EVALUATION OF TRYPANOCIDAL ACTIVITY OF SELECTED MEDICINAL PLANTS IN KENYA AGAINST *TRYPANOSOMA EVANSI*

BY

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156/6236/03

A thesis submitted in partial fulfillment of the requirements for the award of the degree of Master of Science (Medical Biochemistry) of Kenyatta University

2007
Declaration

I hereby declare that this thesis is my original work and has not been presented for a degree in any other University.

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

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Dedication

This thesis is dedicated to my wife Evelyn and children Brian, Maureen and Joseph.
Acknowledgement

My deep appreciation goes to my supervisors Dr. Joseph J. N. Ngeranwa, Dr. Daniel K. Masiga and Professor Peter K. Gathumbi for their constant advice, encouragement and constructive criticism during the course of this study. My sincere thanks also go to the late Dr. Fredrick Thiong’o who provided initial guidance on this project.

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Abstract

Typanosomiasis, a protozoan disease causes morbidity and death to humans and severely limits livestock production in endemic areas. It occurs predominantly in Africa, South America and Asia. Although Chemotherapy has been used to control the disease, cases of resistance have been reported and prospects of short-term vaccine development are remote. To address these challenges, herbal medicines have increasingly been used to control the disease in endemic areas. This study evaluated efficacy of selected indigenous plant extracts on T. evansi. Test extracts included *Azadirachta indica* (neem), *Prunus africana*, *Bidens pilosa*, *Phyalsa peruviana*, *Senna didymobotrya* and *Croton megalocarpus*. 96 well micro titer plates were used and trypanocidal activities evaluated *in vitro* by calculating minimum inhibitory concentrations (MIC) of respective extracts. Safety of extracts was tested in mice (*Swiss white*) by evaluating parasitemia, clinical presentations and histological analysis of liver, kidney, heart, lungs, muscle and brain tissue. Of the 27 extracts evaluated, chloroform extracts of *Azadirachta indica* leaves had the highest activity (minimum inhibitory concentration of 18.75 μg/ml). This extract was toxic to host cells *in vivo* at doses exceeding 500 mg/kilogram body weight and was associated with dyspnoea and lethargy. Histopathology showed damage to kidneys, lungs and liver. Because chloroform extracts of *Azadirachta indica* leaves performed better both *in vitro* and *in vivo* than suramin, a commercial trypanocide, future studies should address purification, structure elucidation and biochemical characteristic of active components of *Azadirachta indica* leaves. This study has confirmed the hypothesis that some Kenyan plants have trypanocidal potential.
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Abbreviations and acronyms

$- Dollar
%

%- Percentage
µg- microgram
µl- Microliter
°C- Degree Celsius
AAT- Africa Animal Trypanosomosis
ANOVA- Analysis of variance
AVE- Average
bw- body weight
CO₂- Carbon dioxide
CNS- Central nervous system
DA- Diminazene aceturate
DDT- Dichlorodiphenyltrichloroethene
DE 52= DEAE- Diethyl amino ethyl cellulose
DFMO- Difluoromethyl ornithine
DPX- Dibutylphthalate xylene
EDTA- Ethylene diamine tetra acetic acid
ESG- EDTA – Saline glucose
EtOH- Ethanol
FBS- Foetal bovine serum
GOK- Government of Kenya
HAT- Human African Trypanosomosis
ICI- Imperial chemical industries
ip- Intraperitoneal
IC₅₀- Concentration that inhibits by 50% the proliferation of parasites
ILRI- International Livestock Research Institute
im- Intramuscular
KARI- Kenya Agricultural Research Institute
KETRI- Kenya Trypanosomiasis Research Institute
Kg- Kilogram
LP- Low power
Mel Cy- Cymelarsan
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CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

1.1.1 Background information

Trypanosomosis is a protozoan disease of both man and animals caused by different species of Trypanosoma. The disease is widespread; it is endemic in Africa, South America and Asia. Tsetse-transmitted human trypanosomosis known as Human African Trypanosomosis (HAT) or sleeping sickness occurs only in Africa. HAT is caused by two sub-species of Trypanosoma brucei namely: T.b. gambiense and T.b. rhodesiense. Livestock are affected by other species of trypanosomes, including T. b. brucei, T. congolense, T. simiae, T. evansi and T. vivax. The disease is a major constraint to development, and limits maximal utilization of livestock in about one-third (over 11 million km²) of the African continent. Livestock production in the endemic areas is constrained by lowered productivity and mortality of affected animals.

Camel trypanosomosis (surra) is the disease caused by T. evansi infection, which in Kenya is the most important single cause of losses in camels (Wilson et al., 1981; Nyanga’o et al., 1994). In Northern Kenya for instance, camel infection rates of 9.7% by direct examination of blood smears and 79% by serological tests have been documented from random samples collected in Ngurunit, Marsabit district (Rutagwenda, 1984). It is notable that rearing of camels in Kenya has moved further into the tsetse infected areas and T. congolense and T. brucei have been isolated from camels (Masiga and Nyanga’o, 2001). Other domestic animals such as cattle, sheep, goats and pigs are susceptible to T. evansi infection, but they develop a less severe disease. This form of disease is however more important than was usually recognized since an animal which is chronically infected can act as a carrier and therefore a potential reservoir of infection (Losos, 1980).
Reports on the pathogenicity of *T. evansi* in sheep and goats are contradictory. Many authors have reported the disease as causing mild and sub-clinical infections in these species (Otieno and Gachanja, 1976; Mahmoud and El Malik, 1978; Rottcher *et al.*, 1987). High pathogenicity has however been reported (Antao *et al.*, 1968), while Hornby (1952) described the disease as being moderate. Antibodies against *T. evansi* have been demonstrated in goats (Boid *et al.*, 1981) an indication of the occurrence of natural infections.

It has also been shown that many members of genus *Trypanosoma* cause fertility problems in several domestic animals, sheep and goats included. Genital lesions occur in rams infected with *T. vivax* (Isoun and Anosa, 1974; Akpavie and Ikede, 1987); *T. brucei* (Ikede and Losos, 1972; Ikede, 1979) and *T. congolense* (Kaaya, 1975; Kaaya and Oduor-Okello, 1980). In zebu bulls infected with *T. vivax* and *T. congolense*, increase in ejaculation time is known to occur (Sekoni *et al.*, 1988). In female animals, abortions have been reported in buffaloes (Parkne and Dhake, 1972); bovines (Eseruoso, 1974; Murray *et al.*, 1977) and goats (Mutayoba *et al.*, 1988). It has also been reported that during infection with *T. congolense* in goats, reduction in progesterone levels occur (Waindi and Gombe, 1985; Mutayoba *et al.*, 1988). Other pathological effects described include degeneration of ovaries (O’hara and Gombe, 1985; Mutayoba *et al.*, 1988) and of pituitary, thyroid and adrenal glands in both sexes (Ikede and Losos, 1972; Mutayoba *et al.*, 1988).

The main control measures of trypanosomosis are based on chemotherapy and chemoprophylaxis and on interventions aimed at control of the vector populations. There is no vaccine against trypanosomosis. While chemotherapy is the most effective method for the control of camel trypanosomosis, resistance to older and commonly used trypanocidal drugs such as quinapyramine and suramin appear to have developed over the years (Schillinger and Rottcher, 1984; Maina *et al.*, 1996). Suramin (Naganol®, Bayer) and quinapyramine (Trypacide®, Rhone
Merieux) are the two common trypanocides in the Kenyan market for the control of cameline trypanosomosis. Both of these drugs are active against *T.evansi* although their effectiveness has been reduced by the development of resistance to both drugs (Gitatha, 1980; Schillinger *et al.*, 1985). Cymelarsan introduced in 1993 is effective against *T.brucel* and *T. evansi* infections (Zweygarth and Kaminsky, 1990) but ineffective against *T. congolense* (Maina *et al.*, 1998). Few chemotherapeutic drugs are available and the presence of strains of parasites resistant to treatments is an increasingly serious problem.


