami, 1992). Intriguingly, over expression of a multidrug resistance protein (MRPA, a putative thiol conjugate transporter) resulted in a ten old greater resistance to melarsoprol (Shashi, 2002), whereas manipulation of trypanothione reductase levels in *T. brucei* had no effect on sensitivity to lysis by arsenicals (Krieger, 2000). Although further work is required to confirm these observations, it could be that a combination of trypanothione depletion and inhibition of trypanothionere ductase is sufficient to kill the cells.

1.1.5 Natural products in drug development

Currently, in industrialized nations, some 50% of all prescribed drugs are derived or synthesized from natural products, the only available sources of which are animals, marine, plants and micro-organism. It is considered that because of the structural and biological diversity of their constituents, terrestrial plants offer unique and renewable resources for the discovering of the potential new drugs and biological entities. However, only 5-15% of the worlds' approximately 250,000 flowering plants have been analyzed for their possible medicinal uses (Black,

1996). The most alarming cause for concern is that, by the turn of this century, it is expected that some 25,000 species of plants will have ceased to exist. This represents about five percent plant species a day between 1998 and year 2000 (Mahindiol, 1998).

Plants have, since time immemorial, been among the common sources of medicaments. Most of the plant derived medicines have been developed on the basis of traditional knowledge in health care moreover, in many cases; there is a correlation between the indications of pure substances and those of their respective crude extracts used in traditional medicine (Farnsworth *et al.*, 1985).

Literature surveys and field studies have shown that plants are used in traditional medicine in Africa to treat trypanosomes in humans and animals (Bizima *et al.*, 1994, Freiburghaus *et al.*, 1996, Youan *et al.*, 1997). So far, only few of these plants have been evaluated for their trypanocidal activity (Asuzu and Chineme *et al.*, 1990, Freiburghaus *et al.*, 1996, 1997, Youan *et al.*, 1997, Adewunmi *et al.*, 2001). Global scenario is now changing towards the use of non toxic plant products having medicinal properties, in development of modern drugs. In this study, *Azadirachta indica*, *Artemesia annua*, *Biden pilosa*, *Kigelia pinnata* and *Senna didymobotrya* were evaluated against *T.b. rhodesiense* KETRI 3798 to determine their potential in treating HAT.

1.2 Statement of the problem

Current chemotherapeutics options are very limited and far from ideal. There are only four approved drugs for HAT, three of which were developed more than a

half a century ago: Suramin, Pentamidine, Melarsoprol and Eflornithine. Other molecules such as Homodium, Isometamidium and Diminazene aceturate are used in animal infections. Only Melarsoprol and Eflorthine, which are able to cross the blood-brain barrier, can be used for the second stage of HAT, with Eflornithine only effective against *T.b. gambiense* infection. However, Melarsoprol and Pentamidine have led to development of resistance due to their frequency and extended usage (Legros, 1999). In addition, the available drugs are expensive, limited, and inaccessible and require hospitalization for administration by parenteral route there is also increasing reports of treatment failure, especially with Melarsoprol (Hoet *et al.*, 2004). The availability of these drugs has not always been guaranteed as drug companies periodically abandon production because of lack of profitability. Therefore, there is urgent need to develop novel, safe, rapidly-acting and inexpensive agents for the treatment of HAT.

1.3 Justification

Nature with its numerous plants, microorganisms and marine organisms is a potential source of new drugs since it contains a countless quantity of molecules with great variety of molecular structures and pharmacological activities (Newman *et al.*, 2003). Through the ages, mineral, plant and animal products have been the main source of medicines for man. It is estimated that two-thirds of world population rely on traditional medical remedies due to the limited availability and affordability of pharmaceutical products (Togboto and Townson, 2001). For instance, *Trachilia emetica* and *Annon senegalensis* extracts from root bark were found to be highly active against *Trypanosoma brucei rhodesiense* and

Trypanosoma brucei brucei respectively (Nakatani and Nakanishi 1993, Igweh *et al*., 1989). Plants were mainly selected on basis of their traditional reputation for efficacy in treatment of trypanosomes and malaria. In this study, 19 extracts from 5 plant species collected in Kenya, which were used in traditional medicine to treat protozoan parasites in both human and animals, were screened for their i*n vitro* and *in vivo* trypanocidal activity, including toxicity levels in mice. The aim of this study was to verify whether the claimed trypanocidal properties of these plants in traditional medicine can be scientifically confirmed.

1.4 Objectives

Broad objective

Screening selected Kenyan medicinal plant extracts for their potential in the treatment of sleeping sickness.

Specific objective

1. Evaluate five selected Kenyan medicinal plant extracts for *in vitro* trypanocidal activity.
2. Determine toxicity levels of plant extracts in mice.
3. Determine efficacy of plant extracts in the experimental mice trypanosomosis.

1.5 Hypothesis

Extracts from the five selected Kenyan medicinal plants have trypanocidal activity.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Plants with antitrypanocidal activity

In countries where sleeping sickness occurs, plants have traditionally been used for centuries and are still widely used to treat this illness and other parasitic diseases. Furthermore, several well-known drugs, such as quinine and artemisinin used as antiprotozoan agents, have their origin in nature (Kirby 1996, Camacho *et al*., 2000, Tagboto and Townson, 2001). *Trichilia emetica* is known as a medicinal plant that is used in various ways in West Africa countries. According to Nakatani and Nakanishi (1993), *Trichilia emetica* is a rich source of limonoids (Trichins) and secolimonoids with antifeedant activity. The study which was done on *Trichilia emetica* extract from root bark was highly active against *Trypanosoma brucei rhodesiense*. It can be speculated that remarkable selectivity for trypanosomes is due to the presence of limonoids, which are contained in the plant.

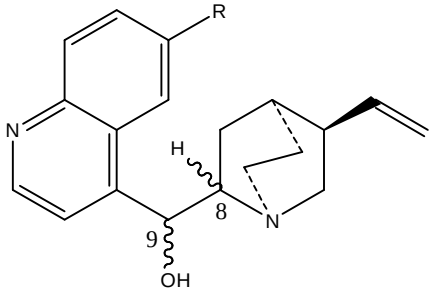
In Kenya and in other third world countries, the use of medicinal plants is widespread and there is real hope of finding an alternative to synthetic drugs in traditional medicines. Exploitation of traditional Africa medicines should be based on a better knowledge of chemical and biological properties of the plant used. The rural populations affected by trypanosomosis are poor and treatment with current drugs is expensive, long lasting and complicated. It is therefore important to exploit the available plants for development of new efficient medicines that are more appropriate and affordable than the few existing drugs.

2.2 Plant compounds with antitrypanosomal activity

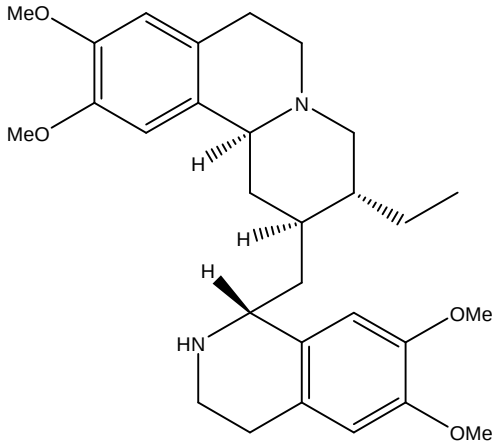
Phytochemical studies of medicinal plants by Hoet *et al.*, 2004, confirmed several compounds with their structures, indicated in bold (numbered from 1 to 46) isolated from a natural source are shown to have antitrypanocidal activity. They are classified as follows:

2.2.1 Alkaloids

Several alkaloids have been tested on trypanosomes *in vitro.* A number of isoquinoline and quinoline alkaloids exhibited high activity. *T. congolense* was always less susceptible to the compounds than *T.b brucei*. The quinoline alkaloids from Cinchona bark (Rubiaceae) (quinidine 1, cinchonine 2, quinine 3, cinchonidine 4) had a significant trypanocidal activity, against *T.b. brucei* (Merschjohann *et al*., 2001).



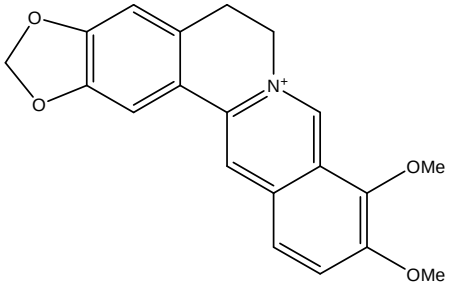
1 R=OMe *8R, 9S*
2 R=H *8R, 9S*
3 R=OMe *8S, 9R*
4 R=H *8S, 9R*



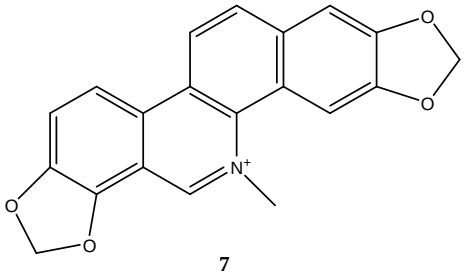
5

Emetine 5, an isoquinoline alkaloid from *Cephaelis ipecacuanha* (Rubiaceae) which has been used in the treatment of amoebiasis, was very trypanocidal

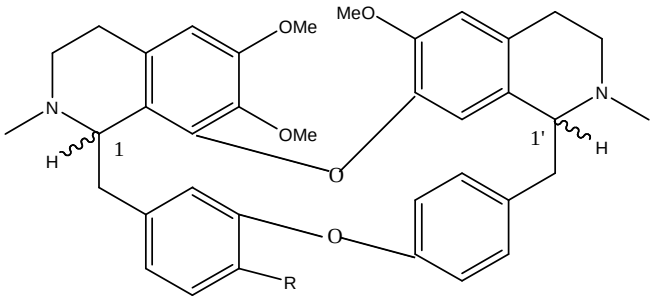
(IC_{50}=0.039µM and 0.43µM for *T.b. brucei* and *T. congolense*) but without any selectivity. Berberine 6 and sanguinarine 7, two quaternary benzylisoquinoline alkaloids found in a number of plant families, and berbamine 8, a bisbenzylisoquinoline (BBIQ), were trypananocidal with little or no selectivity against *T.b. brucei*. DNA intercalation in combination with in inhibition of protein synthesis could be responsible for observed trypanocidal and cytotoxic effects of these alkaloids (Merschjohann *et al*., 2001). Out of the 12 BBIQ alkaloids tested by Camacho *et al*., 2002, on *T.b. brucei* bloodstream trypomastimastigotes, eight had an IC_{50} between 1 and 2µM, but none were as active as pentamidine. Thalisopidine 9 displays the strongest trypanocidal activity. Berbamine 8 was active on *T.b. brucei* in the same concentration range as reported by Hoet *et al*., 2004. Phaeanthine 10, isolated from the leaves of *Triclisia patens* (Menispermaceae) was active against *T.b. brucei in vitro* but in another study it was shown to be inactive in mice infected with the same parasite (Camacho *et* al., 2002 and Dreyfuss *et* al., 1987).



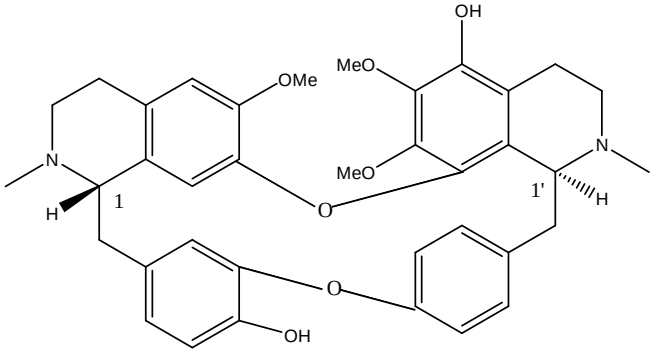
6



7

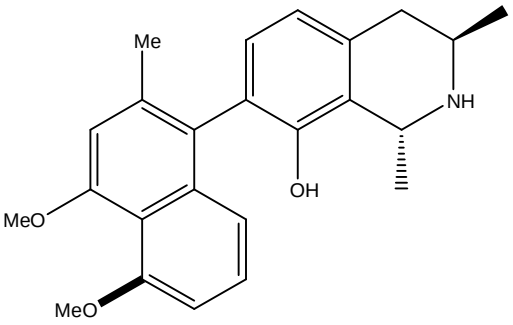


8 R=OH *1R, 1'S*
10 R=OMe *1R, 1'R*

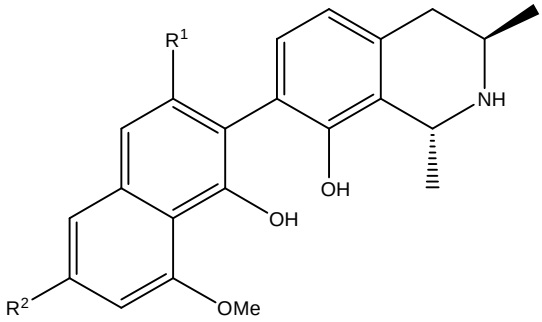


9

Naphthylisoquinoline (NIQ) alkaloids are axially chiral natural biaryls isolated from African plants belonging to the small Ancistrocladaceae and Dioncophyllaceae families which have already shown promising antiprotozoal properties, in particular antiplasmodial activity. Among the monomeric NIQ alkaloids tested up to now, dioncophyllines A, 11, B, 12 and E, 13 were the most active against *T.b. brucei* or *T.b. rhodesiense* bloodstream forms (Bringmann *et al*., 2002 and Bringmann *et al*., 2003).



11

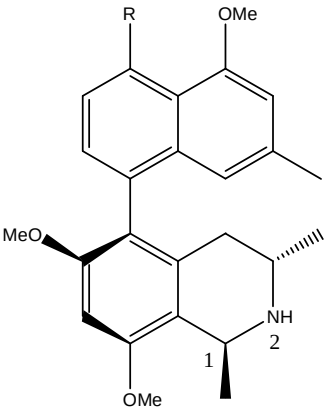


12 R^1=H, R^2=Me
13 R^1=Me, R^2=H

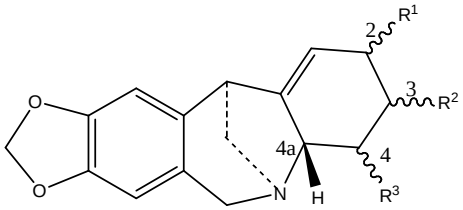
Ancistroealaines A, 14 and B, 15 isolated from the stem bark of *Ancistrocladus ealaensis*, displayed a lower activity on *T.b rhodesiense* but exhibited cytotoxic effects on mammalian L6 cells (rat skeletal myoblasts) at much higher concentrations (Bringmann *et al*., 2000).

The plants of the Amaryllidaceae family are often toxic and contain a special class of isoquinoline alkaloids, especially in bulbs (Nair *et al*., 2000, Herrera *et al*., 2001, Herrera *et al*., 2001, and Labrana *et al*., 2002). Pancracine 16, an alkaloid, with a 5, 11-methanomorphanthridine ring, obtained from the fresh bulb of *Narcissus angustifolius* sub-species *transcarpathicus* was active on the mammalian stage of *T.b. rhodesiense* and was not cytotoxic for L6 cells (Labrana *et al*., 2002). Nangustine 17, a regioisomer of pancracine, was also devoid of cytotoxicity but was almost 14 times less active against the trypanosomes. The antitrypanosomal activity of some Amaryllidaceae alkaloids of the crinane type were evaluated against *T.b rhodesiense* bloobstream forms: haemanthidine 18 was active, oxotidine 19 was weakly active, and maritidine 20 was inactive (Herrera *et*

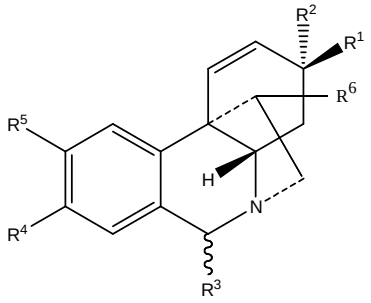
al., 2001, and Herrera *et al*., 2001). Galanthine, an alkaloid of the lycorane type had the same activity on trypanosomes as oxotidine (Herrera *et al*., 2001).



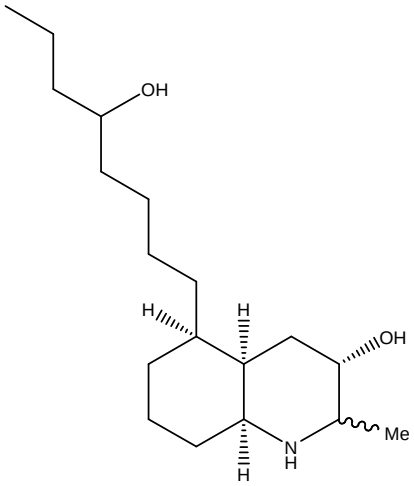
14 R=OMe 1,2 dehydro
15 R=OH



16 R^1, R^2=OH, R^3=H *2S, 3S,* 4a*S*
17 R^1=H, R^2, R^3=OH 3*S*, 4*S*, 4a*R*



18 R^1=OMe, R^2=H, R^3, R^6=OH, R^4-R^5=O-CH_2-O
19 R^1-R^2=O, R^3, R^6=H. R^4, R^5=OMe
20 R^1=OH, R^2, R^3, R^6=H, R^4, R^5=OMe

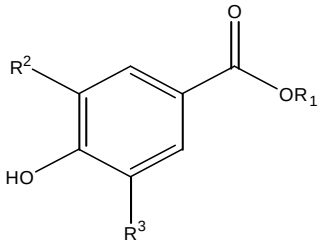


21

2.2.2 Phenolic derivatives

Simple phenolic compounds which are widely distributed in plants have been tested for their antitrypanosomal activity as well. Gallic acid 22, a well-known component of hydrolysable tannins, is equally active on the bloodstream and

procyclic forms of *T.b. brucei*. Gallic acid esters such as ethyl gallate 23 and *n*-propyl gallate 24 were more toxic on bloodstream forms than 22 but their activity on procyclic forms was weaker (32 and 29 µM, respectively). Syringic acid 25 and protocatechuic acid 26, which lack the pyrogallol moiety, were not toxic for bloodstream and procylic forms (Koide *et al*., 1998).

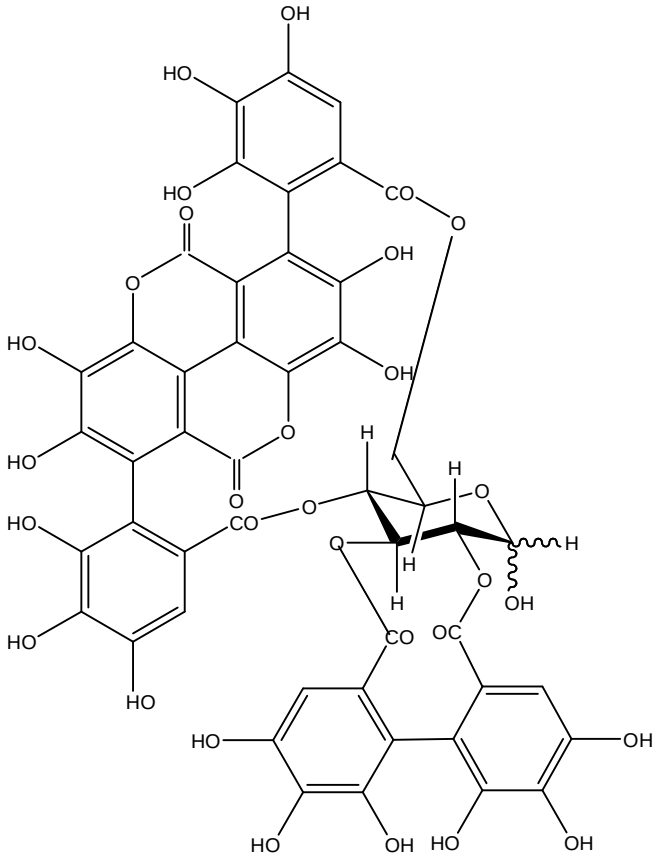


22 R_1=H, R^2,R^3=OH
23 R1=ethyl, R^2, R^3=OH
24 R_1=n-popyl, R^2,R^3=OH
25 R_1=H, R^2, R^3=OMe
26 R_1, R^2=H, R^3=OH

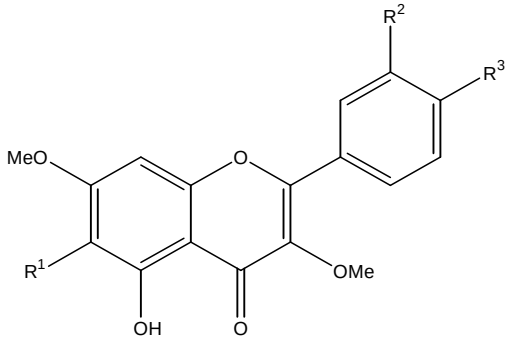
The same researchers suggested that the formation of reactive oxygen species (such as the superoxide anion) might be involved in the gallic-acid induced trypanocidal activity. In other words, it would act as a pro-oxidant (Koide *et al*., 1998). Bio-guided fractionation of a stem bark extract of *Combretum molle* (Combrataceae) led to the isolation of two classes of compounds: two inactive saponins and two hydrolysable tannins, punicalagin 27 and a structurally similar compound for which the full structure has not yet been elucidated. Those two compounds exhibited activity on mammalian stage of *T.b. rhodesiense*. Four methoxylated flavones were isolated by Bio-guided fractionation from leaves of *Ehretia amoena* (Boraginaceae), a plant traditionally used in Uganda to treat

sleeping sickness: chrysosplenetin 28, chrysospenol D 29, resutin 30 and artemetin 31 (Raz, 1998).

Compounds 28 and 29 were the most potent against *T.b. rhodesiense* bloodstream trypomastigotes while 30 was less active (IC_{50}=189 µM). Although 31 was inactive (IC_{50}=189 µM), the author showed that it increases synergistically *in vitro* the trypanocidal activity of the other three flavones.



27



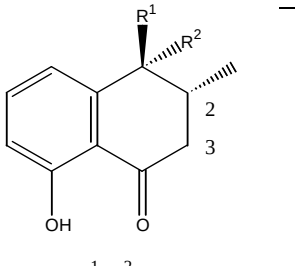
28 R^1, R^2=OMe, R^3=OH
29 R^1=OMe, R^2, R^3=OH
30 R^1=H, R^2, R^3=OMe
31 R^1, R^2, R^3=OMe

2.2.3 Quinones

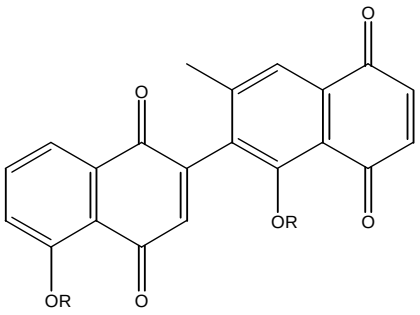
It has been shown that quinines, especially 1, 4-naphthoquinones such as plumbagin 32, can induce oxidative stress in trypanosomes (*T. congolense*, Cenas *et al*., 1994 and *T. cruzi* Salmon-Chemin *et al*., 2001). This may be explained by their reduction to semi-quinone radicals by enzymes such as those present in the mitochondrial electron transport chain and the trypanothione reductase, a key enzyme of the trypanosomal antioxidant thiol metabolism. Compound 32, which can be found in *Drosera* species (Droseraceae), was active on mammalian stage of *T.b. brucei* (Bringmann *et al*., 2003 and Salmon-Chemin *et al*., 2001). While it's reduced derivative, *tran*-isoshinanolone 33 is inactive (Bringmann *et al*., 2003).