The chemotherapeutic agents in current use are either not fully effective or are toxic (Prie *et al.*, 1993) yet except for cymelarsan (Raynaud *et al.*, 1989), no new drug for this disease has been developed for the last 50 years. There is therefore need for a constant search for new antitrypanosome compounds.

1.1.2 Statement of the problem

Although chemoprophylaxis and chemotherapy are the major control methods of trypanosomosis, drug resistance among *T. evansi* isolates is a major constraint (Abebe *et al.*, 1983). The drugs in current use often have severe side effects and are few, costly and their production may eventually be discontinued. In some situations, the resistance has become so prevalent that the drugs concerned have been withdrawn from general use (Leach and Roberts, 1981). Every year, African Animal Trypanosomosis (AAT) causes about 3 million deaths in cattle while approximately 35 million doses of trypanocidal drugs are parenterally administered. Nagana (meaning "loss of spirit" in Zulu language) has a severe impact on agriculture in sub-Saharan Africa with economic losses in cattle production ranging between US$ 1.0-1.2 billion. (http://www.fao.org/ag/againfo/programmes/en/paat/disease.html- 24/08/2005).
1.1.3 Justification

Trypanosomosis is one of the most important diseases of farm animals in tropical countries. African trypanosomosis has continued to disrupt human life, animal husbandry, and wildlife in over 10,000,000 km² of sub-Sahara Africa (Kuzoe, 1993; WHO, 1995). It is estimated that there are approximately 800,000 camels in Kenya (FAO, 1990). Trypanosomosis (surra) due to infection with *T. evansi* has been identified as the most important disease of camels in Kenya (Wilson *et al.*, 1984) and the disease is endemic in all the major camel rearing areas.

Control of surra by chemotherapy has mainly depended on use of quinapyramine and suramin. The former has been in use for more than 30 years while the latter has been in use for 60 years. Suramin and quinapyramine resistant *T. evansi* have been reported from a number of countries including Kenya (Schillinger and Rottcher, 1984). In addition the manufacture of suramin has recently been discontinued. This situation has lead to an urgent need to develop new drugs for use against surra in camels.

Most of the currently available antitrypanosomal drugs are either highly toxic to animals or the parasites have developed resistance to them (Mansfield, 1984; Nantulya and Moloo, 1989). Despite the problems facing chemotherapeutic agents against the disease, pharmaceutical companies are not enthusiastic to develop new drugs for tropical diseases due to anticipated low return on investment yet the number of trypanocides available is limited. *T. evansi*, the causative agent of camel trypanosomosis (surra), is the most widespread pathogenic trypanosome in the world. The disease causes significant morbidity and mortality in camels in arid and semi arid regions in Kenya (GOK, 1997). Since it is difficult to eradicate trypanosomosis through chemotherapy and there are no prospective vaccine to control new outbreaks, alternative control options of trypanosomosis need to be pursued. New potent and safe trypanocidal drugs are therefore urgently needed. Herbal medicines are increasingly being focused on due to their cost-
effective and eco-friendly attributes as alternative Avenue to address this problem (Dwivedi, 1997).

1.1.4 Research question
Are herbal extracts effective and safe for use as trypanocides against *Trypanosoma evansi*?

1.1.5 Research hypothesis
Herbal extracts in common use in Kenya are safe and have trypanocidal effects.

1.1.6 Objectives
1.1.6.1 General objective
To determine the trypanocidal effects and toxicity of plant extracts from selected Kenyan medicinal plants.

1.1.6.2 Specific objectives
1. To determine the trypanocidal effects of some plant extracts against *T. evansi* in *vitro*.
2. To investigate the toxicity of the most active plant extracts in mice.
3. To investigate the trypanocidal effect of the most active and least toxic plant extracts in infected mice (*in vivo*).
1.2.1 The disease

Trypanosomosis is a disease of animals and man caused by microscopic, unicellular and flagellated protozoans, the trypanosomes. The disease has been in existence for millions of years. Over this period, the parasites and their natural host have evolved together to ensure their mutual survival. This is best supported by the fact that the disease is most virulent when the parasite spreads to new hosts.


1.2.2 Aetiology

The parasite in the family Trypanosomatidae constitutes several pathogenic species in animals. The disease in man is caused by *T. gambiense* and *T. rhodesiense*. *T. cruzi* causes Human American Trypanosomosis or Chagas’ disease in South America. In East Africa, *T. congolense* is the most important cause of African Animal Trypanosomosis. Cattle, sheep, goats, horses and pigs are often seriously affected (Cheesborough, 1998). *T. vivax* mainly infects cattle, sheep, and goats and it also causes a mild disease in horses and a chronic disease in dogs. *T. brucei* infects cattle, horses, dogs, cats, camels and pigs. *T. evansi* causes ‘surra’, a disease of horses, camels, dogs, buffaloes and other domestic and wild animals. The disease caused by *T. evansi* is fatal in horses and camels, while wild animals usually appear to act as reservoirs (Hoare, 1972).

1.2.3 Transmission

Except for *T. equiperdum* which is transmitted by coitus in equines, trypanosomes are either transmitted cyclically by tsetse (*T. congolense*, *T. vivax* and *T. brucei*) or non cyclically (mechanically) (*T. evansi* and at times *T. vivax*) by haematophagus flies mostly *Tabanids* and *Stomoxys*. *T. evansi* is unique in being the only trypanosome species which is exclusively mechanically transmitted. Evans (1881) first suggested this kind of transmission by dipteran flies when he noted that “the fly (tabanid) is able to go from one horse to another before the blood
about its mouth is dry." The first scientific evidence of mechanical transmission was however provided by Rogers (1901). Trypanosomes are mechanically transferred from one mammalian host to another by feeding of biting flies (Seifert, 1986). Transmission occurs when an insect feeding on an infected host is interrupted and then feeds upon a susceptible host within periods not exceeding eight minutes (Losos, 1980). These vectors are abundant in camel rearing areas and this makes surra the most important trypanosomosis in camels.

The transmission efficiency of different flies may vary under different geographical conditions (Losos, 1980) and Stomoxys nigra was noted as the principal vector in Mauritius (Moutia, 1928). The infectivity is highest within minutes of feeding and drops quickly with transmission ceasing after 8 minutes (Losos, 1980). Reduviids (Rhodnius prolixus and Depetalogaster maximum) have also been shown to transmit T. evansi in mice and could retain live trypanosomes for up to 9 hours in their guts (Manz, 1985). In South America, the vampire bats get infection, remain carriers and they can transmit the disease when they bite other animals. Because bats can remain infected for a long time, they are more efficient in transmission of the disease than the biting flies (Hoare, 1965; Losos, 1980). Ticks (Ornithodoros cossis) are incriminated in transmission of T. evansi in camels (Cross, 1923).

1.2.4 Epidemiology and occurrence

The epidemiology of African trypanosomosis in animals is dependent on the parasite, vector and host factors. The mammalian T. congolense, T. vivax and T. brucei are normally restricted to tropical Africa roughly between latitudes 15°N and 25°S. According to Hoare (1972), surra originated from Africa, although more recent reports seem to indicate that the disease originated from India and then spread with infected livestock throughout the continent of Asia and the islands in the Indian Ocean (Rottcher et al., 1987). Due to the ability of blood sucking insects
other than *Glossina* to transmit *T. evansi, surra* has been able to spread into Africa North of Sahara desert, Asia and South America. It had been introduced by camels into Australia, North America and South West Africa but in Australia and North America it was rapidly eradicated by control measures (Rottcher *et al.*, 1987). The incidence and severity of the disease varies with localities and depends on the *T. evansi* strain and the animal species. Generally, the disease is more severe in a newly introduced area and less severe in endemic areas. The density of tsetse fly populations determines the rate of spread and the tsetse fly population is dependent on the seasons which govern the availability of water required for their reproduction and survival. The level of animal husbandry practices, nutritional status, workload and stress, exacerbate the severity of the disease (Maclean, 1970).