Diospyrin 34, a bis-naphthoquinone isolated from the bark of *Diospyros Montana* (Ebenaceae), as well as semi-synthetic derivatives, have been investigated for their antitrypanosomal activity *in vitro* against *T.b. brucei* bloodstream forms. The IC_{50} of 34 was 50 µM while diospyrin dimethylether 35 and its hydroquinone form 36 were respectively 24 and 71 times more active than the parent compound (Yardley

et al., 1996). According to the author, the enhanced activity of 36 as compared to the parent compound 34 could be due to the presence of the 4 hydroxyl groups in 36 which generate semi-quinone radicals more easily through one-electron shift thus leading to an increase of the oxidative stress in trypanosomes. On the contrary, in 34, the hydrogen bond between the carbonyl and phenolic groups of one naphthalene ring reduces the electron availability explaining the lower activity of 34 (Yardley *et al*., 1996).

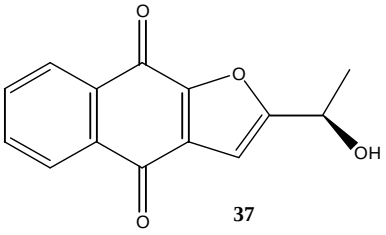


32 R^1, R^2=O 2, 3 dehydro
33 R^1=OH, R^2=H

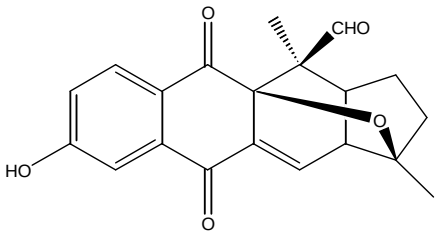


34 R=H
35 R=Me
36 R=Me hydoquinone form

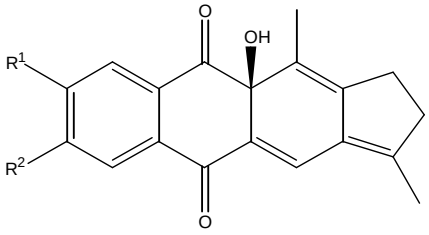
Activity-guided fractionation of the stem bark and root bark extracts of *Kigelia africana* (Bignoniaceae) allowed the isolation of one furanonaphthoquinone, 2-(1-hydroxyether)-naphtho [2, 3-*b*] furan-4, 9-quinone 37 and three naphthoquinoids: isopinnate 38, kigelinol 39, and isokigelinol 40. Compounds 37 and 38 possessed a pronounced activity against both *T.b. brucei* and *T.b. rhodesiense* bloodstream forms with a certain selectivity compared to KB cells. Compounds 39 and 40 had a less potent antitrypanosomal activity depending on the trypanosome tested (Moideneen *et al*., 1999).



37



38



39 R^1=OH $R^{2=}$H
40 R^1=H R^2=OH

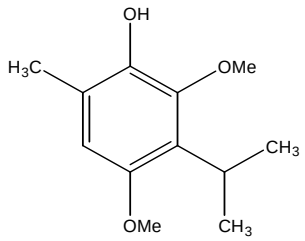
2.2.4 Terpenoids

2.2.4.1 Iridoids

The secoiridoid amarogentin (41) isolated from the upper parts of *Swertia chirata* (Loganiaceae) is a promising compound with leishmanicidal activity. This compound inhibited DNA-Topoisomerase I activity from *Leishmania donovani at* 30 µM (Ray *et al*., 1996). This mechanism of action is quite interesting as most trypanocidal drugs target type II topoisomerases. There are a few compounds that specifically alter biological functions of toposisomerase I with enzyme or DNA-enzyme complex, an exception being camptothecin an antineoplastic drug (Bodley *et al*., 1995). Other natural compounds with the iridoid parent structure also show significant activity against *Leishmania* parasites. A series of iridoids isolated from *Nyctanthes arbortristis* (Oleaceae), arbortristosides A, C (42, 43), have antileishmania activity.

2.2.4.2 Monoterpenes

Monoterpenes are examples of simple antiprotozoal drugs. Espintanol 44 isolated from the bark of *Oxandra espinata* (Annonaceae) (Konig *et al*., 1991), is active against Leishmania.



44

Although, positive antitrypanocidal activity has been shown by various plant extracts and natural products mentioned above, there was need to focus and investigate the effectiveness of A*zidirachta indica*, *Artemesia annua*, *Kigelia pinnata* , *Bidens pilosa* and *Senna didymobotrya* extracts for the treatment of sleeping sickness.

2.3 Medicinal properties and uses of the five selected plant species

2.3.1 *Azidirachta indica* (Neem tree)

For thousands of years, the beneficial properties of Neem (*Azadirachta indica*) have been recognized in the Indian tradition. Each part of the Neem tree has some medicinal property. Recently, Biswas *et al*., 2002, have reviewed the biological activities of some of the Neem compounds, pharmacological actions of the Neem extracts, clinical study and plausible medicinal applications of the Neem along with their safety evaluation.

More than 135 compounds have been isolated from different parts of Neem and several reviews have also been published on the chemistry and structural diversity of these compounds. The compounds have been divided into two major classes; Isoprenoids (like diterpenoids and triterpenoids containing protomeliacin, limonoids, azadirone, and its derivatives, gedunin and its derivatives vilasin type of compounds and C-secomeliacin such as nimbin, salanin and azadirachtin). The second class is non-isoprenoids, which are proteins (amino acids) and carbohydrates (polysaccharides) sulphurous compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalone, coumarin and tannins aliphatic compounds, among others.

Biological activities of Neem compounds

Neem compounds have been shown to have various biological activities. These include:

Aqueous extract of the Neem leaves significantly decrease blood sugar levels and prevents adrenaline as well as glucose-induced hyperglycaemia. Recently, hypoglycemic effect was observed with leaf extract and seed oil, in normal as well as alloxan-induced diabetic rabbits. Neem seed and leaf extracts are effective against both chloroquin-resistant and sensitive strain malaria parasites (Biswas *et al*., 2002).

Extract of Neem leaf, Neem oil seed kernels are effective against certain fungi including; Trichophyton, Epidermophyton, Microsporon, Trichpsporon, geotricun, and Candida Oil from the leaves, seed and bark possesses a wide spectrum of anti-

bacteria action against Gram-negative and Gram-positive micro-organism, including; *M. tuberculosis* and streptomycin resistant strains. *In vitro*, it inhibits *vibrio cholerae*, *klebsiella pneumonia*, *M. tuberculosis* and *M. pyogenes*. Anti-microbial effects of Neem extracts have been demonstrated against *Streptococcus mutans* and *S. faecaus*. Aqueous leaf extract offers anti-viral activity against vaccinia virus, chikungemya and measles virus (Biswas *et al*., 2002).

2.3.2 *Artemesia annua*

Artemesia annua (also known as sweet wormwood) is a highly aromatic annual herb traditionally grown in China as a medicinal plant and more recently in Europe and India for its aromatic leaves, which are used as a source of aroma chemicals for the fragrance industry. Recent research in the Peoples Republic of China has brought Artemesia into the limelight because of the discovery that it yields a compound artemesinin, which shows promise as a potent anti-malaria agent with little or no side effects. Many countries have therefore approved use of artemesinin to treat malaria, in particular one of its most severe forms, cerebral malaria. The use of this drug was recently endorsed for use by the Ethiopian ministry of Health during a recent outbreak of malaria epidemic in the country (Annamed, 2004).

Artemesia is relatively easy to grow and very high biomass yields can be obtained in a period of 5-6 months period. As this temperate plant grows quiet well in wet and cool areas, it has been successfully introduced by German NGO **A**n**n**a**m**ed (Action for Natural Medicine) on the Highlands of Chencha in the Gamo Gofa Region of Ethiopia. **A**n**n**a**m**ed has also shown that taking the herb in the form of

tea cures patient of malaria, with a cure rate of over than 90%. One liter of boiling water is poured onto 5g dried leaves and allowed to brew for 10-15 minutes, and then poured through a sieve. This tea is then drunk by the malaria patient in four portions in the course of the day. The period of treatment is between 5 and 7 days. This experiment has also successfully been done in Uganda, the DRC and Tanzania (Annamed, 2004).

Before embarking on cultivation of this plant, it is important to select a cultivar with good content of artemesinin. This is because wide variation in artemsinin content has been found in different cultivars. Only cultivars with high artemesinin level should be cultivated and used. As the Ethiopian highlands may certainly prove to be suitable sites for cultivation of *Artemesia annua*, the extraction and processing of artemisinin for domestic as well as export markets should be considered as a worthwhile investment area for Ethiopian entrepreneurs. The highly fragrant *Artemesia annua* essential oil is another product from the plant that is also in great demand by the fragrant industry. It is also worthwhile to mention other uses of *Artemesia annua* that would make this plant very attractive for the general public. A concoction of the leaves has been found to be useful to treat hemorrhoids, and because of its positive effects on the immune system the tea has been recommended for use by AIDS patients. Further uses include the vapour against colds and coughs, and chewing the leaves to treat *Candida* infection in the mouth.

2.3.3 *Bidens pilosa* (Black jack)

Bidens pilosa (Black jack) belongs to the family of compositae. The Zulu people chew the young shoot of *Biden pilosa* for the treatment of rheumatism. The Zulu also administer the powdered leaf in water as an enema for abdominal troubles and rub the burnt seed into scarifications on the sides of the trunk for the relief of pain. The flower is a Zulu diarrhea remedy and according to Bryant, an infusion of leaf and root is a Zulu colic remedy (Dounga, *et al*., 1983.

In Tanzania an infusion of the plant, which is bitter, is taken at hourly intervals in treatment of dysentery (Dounga, *et al*., 1983). In West Africa the warm juice of the fresh plant is used as drops for earache and for conjunctivitis and as a styptic on wounds. In Mexico the plant is used as a tonic, a stimulant and a tea substitute. Extracts have shown antibacterial activity against variety of microorganism including five enteric pathogens. *B. pilosa* has been used as remedy for icterus, heartburn and cough and fits in the child (Farmwoth *et al*., 2002)

2.3.4 *Kigelia africana* (African Sausage Tree)

Kigelia (also known as sausage tree) has a long history of use by rural African communities, particularly for its medicinal properties. Most commonly, traditional healers have used the sausage tree to treat a wide range of skin ailments, from fungal infections, boils, psoriasis and eczema, through to the more serious diseases, such as leprosy, syphilis and skin cancer. It also has internal applications, including the treatment of dysentery, ringworm, tapeworm, post-partum hemorrhaging, malaria, diabetes, pneumonia and toothache. The Tonga women of

the Zambezi valley regularly apply cosmetic preparations of *Kigelia* fruit to their faces to ensure a blemish-free complexion.

The fruit is a common ingredient in traditional beer, and is said to hasten the fermentation process. *Kigelia* leaves are an important livestock fodder, and the fruits are much prized by monkeys and elephants. Perhaps not surprisingly, given its suggestive shape, the fruit has also found traditional use as an aphrodisiac. Extract of the Bignoniaceae family such as *Kigelia africana* extracts constitutes iridoids and napthaquinones.

Initial laboratory studies (Akunyili *et al*., 1991 and Akunyili, *et al*., 1993) illustrated the efficacy of an aqueous extract of stem bark and two major iridoids against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and the yeast *Candida albicans*. Kigelinone and isopinnatal were the most active compounds. These tests gave validity to the traditional use of the plant as a natural antibacterial and antifungal agent. Later work also supported the use of *Kigelia* fruit extracts for treating skin cancer, with norviburtinal displaying the greatest activity (Akunyili, 1993,) the sterols are known to help a range of skin conditions, notably eczema, and the flavonoids have clear hygroscopic and fungicidal properties.

2.3.5 *Senna didymobotrya*

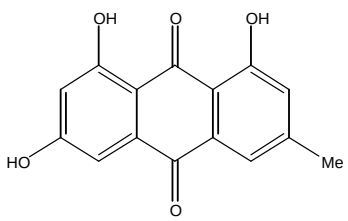
The family Caesalpiniaceae comprises about 150 genera and 2200 species, which are mostly tropical and subtropical trees and shrubs. The leaves are stipulate,

alternate and mostly pinnately compound but may be bipinate. The family is important for both medicinal and pesticidal properties.

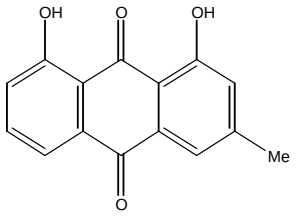
In the Casalpiniaceae family a variety of organic compounds have been encountered for example, anthraquinones, semosides and flavonoids (Seigler *et al*., 1985).

Senna is a genus of over 2200 species, some species are cultivated as ornamental, but some are used as herbal remedies. The dried leaves of *S. anguistifolia* are used as a purgative, anti-malaria medicine, and are also commonly used as a stupefacient for fishing. *An in vitro culture of S. didymobotrya has* compounds that can be converted into low-energy sweeteners and insecticides.

The dried leaves of *S. anguistifolia* Vahl (Caesalpiniacae) are used as a purgative; the part commonly used is the bark which is boiled in water to make a deconcotion used to treat malaria, mumps, pneumonia and other diseases. Many anthraquinones and flavonoids have been isolated, the anthraquinones were found to be active against *Escherichia coli* and *Staphylococcus aurensis*. *S. didymobotrya* bark contains tannins. The leaf infusion is used as emetic against malaria by the Kipsigis. Stems are used against ringworm by the Luyha (Beentje *et al*., 1994). Phytochemical studies carried out have shown that *S. didymobotrya* contain the anthraquinones, among them are emodin 45, chrysophal 46 (Ojewole, 2000).



45



46

Larvicidal assays of aqueous extracts of the leaves stem and root barks of *S. didymobotrya* against malaria vector showed that larval stages are prone to the lethal effects of the plant extracts and the root bark extract possessed the strongest larvicidal activity (Ojewole, 2000).

In conclusion, it is important to note that traditional African medicinal plants have led to several therapeutically and industrially useful preparations and compounds. This should generate interest among the scientists in generating more information about this medicinal plant. As the global scenario is now changing towards the use of non-toxic plant products having traditional medicine use, development of modern drugs from *Azadirachta indica*, *Artemesia annua*, *Kigelia africana*, *Senna didymobotrya* and *B. pilosa* should be emphasized for control of various diseases. In fact, time has come to make good the use of centuries old knowledge on plants through modern approaches of drug development.

2.4 Evaluation of antitrypanosomal herbs

Research on medicinal plants as a source of antitrypanosomal drugs calls for involvement of both pharmacognosists and clinical parasitologists. The plants are mainly selected on the basis of their traditional reputation for efficacy in the

treatment of trypanosomiasis and other disease. Selected plants are subjected to preparation and / or purification of extracts. Initially it is imperative to go for *in vitro* primary screening which reduces the number of laboratory animals used for experiments. Although agents active *in vitro* are often inactive *in vivo* and vice versa, the *in vitro* system can act as a primary screen and helps to identify plants for *in vivo* testing. If a compound kills the parasite *in vitro*, it will also be screened for toxicity against mammalian cells *in vitro*. A plant's active ingredients are isolated and identified with chromatography, mass spectrometry, nuclear magnetic resonance and other techniques (Kirby *et al*., 1996). Identified agents are then further tested for their efficacy and toxicity *in vitro*.

In vivo studies are done in an animal model, preferably mice infected with trypanosomes. The activity of the test material *in vivo* is influenced by a number of factors: The compounds effective *in vitro* may not be effective *in vivo* due to their failure to reach the requisite site of action or they are metabolised too quickly to a less active or inactive form. Or a compound can be more active *in vivo* because it gets metabolized into a more active form. For example, Berbelis alkaloid has little activity against *Entamoeba histolytics* (Phillipsonls *et al*., 1993). When a medicinal plant proves effective *in vivo* and shows no host toxicity, its mechanism of killing parasites is studied through complex and extensive biochemical testing. Also, a likely effect on the host's metabolism must be studied before a drug is released for extensive clinical and field trials. It is also desirable to find out whether a drug has broad spectrum activity by testing it against a range of like organisms.

Standard methods have been used for a long time to screen medicinal plants for activity against various diseases. These have been used in; Anti-viral activity of the extracts of a African medicinal plant *Carissa edulis* against herpes simplex virus (Tolo *et al*., 2005) and Chemotherapy effects of *Annona senegalensis* in *Trypanosoma brucei brucei* **(**Igwe *et al*., 1989). The same approach and methods were used to screen experimental plants for the activity against HAT.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Laboratory animals

Swiss white mice (male) weighing 20 to 30g, aged between 6-8 weeks were used. They were acquired from KARI-TRC colony after obtaining ethical clearance from the institutional animal use and care committee. The animals were kept in an experimental animal room where the temperature was maintained at 22 ± 3^{0}C under a 12hrs light –dark cycle. They were provided with mice cubes and water throughout the experimental period including the 2 weeks acclimatization.

3.2 Trypanosomes

Trypanosoma brucei rhodesiense isolate was obtained from KARI-TRC trypanosome bank. The isolate KETRI 3798, which was isolated from an infected patient in 1977, was used in the study.

3.2.1 Inoculation of mice

Two donor mice were inoculated each by 0.2ml of the stabilate in Phosphoserine glucose (PSG) buffer intraperitoneally. The mice were bled on the tail vein three times a week and the blood examined by the wet film method for the presence of trypanosomes by light microscopy.

3.2.2 Experimental design

Extracts from 5 plant species (*Kigelia africana*, *Bidens pilos*a, *Azadirachta indica*, *Senna didymobotrya* and *Artemesia annua*) were used. Extraction was done at

chemistry laboratory, J.K.U.A.T. *In vitro* and *in vivo* experiments were done at Biochemistry laboratory, TRC-KARI. *In vitro* work was done 96 well plates and in duplicates. In toxicity test, 10 groups of mice (5 per group) were used including the control group. The extract from *Kigelia africana*, *Artemesia annua* and *Azadirachta indica* were used each with the following doses: 250mg/kg, 500mg/kg and 1000mg/kg. The extracts were administered orally using a syringe and i.p. During this period of two months PCV was checked. Efficacy determination was carried out as follows: There were 19 groups, each with 5 mice. Mice were infected as described in subsection 3.2.1 and treatment was begun 24hours post-infection. The plant extracts were administered by i.p, twice daily (9 a.m and 4.30 p.m) for 3 days with the following doses; 1000mg/kg, 2000mg/kg and 5000mg/kg at v/w ratio 1/100 of mouse weight. All extracts were freshly prepared in distilled water (aqueous extracts) and /or with 10% ethanol for dichloromethane and methanol extracts.

3.2.3 Wet film examination

Two microlitre of blood was taken from the cut tail vein of a mouse by pipette and dropped onto a glass slide and covered by cover-slip. The slide was examined using phase contrast/dark ground microscopy at magnification of x400 to determine the presence of trypanosomes..

3.3 Plant materials

Five candidate plants (Table 1), which are used frequently in the treatment of parasitic diseases by traditional practitioners, were used. These were collected in

January 2005 in various locations in Kenya and identified in the Department of botany at Jomo Kenyatta University, using standard taxanomical methods as described in Appendix A. The materials were then taken to the laboratory where they were carefully washed under running tap water (to remove dust and any other foreign material) and left to drain off.

Table 1: Candidate plants studied and localities in Kenya where they were collected and parts used.

Botanical name	Family	Area of collection	Parts used
Azadirachta indica	Meliaeceae	Kajiado	Stem bark/Leaf
Bidens pilosa	Asteraceae	Kasarani	Leaves
Artemesia annua	Asteraceae	Olkurto-Narok	Leaves
Kigelia africa	Bignoniaceae	Gatundu	Fruits
Senna didymobotrya	Fabaceae	Burnt Forest	Root bark/Pods

3.4 Preparation of plant materials for extracts

The size of the plant materials (barks and fruits) were chopped into small bits by a sharp object and leaves were reduced by a knife. They were then spread on laboratory benches and left to air dry for three weeks.

The leaves were ground into powder using a blender while the barks and fruits were ground into powder form using a mortar and thistle. The weight of each plant material in powder form was determined and recorded.

3.5 Extraction techniques and extraction solvents

Five plant materials were extracted using organic and universal solvents. The solvent used include; Water, Methanol, Dichloromethane, Hexane and Ethyl acetate. Four plants (*Artemesia annua*, *Azadirachta indica*, *Kigelia africana* and *Bidens pilosa*) were extracted sequentially by dichloromethane and methanol. On the other hand, *Senna didymobotrya* plant was extracted by hexane and ethyl acetate.

3.5.1 Aqueous extraction

3.5.1.1 Aqueous extraction of plants

The following powdered plants and parts were selected for use; *Azadiradichta indica* (stem barks), *Kigelia africana* (fruits), *Artemesia annua* (leaves), *Bidnes pilosa* (leaves) and *Senna didymobotrya* (pod). One hundred grams (100g) of each of the above was weighed out and soaked separately in 1000ml of distilled water in a conical flask. The contents were warmed in a water bath for 2 hours at 60^{o}C, then left to stand at room temperature for 10 hours, undisturbed. They were subsequently filtered off with sterile filter paper (Whatman No. 1) into a clean conical flask and the filtrate was freeze dried to powder, weighed and recorded.

3.5.1.2 Freeze drying process

The products were pre-frozen in appropriate container (i.e. beaker, tray, flask, and ampoule); temperature controller was used and the product probe was frozen into the product. The freeze dryer base unit was prepared for operation in accordance with operating instructions. When the condenser temperature reached -40^{o}C, the accessory onto the flange of the base unit was assembied and the rack accessory

placed in position over the condenser port. The heater mats were then fitted to the rack by pushing the plug connections into the sockets on the spine and the product trays were loaded onto the rack accessory before fitting acrylic chamber.

Freeze drying process ensured that the samples were completely dry before removing and recording their weights.

3.5.2 Dichloromethane extraction

One hundred grams (100g) of each powdered plant (from subsection 3.5.1) were weighed out and soaked separately in 1000 ml of dichloromethane into a conical flask with a rubber cork. The contents were kept for 5 days away from direct sunlight, undisturbed, then filtered through sterile filter paper into a clean conical flask. The filtrate was transferred into sample holder of rotary vacuum evaporator where the dichloromethane solvent was evaporated at its boiling temperature of 38.5-42^{o}C. The standard extract obtained was then weighed, recorded and stored in refrigerator at 4^{o}C until required for use.

3.5.3 Methanol Extraction

The residues of the dichloromethane extracts were soaked separately in 1000 ml of methanol in a conical flask with a rubber cork for 36 hours away from direct sunlight, undisturbed, then filtered through sterile filter paper into a clean conical flask. The filtrate was transferred into sample holder of rotary vacuum evaporator where the methanol solvent was evaporated at its boiling temperature of 65^{o}C. The standard extracts obtained was then stored in refrigerator at 4^{o} C until required for use.

3.5.4 Hexane and Ethyl acetate extraction

One hundred grams (100g) of *Senna didymobotrya* pod were weighed out and soaked separately in 1000 ml of hexane and acetylacetate into a conical flask with a rubber cork for 5 days away from direct sunlight, undisturbed, then filtered through sterile filter paper into a clean conical flask. The filtrate was transferred into sample holder of rotary vacuum evaporator where the hexane and acetyl acetate solvent were evaporated at its boiling temperature of 38.5-42^{o}C. The standard extract obtained was then weighed, recorded and stored in refrigerator at 4^{o}C until required for use.

3.6 Preparation of Diethylaminoethyl cellulose (DE 52)

DE52 was put in a measuring cylinder up to 300ml mark and topped up with distilled water to 1000 mark. It was stirred and allowed to settle for 30 minutes. The water was poured away and distilled water added again, stirred and allowed to settle for 30 minutes, washed and the sequence was repeated. The column was run as described in appendix C.

3.6 .1 Separation of trypanosomes from red blood cells

An infected mouse was enclosed in a small container with the tail protruding outside. The tail of infected mouse was cleaned with water and cotton wool soaked in methylated spirit. The tail was snipped using scissor and the blood was pipetted from the vein by micro-pipette. The blood was transferred to a small sample vial that contained 840µl of PSG buffer solution and immediately 160µl of 1M NH_4Cl was added and stirred instantly. The PSG buffer with 10% glucose was

added to the column containing DE52 and the cellulose was allowed to settle. When it was compacted, PSG buffer was ran through 3 times the volume. The blood which was collected from mice was diluted with PSG and spun at 1000g for 15 minutes at 4ºC to separate trypanosomes (Supernatant) from red blood cells. The supernatant was further centrifuged at 3000g for 15 min at 4C°. The white fluffy layer was removed and loaded into the column. The PSG buffer with 1% glucose, (pH8) was run through the column until all trypanosomes were washed through.

The PSG buffer was repeatedly added to the column to elute trypanosomes. The trypanosomes were collected in centrifuge tube and counted by Hemocytometer.

3.6.2 Quantification of trypanosomes

Forty five microlitres of staining solution was pipetted into two tubes. Five microlitres of blood was added to the first tube, then, mixed well. A fresh capillary tube was used to transfer further 5 microlitre of dilute blood from the first tube to the second tube and mixed well. The capillary tubes were allowed to stain for 5 minutes. The cover slip was carefully attached to an improved Neubauer counting chamber and before filling side of the counting chamber with a capillary tube and allowing the contents to settle for 2-3 minutes. The trypanosomes were then counted in areas ABCD contained in circles as shown below. The dilution factor of 1/100 was taken into account during the calculation. The trypanosomes present in 1ml were determined as shown below, when for instance the first count was 18 trypanosomes and second count was 16 trypanosomes, then, mean was calculated

as 17 trypanosomes. This mean was multiplied by 5, the number of counted squares to give the total number of trypanosomes present as; $17x5x10^6 = 8.5x10^7$ml

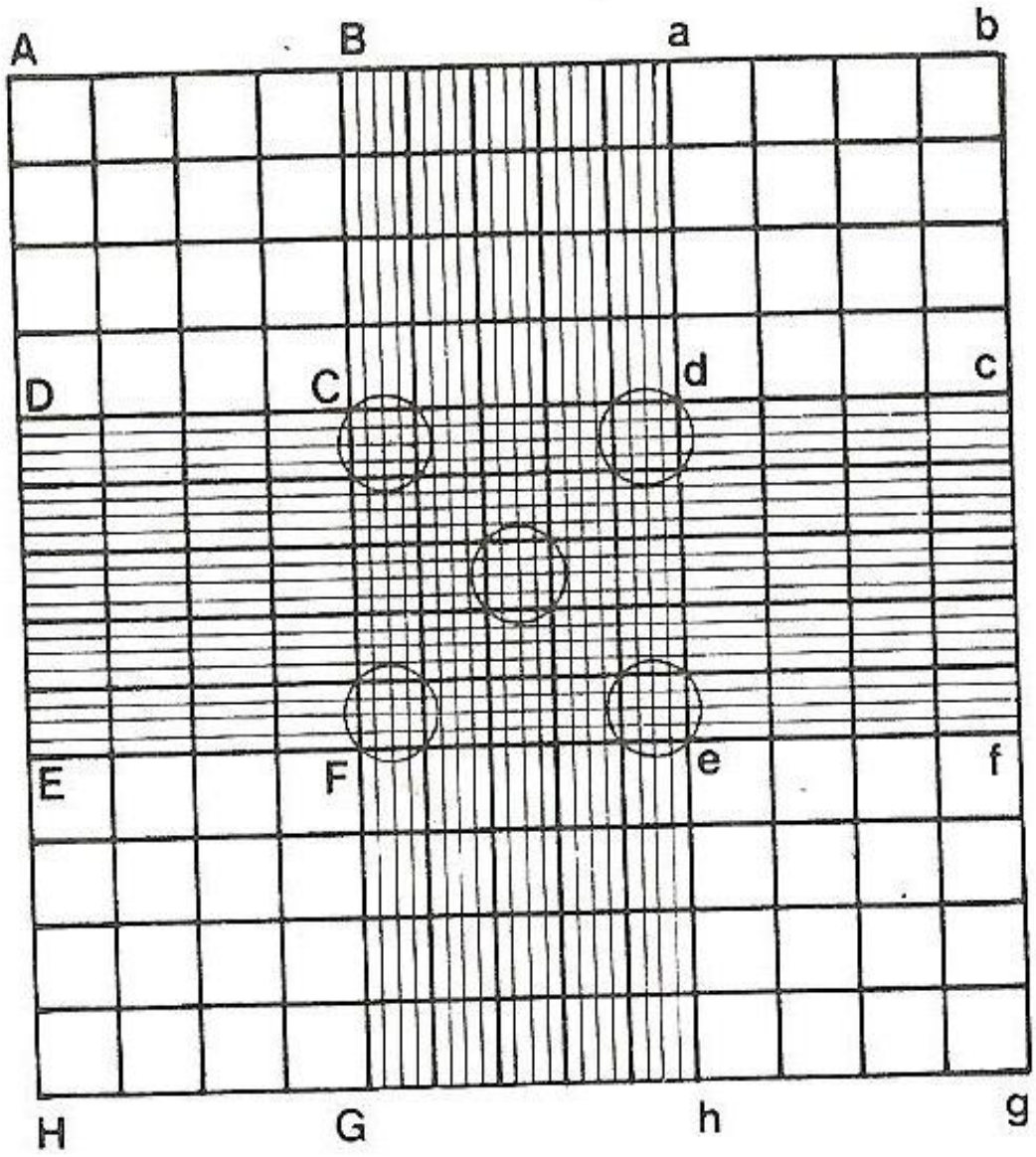


Chart 1: Hemocytometer count chart. The circled areas shows areas where counting of trypanosomes was done.

3.6.3 *In vitro* culturing of parasites

The medium for the cultivation of blood stream trypanosome forms was prepared according to Baltz *et al*., 1985. Seven stock solutions were prepared and stored separately (Appendix B). The working solution was constituted from the species during time of use.

Trypanosome brucei rhodesiense (KETRI 3798 clone) blood stream forms were cultivated *in vitro* in a modified Iscove's medium containing 10% heat-inactivated fetal serum (HI-FCS) and bloodstream form supporting factors; 1.5mM Bathocuproine sulfonate, 1.5mM L-cysteine, 1mM hypoxanthine, 0.2mM 2-mercaptoethanol, 1mM sodium pyruvate, 0.16mM thymidine known as HMI 9 (Hirumi and Hirumi *et al*., 1994). The media with the supporting factors were prepared as follows: 1ml of 1.5mM Bathocuproline sulfonate, 1ml of 1.5mM L-cystein, 1ml of hypoxathine, 1ml of 0.2mM of 2-mercaptoeyhanol, 1ml of sodium pyruvate, 1ml of 0.16 of thymidine and 2mls Gentamycin were transferred into measuring cylinder. And 20 mls of horse serum was added into the measuring cylinder containing 72mls of modified Iscoves' medium and 100ml mark was obtained. The mixture/solution was filtered by sieving pump (carried out in the laminar flow hood) and filtrate was transferred into a small bottle and stored at -20ºC. The trypanosomes were incubated in a humidified atmosphere containing 5% CO_2 at 37°C.

3.7 Determination of PCV

This is the fraction of whole blood volume that consists of red blood cells. The blood was obtained by bleeding tail vein of mice as described in section 3.2.1. The blood was collected in heparinized capillary tubes which were sealed immediately. The capillary tubes with the blood were then centrifuged in a micro-centrifuge for 5 minutes at 10000g rpm. After centrifugation, the height of the red blood cell column was measured by use of haematocrit reader and compared to the total height of the column of the whole blood. The percentage of the total blood volume occupied by RBC mass is the haematocrit which depends mostly on the number of the RBCs. The reference values are 42-52% for males and 36-48% for females. The haemotocrit is usually about 3 times haemoglobin value (assuming there is no marked hypochronia). The average error in haemotocrit is about 1-2%. The haemotocrit may be changed by altitude and position as in the case of hemoglobin. So, decrease in PCV against the reference indicates the presence of infection and also indicated that vital organs/cells are raptured.

3.8 Evaluation of *In vitro* trypanocidal activity of plant extracts

The 19 plant extracts obtained after extraction were assayed alongside the current drugs, Suramin and Melarsoprol. The test material at 10mg/ml concentration was prepared by dissolving 30mg of plant powder (water extracts) into 3ml of distilled water and then filtered through sterilized filters. Serial dilution was then made from stock solution at concentrations 1mg/ml, 0.5mg/ml and 0.25mg/ml. For methanol and dichloromethane extracts, 20mg of each powdered plant material was dissolved in 200µl of ethanol and stirred instantly before adding 1800µl of

distilled water. The extraction solution (stock) at 10mg/ml was obtained. Serial dilution in culture media was made from this stock solution, at concentration of 1mg/ml, 0.5mg/ml and 0.25mg/ml. Each extract was tested in 96 well plates in two-fold serial dilutions in media; each concentration was tested in duplicate. For preliminary screening, each extracts was tested once. One hundred microlitres of trypanosome suspension was added to each well at a parasite density of $1x10^5$/ml. Negative control wells containing media without plant extracts were included. Assays with commercial drugs (Melarsoprol; 7.2µg/ml, 3.6µg/ml, 1.8µg/ml and Suramin; mg/ml, 0.1mg/ml and 0.01mg/ml doses used *in vitro*) were also performed in order to generate reference values.

They were incubated for 72h, after which the test was read under inverted microscope at 100-fold magnification, MIC and test evaluation were determined as described in appendix C.

3.9 *In vivo* evaluation of toxicity of plant extracts

Preliminary evaluation of relative toxicity of plant extracts was performed as follows; Test animals were closely observed throughout the 14 days after administration for overt toxic signs and symptoms. Their overall health and a general well-being was observed and recorded on daily basis for remainder of experiment period. Significant weight loss (more than a two fold loss compared to the physiological saline controls over the 2 week dosing period) was considered a key indicator of declining health due to drug toxicity. After two weeks of close observation of mice, the liver and kidney were removed from each animal and

examined for gross pathology. Subjective scores of toxicities were assigned to the doses/concentration of plant extracts administered.

3.9.1 Determination of *in vivo* efficacy of the plant extracts.

Due to little antitrypanocidal activity observed *in vitro* of the plant extracts, all extracts of (*Kigelia africana*, *Bidens pilos*a, *Azadirachta indica*, and *Artemesia annua*) were evaluated *in vivo* for trypanocidal activity using using the standardized mouse test (Eisler *et al*., 2002). After intraperitoneal inoculation of each mouse with $1.x10^5$ trypanosomes (*T.b.r* KETRI 3798) treatment was administered i.p, 24 hours later. Control and treatment groups consisted of 5 animals each. The animals were injected by i.p with extracts in increasing dose levels, 1000mg/kg, 2000mg/kg and 5000mg/kg, two times daily for three days. All extracts were freshly prepared in distilled water and or ethanol and administered at correct dose, at a v/w ratio of 0.1ml/10g (1/100 mouse weight) mouse weight. Mice were checked daily during 5 days after the first treatment to estimate the number of trypanosomes in their tail blood in a wet blood film. The absolute number of parasites per milliliter of blood was taken as a log using the rapid matching method for estimating the host's parasiteamia according to Herbert and Lumsden, 1976, as shown in appendix D. At higher levels of parasites, this was achieved by matching microscopic fields of wet blood film against charts and, when fewer parasites were present, by counting the number of trypanosomes in 5, 10 or 20 such microscopic fields. For the assessment of antitrypanocidal effect of the extracts, the level of parasitaemia (expressed as log of absolute number of parasites per millimeter of blood) in the animal was compared to that of the control

animals. Animals were checked thrice weekly for parasites in tail vein blood for 60 days. Animals that survived to the end of the experiment, with no parasites in their blood were considered cured. During this period PCV and weights of mice were also determined and recorded.

3.9.2 Statistical analysis

To asses the therapeutic effects, the parasitological data of treated and control animals were statistically analyzed using Student's t-test (Minitab computer program). P-Values <0.05 were considered as significant, whereas those >0.05 as not significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Yields of plant extracts

After drying of the plant leaves of *Artemesia annua*, *Bidens pilosa*, *Azadirachta indica* stem bark, *Kigelia africana* fruits and *Senna didymobotrya* pods, 300g of the material was sieved and 100g was used for extraction. The yields and percentage (%) yields of plant extracts by dichloromethane is shown in Table 2.

Table 2: Yields of plant extracts using various solvents

Plants	*Azadirachta indica*		*Kigelia africana*		*Artemesia annua*		*Bidens pilosa*		*Senna didymobotrya*	
Dry mass(g)	300		300		300		300		300	
Extract	Yield (g)	% Yield	Yield (g)	% Yield	Yield (g)	% Yield	Yield (g)	% Yield	Yield (g)	% Yield
Aqueous	5.1	5.1	3.1	3.1	3.1	3.1	5.7	5.7	nd	nd
MeOH	11.68	11.68	11.7	11.7	10.7	10.7	10.87	10.87	11.2	11.2
Dichloromethane	7.24	7.24	10.21	10.21	9.9	9.9	9.43	9.43	8.72	8.72
Hexane	nd	nd	nd	nd	nd	nd	nd	nd	8	8
Ethyl acetate	nd	nd	nd	nd	nd	nd	nd	nd	7.4	7.4

The % yield was calculated against 100g of the product of the plant material subjected to each extraction technique. nd= not done

4.2 Results of *in vitro* evaluation of trypanocidal activity.

Tables 3, 4, 5 &6 show results of the minimum inhibition concentration (MIC) of all the 19 extracts against *T. b. rhodesiense* isolate KETRI 3798 bloodstream form. Three extracts (*Azadirachta indica* stem bark and *Azadirachta indica* leaves of dichloromethane and *Azadirachta indica* stem bark of methanol respectively)

could be considered as active with MIC value of ≤18.2µg/ml, three extracts (A*zadirachta indica* leaves, *Azadirachta indica* stem bark and *Bidens pilosa* leaves of water) were moderately active with MIC values between 18.2 and 62.5µg/ml, methanol extract of *Artemesia annua* leaves had MIC value of 125µg/ml and twelve extracts were mild at MIC values ≥200µg/ml.

Table 3: Antitrypanocidal activity of water extracts

Plant	Part [a]	Solvent [b]	Activity (µg/ml) [c]
Azadirachta indica	SB	H_2O	31.3±0
Azadirachta indica	LF	H_2O	31.3±0
Bidens pilosa	LF	H_2O	62.5±0
Artemesia annua	LF	H_2O	500±0
Kigelia africana	FT	H_2O	>500
Suramin (µg/ml)		Water	11.2± 0
Melarsoprol (µg/ml)		Water	0.014± 0

Table 4: Antitrypanocidal activity of Methanol extracts

Plant	Part [a]	Solvent [b]	Activity (µg/ml) [c]
Azadirachta indica	SB	CH_3OH	11.42±4.9
Azadirachta indica	LF	CH_3OH	31.3±0
Artemesia annua	LF	CH_3OH	125± 0
Bidens pilosa	LF	CH_3OH	500± 0
Kigelia africana	FT	CH_3OH	>500
Senna didymobortya	PD	CH_3OH	>500
Suramin (µg/ml)		Water	11.2± 0
Melarsoprol (µg/ml)		Water	0.014± 0

Table 5: Antitrypanocidal activity of dichloromethane extracts

Plant	Part [a]	Solvent [b]	Activity (μg/ml) [c]
Azadirachta indica	SB	CH_2Cl_2	3.91
Azadirachta indica	LF	CH_2Cl_2	18.2±11.96
Bidens pilosa	LF	CH_2Cl_2	353.5± 0
Artemesia annua	LF	CH_2Cl_2	500± 0
Kigelia africana	FT	CH_2Cl_2	>500
Senna didymobortya	PD	CH_2Cl_2	>500
Suramin (μg/ml)		Water	11.2± 0
Melarsoprol (μg/ml)		Water	0.014± 0

Table 6: Antitrypanocidal activity of hexane, ethylacetate (*Senna didymobotrya extracts*
and the Standard drugs

Plant extract and commercial drugs	Solvent	Activity (μg/ml) [c]
Senna didymobortya	Hexane	>500
Senna didymobortya	Ethyl acetate	>500
Suramin (μg/ml)	Water	11.2± 0
Melarsoprol (μg/ml)	Water	0.014± 0
Dihydroartemesinin	Water	125

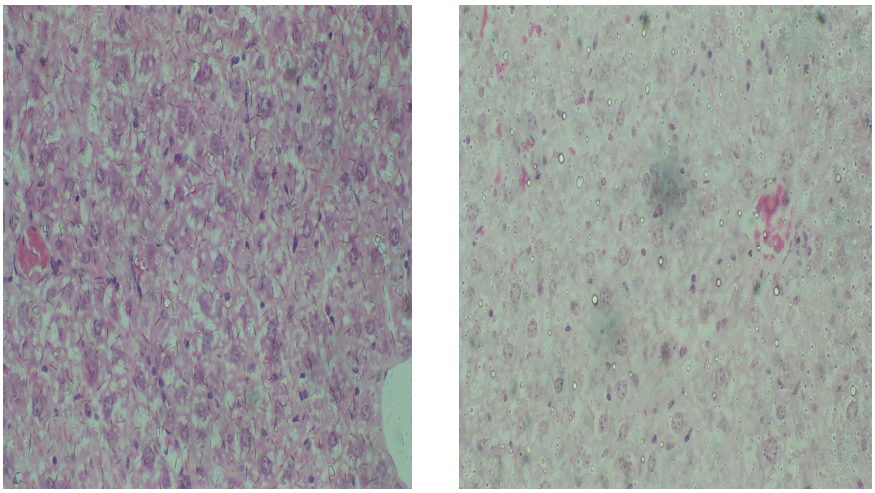
[a]Plant part used: SB=Stem bark, LF=Leaves, FT=Fruits, PD=Pod, RB=Root bark.

[b]Extraction solvent. [c] Activity =MIC (x±s.d) - Minimum inhibition concentration means the concentration at which no cell with normal morphology and / or motility is found in comparison with the control. nd = not done

4.3 Results of acute toxicity studies on the kidney and the liver of mice treated with plant extracts.

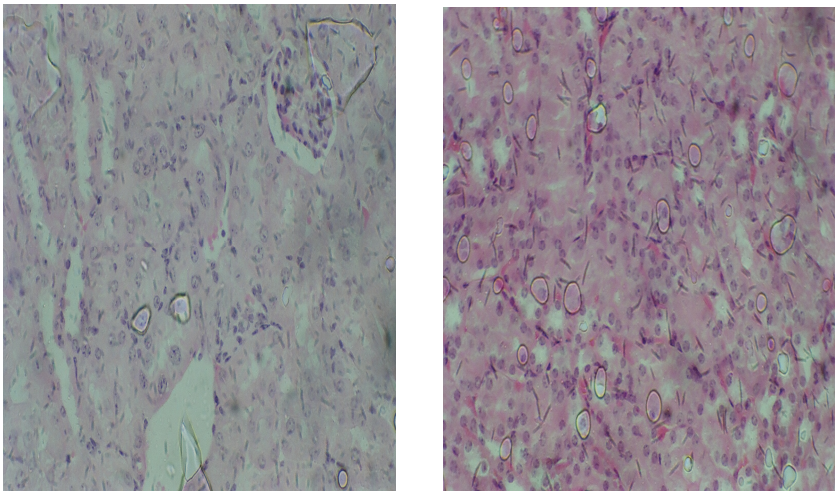
The plates 1-8 below shows longitudinal sections of pathological examinations of the liver and kidney of mice administered with dichloromethane extracts of ***Azadirachta indica*, *Kigelia africana*, *Artemesia annua*** and the control in a single

oral dose (1000mg/kg) by a syringe. Pathological examinations of the tissues on gross and microscopic basis indicated that there were no detectable abnormalities of the animals treated when compared to controls and the magnification used was x400.