1.2.5 Control of trypanosomosis

There are three main methods of control of trypanosomosis, those that target the vector, parasite and the host.

1.2.5.1 Vector control

The use of mechanical devices for tsetse control usually employs traps or targets. Targets consist of a piece of black cloth, flanked by netting which is mounted on a metal frame (Vale *et al.*, 1988). Targets are usually impregnated with chemicals, which kill tsetse flies that land on them. Recent methods of chemical control involve the baiting of tsetse flies by the use of odours that are known to attract the flies. Known attractants include octanols, phenols, acetone, and carbon dioxide, which were identified as a result of observation that ox breath and cow urine are attractive to tsetse. The uses of such attractants near traps and targets have been shown to increase the number of tsetse entering them (Vale *et al.*, 1988; Laveissiere *et al.*, 1989). Insecticide impregnation on targets together with the use of attractants has been used to control or eradicate tsetse populations in Zimbabwe (Vale *et al.*, 1986) and Kenya (Opiyo *et al.*, 1987).
In order to increase the killing efficiency of traps and targets, insecticides are usually impregnated onto the cloth so that the tsetse flies that land on the cloth receive a lethal dose of insecticide. This method results in an increase of the efficiency of traps as control devices (Vale and Hargrove, 1979).

Vector control, using chemicals has been the most widely used method. It utilizes insecticides, mainly pyrethroids or organochlorides. The first insecticide used for the control of tsetse was dichlorodiphenyltrichloroethane (DDT). Other organochlorides which have been used are dieldrin and endosulfan but these are toxic to the environment which limits their widespread use. Although natural pyrethroids have been shown to have activity against tsetse, it is the synthetic pyrethroids, deltamethrin, cypermethrin and permethrin which have been widely used for tsetse control (Jordan, 1986). Different chemical methods have been used to control some insects which acts as vectors of T. evansi (Soulsby, 1968). These methods are applicable where animals are housed but are not useful in the control of cameline T. evansi in pastoral systems.

1.2.5.2 Chemotherapy
1.2.5.2.1 Arsenicals

After intravenous administration of arsenicals, drug concentration in blood reaches maximum levels within 24hrs (Hawking, 1963). These drugs also cross the blood-brain barrier which makes them important in the treatment of infections caused by Trypanozoon parasites such as T. evansi which invade the CNS. The commonly used arsenical is cymelarsan, a compound recently developed by Rhone Merieux. Cymelarsan is used against trypanosomes of the brucei group mainly in humans but was also proposed for use on T. evansi infections (Raynaud et al., 1989).
Studies in Ethiopia (Zelleke et al., 1989), Mali (Tager-Kagan et al., 1989) and Kenya (Otsyula et al., 1992) indicate that cymelarsan is active against cameline T. evansi infection at dose rates of 0.3 to 1.25mg/kg bw administered subcutaneously.

The efficiency of cymelarsan, administered by intramuscular (im) injection in the treatment of chronic cases of camel trypanosomosis due to Trypanosoma evansi, was tested under controlled laboratory conditions. It was confirmed that cymelarsan is a safe drug for use in dromedary camels when administered im at dose rate of 0.25 or 0.5 mg/kg body weight (Musa et al., 1994).

In a study carried out by Zweygarth et al. (1992) on evaluation of trypanocidal activity of an arsenical compound (Rm 110; Mel Cy; Cymelarsan) against Trypanosoma brucei and T. brucei evansi in goats and pigs the following was revealed. Goats infected with a susceptible T. b. evansi stock were cured after a single injection of 0.3 mg/kg cymelarsan. In three out of four goats chronically infected with the same stock, a single injection of 0.625 mg/kg cymelarsan effected a cure, whereas the goat in which the infection relapsed was finally cured after injection of 0.625 mg/kg on each of three consecutive days. Although the recommended dose for this drug is 0.25mg/kg, both Zweygarth et al. (1992) and Lun et al. (1991) found that this dose was not effective in curing infections involving the CNS in goats and buffaloes, respectively. An efficacy trial of cymelarsan on a Zambian strain of Trypanosoma brucei brucei was done by Syakalima et al. (1995). Five groups of 5 mice were treated with 0 (control), 0.25, 0.5, 1.0, and 2.0 mg/kg cymelarsan, respectively. The 0.25 and 0.5 mg/kg groups remained parasitaemic although the parasitaemia levels were reduced. The 1.0 mg/kg group had a proportion of aparasitaemic mice. However, all mice in the 2.0 mg/kg group remained aparasitaemic until day 20 when 2 mice relapsed. These results suggested that more than 2.0 mg/kg was required to eliminate this strain. Melarsoprol (Arsobal\textsuperscript{R}, SPECIA) is curative when used at a dosage of 3.6mg/kg bw which must not be exceeded (Schillinger, 1984). This drug both overcomes resistance and cures the disease in later stages.
1.2.5.2.2 Suramin

It took eight years for Bayer workers in Germany to develop and produce this compound before it became available in 1920 for the treatment of human and cameline trypanosomosis (Williamson, 1978; Apted, 1980; Leach and Roberts, 1981). Suramin (Naganol \textsuperscript{R}, Bayer) has also been available under several trade names such as Germanin \textsuperscript{R}, Bayer 205\textsuperscript{R}, Antrypol \textsuperscript{R}, Moranyl' \textsuperscript{R} and Naganin \textsuperscript{R}. Although suramin is active against infections caused by Trypanozoon parasites, it is ineffective against \textit{T. vivax}, \textit{T. simiae} and \textit{T. congolense}. For human-infective trypanosomes, this drug is recommended for intravenous use in early stage sleeping sickness, before the CNS is involved (Hawking, 1963). For animal trypanosomosis, suramin is recommended for curative use at 10 to 12mg/kg bw and for prophylactic use at 5mg/kg bw administered intravenously as a 10\% solution in camels and equids infected with \textit{T. evansi}, \textit{T. brucei} or \textit{T. equiperdum}. The drug gives prophylactic cover for 6 to 12 weeks depending on dosage and disease challenge (Losos, 1980; Rottcher \textit{et al.}, 1987). It is believed that highly polar suramin does not cross the blood – brain barrier (Apted, 1980).

1.2.5.2.3 Diminazene aceturate (Berenil)

The drug, marketed as Berenil (Hoechst\textsuperscript{R}) was shown to have activity against \textit{T. congolense}, \textit{T. evansi}, \textit{T. vivax} and to a lesser extent \textit{T. brucei} (Hawking, 1963; Williamson, 1970). It is recommended for use at a dose rate of 3.5 to 7mg/kg bw administered through the intramuscular route. Berenil\textsuperscript{R} up to 10mg/kg has been used successfully in calves (Verma \textit{et al.}, 1976). Berenil has little prophylactic activity for it is either excreted quickly or metabolized within 24hrs of administration (Hawking, 1963). It is commonly used to control trypanosomosis in cattle, sheep and goats. Its use however is contraindicated in the dromedary camel because it is highly toxic and believed to be ineffective (Leach, 1961). However, diminazene aceturate has been used successfully in the camel at a dosage of 2.85mg/kg intramuscularly (Raisinghani and Lodha, 1980).
1.2.5.2.4 Isometamidium chloride

Isometamidium chloride (Samorin\textsuperscript{R}-May and Baker) or (Trypaidium\textsuperscript{R}-SPECIA) is used when resistance occurs to suramin and quinapyramine (Rottcher \textit{et al.}, 1987). However, the drug does not cure the disease in the chronic state after trypanosomes enter other body compartments such as joints of camels (Schillinger, 1984) and cerebrospinal fluid of ponies (Schonefeld, 1979; Wust, 1980; Horchner \textit{et al.}, 1983). This would render \textit{T. evansi} inaccessible by trypanocidal drugs other than the arsenicals since the former do not cross the blood brain barrier. Isometamidium is indicated for \textit{T. congolense} and \textit{T. vivax} infections at a dose rate of 1-2 mg/kg bw administered intramuscularly and is prophylactic for a period of 2-3 months. Although this drug has been shown to have activity against \textit{T. evansi} at 1.0mg/kg bw administered intramuscularly (Petrovskii, 1974), its use in camels has been reported to result to toxic reactions (Schillinger and Rottcher, 1984; Schillinger \textit{et al.}, 1985; Ali and Hassan, 1986). When administered subcutaneously or intramuscularly pain and lameness are observed while intravenous administration results in pronounced shock or other fatal systemic symptoms.