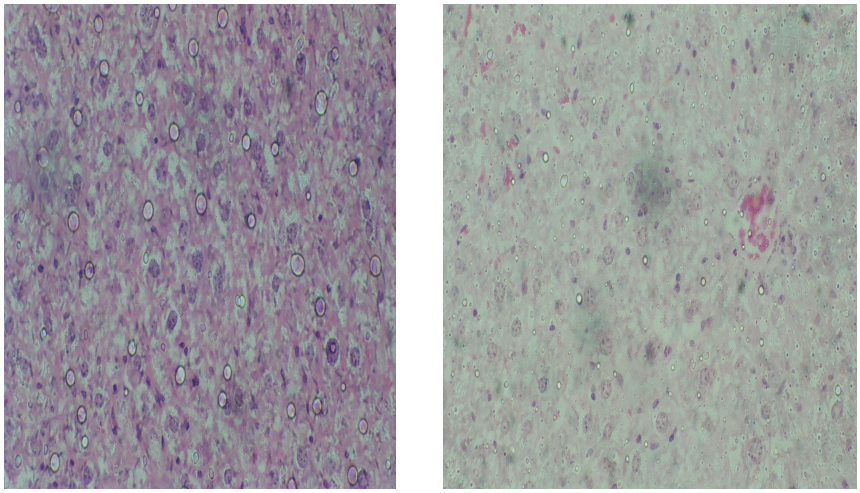
Treatment control

Plate 1: Longitudinal section of the liver of mouse subjected to *Azadirachta indica* extract



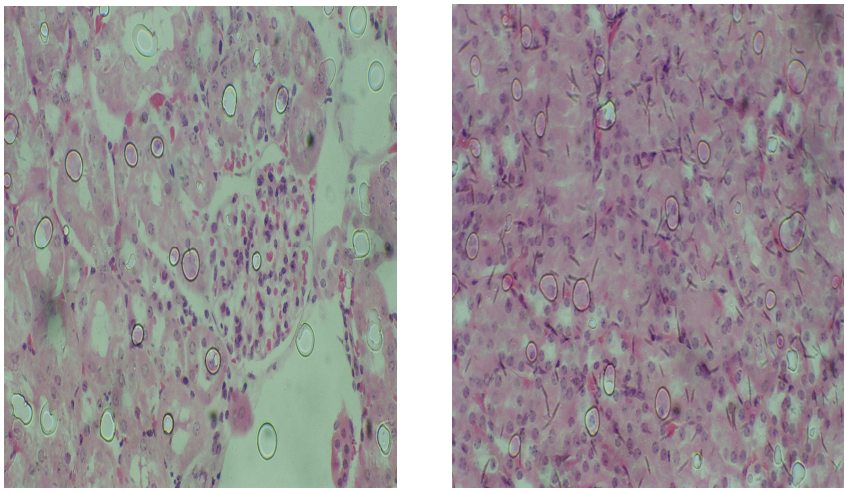
Treatment Control

Plate 2: Longitudinal section of the kidney of mouse subjected to *Azadirachta indica* extract



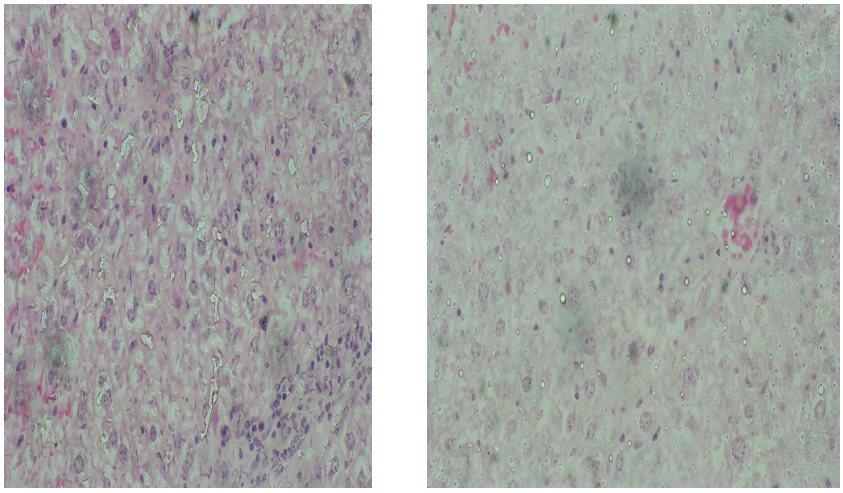
Treatment Control

Plate 3: Longitudinal section of the liver of mouse subjected to *Kigelia africana* extract



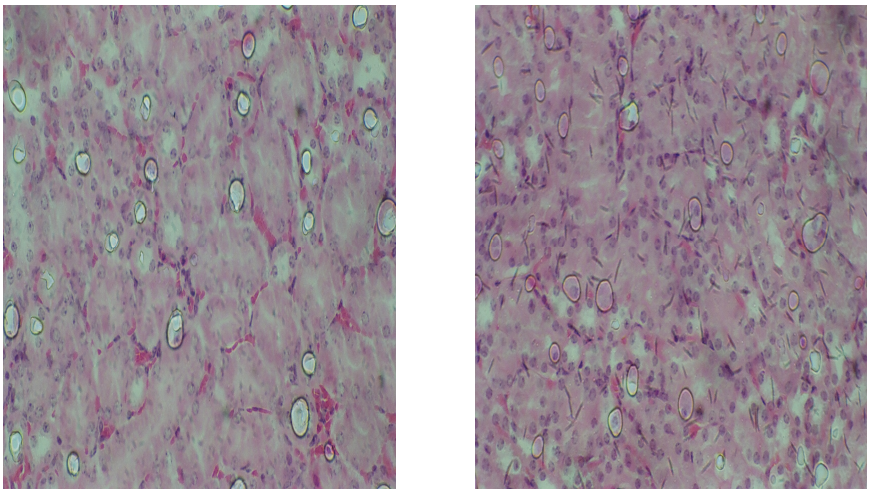
Treatment Control

Plate 4: Longitudinal section of the Kidney of mouse subjected to *Kigelia africana* extract



Treatment Control

Plate 5: Longitudinal section of the liver of mouse subjected to *Artemesia annua* extract



Treatment Control

Plate 6: Longitudinal section of the kidney of mouse subjected to *Artemesia annua* extract

No death was recorded during the treatment period in either control or treated groups given the single dose of 1000mg/kg of dichloromethane extracts of *Kigelia african*, *Azadirachta indica* and *Artemesia annua* respectively by both oral and i.p. The animals did not show any changes in general behavior or other physiological activities. After oral /i.p administration of plant extracts into test animals for signs

of acute toxicity, it was noted that, after close monitoring for 10-15 minutes, all the mice were active, aggressive and with no sign of apparent ill effects.

At the conclusion of the experiment, the surviving animals were sacrificed in CO_2 chamber and their organs such as liver and kidney were excised. The pathological observations of these tissues were performed on gross and microscopic basis and indicated that there were no abnormalities. The organs of both the treatment and control groups (plates 1-6) were a justification of safety levels of the plant extracts used. No further evidence of histopathological changes was seen.

4.4 *In vivo* evaluation of trypanocidal activity

Mice were infected as described under subsection 2.2.1 and treatment was administered 24 hours post-infection. The plant extracts shown below were administered i.p, twice daily (9 a.m and 4.30 p.m) for 3 days at the total dose shown in Table 7. The survival time (days) was the total number of days (2 months and beyond) the mice survived before death over the number of mice in experimental group i.e. 5 mice per group. The figures in the brackets below indicate the number of days, each mouse survived before death occurred. Those that lived up to and beyond 60 days were considered cured. These were 21 in number.

Table 7: Activity of herbal/plant extracts administered by i.p injection against *T.b.rhodesiense* KETRI 3798 in Swiss white mice

	Survival time (days)		No. mice cured/Total No. tested (survival period)	Cured rate (%)
Drug (dose)	Mean	StDev		
Control	11.6	±2.5	0/5 (9,9,12,14,14)	0
Ethanol control	14.2	±3.0	0/5 (9,14,16,16,16)	0
A.indica (DCM extract))				
1000mg/kg	18	±2.0	0/5 (16,16,18,20,20)	0
2000mg/kg	23.4	±3.4	0/5 (20,22,22,24,29)	0
5000mg/kg	27	±8.9	0/5 (20,20,29,31,35)	0
A. annua (water extract)				
1000mg/kg	17.6	±6.8	0/5 (12,16,18,18,24)	0
2000mg/kg	17.6	±3.0	0/5 (14,16,18,18,22)	0
5000mg/kg	18	±3.7	0/5 (12,18,18,20,22)	0
A. annua (methanol extract)				
1000mg/kg	18	±4.9	0/5 (12,14,20,20,24)	0
2000mg/kg	19.2	±3.0	0/5 (14,16,18,18,22)	0
5000mg/kg	15.6	±3.3	0/5 (12,14,14,18,20)	0
K. africana (DCM extract)				
1000mg/kg	41.8	±16.7	2\5 (29,29,31,60,60)	40
2000mg/kg	50.8	±12.0	9/15 (31,31,37,39,42,42,60{9})	60
5000mg/kg	18.4	±4.8	0/5 (14,16,16,,20,26)	0
K.africana (Methanol extract)				
1000mg/kg	17.6	±5.4	0/5 (12,12,20,20,24)	0
2000mg/kg	17.6	±5.2	0/5 (12,12,20,22,22)	0
5000mg/kg	23	±3.7	0/5 (20,20,22,24,29)	0
Melarsoprol(36mg/kg)	cured	N/A	5/5	100
Suramin(5mg/kg)	cured	N/A	5/5	100

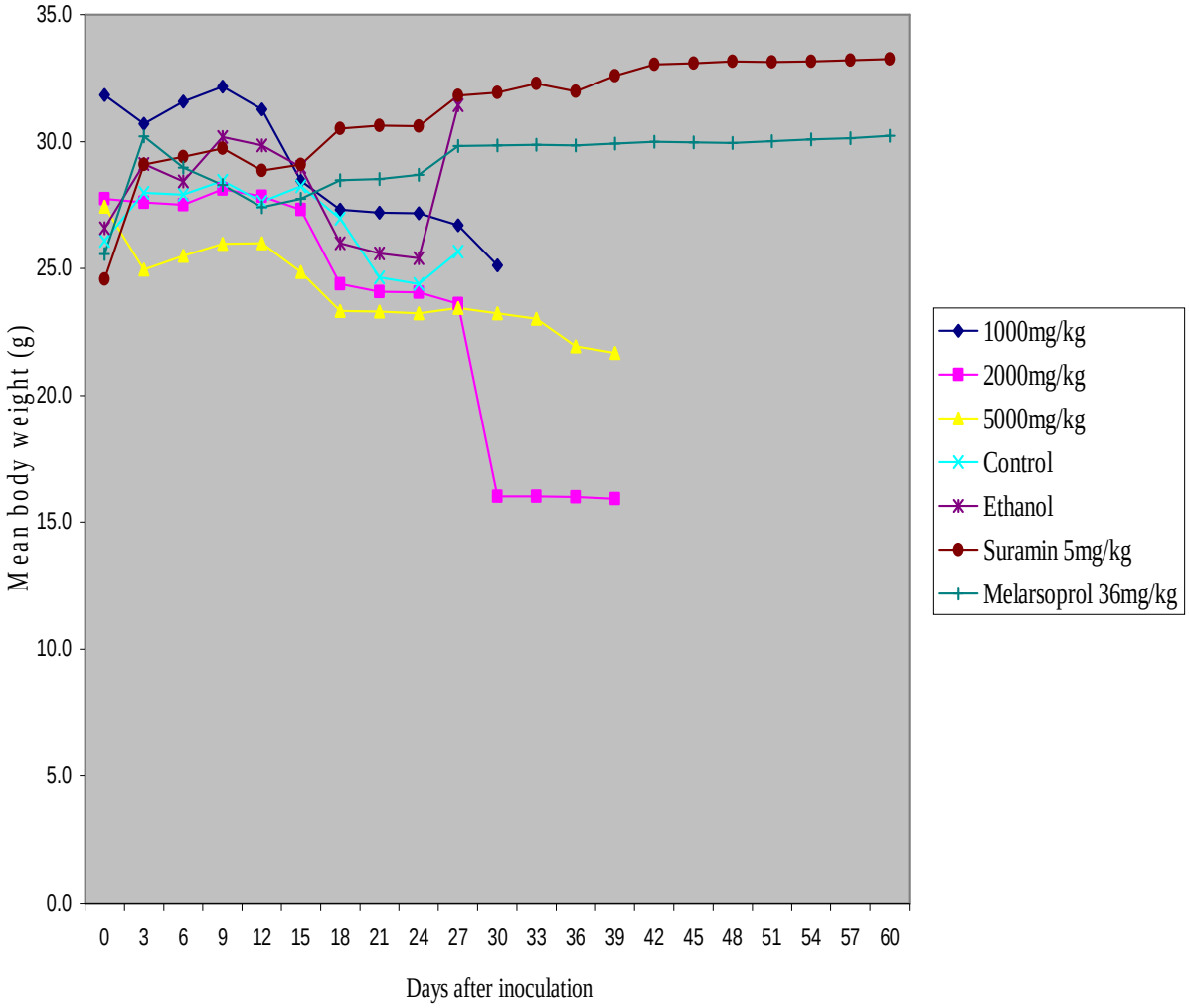
4.4.1 Trends and variations of parasiteamia levels, weights and PCV of mice treated with plant extracts and controls.

The figure(2,3,4), figure(5,6,7) and figure(8,9,10) shows the trends and variations of mean body weights (g), change of parasitaemia levels (mean log) and packed cell volume (percentage %) respectively of mice infected and treated by i.p

injection with plant extracts at various dose rates indicated along side the graphs, besides the controls.

Following the infection and the treatment of mice, the antitrypanosomal activity was checked during the first 5 days and thereafter, thrice a week for 60 days. It was observed that from the fourth or fifth day, after treated both the treatment and the control groups were negative, possibly, the extracts were either metabolized to less active form or did not reach the target site. After the fifth day, groups of mice treated with *Azadirachta indica* and *Artemesia annua* extracts became parasite positive. The parasitaemia levels kept increasing each day as shown in Fig. 5 and 6. There was continuous weight lose with time post-treatments as demonstrated in Fig. 2 and 3 respectively. The survival time of mice treated by these extracts was close to that of control group that did not receive this treatment. This implied that there was no significant difference between the treatment groups and the control groups. No mice were cured by these extracts. *Azadirachta indica* and *Artemesia annua* extracts were not effective in mice model experiment. The ingredient in the plant material may not be effective against trypanosomes. Tables 11, 12 and 13 are indicated as Appendix E and these tables somehow indicates that the experiments were terminated premature, that was not case but shows that the mice died on day of termination. However, others survived upto 60 days which implied that the mice were cured. *Azadirachta indica*, *Artemesia annua* and *Kigelia africana* extracts indicated in the 3 tables were extracted by dichloromethane, water and dichloromethane respectively.

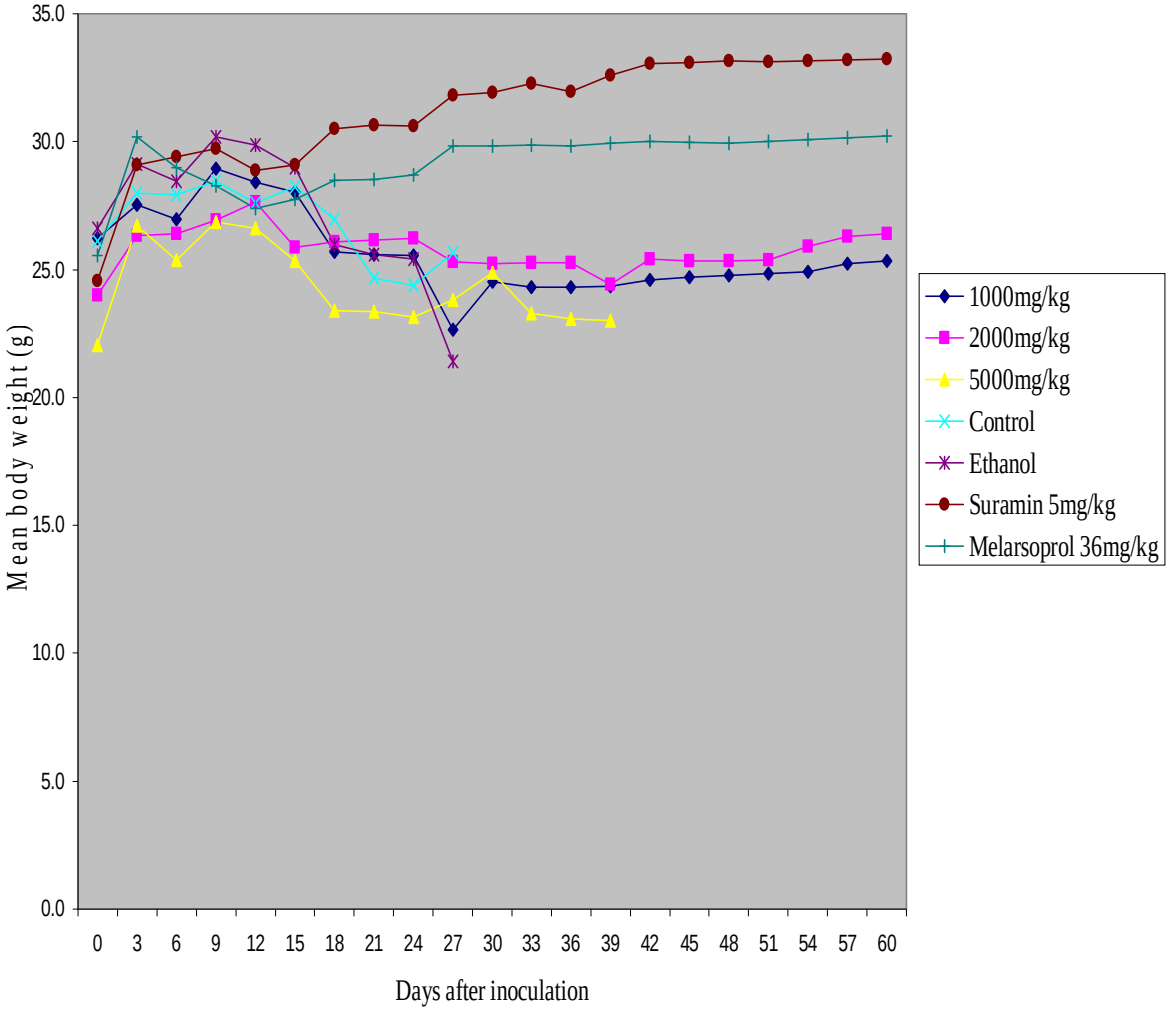
Fig 2: Change in mean body weights (g) of mice treated with *Azadirachta indica* extract



Change in body of the treated animals was monitored during the period of the experiment (figure 2). It was observed that the mice treated with 2000mg/kg of dichloromethane extracts displayed tremendous weight lose, followed by 5000mg/kg and both lived for 39 days longer than the dichloromethane extract of 1000mg/kg. There was significant difference between 2000mg/kg and 1000mg/kg ($p \leq 0.233$). For the negative controls (ethanol and water) the animals lost a lot of

body weights and survived for only 27 days. In contrast, the animals treated with Melarsoprol and Suramin slightly increased in body weights.

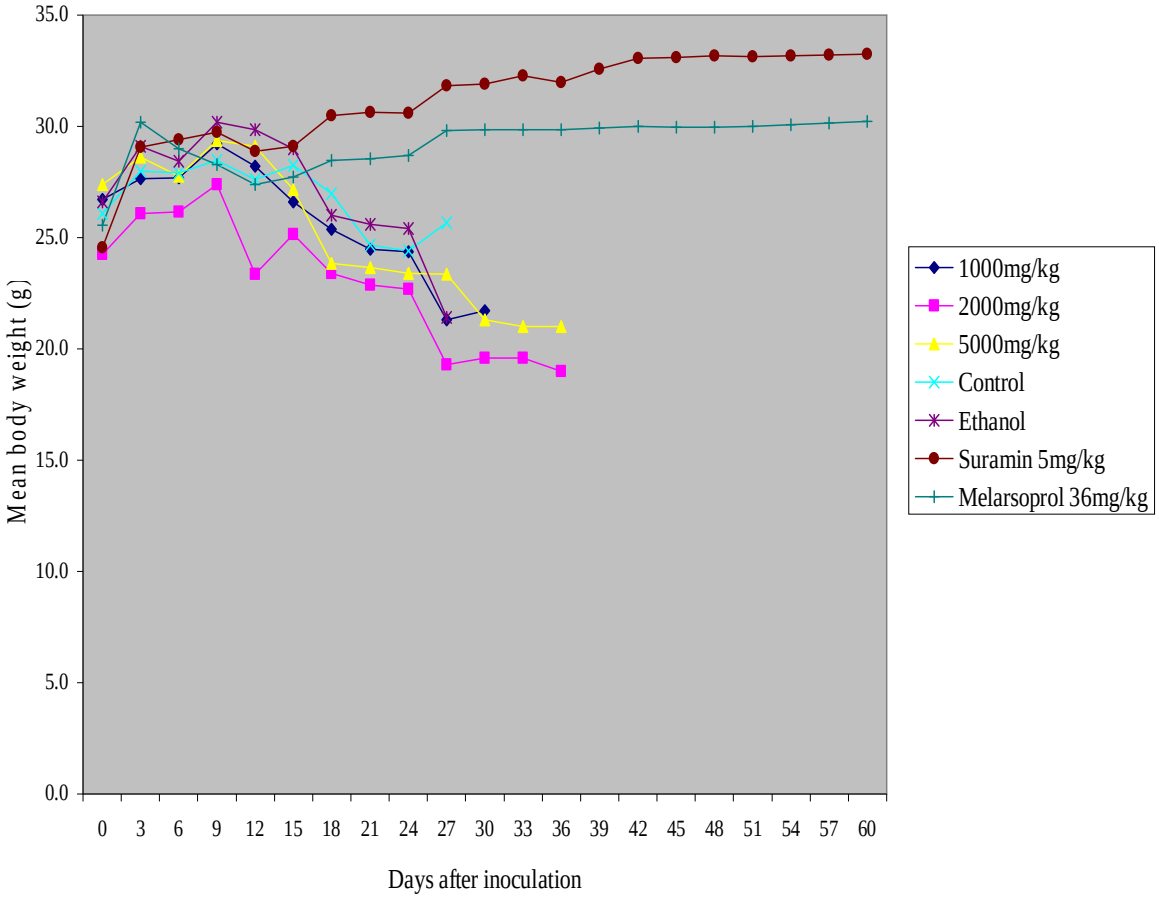
Fig 3: Change in mean body weights (g) of mice treated with *Kigelia africana* extract



Change in body of the treated animals was monitored during the period of the experiment (figure3). It was observed that animals treated with 2000mg/kg of the plant extract on average maintained their body weight post treatment while the animals treated with 1000mg/kg showed reduced body weights. There was significant difference between 2000mg/kg and 1000mg/kg ($p \leq 0.172$). For the negative controls (ethanol and water) the animals lost a lot of body weights and survived for only 27 days. In contrast, the animals treated with Melarsoprol and

Suramin slightly increased the body weights and maintained their weights after 27 days.