1.2.5.2.5 Quinapyramine

This compound was synthesized by Barret \textit{et al.} (1953) from the original quinoline which had been shown to have trypanocidal activity. Quinapyramine was then developed by Imperial Chemical Industries (I.C.I) and introduced for cattle trypanosomosis in 1949 (Curd and Davey, 1949). It is available as a sulphate used for therapy or as a sulphate-chloride combination used for prophylaxis. Quinapyramine is active against \textit{T. congolense}, \textit{T. simiae}, \textit{T. vivax} as well as trypanosomes of the \textit{brucei} group (Williamson, 1970). Quinapyramine is only effective during the early stages of infection because it does not cross the blood-brain barrier. When first produced by I.C.I, quinapyramine sulphate (Antrycide\textsuperscript{R}) was recommended for use at a dose rate of 4.4mg/kg bw subcutaneously. The initial prophylactic compound consisting of a 3:4 sulphate chloride ratio was recommended for use at a dose rate of 11mg/kg bw subcutaneously.
A revised formula was developed which consisted of only half the original chloride and was recommended for use at 7.4mg/kg bw. I.C.I withdrew quinapyramine from the market in 1974 due to widespread resistance developed by trypanosomes. In 1984, quinapyramine was however re-introduced by May and Baker as Trypamide<sup>8</sup>. The prophylactic compound, Trypamide Prosalt, has a 3:2 sulphate-chloride ratio. Both the sulphate and the prosalt are recommended for subcutaneous administration at 5mg/kg. Since 1984, May and Baker has placed special emphasis on the use of quinapyramine in the control of <i>T. evansi</i> infections of camels.

1.2.5.2.6 Homidium

The use of this drug is recommended at a dose rate of 1mg/kg bw intramuscularly for the cure of infections caused by <i>T. congolense</i> and <i>T. vivax</i> (Hawking, 1963).

1.2.5.2.7 Prothidium

Unlike homidium, this compound is not quickly eliminated from the body for it binds strongly to proteins, thus resulting in a prophylactic activity (Taylor, 1960). It is recommended for use as a single intramuscular or subcutaneous dose of 2-4 mg/kg bw which provides a prophylactic activity for 6-8 weeks (Hawking, 1963). Like homidium, local irritation at the site of injection have been observed as well as delayed reactions resulting in deaths several months after treatment.

It has been found that DL-alpha difluoromethyl ornithine (DFMO) is able to suppress isometamidium chloride and diminazene aceturate resistant strains of <i>T. congolense</i> (Schillinger and Gorton, 1984). This drug then could be a potential remedy in case of multiple drug resistance as it does not share resistance pattern with isometamidium chloride and diminazene aceturate (Schilliger and Gorton, 1984).
The same drugs used in camels, are used in treatment of trypanosomosis in other animals. In cattle and horses, a combination of Suramin and Berenil gives better results (Hu et al., 1985). Quinapyramine-Prosalt has been reported to protect cattle for periods of up to 10 months in the Philippines (Castillo, 1962). The exposure of parasites to subtherapeutic drug concentrations owing to the commonly practiced under dosing is the most important factor for the development of drug resistant trypanocides (Whiteside, 1962).

1.2.6 Medicinal herbs for treatment of trypanosomosis
1.2.6.1 In vitro studies on trypanocidal activity of some medicinal plants

Dwivedi in 1997 tested 23 different plants in India and observed that fresh juice of Xanthium strumarium leaves, Parthenium hysterophorus flowers and Aristolochia indica stems possessed 100% trypanocidal activities in vitro at doses of 100, 500 and 1000μg/ml. He found out that only the alcoholic extracts of three plants, i.e. Xanthium strumarium leaves, Parthenium hysterophorus flowers, and Nyctanthes arbor-tristis leaves, were effective in vitro. Other plants exhibiting mild to moderate trypanocidal activity in vitro at similar doses included root, bark and leaves of Azadirachta indica, leaves of Senna occidentalis, rhizomes of Cyperus rotundus and seed of Cannabis indica.

An in vitro trypanocidal effect of methanolic extract of some Nigerian Savannah plants was noted in a study by Atawodi et al. (2003). Methanol extracts from twenty three plants harvested from the Savannah vegetation belt of Nigeria were analyzed for in vitro trypanocidal activity against T.b. brucei and T. congolense at concentrations of 4mg/ml, 0.4mg/ml and 0.04mg/ml, with following results; extracts of Khaya senegalensis, Piliostigma reticulata, Securidaca longependunculata and Terminalia avicennodes were strongly trypanocidal to both organisms. Promising trypanocidal activity with IC50 value below 10μg/ml was found in 32 extracts of
Annona senegalensis, Bussea occidentalis and Physalis angulata (Freiburghaus et al., 1996). Study by Hoet et al. (2004) reported in vitro antitrypanosomal activity of methylene chloride, methanol, and aqueous extracts of the leaves and twigs of five plant species traditionally used in Benin for treatment of trypanosomosis. The results showed that the four most active extracts (Hymenocardia acida twig and leaf, Strychnos spinosa leaf, Trichilia emetica leaf) had MIC values ≤ 19µg/ml. Determination of the IC₅₀ values of the methylene chloride leaf extracts on two strains of trypanosomes (T. b. brucei and T. b. rhodesiense) and two mammalian cell lines (L6 and J774 cells) showed that all extracts possessed some antitrypanosomal activity with IC₅₀’s ranging from 1.5 to 39µg/ml (Hoet et al., 2004). The extracts of Enantia polycarpa and Trichilia emetica were reported to possess the most promising antitrypanosomal activity with IC₅₀ values of 0.5 and 0.04µg/ml, respectively. The study was done with 101 crude ethanol extracts derived from 88 medicinal plants from Cote d’Ivoire using Trypanosoma brucei rhodesiense (Atindehou et al., 2003). Recent observations indicate that aqueous, extract of Azadirachta indica stem bark inhibit growth of T. brucei and T. congoense at in vitro concentration of 62.5µg/ml and 125µg/ml respectively (Gathumbi et al., 2007). However, aqueous extract of its leaf was not effective (Gathumbi, personal communication).

1.2.6.2 Toxicity tests on some medicinal plants on animals

The toxicity trials revealed that 50% ethanoic extract of xanthium strumarium leaves was hepatotoxic and killed 50% of the mice within 4-12 days when given at doses of 1000 mg/kg bw intraperitoneally. At a similar dose and administration route, 50% ethanolic extract of Parthenium hysterophorus flowers killed all the six test mice within four hours. The alcoholic extract of Nyctanthes arbor-tristis leaves was less toxic and caused 33.4% mortality in rats. Alcoholic extracts of three plants (Xanthium strumarium leaves, Parthenium hysterophorus flowers and Nyctanthes arbor-tristis leaves) that were effective both in vitro and in vivo were
toxic to mice (Dwivedi, 1997). Ethanolic extract of *Xanthium strumarium* leaves which exerted antitrypanosomal effect at dosage of 1000mg/kg bw *in vivo* trial was found to be toxic to the mice at the same dosage (Talakal *et al.*, 1995). The *Annona senegalensis* root extract possesses different margins of safety in the mice depending on the route of administration. Adverse reactions, especially to doses of 2.3 – 5.76mg/kg, were noted in animals that received the drug parentally but not when the drug was administered orally (Igweh and Onobanjo, 1989).