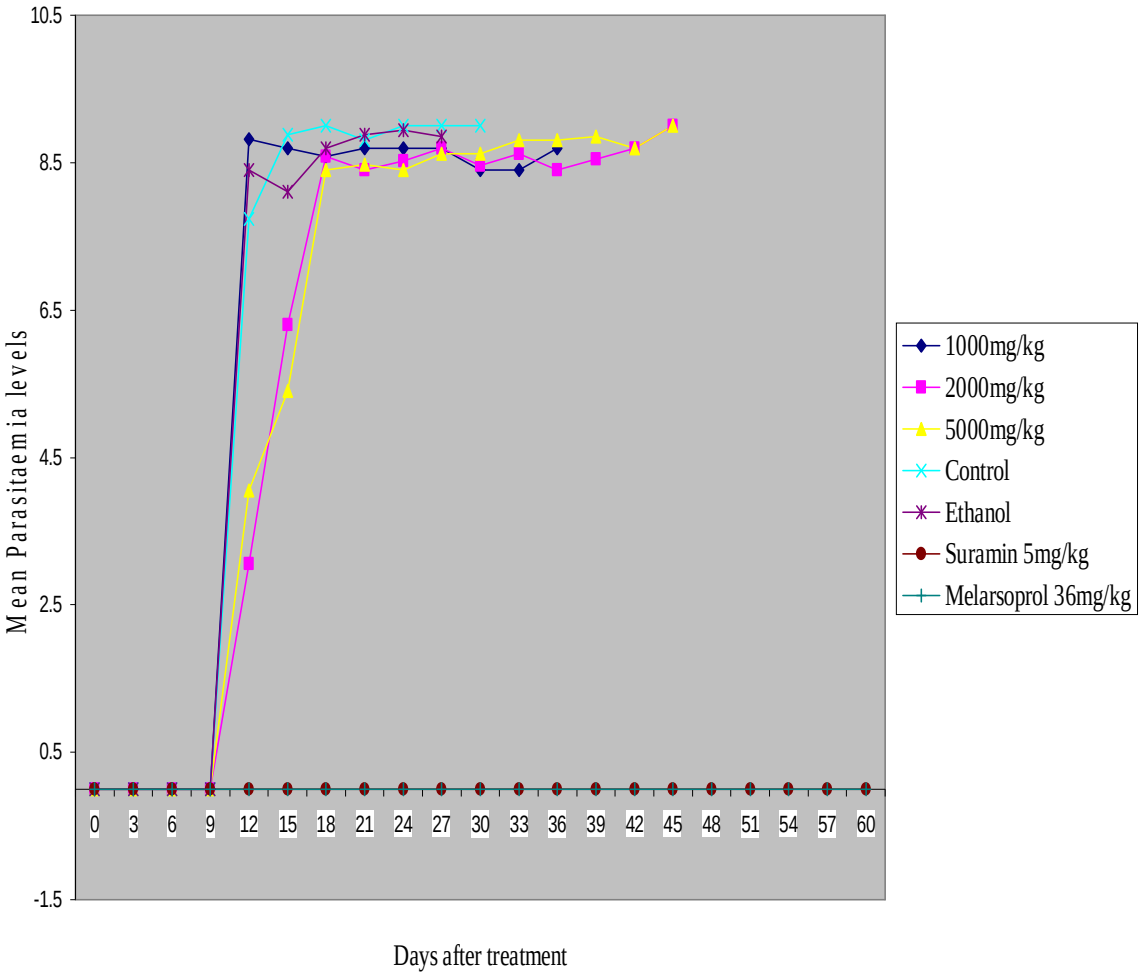
Fig 4: Change in mean body weights (g) of mice treated with *Artemisia annua* extract



Change in body of the treated animals was monitored during the period of the experiment (figure 4). The animals treated with the three concentrations (1000mg/kg, 2000mg/kg and 5000mg/kg) of aqueous extract indicated treamendous reduction of weight while the ethanol and water (control), there was slight decrease in weight in mice but survived for 27 days compared 36 days of

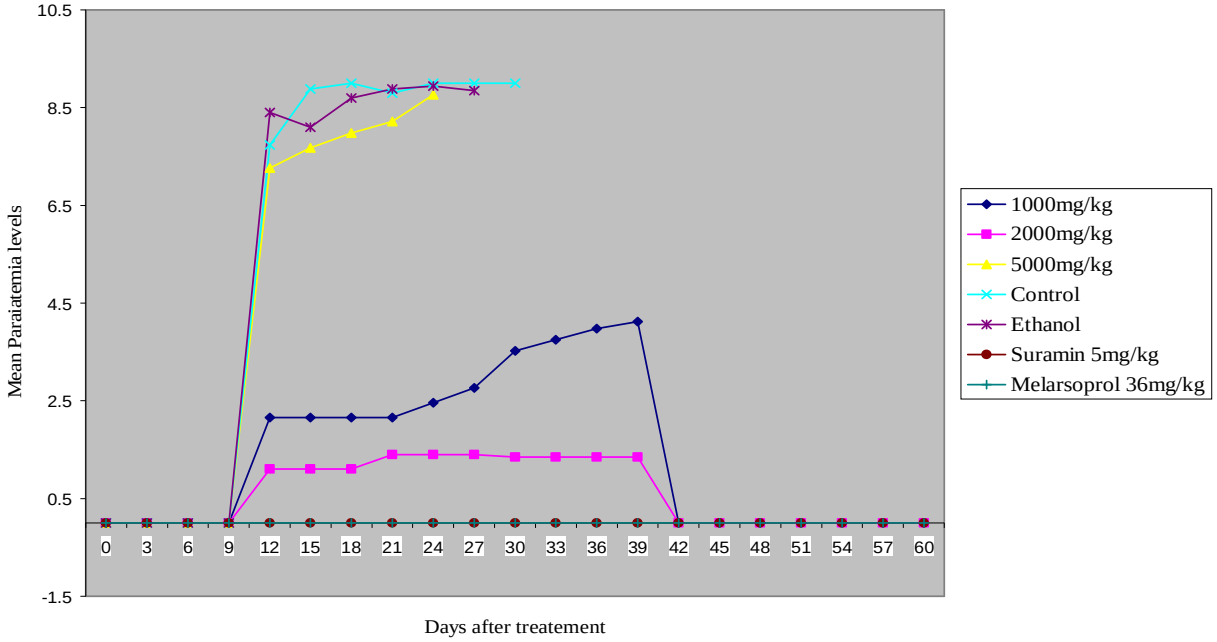
extract. In contrast, the animals treated with Melarsoprol and Suramin slightly increased the body weights and maintained their weights after 27 days.

Fig 5: Change in mean Parasitaemia levels in mice after treatment with *Azadirachta indica* extract



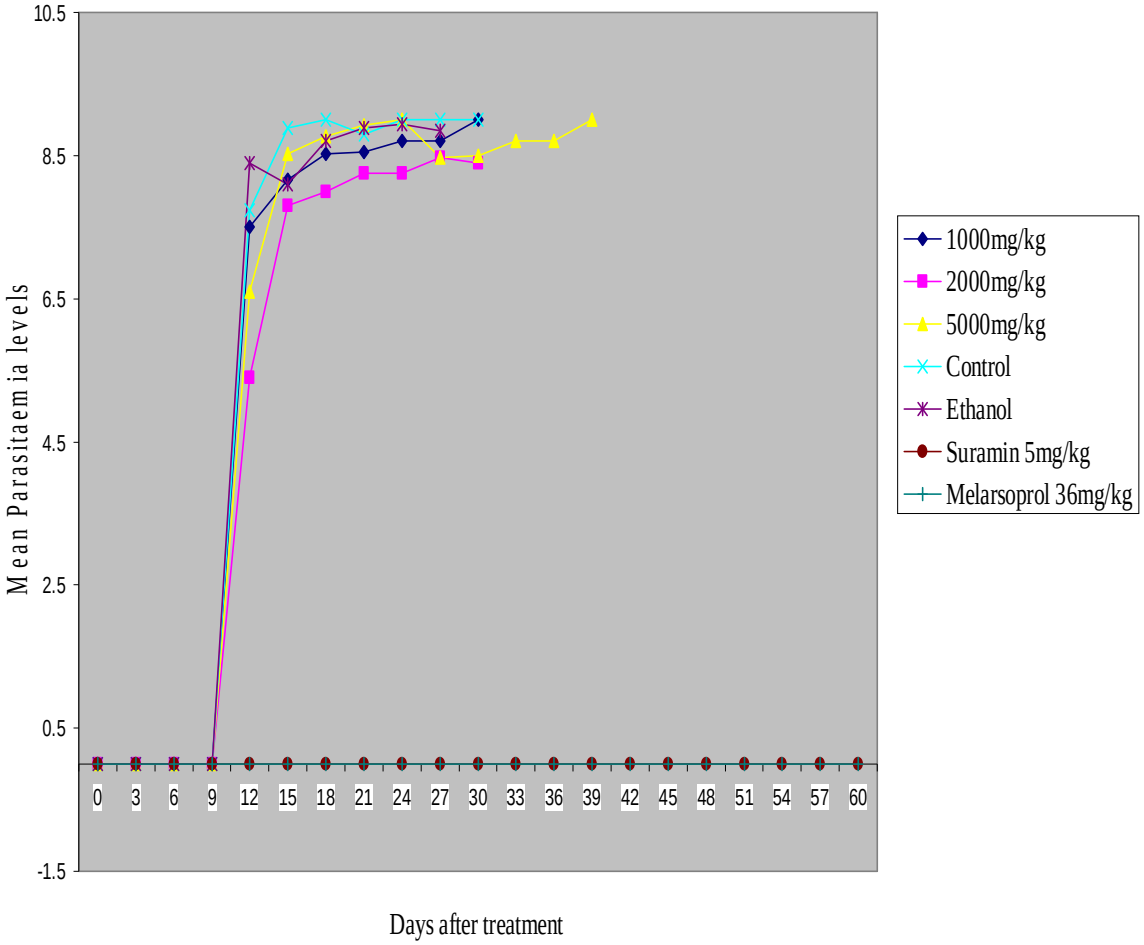
The results of parasiteamia levels as indicated in figure 5 showed that there was rapid increase of parasites in mice irrespective of treatment with dichloromethane extract. However, the animals survived for 42 days at most. Melarsoprol and Suramin cleared the parasites and the animals survived up to and beyond 60 days.

Fig. 6 Change in parasitaemia levels mice after treatment with *Kigelia africana*



The results on parasitaemia as presented in fig. 6 showed that mice treated with 2000mg/kg of DCM extract on average had low parasitemia level since the majority of mice did not have trypanosomes after post treatment and they survived up to and beyond 60 days. The mice in the same group that showed high levels of parasitaemia survived for at least 39 days, showing that they resisted the parasites for appreciable period of time. On the other hand, the mice treated with 1000mg/kg had higher parasitaemia levels than that of 2000mg/kg and only 40% of infected mice survived. It was further observed that the mice treated with 5000mg/kg had the highest parasites and none of the animals was cured. They survived for only 24 days. Negative controls (water and ethanol) survived for 30 and 27 days respectively and parasitaemia levels were high. Melarsoprol and Suramin cleared the parasites and the animals survived up to and beyond 60 days.

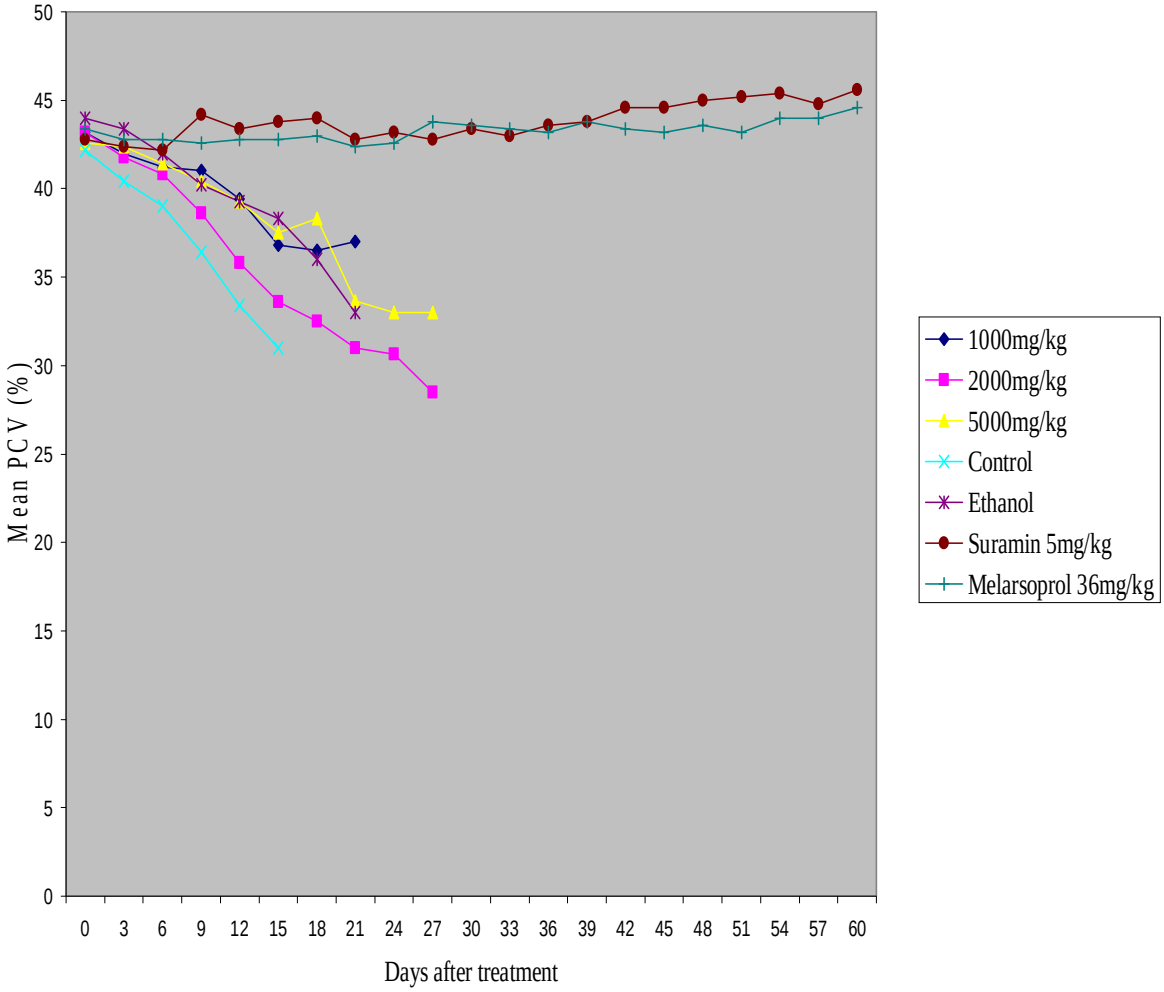
Fig 7: Change in mean Parasitaemia levels in mice treated with *Artemesia annua* extract



The results on parasitaemia as presented in figures 7, showed that mice treated with aqueous extract on average had high parasitemia level since the majority of mice did have trypanosomes after post treatment and they survived for 31, 31 and 39 days for the doses, 1000, 2000 and 5000mg/kg respectively. Negative controls (water and ethanol) survived for 29 and 31 days respectively and parasitaemia

levels were high. Melarsoprol and Suramin cleared the parasites and the animals survived up to and beyond 60 days.

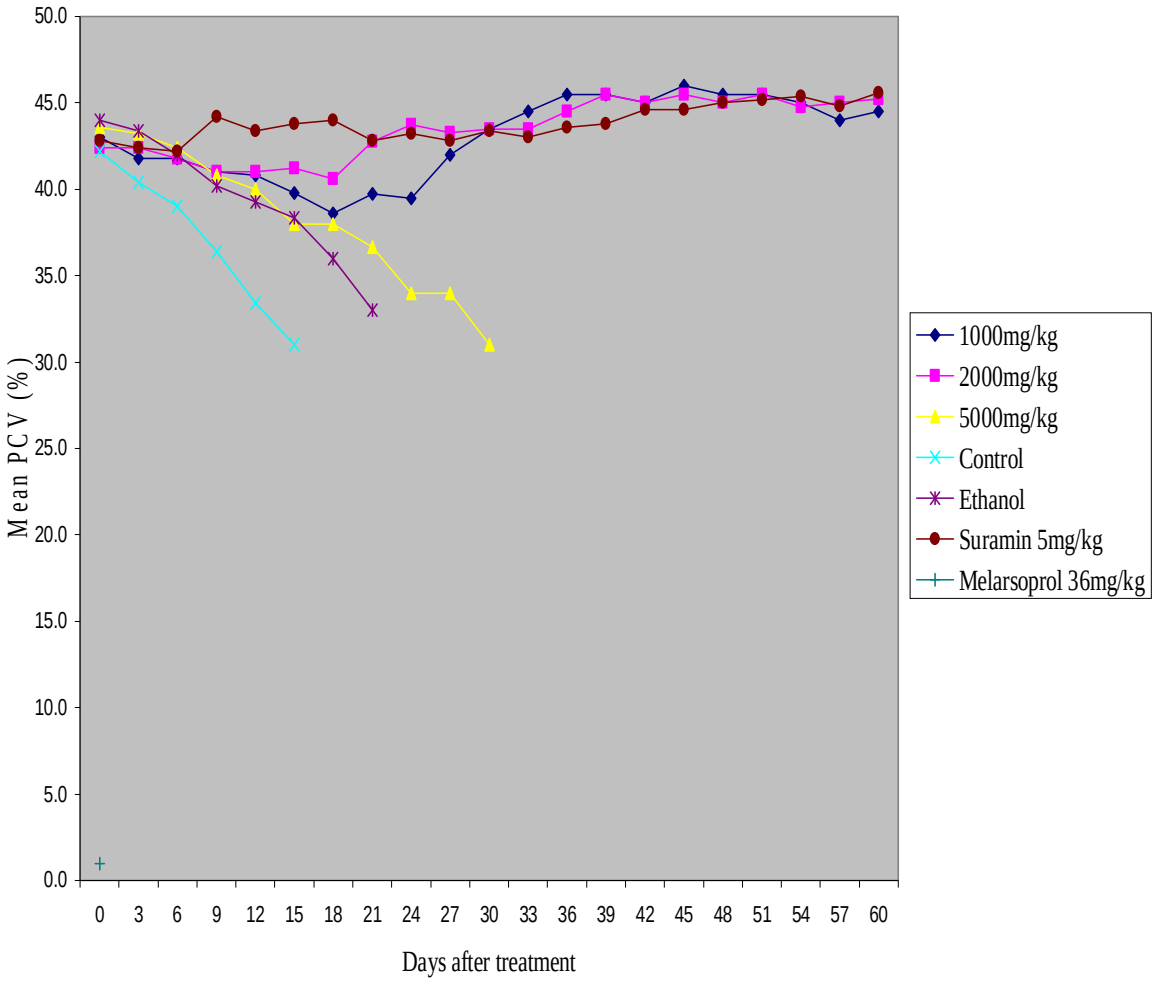
Fig 8: The trend of mean PCV of mice after treatment with *Azadirachta indica* extract



The experiment on packed cell volume (PCV) analysis gave results that were consistent with the observations made on parasitaemia. As shown in graph above, the PCV of mice treated with dichloromethane extracts had sharp reduction of PCV below reference values (42-52%). Ethanol and water extracts had PCV below

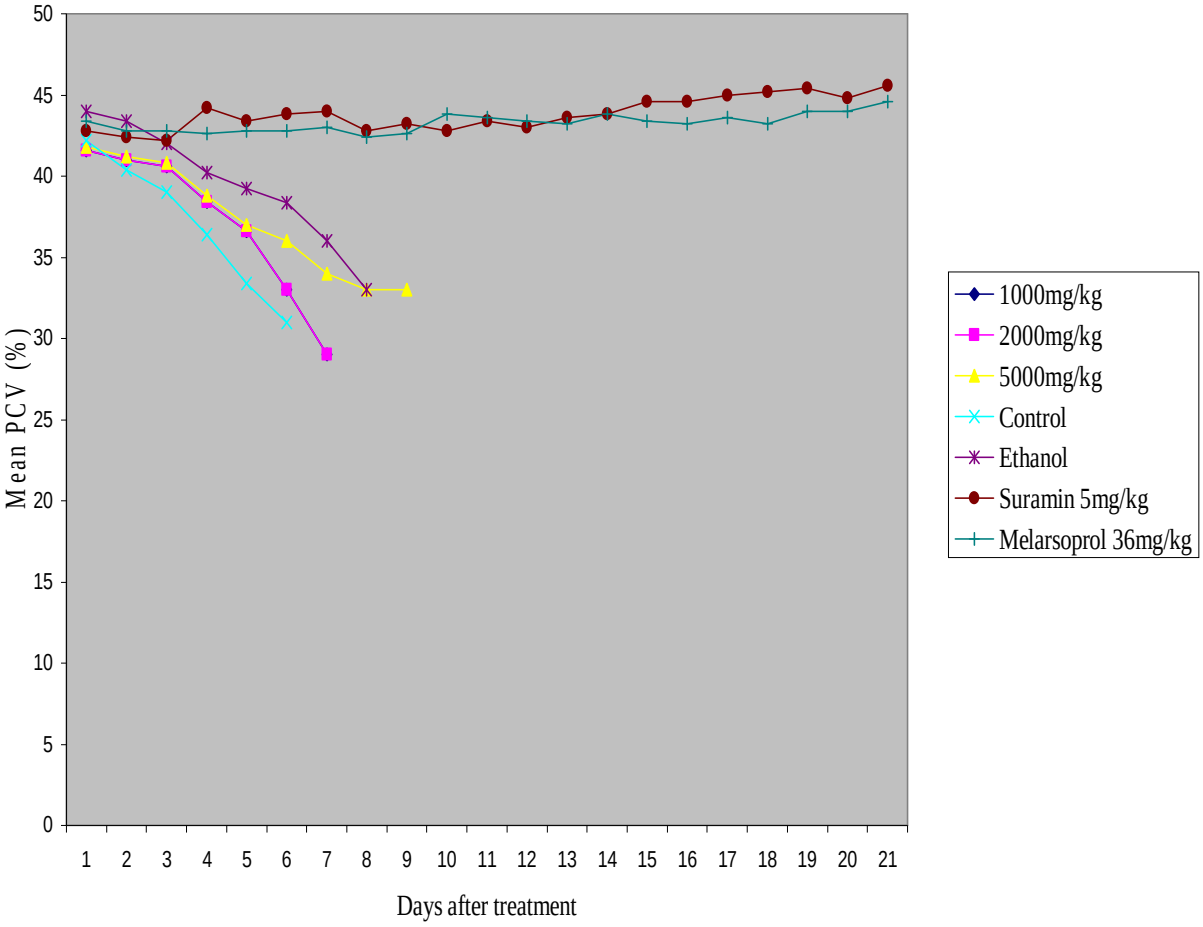
the reference. The animals treated with Melarsoprol and Suramin their PCVs were within the limits (43-44%) (Fig 8)

Fig 9: The trend of mean PCV of mice treated with *Kigelia africana* extract



The experiment on packed cell volume (PCV) analysis gave results that were consistent with the observations made on parasitaemia. As shown in Figure 9, the PCV of mice treated with 2000mg/kg was on average above 43% which was fairly within the reference values of 42-52 for males. The PCV of animal treated with 1000mg/kg was 42% and there is no significant difference between 1000mg/kg and 2000mg/kg ($p=0.004$).The mice treated with 5000mg/kg relative to negative controls, their PCVs were below the reference values (42-52%). The animals treated with Melarsoprol and Suramin their PCVs were within the limits (43-44%) (Fig. 9)

Fig 10: The trend of mean PCV of mice treated with *Artemisia annua* extract



The experiment on packed cell volume (PCV) analysis gave results that were consistent with the observations made on parasitaemia. As shown in graph above, the PCV of mice treated with aqueous extracts had sharp reduction of PCV below reference values (42-52%). Ethanol and water extracts had PCV below the reference. The animals treated with Melarsoprol and Suramin their PCVs were within the limits (43-44%).

4.5 Statistical analysis

This study was set primarily as descriptive. Descriptive study was to describe tolerability, safety and efficacy. To asses the therapeutic effects, the parasitological data of treated and control animals were statistically analyzed using Student's t-test (Minitab computer program). P-Values <0.05 were considered as significant, those>0.05 as not significant. During the experiment, three key parameters were considered; parasitaemia levels, weights and packed cell volume. There was significant reduction in parasitaemia level when concentration of 1000mg/kg and 2000mg/kg of extract as compared to 5000mg/kg of the extract which did not yield significant reduction in parasitaemia level. The statistical summaries of trypanocidal activity see Table 8. Detailed analysis is displayed on Appendix F.