Methylene chloride extracts from *Hymenocardia acida* twig and leaf, *Strychnos spinosa* leaf and *Trichilia emetica* leaf, were all found to possess some antitrypanosomal activity with IC$_{50}$’s ranging from 1.5 to 39 µg/ml, were toxic to mammalian cell line (J774 cells) with only exception of *Strychnos spinosa* (Hoet *et al.*, 2004).

Cytotoxicity of active extracts prepared from *Albizia gummifera, Ehretia amoena, Entada abyssinica, Securinega virosa* and *Vernonia subuligera*, which revealed very promising antitrypanosomal activity with IC$_{50}$ values between 1-2 µg/ml when tested on a human fibroblast cell line (W1-38) and were found to be highly toxic (Freiburghaus *et al.*, 1996).

1.2.6.3 *In vivo* trials on trypanocidal activity of some medicinal plants

Mice infected with *Trypanosoma brucei brucei* were treated orally and intramuscularly (im) with aqueous root extracts of *Annona senegalensis*, in doses of 27.8mg/kg and 9.5mg/kg respectively, for four consecutive days commencing 72 hours after they were infected. At these dosages the parasites were cleared from the circulation and no relapse was recorded over 60 days. The plant extract, however, had no effect on the trypanosomes when therapy was initiated at the late stages of infection about the sixth day when the parasitaemia level was 0.9 x 10$^6$, and all the animals died a day or two later. However, *A. senegalensis* has been shown to be therapeutically effective against *T.b. brucei* in mice, which agrees with the claims of Nigerian practitioners of traditional medicine that it is effective against trypanosomosis (Igweh *et al.*, 1989).
In vivo trial revealed that 50% ethanolic extract of *Xanthium strumarium* leaves exerted antitrypanosomal effect on mice at dosage of 100, 300 and 1000 mg/kg intraperitoneally. At 100 and 300mg/kg dosages the survival period of the *Trypanosoma evansi* infected mice was significantly prolonged (Talakal *et al.*, 1995). The alcoholic extracts of *Xanthium strumarium* leaves, *Parthenium hysterophorus* flower and *Nyctanthes arbor-tristis* leaves were found effective *in vivo* at dosages of 100 and 300mg/kg bw (Dwivedi, 1997).

### 1.2.6.4 In vivo methods

Due to the cost and technical difficulties of maintaining large animals in laboratory conditions, the traditional method of testing for drug resistance has been performed using rodents (Godfrey and Killick-Kendrick, 1962). This is however limited to trypanosomes of the *Trypanozoon* and *Nannomonas* subgenera, which are infective to rodents. Parasites are inoculated into the rodents mainly by the intraperitoneal route at an inoculum dose ranging from $1 \times 10^5$ to $1 \times 10^6$ trypanosomes (Abebe *et al.*, 1983; Schillinger *et al.*, 1985; Peregrine *et al.*, 1990; Zhang *et al.*, 1992). The duration between infection and treatment varies from immediately after infection up to 48 hours post-infection (Jones and Folkers, 1966; Abebe *et al.*, 1983; Schillinger *et al.*, 1985; Peregrine *et al.*, 1990; Zhang *et al.*, 1992). The rodents are then bled regularly and the blood examined microscopically for parasitaemia using microscopic examination of wet blood smears or the haematocrit centrifugation technique (Woo, 1970). Persistence of parasitaemia or relapse after treatment is taken to be an indication of resistance to the trypanocide used. Variables such as the parasite inoculum dose and the timing of treatment may be important, especially in *T. brucei* family parasites which invade the central nervous system and the drugs used do not cross the blood-brain barrier.
1.2.6.5 Extract preparation and screening for biological activity

After securing the plant material, the next step in the process is to prepare extracts for biological activity screening. There are no laid down procedures for the extraction process, but as a guide the procedure should enable maximum extraction of secondary metabolites. It is desirable to include an aqueous extract prepared in the same way as herbalists do to enable an evaluation of the original drug.

It should also be recognized that certain processes used in the preparation of extracts, e.g., drying of plant material before extraction or extraction at high temperatures such as boiling in water, may alter the chemical nature of compounds within a plant material. Inadequate drying, may lead to microbial contamination and post harvest infections could also influence the chemical composition. An example is the antibiotic anthraquinones once isolated from Cinchona bark that were later found to have been formed in response to fungal attack (Chapman, 2002).
The present study evaluated the efficacy and safety of some plant extracts that are traditionally used to treat animal trypanosomosis in Kenya.

Figure 1: Procedure for obtaining bioactive compound from plants adopted from (Hamburger et al., 1991).
CHAPTER TWO
MATERIALS AND METHODS

2.1 Plant materials

In this study, ethnopharmacological information from literature of other researchers was used to select plants for analysis of trypanocidal activity against the *T. evansi*, in both *in vitro* and *in vivo* studies. The selection of plants was based on the fact that they belong to the same family with other plants reported to have trypanocidal activity elsewhere. A total of six medicinal plants were screened for antitrypanosomal activity (Table 1). Only the plant parts used for preparation of herbal medicines were analyzed and as such only these parts were collected. The parts of the plants collected from plants from their natural habitat were stem barks, leaves or the whole plant. *Azadirachta indica* A. Juss. (Neem) leaves and stem bark was collected from the National Museums of Kenya, Gede, Malindi Coast Province. *Prunus africana* (Hook, f.) Kalkm (stem bark), *Bidens pilosa* L. (whole plant), *Physalis peruviana* L. (whole plant), *Senna didymobotrya* (Fresen) Irwin & Barneby., (Syn. *Senna didymobotrya*) (stem bark and leaves) and *Croton megalocarpus* Hutch. (leaves) were collected during the day on a dry season from Murang’a District, Central Province. An acknowledged authority, Chief botanist, authenticated the botanical identity of the plants and a voucher specimen was deposited at the National Museums of Kenya Herbarium, Nairobi.
Table 1: Plants selected for in vitro antitrypanosomal activity screening

<table>
<thead>
<tr>
<th>Plant name &amp; family</th>
<th>Sample</th>
<th>Part Collected</th>
<th>Collection site</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bidens pilosa</td>
<td>A</td>
<td>Whole</td>
<td>Central Province</td>
<td>Jan, 2005</td>
</tr>
<tr>
<td>Compositae</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Physalis peruviana</td>
<td>B</td>
<td>Whole</td>
<td>Central Province</td>
<td>Jan, 2005</td>
</tr>
<tr>
<td>Solanaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prunus africana</td>
<td>C</td>
<td>Stem bark</td>
<td>Central Province</td>
<td>Jan, 2005</td>
</tr>
<tr>
<td>Rosaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azadirachta indica</td>
<td>D</td>
<td>Mature plant</td>
<td>Coast Province</td>
<td>Dec, 2004</td>
</tr>
<tr>
<td>Meliaceae</td>
<td></td>
<td>stem bark</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azadirachta indica</td>
<td>E</td>
<td>Leaves</td>
<td>Coast Province</td>
<td>Dec, 2004</td>
</tr>
<tr>
<td>Meliaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azadirachta indica</td>
<td>F</td>
<td>Young plant</td>
<td>Coast Province</td>
<td>Dec, 2004</td>
</tr>
<tr>
<td>Meliaceae</td>
<td></td>
<td>stem bark</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Senna didymobotrya</td>
<td>G</td>
<td>Stem bark</td>
<td>Central Province</td>
<td>Jan, 2005</td>
</tr>
<tr>
<td>Caesalpinioideae</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(formerly Leguminosae)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Senna didymobotrya</td>
<td>H</td>
<td>Leaves</td>
<td>Central Province</td>
<td>Jan, 2005</td>
</tr>
<tr>
<td>Caesalpinioideae</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(formerly Leguminosae)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Croton megalocarpus</td>
<td>I</td>
<td>Leaves</td>
<td>Central Province</td>
<td>Jan, 2005</td>
</tr>
<tr>
<td>Euphorbiaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.2 Experimental animals

Swiss white mice, about 6-8 weeks old were obtained from Kabete Veterinary Laboratories. They were housed 5 per cage, male and female separately and fed on commercial pellets and given water ad libitum. Wood shavings were provided as bedding material and changed frequently to maintain a dry housing environment. The mice were allowed to acclimatize for one week in the animal unit of the department of Biochemistry Trypanosomosis Research Centre (TRC) Muguga before any experimental procedures were conducted.

2.3 The parasite (Trypanosoma evansi)

Trypanosoma evansi, KETRI 2450 isolated from a camel at Galana ranch in 1979 was obtained from the trypanosome bank at the TRC of the Kenya Agricultural Research Institute (KARI), Muguga. The T. evansi was available as a population of trypanosomes preserved alive as a stabilate cryopreserved in liquid nitrogen.
2.4 Plant extraction procedure

Plant extraction apparatus and equipment are described in Appendix 3. All plant materials were collected from the field, chopped into small sizes and air dried for 3 weeks. The material was then milled into a powder. The powder was kept dry in polythene bags at room temperature away from direct sunlight until used.

2.4.1 Water extraction

An aqueous extract was prepared by soaking 10g powder in 100 ml distilled water, then heating in a water bath for 2 hours at 60°C. The mixture was left at room temperature for 10 hours. The extract was filtered and freeze dried. The yield of each plant extract was then recorded. The dried extracts were weighed and stored in cool dry conditions at 0-4°C until required for use.

2.4.2 Organic extraction (chloroform, methanol)

A 10g portion of plant material was soaked in 250 ml chloroform and left for 5 days at room temperature away from direct light. The material was then filtered and the filtrate concentrated in a rotary evaporator. The remaining chloroform was then blown out using nitrogen gas. After chloroform extraction the residue was spread out in the hood for the solvent to evaporate before extraction with the next organic solvent (methanol) which was done exactly as with chloroform. The yield in solvent extractions was then calculated.
Figure 2: Preparation of extracts for preliminary antitrypanosomal activity tests

1. Sequential extraction of plant powder soaked in organic solvent for 5 days. Filtrates were pooled and concentrated in a rotary evaporator. The residue was allowed to dry before the next extraction.

2. Plant powder soaked in a water bath at 60°C for 2 hours and left at room temperature for 10 hours. The preparation was then filtered and the filtrate freeze dried. The powder residue was discarded.
2.5 *In vitro screening*

The trypanosome stabilate was diluted in phosphate saline glucose (PSG) pH 8.0 (Appendix 4) and then injected intraperitoneally into two donor mice for multiplications. The trypanosomes whose parasitaemia was 5 per field (equivalent to $1.58 \times 10^7$ tryps/ml) was inoculated at 0.2ml/mouse into the donor mice. At the log phase of parasitaemia a drop of blood (5μl to 10μl) was obtained from the tail and trypanosomes separated from erythrocytes using the ion-exchange chromatography method of Lanham and Godfrey (1970). Infected mouse blood previously diluted with PSG was then applied to the top of the column and allowed to run into the cellulose (DE 52). Trypanosomes were eluted from the column with several volumes of PSG which was collected into a centrifuge tube cooled on ice. The trypanosome (tryps) concentration in the eluate was determined using the haemocytometer. The trypanosome separated was diluted to $1 \times 10^5$ tryps/ml in the medium.

A stock solution of 10 mg/ml in ethanol of each chloroform and methanol extracts was diluted with medium to achieve final well concentrations of 1.0, 0.5 and 0.1 mg/ml. Water extracts were also made into three different concentrations as follows, 2.0, 1.0 and 0.5 mg/ml from a stock solution of 20 mg/ml in water. A stock solution of 1.0 mg/ml in water of cymelarsan and berenil was diluted with medium to achieve final well concentrations of 1.0, 0.5 and 0.1 μg/ml. The three different concentrations tested with suramin (1.0, 0.5 and 0.1 mg/ml) was made by diluting the stock solution of 1.0 mg/ml with the medium. The stock solutions were first filter sterilized using 0.2 μm syringe filter before preparation of working dilutions. Each sample was tested in a one 96 well micro titer plate in 3 different concentrations. Thus a total of 31 plates were used, 27 for each herbal extract, 3 for each standard drug and one for ethanol as the diluent.
The assays on *in vitro* determination of anttrypanosomal activity were performed according to the procedures described by Freiburghaus *et al.* (1996). A 100 μl of media was added into wells of column 2 to column 10 in duplicate in 96 well plates (Appendix 3). 200 μl of the crude plant extracts, standard drugs and diluent were added into wells of column 11. Serial dilution of the initial extract concentration in column 11 was done by transferring 100 μl from wells of column 11 to column 10 after mixing up and down of the solution using a multi channel pipette. Then again after mixing up and down of solution in column 10, 100 μl were transferred from column 10 to column 9. This continued up to column 4, where the 100 μl were discarded from these wells. The wells of column 2 and column 3 contained 100 μl of media and they served as the control. To each well of column 2 to column 11 a 100μl of $1 \times 10^5$ tryps/ml was then added. The plates were placed in a modular chamber gassed with 5% CO$_2$ incubated at 37°C as described by Sutherland *et al.* (1993). The test was read using an inverted microscope at 100-fold magnification. In every row the highest dilution of either standard drug or sample extracts with no motile trypanosomes was determined. The concentration in this well was defined as the minimum inhibitory concentration (MIC).

### 2.5.1 Haemocytometry

The concentration of trypanosomes used for infection was determined by counting the parasites in a haemocytometer. Ten μl of a trypanosome suspension in culture medium was carefully transferred by touching a pipette tip onto the cover slip edge, the counts were made in duplicate using both chambers on either sides of the haemocytometer. The number of trypanosomes in four corner squares and the centre were counted using a microscope (x 400 magnification, Leitz Wetzlar SM- Lux, Germany). All trypanosomes touching the left and upper borders were included.
The number of trypanosomes per ml was determined using the following formula:

\[ \text{No. of tryps} = \text{the average count per square} \times \text{dilution factor} \times 10^4 \] (Appendix 6).

Appropriate dilutions of the trypanosome suspension were prepared.

### 2.5.2 Preparation of parasite

Trypanosomes were separated from the red blood cells on DE 52 column in PSG, pH 8.0. The concentration of trypanosomes was carried out by spinning at 3,000 rpm for 10 min (using burkard scientific UK, radius 115 mm centrifuge) and supernatant discarded. The pellet was resuspended in media and parasite counted.

Below is an example of one of the tests performed.

\[ 20 \times 5 = 100 \times 10^4 \times 10 \text{ (dilution factor)} \]

\[ = 1 \times 10^7 \text{ tryps/ml} \]

6 plates were used each 6 ml/plate = 36 ml. Thus at least 36 ml of media were required.

40 ml of \(1 \times 10^5\) tryps/ ml were made

\[ C_1V_1 = C_2V_2 \quad \text{Where } C = \text{Concentration and } V = \text{Volume.} \]

\[ 1 \times 10^7 \times \text{Volume} = 40 \times 1 \times 10^5/\text{ml} \]

\[ \text{Volume} = 40 \times \frac{10^5}{10^7} = 0.4 \text{ ml} \]

Thus 0.4 ml tryps suspension was added into 40 ml of the media. This was plated into each well at 100 µl per well. The efficacy of the plant extracts against \(T. evansi\) was evaluated by determining the minimum inhibitory concentration (MIC). The minimum inhibitory
concentration is the lowest concentration of an antitrypanosomal agent which inhibits the growth of the test trypanosomes.

2.6 Preliminary test for acute toxicity

The two most active plant extracts (*A. indica* and *P. peruviana*) *in vitro* were screened for acute toxicity in mice. The extract was dissolved in absolute alcohol at various concentrations and injected intraperitoneally into the groups of five healthy mice. The highest dose that did not kill mice was used as the highest therapeutic trial dose (dose X). Each mouse received one hundredth of its body weight as drug dose volume.