Table 8: Summary of statistical analysis of *Kigelia africana* dichloromethane extract

Parasitaemia levels (log)		
Statistical parameters	Concentration	
	1000mg/kg	2000mg/kg
N	12	18
Mean	2.77	0.688
StDev	2.20	0.922
SE Mean	0.63	0.22
P-Value	0.008	
Body weights (g)		
Statistical parameters	Concentration	
	1000mg/kg	2000mg/kg
N	12	18
Mean	26	25.13
StDev	1.93	1.28
SE Mean	0.54	0.30
P-Value	0.172	
Packed cell volume (PCV) (%)		
Statistical parameters	Concentration	
	1000mg/kg	2000mg/kg
N	12	18
Mean	38.63	41.65
StDev	3.05	0.57
SE Mean	0.85	0.14
P-Value	0.004	

Table 9: Summary of statistical analysis of *Azadirachta indica* dichloromethane extract

Parasitaemia levels (log)		
Statistical parameters	Concentration	
	1000mg/kg	2000mg/kg
N	13	15
Mean	5.98	5.74
StDev	4.15	3.87
SE Mean	12	1.0
P-Value	0.879	
Body weights (g)		
Statistical parameters	Concentration	
	1000mg/kg	2000mg/kg
N	11	14
Mean	29.05	23.29
StDev	2.51	5.05
SE Mean	0.76	1.4
P-Value	0.001	
Packed cell volume (PCV) (%)		
Statistical parameters	Concentration	
	1000mg/kg	2000mg/kg
N	8	10
Mean	39.64	35.65
StDev	2.60	5.16
SE Mean	0.92	1.6
P-Value	0.054	

Table 10: Summary of statistical analysis of *Artemesia annua* aqueous extract

Parasitaemia levels		
Statistical parameters	Concentration	
	1000mg/kg	2000mg/kg
N	10	11
Mean	5.01	4.96
StDev	4.32	4.02
SE Mean	1.4	1.2
P-Value	0.979	
Body weights		
Statistical parameters	Concentration	
	1000mg/kg	2000mg/kg
N	11	10
Mean	24.85	27.12
StDev	2.73	1.86
SE Mean	0.82	0.59
P-Value	0.039	
Packed cell volume (PCV)		
Statistical parameters	Concentration	
	1000mg/kg	2000mg/kg
N	7	8
Mean	37.17	37.88
StDev	4.69	3.9
SE Mean	1.8	1.4
P-Value	0.760	

CHAPTER FIVE

5.0 DISCUSSION

5.1 Results of *In vitro* antitrypanocidal activity

Two of the five plants had at least one extract, often the leaf/ stem bark dichloromethane extract with MIC values≤18.2µg/ml against trypanosomes. The dichloromethane *extracts* were most active followed by methanol extracts and aqueous extracts in that order. The hexane extracts and ethyl acetate extracts were mild /inactive against trypanosomes.

All the 3 extracts of *A. indica* showed some activity against *T.b. rhodesiense.* No marked differences in the sensitivities of the extracts against trypanosomes were observed. However, for *Azadirachta indica* extract from leaves gave MIC value of 18.2µg/ml. All the MIC values for extracts were observed to be high compared to the values obtained for commonly used trypanocidal drugs such as Suramin. The *Azadirachta indica* dichloromethane extract had marked activity when compared to the standard drug used as a reference, Suramin had MIC value of 11.72µg/ml while the extract had 3.91µg/ml. However, Melarsoprol was more active than both, with MIC value of 0.014µg/ml.

The best extracts against *T.b. rhodesiense* KETRI 3798 were those obtained from *Azadirachta indica.* These extracts gave MIC values of 3.91, 11.42 and 18.2µg/ml with dichloromethane (stem barks), methanol (stem bark) and dichloromethane (leaves) extracts respectively. Limonoids have been identified in both stem bark and leaf of *Azadirachta indica* (Biswas, *et al*., 2002). Literature survey indicated

that limonoids have antitrypanosomal activity, which was demonstrated by study which was carried out on *Trachilia emetica* extract from root barks (Newman, *et al*., 2003). This extract was highly active against *T.b. rhodesiense*. It can be thought that the remarkable selectivity against trypanosomes is due to the presence of limonoids, which is contained in the plant (Newman, *et al*., 2003). These values are in line with what Hoet *et al*., 2004 reported. They observed the MIC values of the crude extracts of *Trachilia emetica* and *Hymenocardia acida* (MIC≤19µg/ml) against *T. b. brucei*.

Bidens species are largely used in traditional medicine as a source of antibiotics, liver protection, antimalaria and their activities are explained by abundant production of phenyl acetylenes. Biological evaluations have been performed with 1-phenyl-3,5,7-heptatriyne, present in the leaves of *Bidens pilosa* plants (Dounga *et al*., 1983) as well as other phenyl acetylene compounds, some of which required ultraviolet light for expression of the activity. The phenyl acetylene compounds and derivatives (thiophene), for instance, present potent phototoxic activity against a range of organisms, including fungi, bacteria, nematodes, insects and protozoan (Arnason *et al*., 1980, Wat *et al*., 1979).

Bidens pilosa aqueous extracts (leaves) showed moderate activity (MIC=62.5µg/ml). It is noteworthy that *B. pilosa* has been investigated for other antiparasitic activities. Ethanolic extract of the plant was active against *P. falciparum* drug resistant parasites *in vitro* and in rodent malaria *in vivo* making it a good candidate for further development as phytotherapeutic agent (Andrade-

Neto *et al.*, 2004). It is possible that the plant extracts may have shown more activity against other subspecies of trypanosomes as opposed to the low activity seen with *T.b. rhodesiense* in this study. Also the mode of preparation of extracts and the period the plant materials were collected could partly be responsible for inactivity observed.

Tannins and flavonoids are contained in *Bidens pilosa* from the literature survey (Biswas *et a*l., 2003). However, phytochemical screening has not been done. The observed activity may be due to tannins and flavanoids. *Annona senegalensis* root extract has been shown to possess antitrypanosomal activity due to tannins contained in the plant**.** *Cassia sleberiana* DC have flavanoids which have been demonstrated to have antitrypanocidal activity (Duquenols and Anto, 1968, Kerharo and Adam, 1974, Sultana and llyas, 1987). These evidences may explain the observation that *Bidens pilosa* has activity against trypanosomes.

Of the five plants species screened against *T.b. rhodesiense* KETRI 3798, only three plants species showed negligible or no antitrypanosomal activity (MIC≥100µg/ml) for all the extracts (*Kigelia africana*, *Artemesia annua* and *Senna didymobotrya*).

The methanol, dichloromethane and water extracts of *Artemesia annua* had the following activities, 125µg/ml, 500µg/ml and 500µg/ml compared to the commercial drug Dihydroartesinin which had antitrypanocidal activity of 125µg/ml. When the activities of the extracts of *Artemesia annua* and the artemisinin drug (Dihydroartemesinin) were compared there were no significant

differences. With such high values, it can be concluded that the plant had no effect against *T.b. rhodesiense*. The major challenge experienced with *Artemesia annua* extracts was solubility factor. The extracts of *Artemesia annua* and dihydroartemesinin drug did not dissolve in water and other solvents used. This may have affected bioavailability of the drug. However, Annamed (2004) has showed that taking that herb in form of tea cures patients of malaria, with a cure rate of better than 90%.

The lack of an *in vitro* antitrypanosomal activity in these plants (*Kigelia africana*, *Artemesia annua* and *Senna didymobotrya*) may not necessarily imply the same *in vivo* since compounds may either act as pro-drugs (which must undergo metabolic changes to achieve the required activity). Besides, the presence of bioactive compounds depends on many factors such as the season, age, intra-species variation, part collected, soil and climate. Whereas some plant extracts may not display *in vitro* activity they may display *in vivo* activity (Gessier *et al*., 1995) or vice versa.

The *in vitro* and *in vivo* results of this study are comparable with those from trials by Freiburhaus *et al.*, 1996 who revealed a higher antitrypanosomal activity where 15% of 178 extracts tested in LILIT had MIC values of <19µg/ml . They were however, less active than the commercially available trypanocidal drugs suramin and melasoprol. All the 15 extracts tested in LILIT by Hoet, *et al*., (2004) also showed a higher trypanocidal activity with MIC <19µg/ml, but they were all toxic to mammalian cells. Like our *in vivo* studies, other authors also reported that

it was not possible to completely eliminate the trypanosomes from blood of infected animals. The drugs only affected the prolongation of the life of the infected experimental animals (Asuzu & Chineme, 1990).

However, due to the complex composition of the extracts, it is not possible at this stage to identify the compounds which may be responsible for the observed activities. This should be done by bio-guided fractionation, isolation and characterization of pure compounds.

5.2 *In vivo* trypanocidal activity

The results of *in vivo* studies showed that only one of the three extracts tested was able to stop trypanosomes from establishing and multiplying in the animals. The extract of *Kigelia africana* fruits *(*dichloromethane) tested at two doses was effective, curing 60% of the animals treated. The treatment was administered 24hours post- infection, therefore, no trypanosomes in circulation after treatment. There were no significant differences between 1000 mg/kg and 2000 mg/kg ($p=0.008$). However, the administration of this extract at dose of 5000mg/kg (i.p, twice daily for three days) formed bolus at the point of injection. The drug failed to reach the target sites or organ possibly due to low absorption. Dilutions were made to reduce viscosity and possibly enhanced absorption. Also, the administration of this extract at dose of 1000mg/kg (i.p. twice daily for three days) was observed to have low activity and hence not effective compared to the dose of 2000mg/kg, body weight (bwt). However, the administration of this extract at dose of 2000mg/kg, bwt, was found effective against early stage of the disease in 60% of the animals with no detectable side effects.

Kigelia africana extract was evaluated at three concentrations; 1000mg/kg, 2000mg/kg and 5000mg/kg. The survival time were 41.5 days, 50.8 days and 18.4 days, respectively compared to 11.4 and 14.2 days of the untreated controls (Table 8). The survival time of 50.8 days compared with the survival time of 60 day of mice treated with extract and Melarsoprol respectively, were not significantly different($p=0.012$). Of the doses tested, 2000mg/kg was the most effective with curative rate of 60%. The experiment were initially carried out with 5 mice and repeated with 10 mice at the same dose of 2000mg/kg, bwt. Six mice were cured and this translated to cure rate of 60%. The two experiments gave similar results and this confirmed that the experiment was reproducible under the same conditions. The cured animals were held and observed for 45 to 60 days beyond the 60 days necessary to demonstrate cures. All the mice had normal weight gains and no apparent ill effects. Packed cell volume was determined and the values obtained to which drug was administered in mice orally and i.p was more than 43%. The reference values were 42-52% for males and 36-48% for females. The extracts used did not affect packed cell volume values. The levels of certain plasma constituents decrease due to damage to the organ or tissues responsible for their synthesis. This was true for PCV values of mice treated with *Azadirachta indica* dichloromethane extract and *Artemesia annua* aqueous extract were less than 40% on average. This activity of *Kigelia africana* extract is thought to be due to the increase of oxygen consumption and stimulation of hydrogen peroxide production in the protozoa cell. Protozoa do not have the same biochemical mechanism as mammalian cells for dealing with excess peroxide and consequent

oxygen free radicals, and so this process is used as a target in the search for novel antiprotozoal compounds. The dichloromethane extract showed no toxicity when it was administered to the mice and so these studies provide further evidence of the safety and efficacy against sleeping sickness of the extract of the *Kigelia africana* fruits. Further evaluation is required using purified fractions. This should be done by bio-guided fractionation and isolation and characterization of pure compounds.

Most of the studies on the biological activity of *Kigelia africana* extracts and constituents have been connected in some way to its traditional uses. In many parts of Africa the extracts of *Kigelia africana* bark have been used as a treatment for sexually transmitted Diseases (STDs). An unpublished ethnobotanical survey amongst traditional healers of the Ibos in south eastern Nigeria conducted by Dr. Akunyili from University of Nigeria revealed that they use an aqueous or dilute alcohol extract of *Kigelia pinnata* roots for a treatment of STDs. Extracts of the roots equivalent to those obtained using the traditional methods were found to contain the iridoids. The extracts, as well as two of the isolated iridoids, were tested and also their 1/10 and 1/100 dilutions, against four representative species of bacteria viz. *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli and Pseudomonas aeruginosa*, and the yeast *Candida albicans* in the absence and presence of the enzyme emulsion (Akunyili *et al*., 1991 and 1993). This enzyme converts catalpol-type iridoids to their more antimicrobially active non-sugar containing aglycones. The growth of the organism in culture broth was assessed by measuring the turbidity of the solution. The results showed that the aqueous extract had strong activity, even in the absence of emulsion, against all the bacteria tested

but especially against the yeast *C. albicans.* This was of interest since Candida infections are common opportunistic infections of genito-urinary tract and the traditional use of this plant extract might alleviate this in sexually transmitted diseases. Both isolated iridoids also showed activity in the same way but did not seem to account for all the activity shown by the extracts.

Further studies using a wider range of micro-organisms and compounds showed that the naphthoquinoides from the stem bark also had a moderate antimicrobial effect.

Kigelinone 7 and Isopinnatal 11 were the most active compounds with minimum inhibitory concentrations (MIC) not less than 50 µg mL^{-1} against any other bacteria or yeasts tested (Akunyili, *et al*., 1991 and 1993). However, it should be pointed out that the naphthoquinoids are unlikely to exist in sufficiently high concentrations in aqueous or dilute alcohol extracts such as those used in traditional medicine, particularly amongst the white population in southern Africa. From the above reports, point to evidence that the extracts of the roots and stem bark of *Kigelia africana* possess antimicrobial activity. In addition to the traditional uses against genito-urinary infections, this observed effect may also be relevant to the use of such preparations gainst skin conditions which might be caused by micro-organisms such as dermatophytes (ringworm and associated conditions) or bacteria (complications of acne, boils and similar diseases). Apart from reports cited above, several independent anecdotal reports were received by our group of the usefulness of *Kigelia* extract in treating skin cancer

Although the nature of the extract and the part of the plant from which it was derived were at first unclear, further investigations revealed that a 50% ethanolic extract of the fresh fruits was most commonly used (Akunyili, 1991 and 1993). Comparison of the activity of tested extracts with commercial Suramin and Melarsoprol *in vitro* and *in vivo* studies showed that the classical drugs were much more active than some extracts. For instance, the activity of the most active extracts in 72 hours incubation test was up to 3.9µg/ml and 18.9µg/ml, respectively less effective than Melarsoprol but slightly more effective than Suramin. In mice the activity of dichloromethane extract of *Kigelia africana* was more effective than other extracts.

However, due to difference in study protocol, it is difficult to compare results between the different studies. For instance, the trypanocidal activity was expressed by some authors by MIC values and by other authors by the trypanocidal inhibition concentration (e.g. IC_{50}) (Freiburghaaus, *et al*., 1997). Besides, the duration of the incubation of the extracts differed as well. Therefore, the protocols for evaluation of trypanocidal activity of crude extracts and active principal should be standardized to allow comparisons of results of different studies.

5.3 Acute toxicity

A good drug is one that distributes to all potential sites of infection in the body. If toxic, the drug will therefore exert its effects on multiple tissues and body organs. This is especially true for the liver and the kidney, the main sites of metabolism and drug excretion respectively. The idea behind the oral administration of these plant extracts to mice was that if the doses prescribed by the traditional

practitioners are not toxic to the patients, they should not be toxic to mice, under the same conditions, and may even be active. Based on the results in acute toxicity studies, It was concluded that the doses 250mg/kg, 500mg/kg and 1000mg/kg of the plant extracts given oral/i.p appeared to be non-toxic. It was shown that the doses administered did not produce any toxic symptoms. Besides, pathological analysis indicated that the observed tissues and organs had no abnormalities or histopathological changes were not seen.

5.4 Conclusion

In conclution, the results of the present study showed that *Kigeli africana* dichloromethane extract out of the 5 plants screened had antitrypanocidal activity in mice against *T.b. rhodesiense* KETRI 3798. This requires further purifications and evaluations. The observations of this study indicate that *Kigelia africana* should be further investigated as a lead compound in treatment of early stage of HAT.

However, other extracts obtained from these plants had low activity in comparison with conventional trypanocides as observed in our studies. This problem could be overcome by isolating and preparing drugs with high concentration of active ingredients.

This method of testing the sensitivity of trypanosomes against plant extracts is easy and inexpensive, and could be applied to other areas of research on tropical diseases.

5.5 Recommendations

Following the identification, extraction and testing of methanol, dichloromethane and water extracts of various parts of *Azadirachta indica*, *Artemesia annua*, *Senna didymobotrya*, *Bidens pilosa* and *Kigelia africana*, it was demonstrated that the dichloromethane extract of *Kigelia africana* was most active, with cure rate of 60%.

It is therefore recommended that:

1. The extract be further purified using the activity guided fractionation, isolation and characterization of pure compounds
2. That the extract, once purified, be tested against other trypanosome species that affect both humans and animals both *in vitro* and *in vivo*.
3. Biochemical investigations, be carried out to enhance understanding of mechanisms of action.
4. That the pure compound be tested against drug resistant trypanosome isolates.

REFERENCES

Action for **N**atural **M**edicine **(A**n**n**a**m**ed**), 2004.** The wondrous antimalaria plant comes to Ethiopia. *J. Planta medica* 18, 122-9

Akunyili, N. D., Houghton, P. .J., Amala Kama**, 1991.** Antimicrobial activities of stem bark of *Kigelia pinnata*. *J. Ethnopharmacology* 35, 173-177.

Akunyili, **1993**. Monoterpenoids from *Kigelia pinnata*. *J. Phytochemistry* 32(4), 1015-1018**.**

Adewumi C. O Agbedahunsi J. M, Adebajo A. C, Aladesanmi A. J, Murphy N., Wando, **2001**. Screening of Nigeria medicinal plants for trypanocidal properties. *J. Ethnopharcology*, 77, 19-24.

Adrade-Neto V. F,, Brand M. G,, Oliveira F. Q,, Casali V. W,, Njaine B,, Zali M. G,, Oliveira L. A,, Krettle A. U,, **2004.** ***J.Phytother Ras*,**18 (8), 634-9.

Asuzu I. U, Chineme C. N**., 1990.** Effects of Murinda lucida leaf extract on ***T.b.brucei*** infection in mice**.** *J. Ethnopharmacology***,** 30, 307-313.

Awadzi, K., **1995.** The chemotherapy of onchocerciasis XVIII. Aspects of treatment with suramin. *J.Trop. Med. Parasitol*. 46, 19–26.

Baltz, T., Baltz, D., Giroud, C. H., Crockett, J., **1985**. Cultivation in semi-defined medium of animal infective forms of *T.brucei*, *T.equiperdum*, *T.evansi*, *T.rhodesiense* and *T.gambiense*, *EMBO J.* 4, 1273-1277.

Barrett, M. P., **1999.** The fall and rise sleeping sickness. Lancet 353, 1113-1114.

Barret M. P, Burchmore R. I. S, Stich A., Lazzari J. O., Frasch A. C., Cazzulo J. J. and Krishna S., **2002**. *Lancet* .362, 1469.

Beentje H. J., **1994.** Kenya trees, shrubs and Lianas National museums of Kenya, 552-560.

Benaim G., **1993**. A calmodulin-stimulated $Ca_2þ$ pump in plasma membrane vesicles from Trypanosoma brucei: selective inhibition by pentamidine. *Biochem. J*. 296, 759–763.

Berger B. J., **1993.** Polyamine and pentamidine metabolism in African trypanosomes. *Acta Trop*. 54, 215–224.

Berger B. J., **1995.** Characterization of pentamidine-resistant *Trypanosoma brucei brucei. Mol. Biochem. Parasitol*. 69, 289–298.

Biswas, Kausik, Ishita, Chattopaday, Ranajit K, Banerjee and Uday Bandyopadhyay,, **2002**. Biological activities and medicinal properties of neem *(Azadirachtaindica)*. *Current science*, 82(11), 1336-1345.

Bizimana N., **1994**. Traditional vertinery practice in Africa, Deutsche Gasellschaft Fur Technische Zusammerbeit(GTZ). Gmbh(Ed.), Schriftereihe der GTZ No.243,ISBN 3-88085-502.Eschborn 917.

Black, J.G., **1996**. Microbiology; Principles and application. Hall.N.J, 260

Bodley, Shapiro, A.L, T.A, **1995**. *Proc.Natl.Acad.Sci.*USA, 92, 3726**.**

Borst, P. and Fairlamb, A.H., **1998**. Surface receptors and transporters of *Trypanosoma brucei*. *Annu. Rev. Microbiol*. 52, 745–778.

Bray, P.G., **2003.** Pentamidine uptake and resistance in pathogenic protozoa: past, present and future. *Trends Parasitol* 19, 232–239.

Bringmann G, Hamm A, Gunther C, Michael M, Brun R, and Mudogo V., **2000.** *J. natural product*,63,1465**.**

Bringmann G, Messer Wolf K, Muhlbacher J, Grune M, Brun R, and Louis A. M., **2002**. *Phytochemistry*,60,389.

Bringmann. G, Hoerr, V. Holzgrabe, U and Stich.A**., 2003**,. *Pharmazie* 58, 243.

Burri C., **1993.** Pharmacokinetic properties of the trypanocidal drug melarsoprol. *J.Chemotherapy* 39, 225–234.

Burri C.**, 2000.** Efficacy of new, concise schedule for melarsoprol in treatment of sleeping sickness caused by *Trypanosoma brucei gambiense*: a randomised trial. Lancet 355, 1419–1425.

Camacho M. D. R., Crof S. L., Phillipson J. D., **2000.** Natural products as source of antiprotozoal drugs. Current Opinion in Anti-infective Investigational Drugs, 2, 47-62.

Camacho M. D, R., Croft S. L., Phillipson J. D, Rocks P, Marshall S. J and Schiff Jr., **2002.** *Phytother.Res*, 16,432

Carter N. S. and Fairlamb, A. H., **1993.** Arsenicalresistant trypanosomes lack an unusual adenosine transporter. *Nature* 361, 173–176.

Carter N. S. and Allen and Unwin, **1995**. Uptake of diamidine drugs by the P2 nucleoside transporter in melarsen-sensitive and resistant *Trypanosoma brucei brucei. J. Biol. Chem*. 270, 28153–28157.

Cenas N .K, Arscott D, Williams C. H., Jr. and Blanchard J. S., **1994.** *Biochemistry*, 33, 2509.

Clarkson, A. B., **1984.** New drug combination for experimental late-stage African trypanosomiasis: DL-a-difluoromethylornithine (DFMO) with suramin. *Am. J. Trop. Med. Hyg.* 33, 1073–1077.

Deborah W. Kioy, Grace Murilla, Mawuli W. Koffi Tsepo**, 1998.** *Africa Journal of Health science*, volume 5 No. 1.

De Koning H. P., 2001. Uptake of pentamidine in *Trypanosoma brucei brucei* is mediated by three distinct transporters: Implications for cross-resistance with arsenicals. *Mol. Pharmacol*. 59, 586–592.

De Koning H. P. and Jarvis, S.M., **2001.** Uptake of pentamidine in Trypanosoma brucei brucei is mediated by the P2 adenosine transporter and at least one novel, unrelated transporter. *Acta Trop*.80, 245–250.

Dreyfuss. G, Allais.D.P, Guinaudeau.H and Brunelon.J., **1987.** *Ann. Pharm. Fr*., (45), 243.

Dounga M., Balansard G., Babadjamian A., David P. T., and Gasquet, M., **1983**. Contribution of letudede *Bidens pilosa* . Identification and activity antiparasitaire De La phenyl heptatriyn-1,3,5. Plantes medicinales ed phenytotherapier 2, 64-75.