To determine toxicity of the most active plant extract in normal health mice, the mice were divided into 8 groups of 5 mice each. Group VII served as the control, and was treated with 0.2 ml ethanol, while Group VIII was untreated. Each mouse in group I - VI was intraperitoneally injected with 0.2 ml of Neem leaves chloroform extract at various dosages (900 mg/kg, 800 mg/kg, 700 mg/kg, 600 mg/kg, 500 mg/kg and 400 mg/kg body weights) (Table 11). The plant extract was administered once. The animals were kept under close observation and fed on pellet diet and water *ad libitum*. Clinical signs such as dyspnoea and lethargy were observed and recorded daily. The animals were weighed after every 2 days for 2 weeks. Soon after death, each animal was dissected and the liver, kidney, heart, lungs, spleen, brain and muscles removed. The organs were preserved in 10% buffered formalin solution for histopathology analysis. After 14 days the surviving mice were sacrificed and subjected to autopsy as for the dead mice. The experimental protocol for the second most active plant extract *P. peruviana* whole plant chloroform extract was done as described above, at three different dosages i.e. 1,000 mg/kg bw, 300 mg/kg bw and 100 mg/kg bw (Table 12).
2.6.1 Histopathology

The formalin-fixed organs were trimmed to include all the major parts to be investigated and washed in running water overnight to remove excess formalin. The tissues were then processed using an automatic tissue processor (Histokinette). The tissues were loaded into cassette bearing labels of the organ and closed safely. They were then dehydrated sequentially in increasing concentrations of alcohol i.e. 70% and 90% at hourly stepped intervals. Tissues were washed for 2 hours in 100% alcohol. Tissues were then cleared of alcohol twice in two changes of xylene. The tissues were infiltrated by passing them through molten paraffin wax for 6 hours, 3 hours in each wax bath. The tissues were then embedded in fresh molten wax in embedding moulds. Embedded tissues were then attached to the wooden block and sectioned at 0.5 μm thicknesses with a microtome and floated in warm water bath at 54°C to spread out, then attached onto a clean microscopic slide. After holding in hot oven for 15 minutes, the tissues sections were dewaxed in xylene and then stained with haematoxylin and eosin dyes using standard histological protocols. The stained tissues were cover slipped with DPX mountant, dried and examined microscopically for any pathological changes.

2.7 In vivo efficacy trials

The extracts with highest in vitro trypanocidal activity were tested in vivo. The highest dose that did not kill the mice during acute toxicity screening was chosen as the highest therapeutic trial dose for in vivo testing. *T.evansi* isolate KETRI 2450 that was used in the in vitro studies was used in vivo tests. The parasites were injected into two immunosuppressed (by irradiation) donor mice for propagation. At the first peak of parasitaemia donor mice were bled intra-cardially into cold PSG, pH 8.0 and parasitaemia quantified using a haemocytometer (Appendix 6). The parasites were diluted to 1 x 10⁴ trypanosomes / ml using cold PSG, pH 8.0.
Two extracts were tested each at three different concentrations, 500, 250 and 125 mg/kg for *A. indica* and 1000, 500 and 250 mg/kg bw for *P. peruviana* using five mice per concentration against *T. evansi*. The infected mice were treated at the onset of parasitaemia. The experiment was run alongside the standard drugs berenil at a dose of 7 mg/kg and cymelarsan together with suramin both at a dosage of 5mg/kg bw. The control groups (injected with ethanol as a diluent and untreated) were also set to run together with both plant extracts and standard drugs. The animal were monitored for parasitaemia for 42 days post treatment, daily for the first 2 weeks and thereafter twice per week. Weekly body weights were also recorded.

2.7.1 Experimental design

The experiment comprised of 11 groups of 5 mice each giving a total of 55 mice. All mice were infected with *T. evansi* at 1 x 10^4 tryps/ml intraperitoneally and then monitored by wet blood films for presence of parasite in the blood upon which the drug was administered through the same route (ip). Groups 1- 6 were treated with different concentrations of the plant extracts. Groups 7- 9, treated with each of the standard drug. Group 10, mice were infected and not treated. Group 11 mice were infected and then treated with diluent (ethanol), (Table 2).
Table 2: Experimental design for \textit{in vivo} evaluation on efficacy of plant extracts against \textit{T. evansi} infection in mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug</th>
<th>Dosage (mg/kg bw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bc</td>
<td>250</td>
</tr>
<tr>
<td>2</td>
<td>Bc</td>
<td>500</td>
</tr>
<tr>
<td>3</td>
<td>Bc</td>
<td>1000</td>
</tr>
<tr>
<td>4</td>
<td>Ec</td>
<td>125</td>
</tr>
<tr>
<td>5</td>
<td>Ec</td>
<td>250</td>
</tr>
<tr>
<td>6</td>
<td>Ec</td>
<td>500</td>
</tr>
<tr>
<td>7</td>
<td>Berenil</td>
<td>7</td>
</tr>
<tr>
<td>8</td>
<td>Cymelarsan</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>Suramin</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>Control</td>
<td>No treatment</td>
</tr>
<tr>
<td>11</td>
<td>Diluent (ethanol)</td>
<td>Ethanol</td>
</tr>
</tbody>
</table>

Key
Bc – \textit{Physalis peruviana} chloroform extract
Ec – \textit{Azadirachta indica} leaves chloroform extract

2.7.2 Mice infection, monitoring and treatment

Column-separated trypanosome suspensions provided as described in section 2.5.2 were diluted in phosphate saline glucose (PSG) (pH 8.0) to a concentration of $1 \times 10^4$ tryps/ ml each experimental mouse was infected intraperitoneally with 0.2 ml of this parasite dosage.

In order to monitor the course of infection, tail blood samples were collected daily from experimental animals. Blood was examined microscopically as a wet blood smear on a slide (Baker, 1970). Wet blood films were prepared by placing a small drop of blood on clean, grease - free glass slide (75 x 25mm) and overlaid with a cover slip (22 x 22 mm). The slide was then examined for the presence of trypanosomes in 20 fields at x 400 magnification. The number of
parasites was determined microscopically at x 400 magnification as outlined in the "Rapid Matching" method of Herbert and Lumsden (1976). The method involves microscopic counting of parasite per field in a drop of blood from the tail, pre-sterilized with methylated spirit. Logarithm values of these counts obtained by matching with the table of Herbert and Lumsden (1976) is converted to antilog to provide absolute number of trypanosomes per ml of blood. When the parasitaemia became patent, the infected animals were treated intraperitoneally where each mouse received a dosage equivalent to its body weight.

2.8 Data analysis

The trypanocidal activity of the crude plant extracts in the in vitro test was evaluated by determining the minimum inhibitory concentration (MIC). To test the reliability of the assay for the test extracts and standard drugs, the coefficient of variation, a measure for the precision of the technique, was calculated by comparing the actual content of the reference samples and the values obtained by the bioassay. Toxicity of the plant extracts was determined by comparing body weight changes, mortality rate, and survival period with histological data. Clinical signs of acute toxicity were also used to assess toxicity of the extracts. The in vivo effects of plant extracts on trypanosomes were assessed using mice mortality data, number of mice with relapsing infections, period of relapse, and days after inoculation to expression of infection and percentage of mice cured of T. evansi infection. Levels of parasitaemia and change in body weight were also used to monitor the test drugs efficacy. Regression analysis was performed with level of parasitaemia as the response variable, days after treatment as the explanatory variable and the extracts as the groups. The variation between and within treatment groups were determined using ANOVA. Statistical analysis was performed using SPSS.
CHAPTER THREE

RESULTS

3.1 Yield of herbal extracts

Nine samples from six medicinal plants (Table 1) were extracted using three different solvents and the calculated yield of plant extract per sample is shown in Tables 3, 4 and 5.