Duquenois, P., Anton, R., **1968**. Chemical study of *Cassia sieberiana* leaves. *Planta Medica* 16, 184-10.

Eisler M. C., Brandt J. J., Bauer B., Clausen P. H., Delespaux V., Holmes P.H., Machilla N., Mbwambo H., Mc Dermott J., Mehlits D., Murilla G., Ndungu' J.M,. Peregrine A. .S., Sidibe I., Sinyanywe L., Greerts S.,, **2001**. Standardised tests in mice and cattle for detection of drug resistance in tsetse-transitted trypanosomes of Africa domestic cattle vertinary parasitology, 97, 171-182.

Fairlamb A. H., **1989.** Trypanothione is the primary target for arsenical drugs against African trypanosomes. *Proc. Natl. Acad. Sci.* U. S. A. 86, 2607–2611.

Fairlamb A. H. and Cerami A., **1992.** Metabolism and functions of trypanothione in the Kinetoplastida. *Annu. Rev. Microbiol*. 46, 695–729.

Fairlamb A. H. and Le Quesne, S.A., **1997**. Polyamine metabolism in trypanosomes. In Trypanosomiasis and Leishmaniasis: Biology and Control,pp. 149–161..

Fairlamb A. H. and Bowman, **1998**. Control and surveillance of Africa trypanosomiasis. Report of a WHO expert committee. Geneva WHO, (WHO Technical report series No.881).

Farmworth N. M, Akerele O, Bingel A. S, Soejarto D. D, Guo Z., **1985**. Medicinal plants in therapy, Bulletin of the WHO, 63, 965-981.

Freiburghaus F, Kaminsky R, Brun R., **1996**. Evaluation of Africa medicinal plants for their *in vitro* trypanocidal activity; *Journal of ethnopharmacol*, December, 55(1):1-11.

Freiburghaus F., Jonker S. A., Nkunya M. H. H., Mwasumbi L. B., Brun, R.,, **1997**. *In vitro* trypanocidal activity of some rare Tanzanian medicinal plants. *Acta tropica*, 67,181-185.

Fries D. S. and Fairlamb, A.H., **2003**. Antiprotozoal agents. In Burger's Medicinal Chemistry and Drug Discovery: Chemotherapeutic Agents pp. 1033–1087,

Gessier M. C., Tanner, M., Chollel J., Nkunya M. H. H., Heinnack M., **1995**. Tanzania medical plants used traditionally for the treatment of malaria *In vivo* anti-malaria and *in vitro* cytotoxicity activities. *Phytotherapy Research*, 9, 504-508.

Heby O. **2003.** Polyamine biosynthetic enzymes as drug targets in parasitic protozoa. *Biochem. Soc. Trans*. 31, 415–419.

Herbert, W. J, Lumsden, W. H. R., **1976**. *Trypanosoma brucei*; Rapid "matching"method of estimating the host parasitaemia. Experimental parasitology, 40, 425-431.

Herrera M. R, Machocho A. K, Nair J. J., Campbell E. W, Brun R, Viladomat C, and Bastida J., **2001.** *J. Fitoterapia*, 72, 444.

Herrera M. R, Machocho A. K, Nair J. J, Campbell.E.W, Brun.R, Viladomat.C, and Bastida.J, **2001**. *Planta Med*, 67, 191.

Hirumi Hiroyuki and Kazuko Hirumi., **1994,Feb**. Axenic culture of African trypanosome blood stream form. *Parasitol Today*, 10(2):80-84.

Hoet S., Opperdoes F., Brun R., Adjakidje V., Qwetin-Lacleriq J., **2004**. *In vitro* antitrypanosomal activity of ethnopharmacologically selected Beninese plants. *Journal of Ethnopharmacology*, 91, 37-42.

Hoet, S., Fredricd, Opperdoes, Peto, Brun and Joell Qwetin-Laclerig., **2004**, *Nat.prod.Rep*, 21, 353-364.

Igwe A. C, Onabanjo A. O., **1989**, **Oct**. Chemotherapeutic effects of *Annona senegalensis* in *Trypanosoma brucei brucei*. *Ann Trop Med parasitol*, 83(5): 527-534.

Kaur M., 2002. Suramin's development: what did we learn? *Invest. New Drugs* 20, 209–219.

Keiser J., **2000**. Investigations of the metabolites of the trypanocidal drug melarsoprol. *Clin. Pharmacol. Ther*. 67, 478–488.

Kerharo J. and Adam J. G., **1974**. La Pharmacopee Senegalaise Traditionnelle: *Plants Medicinal et Toxiques. Vigor Freres, Paris*.

Kirby G. C., **1996**. Medicinal plants and the control of parasites: medicinal plants and control of protozoal disease with particular reference to malaria. *Transactions of the Royal Society of Tropical medicine and Hygiene*, 90, 605-609.

Kirchhoff L. V., G. L.Mandell, J. E. Benett, and R. Dolin, Churchill Livingstone, Philadelphia**, 2000**. Principles and practice of effections diseases, ed. 2853-2858.

Krieger S., **2000.** Trypanosomes lacking trypanothione reductase are virulent and show increased sensitivity to oxidative stress. *Mol. Microbiol*. 35, 542–552.

Koide T, Nose M, Inoue M, Ogihara Y, Yabu Y. and Ohta N., **1998.** *Planta Med*., 64,

Konig G. M., Wright A. D., Sticher O., Jurcic K., Offermann F,, Redl K., Wagner, 1998 H., Angerhofer C. K., Pezzuto J. M., **1991**. *Planta Med.*, 57 Supplement, issue 2, A5

Labrana J, Machocho K. A., Kricsfalusy V., Brun R., Codina C., Viladomat F and Bastida, **2002**. *J. Phytochemistry*, 60, 847.

Lan R. Freshney. Culture of animal cells (A manual of Basic techniques), second edition; 43-47.

Legros D., **1999**. Therapeutic failure of malarsoprol among patients treated for late stage of *T.b.g* HAT in Uganda, 92(3), 171-172.

Legros D., **2002.** Treatment of human African trypanosomiasis- present situation and needs for research and development. *The Lancet Infectious Diseases*, 20, 437-440.

Liu Y., **1994.** Inhibition of *In vitro* splicing of a group I intron of *Pneumocystis carinii*. *J. Eukaryot. Microbiol*. 41, 31–38.

Mahidol C., **2005**. Biodiversity and natural product discovery. *Intl. J. mol.med, Adv.sci*. 1(4): 392-397.

Merschjohann K, Sporer F, Steverding D and Wink M., **2001**. *Planta Med*., (67), 623.

Moideen K .S. V., Houghton J. P, Rocks.P, Croft.L.S and Aboagye-Nyame.F., **1999**. *Planta Med.*, 65, 536.

Nakatani and Nakanishi, **1993**. Aspects of treatment with Trachila emetica extracts. *J. Nat. prod.*, 16, 203-7.

Nair J. J., Machocho K. A, Campbel E. W, Brun R, Viladomat F, Codina C and Bastida., **2000**. *J. Phytochemistry*, 54,945.

Neuwinger H. D., **2000**. African Traditional Medicine: A Dictionary of plants Use and Application. *Medpharm*.

Newman D. J., Cragg, G. M., Snader, K. M., **2003.** *J. Nat. prod.*, 66, 1022.

Ojewole, J. K.**, 2000.** Mosquito larvicidal properties of aqueous extract of *Senna didymobotrya*, *Nigeria J. Nat. Prod. and Med*. 2000, 4, 46-47.

Opperdoes F. R., **1989.** Unique properties of glycosomal enzymes. In Parasites: Molecular Biology, Drug and Vaccine Design, pp. 233–246.

Pal A., 2002**.** Evidence for a non-LDL-mediated entry route for the trypanocidal drug suramin in *Trypanosoma brucei*. *Mol. Biochem. Parasitol*. 122, 217–221.

Pépin J. and Milord, F., **1994.** The treatment of human African trypanosomiasis. *Adv.Parasitol*. 33, 1–47.

Phillipson J. D., Wrigth, C .W., Kirby. C. and Warhust., **1993.** Tropical plants as source of antiprotozoal agents. *Recent adv. phytochemistry***,** 27, 1-40.

Ras B., **1998**. Isolation and evaluation of antitryparasitic lead compounds from African medicinal plants, Ph.D. Thesis, Universitat Basel, 216.

Ray J. S., Majunder H. K, Chakaryvarty A. K, Mukhopa-Dhyay S., **1996**. *J. Nat.Prod*., 56, 27.

Roberts S. C., **2002**. S-adenosylmethionine decarboxylase from Gaps and obstacles in the drug discovery process. Leishmania donovani molecular, genetic, and biochemical characterization of null mutants and overproducers. *J. Biol. Chem*. 277.

Salmon-Chemin L, Buisine E, Yardley V, Kohler S, Debreu A. M, Landry V, Sergheraert C, Croft L. S, Krauth-Siegel L. R and Darviuod-Chevert E., 2001, *J.Med.Chem*.,44, 548.

Seigler, D.S., **1985.** Isolation of the constituents terpenoids. *Fitoterapia*, 71, 600.

Scientific working group-Report on Africa Trypanosomiasis 4-8, **2001, June**, Geneva Switzerland**.**

Shahi S. K., **2002.** Over expression of the putative thiol conjugate transporter TbMRPA causes melarsoprol resistance in *Trypanosoma brucei*. *Mol. Microbiol*. 43, 1129–1138.

Shapiro T. A. and Englund, P.T., **1990**. Selective cleavage of kinetoplast DNA minicircles promoted by antitrypanosomal drugs. *Proc. Natl. Acad. Sci*. U. S. A. 87, 950–954.

Shaw A. M. P, Cattand P., **1985.** The costs of different approaches towards HAT. Paper prepared for WHO experts committee on the epidemiology and control of Africa Trypanosomiasis, Ref.TRY/EC/WP/85.17.

Staff. "Clinical Trials Update", Genetic engineering News, 2005, Sept. 15, 5.

Soignet S. L., **1999**. Clinical study of an organic arsenical, melarsoprol, in patients with advanced leukemia. *Cancer Chemother*, *Pharmacol*. 44, 417–421.

Sultana S., Llyas, M., **1987.** A new isoflavone from leaves of *Afrormosia laxiflor*a Harms. Indian Journal of Chemistry, Section B: Organic Chemistry Including Medicinal Chemistry 26B, 799.

Tandon J. S., Srivastava, V., Guru, P. Y., **1991**. *J.Nat. Prod*., 54, 1102.

Tagboto S. and Townson S., **2001**. Antiparasitic properties of medicinal plants and other naturally occurring products. *Advanced parasitol*, 50,199-295.

Tolo F. M, Rukunga G. M, Muli F. W, Njagi E. N,, Njue W, Kumo K, Mungai G. M, Muthaura C. N, Muli J. M, Keter L. K, Oishi E, Kofi-Tsekpo M.W., **2005 Sept**. Antiviral activity of extracts of a Kenyan medicinal plant *Carisa edulis* against herpers simplex virus. *J. Ethnopharmacol*, *27.*

Van Schaftingen, **1987**. Effects of various metabolic conditions and of the trivalent arsenical melarsen oxide on the intracellular levels of fructose 2, 6-bisphosphate and of glycolytic intermediates in *Trypanosoma brucei*. *Eur. J. Biochem*. 166, 653–661.

Vansterkenburg E. L. M., **1993**. The uptake of the trypanocidaldrug suramin in combination with low-density lipoproteins by *Trypanosoma brucei* and its possible mode of action. Acta Trop. 54, 237–250.

Vercesi A. E. and Docampo, R., **1992**. $Ca_2þ$ transport by digitonin permeabilized Leishmania donovani. Effects of $Ca_2þ$ pentamidine and WR-6026 on mitochondrial membrane potential in situ. *Biochem. J*. 284, 463–467.

Vickerman K. and Cox F. G. E., **1967**. The protozoa, in Introductory studies in biology, 1-57.

Waddy B. B., **1970.** Chemoprophylaxis of human trypanosomiasis. In The African Trypanosomiases (Mulligan, H.W., ed.), pp. 711–725.

Walther M. M., **1996**. Intravesical suramin: a novel agent for the treatment of superficial transitional-cell carcinoma of the bladder. *J. Urol*. 14 (Suppl. 1), S8–11.

Wang Z. F., **2000**. Inhibition of Trypanosoma brucei gene expression by RNA interference using an integratable vector with opposing T7 promoters. *J. Biol. Chem*. 275, 40174–40179.

Williamson, David, **2005**, August." Compound migth defeat African sleeping sickness, clinical trial beginning this month", University of North Carolina, 25.

World Health Organisation (WHO), **1996**. Control and surveillance of African trypanosomiasis. Report of a WHO Experts committee, World Health Organisation technical report series, World Health Organisation, Geneva, 1998.Yardley.V, Snowdon.D, Croft.L.S and Hazra.B. *Phytother. Res*, (10), 559.

WHO. **1998**. Control and surveillance of Africa trypanosomiasis. Control and surveillance of Africa trypanosomiasis. Report of a WHO expert committee. Geneva WHO, 1998 (WHO Technical report series No.881).

Youan B. B. C, Coulibaly S., Miezen, T.B., Doua, F., Bamba, M., **1997**. *In vivo* evaluation of 16 plant extracts on mice inoculated with *T.b. gambiense*. Bulletin of WHO, 75, 343-348.

Zhang Y., **2002**. Pentamidine inhibits catalytic activity of group I intron Ca.LSU by altering RNA folding. Nucleic Acids Res. 30, 2961–2971, 5902–5909.

APPENDICES

Appendix A: Classification of plants

(a) ***Azadirachta indica***

Kingdom – Plantae – Plants

Sub-Kingdom- Tracheobionta – Vascular plants

Super-Division – Spermatophyta – Seed plants

Division – Magnaliophyta – flowering plants

Class – Magnaliopsida – Dicotyledons

Sub-Class – Rosidae

Order – Sapindales

Family – Melieaceae – Mohony family

Genus – *Azadirachta*

Species – *indica*

(b**) *Bidens pilosa***

Kingdom – Plantae – Plants

Sub-Kingdom- Tracheobionta – Vascular plants

Super-Division – Spermatophyta – Seed plants

Division – Magnaliophyta – flowering plants

Class – Magnaliopsida – Dicotyledons

Sub-Class – Asteridae

Order –Asterales

Family – Asteraceae – Aster family

Genus – *Bidens*

Species – *pilosa*

(c) ***Artemesia annua***

Kingdom – Plantae – Plants

Sub-Kingdom- Tracheobionta – Vascular plants

Super-Division – Spermatophyta – Seed plants

Division – Magnaliophyta – flowering plants

Class – Magnaliopsida – Dicotyledons

Sub-Class – Asteridae

Order – Asterales

Family – Asteraceae – Aster family

Genus – *Artemesia – L-soge brush*

Species – *annua*

(d) ***Kigelia africana***

Kingdom – Plantae – Plants

Sub-Kingdom- Tracheobionta – Vascular plants

Super-Division – Spermatophyta – Seed plants

Division – Magnaliophyta – flowering plants

Class – Magnaliopsida – Dicotyledons

Sub-Class – Asteridae

Order – Scrophyilariales

Family –Bignoniaceae –Trumpet family- creep

Genus – *Kigelia*

Species – *africana*

(e) ***Senna didymobotrya***

Kingdom – Plantae – Plants

Sub-Kingdom- Tracheobionta – Vascular plants

Super-Division – Spermatophyta – Seed plants

Division – Magnaliophyta – flowering plants

Class – Magnaliopsida – Dicotyledons

Sub-Class – Rosidae

Order – Fabales

Family – Fabaceae – Pea family

Genus – *Senna*

Species – *didymobotrya*

Appendix B: Baltz (B and B modified) medium

Seven stock solutions were prepared and stored separately (as below) and mixed only when ready to use the medium.

Stock solution 1- MEM medim

1. The following were weighed as indicated below;

L-Threonine -48mg

D-Glucose - 1g

HEPES 5.95g

$NAHCO_3$ 2.2g

MEM-NEAA 10ml

MEM powder 100ml

2. The above were dissolved in sterile reagent water and made to 1 litre mark in volumetric flask.
3. Adjusted the solution pH to 7.2 (using 1N NaOH or conc.HCl)
4. The sterile was filtered using 0.22µm pore size filter.
5. Made aliquots 250ml or appropriate aliquots to avoid frequent thawing and freezing.
6. Stored at -20ºC

Stock solution 2 – Nucleosides

1 The following salts were weighed as below;

Chemical	Weight
Hypoxanthine	3.6mg
Thymidine	3.9mg
Adenosine	21.4mg

2. All these powders were transferred into a 5ml/10ml beaker with magnetic stirrer.

3. Added approximately 5ml reagent water and while stirring, added a few drops of 1N NaOH solution till the powder completely dissolved.

4. Transferred the solution into a volumetric flask.

5. Rinsed the beaker using little amounts of reagent water and put the water in the flask.

6. The volume was made to10ml in volumetric flask by reagent water and stored at -20ºC until required.

Stock solution 3 –Pyruvate

1. 220mg of sodium pyruvate was weighed accurately.
2. Transferred into 10ml volumetric flask.
3. And dissolved with a few mililitres of reagent water.
4. The volume was made to 10ml in volumetric flask.
5. And stored at -20ºC until required.

Stock solution 4 – L-Glutamine

1. 293mg/10mls of solution required was weighed accurately.

2. 50mls of MEM was added to rehydrate lyophilized L-glutamine.

3. 5mls solution aliquots were made into bijou bottle and stored at -20ºC until required for use.

Stock solution 5 – 2-Mercaptoethanol (0.2mM final conc.) (Fresh solution was prepared each time)

1. 10mls of reagent water was measured into capped tube and added 14µl of 2-Mercaptoethanol and mixed thoroughly.
2. And used it immediately.

Stock solution 6 – Gentamycin (optionl)

1. Weighed accurately 50mg/ml solution, dissolved in water and stored at +4ºC.

Stock solution 7 – Serum

1. Obtained horse blood from jagular vein using 50ml centrifuge tube.
2. The blood was left in the room temperature to separate.
3. Centrifuged at 3000rpm for 10 minute.
4. Removed the supernatant (serum).
5. Pooled all the serum into a filter unit and allowed to filter.
6. Heated the filtrate to inactivate.
7. Aliquoted into tubes small amounts approximately 50mls serum.
8. And stored at -20ºC until required.

Mixing stock solution when in use

1ml of 1.5mM Bathocuproline sulfonate, 1ml of 1.5mM L-cystein, 1ml of hypoxathine, 1ml of 0.2mM of 2-mercaptoeyhanol, 1ml of sodium pyruvate, 1ml of 0.16 of thymidine and 2mls Gentamycin were transferred into measuring cylinder. And 20 mls of horse serum was added into the measuring cylinder. 72mls of modified Iscoves' medium and 100ml mark was obtained. The mixture/solution was filtered by sleving pumb (Done in the laminar flow hood) and filtrate was transferred into a small bottle and stored at -20ºC.

Appendix C: Preparation of DE 52 and test evaluation

DE52 was put in a measuring cylinder up to 300ml mark and to up by distilled water to 1000 mark. It was stirred and allowed to settle for 30 minutes. The water was pored away and added distilled water again, stirred and allowed to settle for 30 minutes, washed and the sequence was repeated again, off the fines in buffer times 1 and added heparin at 10 units/ml.

Running DE 52 Column

To the PSG buffer with 10% glucose, DE 52 was added to the column and allowed the cellulose to settle. When it was compacted, PSG buffer was ran through 3 times s the volume. The blood which was collected from mice was spun at 3000 rpm for 15 minutes at 4ºC. Trypanosomes layer was carefully removed using a needle and syringe (Red blood cells were also gotten as well). Respun in 15ml centrifuge tubes for 15 minutes at 3000 rpm. White fluffy layer was again removed and loaded into the column. The column was ran through until PSG 1% glucose, pH8 had washed through trypanosomes.

Test evaluation

The test was read under inverted microscope at 100-fold magnification. In every row the highest dilution of either standard or samples with no motile trypanosomes of normal shape was determined. The concentration in this well defined as the minimum inhibitory concentration (MIC).

Determination $MIC*D*2^n=C_x$

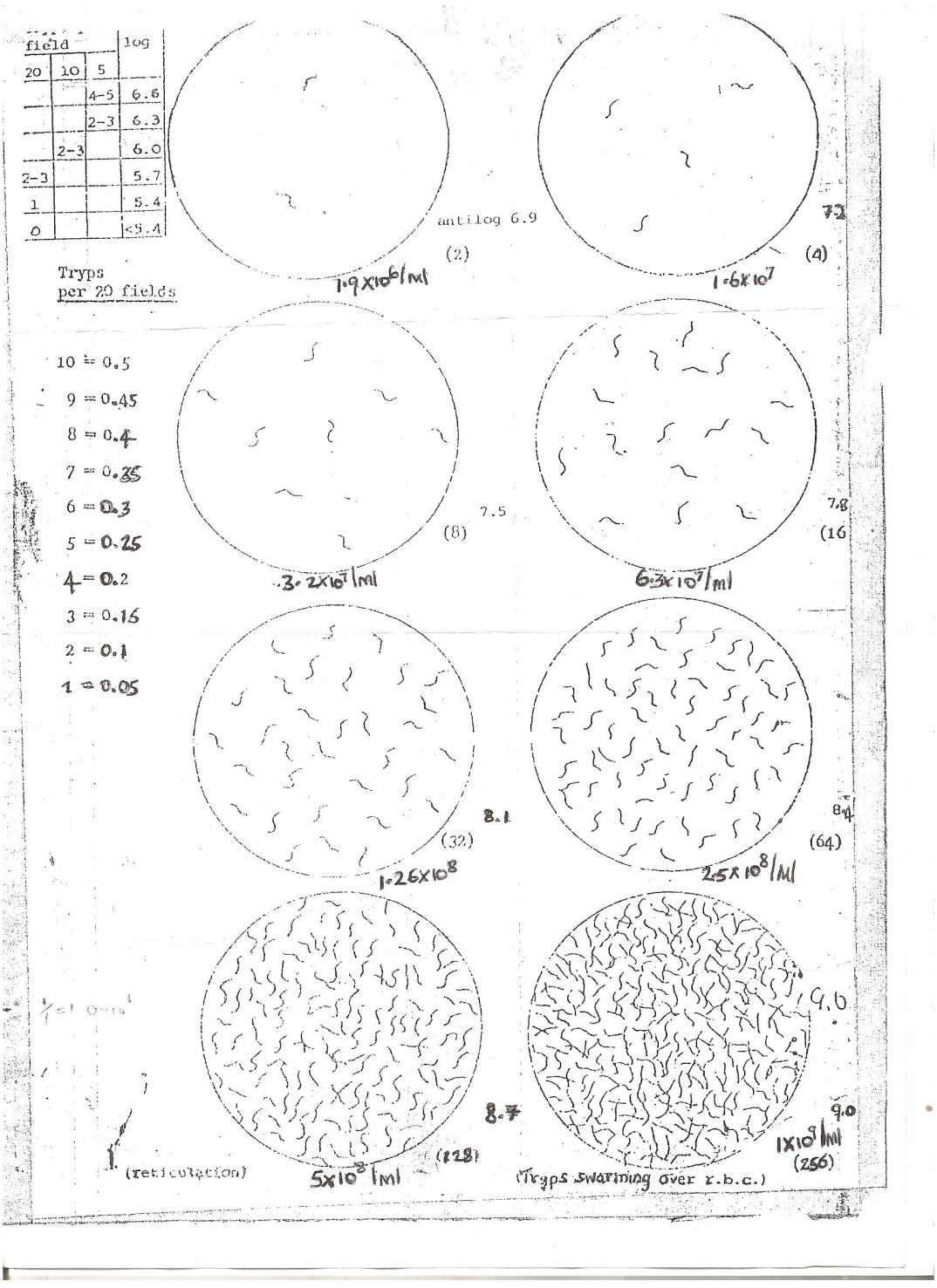
MIC= Minimum Inhibitory Concentration.

D= Dilution of drug by medium in the wells of column 11 (Maximum concentration).

n= Steps of dilution to the first well with no living trypanosomes.

C_x= Concentration of drug solution.

Appendix D: Parasitaemia matching/



Appendix: E

Table 11: Change in mean body weights of mice treated with various plant extracts

	Days after treatment/body weights																				
Drug name	0	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48	51	54	57	60
K.africana 1000mg/kg	26.2	27.5	26.9	28.9	28.4	28.0	25.7	25.5	25.5	22.6	24.5	24.3	23.4	25.9	24.6	24.7	24.8	24.9	24.9	25.2	25.3
K.africana (2000mg/kg	23.9	26.3	26.4	26.9	27.6	25.8	26.0	26.1	26.2	24.5	24.1	24.1	24.1	24.0	24.1	23.9	25.4	25.4	25.9	26.3	26.4
K. africana 5000mg/kg	22.0	26.7	25.3	25.3	23.3	23.3	23.1	23.8	24.8	23.2	23.0	23.0	23.1	23.0							
A. indica 1000mg/kg	31.8	30.7	31.5	32.1	31.2	28.4	27.3	27.2	27.1	26.7	25.1										
A. indica 2000mg/kg	27.7	27.6	27.5	28.1	27.8	27.3	24.3	24.0	24.0	23.6	16.0	16.0	15.9	15.9							
A. indica 5000mg/kg	27.4	24.9	25.5	25.9	26.0	24.8	23.3	23.3	23.2	23.4	23.2	23.0	21.9	21.6							
A.annua 1000mg/kg	27.9	26.8	27.2	28.4	27.3	24.3	23.6	22.0	21.9	21.8	21.7										
A.annua 2000mg/kg	27.9	27.6	27.4	28.5	27.5	27.1	25.0	24.5	24.6	30.5	19.6	19.0									
A. annua 5000mg/kg	29.3	30.2	29.1	29.4	29.1	27.2	23.8	23.7	23.4	23.4	21.3	21.0	21.0								
Control	26.1	27.9	27.9	28.4	27.6	28.2	26.9	24.6	24.3	28.2											
Ethanol control	26.6	29.1	28.4	30.1	29.8	28.9	25.9	25.6	25.4												
Suramin 5mg/kg	24.6	29.0	29.4	29.7	28.8	29.0	30.5	30.6	30.6	31.8	31.9	32.2	32.4	32.1	32.6	33.1	33.2	33.1	33.3	33.2	33.2
Melarsoprol 36mg/kg	25.5	30.2	28.9	28.2	27.4	27.7	28.4	28.5	28.6	29.8	29.8	29.8	29.8	29.9	29.9	29.9	29.9	30	30.1	30.1	30.2

Table 12: Change in mean Parasitaemia levels in mice treated with various plant extracts

	Days after treatment/log parasitaemia																				
Drug name	0	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48	51	54	57	60
K.africana 1000mg/kg	0	0	0	0	2.2	2.2	2.2	2.2	2.5	2.8	3.5	3.8	4.0	4.1	0	0	0	0	0	0	0
K.africana (2000mg/kg	0	0	0	0	1.4	1.1	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	0	0	0	0	0	0	0
K. africana 5000mg/kg	0	0	0	0	7.3	7.7	8.0	8.2	8.8												
A. indica 1000mg/kg	0	0	0	0	8.8	8.7	8.6	8.7	8.7	8.7	8.4	8.4	8.7								
A. indica 2000mg/kg	0	0	0	0	3.1	6.3	8.6	8.4	8.5	8.7	8.5	8.6	8.4	8.6	8.7	9.0					
A. indica 5000mg/kg	0	0	0	0	4.1	5.4	8.4	8.5	8.4	8.6	8.8	8.8	8.9	8.7	9.0						
A.annua 1000mg/kg	0	0	0	0	7.5	8.2	8.5	8.6	8.7	8.7	9.0										
A.annua 2000mg/kg	0	0	0	0	5.4	7.8	8.0	8.3	8.3	8.5	8.4										
A. annua 5000mg/kg	0	0	0	0	6.6	8.5	8.8	8.9	9.0	8.5	85	8.7	8.7	9.0							
Control	0	0	0	7.7	8.9	9.0	8.8	9.0	9.0	9.0											
Ethanol control	0	0	0	8.4	8.1	8.7	8.9	8.9	8.9	9.0											
Suramin 5mg/kg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Melarsoprol 36mg/kg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 13: The trend of mean PCV of mice treated with various plant extracts

	Days after treatment/PCV																				
Drug name	0	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48	51	54	57	60
K.africana 1000mg/kg	43	41.8	41.8	41	40.8	40.8	39.8	36.6	39.8	39.5	42	43.5	45.5	45.5	45	46	45.5	45.5	45	44	44.5
K.africana (2000mg/kg	42.4	42.4	41.8	41	41	40.6	42.8	43.8	43.3	43.5	43.5	44.5	45.5	45	45.5	45	43.5	44.8	45	45.3	45.3
K. africana 5000mg/kg	43.6	43.2	42.4	40.8	38	38	36.7	34	34	31											
A. indica 1000mg/kg	43.2	42	41.2	41	39.4	36.5	37														
A. indica 2000mg/kg	43.2	41.8	40.8	38.6	35.8	33.6	32.5	31	30.7	28.5											
A. indica 5000mg/kg	42.6	42.4	41.4	40.4	39.4	37.5	38.3	33	33												
A.annua 1000mg/kg	41.6	41	40.6	38.4	36.6	33	29														
A.annua 2000mg/kg	41.6	41	40.6	38.4	36.6	33	29														
A. annua 5000mg/kg	41.8	41.2	40.8	38.8	37	36	34	33	33												
Control	42.2	40.4	39	36.4	33.4	31															
Ethanol control	44	43.4	42	40.2	39.3	38.3	36	33													
Suramin 5mg/kg	42.8	42.4	42.2	42.2	43.4	43.8	44	42.8	43.2	42.8	43.4	43	43.6	43.8	44.6	44.6	45	45.2	45.4	44.8	45.6
Melarsoprol 36mg/kg	43.4	42.8	42.8	42.6	42.8	42.8	43.0	42.4	42.6	43.8	43.6	43.4	43.2	43.6	43.4	43.2	43.6	43.2	44.0	44.0	44.6

Appendix F: Statistical analysis of Parameters

Parasitaemia levels

- Two-Sample T-Test and CI: *Kigelia africana* 2000mg/kg, *Kigelia africana* 1000mg/kg

Two-sample T for 2000mg/kg vs 1000mg/kg

	N	Mean	StDev	SE Mean
2000mg/kg	18	0.688	0.922	0.22
1000mg/kg	12	2.77	2.20	0.63

Difference = mu 2000mg/kg - mu 1000mg/kg

Estimate for difference: -2.083

95% CI for difference: (-3.532, -0.633)

T-Test of difference = 0 (vs not =): T-Value = -3.10 P-Value = 0.008 DF = 13

- Two-Sample T-Test and CI: *Kigelia africana* 2000mg/kg, *Kigelia africana 5000mg/kg*

Two-sample T for 2000mg/kg vs 5000mg/kg

	N	Mean	StDev	SE Mean
2000mg/kg	18	0.688	0.922	0.22
5000mg/kg	8	1.80	3.33	1.2

Difference = mu 2000mg/kg - mu 5000mg/kg

Estimate for difference: -1.11

95% CI for difference: (-3.95, 1.72)

T-Test of difference = 0 (vs not =): T-Value = -0.93 P-Value = 0.384 DF = 7

- Two-Sample T-Test and CI: *Kigelia africana* 1000mg/kg, *Kigelia africana 5000mg/kg*

Two-sample T for 1000mg/kg vs 5000mg/kg

	N	Mean	StDev	SE Mean
1000mg/kg	12	2.77	2.20	0.63
5000mg/kg	8	1.80	3.33	1.2

Difference = mu 1000mg/kg - mu 5000mg/kg

Estimate for difference: 0.97

95% CI for difference: (-1.98, 3.92)

T-Test of difference = 0 (vs not =): T-Value = 0.72 P-Value = 0.484 DF = 11

- Two-Sample T-Test and CI: *Azadirachta indica* 1000mg/kg, *Azadirachta indica* 2000mg/kg

Two-sample T for1000mg/kg vs 2000mg/kg

	N	Mean	StDev	SE Mean
1000 mg/kg	13	5.98	4.15	1.2
2000 mg/kg	15	5.74	3.87	1.0

Difference = mu 1000mg/kg - mu 2000mg/kg

Estimate for difference: 0.23

95% CI for difference: (-2.91, 3.38)

T-Test of difference = 0 (vs not =): T-Value = 0.15 P-Value = 0.879 DF = 24

- Two-Sample T-Test and CI: *Azadirachta indica* 1000mg/kg, *Azadirachta indica* 5000mg/kg

Two-sample T for 1000 mg/kg1 vs 5000mg/kg

	N	Mean	StDev	SE Mean
1000 mg/kg	13	5.98	4.15	1.2
5000mg/kg	15	5.81	3.87	1.0

Difference = mu 1000mg/kg – 5000mg/kg

Estimate for difference: 0.17

95% CI for difference: (-2.98, 3.32)

T-Test of difference = 0 (vs not =): T-Value = 0.11 P-Value = 0.913 DF = 24

- Two-Sample T-Test and CI: *Azadirachta indica* 2000mg/kg, *Azadirachta indica* 5000mg/kg

Two-sample T for 2000mg/kg vs5000mg/kg

	N	Mean	StDev	SE Mean
2000 mg/kg	15	5.74	3.87	1.0
5000 mg/kg	15	5.81	3.87	1.0

Difference = mu 2000mg/kg - mu 5000mg/kg

Estimate for difference: -0.07

95% CI for difference: (-2.97, 2.84)

T-Test of difference = 0 (vs not =): T-Value = -0.05 P-Value = 0.963 DF = 27

- Two-Sample T-Test and CI: *Artemesia annua* 1000mg/kg, *Artemesia annua* 2000mg/kg

Two-sample T for 1000mg/kg vs 2000mg/kg

	N	Mean	StDev	SE Mean
1000mg/kg	10	5.01	4.32	1.4
2000mg/kg	11	4.96	4.02	1.2

Difference = mu 1000 mg/kg – 2000mg/kg

Estimate for difference: 0.05

95% CI for difference: (-3.79, 3.89)

T-Test of difference = 0 (vs not =): T-Value = 0.03 P-Value = 0.979 DF = 18

- Two-Sample T-Test and CI: *Artemesia annua* 1000mg/kg, *Artemesia annua* 5000mg/kg

Two-sample T for1000 mg/kg vs 5000 mg/kg

	N	Mean	StDev	SE Mean
1000 mg/kg	10	5.01	4.32	1.4
5000 mg/kg	12	1.18	2.74	0.79

Difference = mu 1000mg/kg – mu 5000mg/kg

Estimate for difference: 3.83

95% CI for difference: (0.44, 7.22)

T-Test of difference = 0 (vs not =): T-Value = 2.43 P-Value = 0.029 DF = 14

- Two-Sample T-Test and CI: *Artemesia annua* 2000mg/kg, *Artemesia annua* 5000mg/kg

Two-sample T for 2000mg/kg vs 5000mg/kg

	N	Mean	StDev	SE Mean
2000mg/kg	11	4.96	4.02	1.2
5000mg/kg	12	1.18	2.74	0.79

Difference = mu 2000mg/kg - mu 5000mg/kg

Estimate for difference: 3.79

95% CI for difference: (0.73, 6.84)

T-Test of difference = 0 (vs not =): T-Value = 2.61 P-Value = 0.018 DF = 17

Weights

- Two-Sample T-Test and CI: *Azadirachta indica* 1000mg/kg, *Azadirachta indica* 1000mg/kg

Two-sample T for 1000mg/kg vs 2000mg/kg

	N	Mean	StDev	SE Mean
1000mg/kg	11	29.05	2.51	0.76
2000mg/kg	14	23.29	5.05	1.4

Difference = mu 1000mg/kg – mu 2000mg/kg

Estimate for difference: 5.76

95% CI for difference: (2.52, 9.00)

T-Test of difference = 0 (vs not =): T-Value = 3.72 P-Value = 0.001 DF = 19

- Two-Sample T-Test and CI: *Azadirachta indica* 1000mg/kg, *Azadirachta indica* 5000mg/kg

Two-sample T for 1000mg/kg vs 5000mg/kg

	N	Mean	StDev	SE Mean
1000 mg/kg	11	29.05	2.51	0.76
5000 mg/kg	14	24.13	1.68	0.45

Difference = mu 1000 mg/kg - mu 5000mg/kg

Estimate for difference: 4.926

95% CI for difference: (3.059, 6.793)

T-Test of difference = 0 (vs not =): T-Value = 5.59 P-Value = 0.071 DF = 16

- Two-Sample T-Test and CI: *Azadirachta indica* 2000mg/kg, *Azadirachta indica* 5000mg/kg

Two-sample T for 2000mg/kg vs 5000mg/kg

	N	Mean	StDev	SE Mean
2000 mg/kg	14	23.29	5.05	1.4
5000 mg/kg	14	24.13	1.68	0.45

Difference = mu 2000 mg/kg - mu 5000mg/kg

Estimate for difference: -0.84

95% CI for difference: (-3.87, 2.20)

T-Test of difference = 0 (vs not =): T-Value = -0.59 P-Value = 0.566 DF = 15

- Two-Sample T-Test and CI: *Kigelia africana* 1000mg/kg, *Kigelia africana* 2000mg/kg

Two-sample T for 1000 mg/kg vs 2000mg/kg

	N	Mean	StDev	SE Mean
1000 mg/kg	13	26.00	1.93	0.54
2000 mg/kg	18	25.13	1.28	0.30

Difference = mu 1000 mg/kg - mu 2000 mg/kg

Estimate for difference: 0.872

95% CI for difference: (-0.415, 2.160)

T-Test of difference = 0 (vs not =): T-Value = 1.42 P-Value = 0.172 DF = 19

- Two-Sample T-Test and CI: *Kigelia africana* 1000mg/kg, *Kigelia africana* 5000mg/kg

Two-sample T for 1000 mg/kg vs 5000 mg/kg

	N	Mean	StDev	SE Mean
1000 mg/kg	13	26.00	1.93	0.54
5000 mg/kg	14	24.29	1.57	0.42

Difference = mu 1000 mg/kg - mu 5000mg/kg

Estimate for difference: 1.707

95% CI for difference: (0.298, 3.116)

T-Test of difference = 0 (vs not =): T-Value = 2.51 P-Value = 0.020 DF = 23

- Two-Sample T-Test and CI: *Kigelia africana* 2000mg/kg, *Kigelia africana* 5000mg/kg

Two-sample T for 2000 mg/kg vs 5000 mg/kg

	N	Mean	StDev	SE Mean
2000 mg/kg	18	25.13	1.28	0.30
5000 mg/kg	14	24.29	1.57	0.42

Difference = mu 2000 mg/kg - mu 5000 mg/kg

Estimate for difference: 0.835

95% CI for difference: (-0.235, 1.905)

T-Test of difference = 0 (vs not =): T-Value = 1.61 P-Value = 0.120 DF = 24

- Two-Sample T-Test and CI: *Artemesia annua* 1000mg/kg, *Artemesia annua* 2000mg/kg

Two-sample T for 1000 mg/kg vs 2000 mg/kg

	N	Mean	StDev	SE Mean
1000 mg/kg	11	24.85	2.73	0.82
2000 mg/kg	10	27.12	1.86	0.59

Difference = mu 1000 mg/kg - mu 2000 mg/kg

Estimate for difference: -2.27

95% CI for difference: (-4.40, -0.13)

T-Test of difference = 0 (vs not =): T-Value = -2.24 P-Value = 0.039 DF = 17

- Two-Sample T-Test and CI: *Artemesia annua* 1000mg/kg, *Artemesia annua* 5000 mg/kg

Two-sample T for 1000mg/kg vs 5000 mg/kg

	N	Mean	StDev	SE Mean
1000 mg/kg	11	24.85	2.73	0.82
5000 mg/kg	13	30.023	0.844	0.23

Difference = mu 1000 mg/kg - mu 5000 mg/kg

Estimate for difference: -5.169

95% CI for difference: (-7.053, -3.284)

T-Test of difference = 0 (vs not =): T-Value = -6.04 P-Value = 0.000 DF = 11

- Two-Sample T-Test and CI: *Artemesia annua* 2000mg/kg, *Artemesia annua* 5000mg/kg

Two-sample T for 2000 mg/kg vs 5000 mg/kg

	N	Mean	StDev	SE Mean
2000 mg/kg	10	27.12	1.86	0.59
5000 mg/kg	13	30.02	0.84	0.23

Difference = mu 2000 mg/kg - mu 5000 mg/kg

Estimate for difference: -2.903

95% CI for difference: (-4.298, -1.509)

T-Test of difference = 0 (vs not =): T-Value = -4.58 P-Value = 0.001 DF = 11

Packed cell volume

- Two-Sample T-Test and CI: *Azadirachta indica* 1000mg/kg, *Azadirachta indica* 2000mg/kg

Two-sample T for 1000 mg/kg vs 2000 mg/kg

	N	Mean	StDev	SE Mean
1000 mg/kg	8	39.64	2.60	0.92
2000 mg/kg	10	35.65	5.18	1.6

Difference = mu 1000 mg/kg - mu 2000 mg/kg

Estimate for difference: 3.99

95% CI for difference: (-0.07, 8.05)

T-Test of difference = 0 (vs not =): T-Value = 2.12 P-Value = 0.054 DF = 13

- Two-Sample T-Test and CI: *Azadirachta indica* 1000mg/kg, *Azadirachta indica* 5000mg/kg

Two-sample T for 1000 mg/kg vs. 5000 mg/kg

	N	Mean	StDev	SE Mean
1000 mg/kg	8	39.64	2.60	0.92
5000 mg/kg	10	38.16	3.77	1.2

Difference = mu 1000 mg/kg - mu 5000 mg/kg

Estimate for difference: 1.48

95% CI for difference: (-1.73, 4.69)

T-Test of difference = 0 (vs not =): T-Value = 0.98 P-Value = 0.342 DF = 15

- Two-Sample T-Test and CI: *Azadirachta indica* 2000mg/kg, *Azadirachta indica* 5000mg/kg

Two-sample T for 2000 mg/kg vs. 5000 mg/kg

	N	Mean	StDev	SE Mean
2000 mg/kg	10	35.65	5.18	1.6
5000 mg/kg	10	38.16	3.77	1.2

Difference = mu 2000 mg/kg - mu 5000 mg/kg

Estimate for difference: -2.51

95% CI for difference: (-6.81, 1.79)

T-Test of difference = 0 (vs. not =): T-Value = -1.24 P-Value = 0.233 DF = 16

- Two-Sample T-Test and CI: *Kigelia africana* 1000mg/kg, *Kigelia africana* 2000mg/kg

Two-sample T for 1000 mg/kg vs. 2000 mg/kg

	N	Mean	StDev	SE Mean
1000 mg/kg	13	38.63	3.05	0.85
2000 mg/kg	16	41.65	0.57	0.14

Difference = mu 1000 mg/kg - mu 2000 mg/kg

Estimate for difference: -3.019

95% CI for difference: (-4.890, -1.149)

T-Test of difference = 0 (vs. not =): T-Value = -3.52 P-Value = 0.004 DF = 12

- Two-Sample T-Test and CI: *Kigelia africana* 1000mg/kg, *Kigelia africana* 5000mg/kg

Two-sample T for 1000mg/kg vs. 5000mg/kg

	N	Mean	StDev	SE Mean
1000 mg/kg	13	38.63	3.05	0.85
5000 mg/kg	11	38.37	4.13	1.2

Difference = mu 1000 mg/kg - mu 5000 mg/kg

Estimate for difference: 0.26

95% CI for difference: (-2.91, 3.42)

T-Test of difference = 0 (vs. not =): T-Value = 0.17 P-Value = 0.866 DF = 18

- Two-Sample T-Test and CI: *Kigelia africana* 2000mg/kg, *Kigelia africana* 5000mg/kg

Two-sample T for 2000mg/kg vs. 5000mg/kg

	N	Mean	StDev	SE Mean
2000 mg/kg	16	41.650	0.574	0.14
5000 mg/kg	11	38.37	4.13	1.2

Difference = mu 2000 mg/kg - mu 5000 mg/kg

Estimate for difference: 3.28

95% CI for difference: (0.48, 6.07)

T-Test of difference = 0 (vs. not =): T-Value = 2.61 P-Value = 0.026 DF = 10

- Two-Sample T-Test and CI: *Artemesia annua* 1000mg/kg, *Artemesia annua* 2000mg/kg

Two-sample T for 1000 mg/kg vs 2000 mg/kg

	N	Mean	StDev	SE Mean
1000 mg/kg	7	37.17	4.69	1.8
2000 mg/kg	8	37.88	3.90	1.4

Difference = mu 1000 mg/kg - mu 2000 mg/kg

Estimate for difference: -0.70

95% CI for difference: (-5.65, 4.24)

T-Test of difference = 0 (vs not =): T-Value = -0.31 P-Value = 0.760 DF = 11

- Two-Sample T-Test and CI: *Artemesia annua* 1000mg/kg, *Artemesia annua* 5000mg/kg

Two-sample T for 1000 mg/kg vs 5000 mg/kg

	N	Mean	StDev	SE Mean
1000 mg/kg	7	37.17	4.69	1.8
5000 mg/kg	9	37.24	3.54	1.2

Difference = mu 1000 mg/kg - mu 5000 mg/kg

Estimate for difference: -0.07

95% CI for difference: (-4.82, 4.67)

T-Test of difference = 0 (vs not =): T-Value = -0.03 P-Value = 0.973 DF = 10

- Two-Sample T-Test and CI: *Artemesia annua* 2000mg/kg, *Artemesia annua* 5000mg/kg

Two-sample T for 2000 mg/kg vs 5000mg/kg

	N	Mean	StDev	SE Mean
2000 mg/kg	8	37.88	3.90	1.4
5000 mg/kg	9	37.24	3.54	1.2

Difference = mu 2000 mg/kg - mu 5000 mg/kg

Estimate for difference: 0.63

95% CI for difference: (-3.26, 4.52)

T-Test of difference = 0 (vs not =): T-Value = 0.35 P-Value = 0.733 DF = 14