*Prunus africana* (stem bark) yielded the highest amount of crude aqueous extract (11%), followed by *Senna didymobryta* (leaves), *A. indica* (stem bark) with 6.5% and 6.27% respectively. *Senna didymobryta* (stem bark) had the lowest yield of crude extract, 1.37% (Table 3).

Table 3: Percentage yield of crude water extracts

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight (grams)</th>
<th>% yield</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. pilosa</em> (whole plant)</td>
<td>0.55</td>
<td>1.83</td>
</tr>
<tr>
<td><em>P. peruviana</em> (whole plant)</td>
<td>1.06</td>
<td>3.53</td>
</tr>
<tr>
<td><em>P. africana</em> (stem bark)</td>
<td>3.30</td>
<td>11.0</td>
</tr>
<tr>
<td><em>A. indica</em>/Neem (stem bark)</td>
<td>1.88</td>
<td>6.27</td>
</tr>
<tr>
<td><em>A. indica</em>/Neem (leaves)</td>
<td>0.83</td>
<td>2.77</td>
</tr>
<tr>
<td><em>A. indica</em>/Neem (young plant stem bark)</td>
<td>0.59</td>
<td>1.97</td>
</tr>
<tr>
<td><em>S. didymobryta</em> (stem bark)</td>
<td>0.41</td>
<td>1.37</td>
</tr>
<tr>
<td><em>S. didymobryta</em> (leaves)</td>
<td>1.95</td>
<td>6.5</td>
</tr>
<tr>
<td><em>C. megalocarpus</em> (leaves)</td>
<td>1.15</td>
<td>3.83</td>
</tr>
</tbody>
</table>

Among the methanol extracts, *Physalis peruviana* (whole plant) had the highest yield (16.47%), followed by *Prunus africana* (stem bark) and *Azadirachta indica* (stem bark) which yielded
15.33% and 15.17% respectively. *Bidens pilosa* (whole plant) yielded the lowest with 1.13% (Table 4).

**Table 4: Percentage yield of crude methanol extracts**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight (grams)</th>
<th>% yield</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. pilosa</em> (whole plant)</td>
<td>0.34</td>
<td>1.13</td>
</tr>
<tr>
<td><em>P. peruviana</em> (whole plant)</td>
<td>4.94</td>
<td>16.47</td>
</tr>
<tr>
<td><em>P. africana</em> (stem bark)</td>
<td>4.60</td>
<td>15.33</td>
</tr>
<tr>
<td><em>A. indica</em> (stem bark)</td>
<td>4.55</td>
<td>15.17</td>
</tr>
<tr>
<td><em>A. indica</em> (leaves)</td>
<td>0.43</td>
<td>1.43</td>
</tr>
<tr>
<td><em>A. indica</em> (young plant stem bark)</td>
<td>2.98</td>
<td>9.93</td>
</tr>
<tr>
<td><em>S. didymobotrya</em> (stem bark)</td>
<td>3.63</td>
<td>12.1</td>
</tr>
<tr>
<td><em>S. didymobotrya</em> (leaves)</td>
<td>0.46</td>
<td>1.53</td>
</tr>
<tr>
<td><em>C. megalocarpus</em> (leaves)</td>
<td>0.45</td>
<td>1.50</td>
</tr>
</tbody>
</table>

*A. indica* (leaves) yielded the highest amount of crude chloroform extract (3.63%), followed by *A. indica* (young plant stem bark) and *A. indica* (mature plant stem bark) which yielded 1.79% and 1.57%, respectively while *Prunus africana* (stem bark) had the lowest yield (0.20%) (Table 5).
### Table 5: Percentage yield of crude chloroform extracts

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight (grams)</th>
<th>% yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. pilosa (whole plant)</td>
<td>0.20</td>
<td>0.67</td>
</tr>
<tr>
<td>P. peruviana (whole plant)</td>
<td>0.18</td>
<td>0.60</td>
</tr>
<tr>
<td>P. africana (stem bark)</td>
<td>0.03</td>
<td>0.20</td>
</tr>
<tr>
<td>A. indica/Neem (stem bark)</td>
<td>0.47</td>
<td>1.57</td>
</tr>
<tr>
<td>A. indica/Neem (leaves)</td>
<td>1.09</td>
<td>3.63</td>
</tr>
<tr>
<td>A. indica/Neem (young plant stem bark)</td>
<td>0.54</td>
<td>1.79</td>
</tr>
<tr>
<td>S. didymobota (stem bark)</td>
<td>0.32</td>
<td>1.07</td>
</tr>
<tr>
<td>S. didymobotrya (leaves)</td>
<td>0.38</td>
<td>1.27</td>
</tr>
<tr>
<td>S. megalocarpus (leaves)</td>
<td>0.40</td>
<td>1.33</td>
</tr>
</tbody>
</table>

#### 3.2 In vitro trypanocidal activity

The trypanocidal activity of twenty seven crude plant extracts from six plants was analyzed *in vitro* against *T. evansi*, 9 each of chloroform, methanol and water. The test was run for 24 hours with all the 27 extracts and results obtained are shown in Table 6.

Attempts to adapt the trypanosomes to the culture media were unsuccessful after several trials. In one of the trials, parasites incubated with the control, chloroform and methanol extracted samples and cymelarsan were able to survive for a period of up to 48 hours while those incubated with water extract and standard drugs berenil and suramin survived for up to 24 hours. However, in order to assure uniformity of the results only data generated over the 24 hour period of testing were selected for *in vitro* evaluation of efficacy (Table 6).
<table>
<thead>
<tr>
<th>Extract</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Azadirachta indica /Neem (leaves) - Chloroform.</td>
<td>18.7</td>
</tr>
<tr>
<td>2 Azadirachta indica /Neem (stem bark) - Chloroform.</td>
<td>37.5</td>
</tr>
<tr>
<td>3 Physalis peruviana (whole plant) - Chloroform.</td>
<td>37.5</td>
</tr>
<tr>
<td>4 Prunus africana (stem bark) - Methanol.</td>
<td>37.5</td>
</tr>
<tr>
<td>5 Azadirachta indica /Neem (stem bark) - Chloroform.</td>
<td>41.8</td>
</tr>
<tr>
<td>6 Physalis peruviana (whole plant) - Methanol.</td>
<td>42.7</td>
</tr>
<tr>
<td>7 Croton megalocarpus (leaves) - Chloroform.</td>
<td>52.2</td>
</tr>
<tr>
<td>8 Bidens pilosa (whole plant) - Chloroform.</td>
<td>58.3</td>
</tr>
<tr>
<td>9 Azadirachta indica /Neem (young plant stem bark) - Methanol.</td>
<td>58.3</td>
</tr>
<tr>
<td>10 Azadirachta indica /Neem (stem bark) – Water.</td>
<td>62.5</td>
</tr>
<tr>
<td>11 Prunus africana (stem bark) – Water.</td>
<td>62.5</td>
</tr>
<tr>
<td>12 Senna didymobotrya (stem bark) - Chloroform.</td>
<td>67.0</td>
</tr>
<tr>
<td>13 Senna didymobotrya (leaves) - Chloroform.</td>
<td>93.8</td>
</tr>
<tr>
<td>14 Azadirachta indica (Stem bark) - Methanol.</td>
<td>93.8</td>
</tr>
<tr>
<td>15 Prunus africana (stem bark) - Chloroform.</td>
<td>104</td>
</tr>
<tr>
<td>16 Senna didymobotrya (stem bark) - Methanol.</td>
<td>125</td>
</tr>
<tr>
<td>17 Azadirachta indica /Neem (young plant stem bark) – Water.</td>
<td>177</td>
</tr>
<tr>
<td>18 Croton megalocarpus (leaves) - Methanol.</td>
<td>213</td>
</tr>
<tr>
<td>19 Senna didymobotrya (leaves) - Methanol.</td>
<td>250</td>
</tr>
<tr>
<td>20 Azadirachta indica /Neem (leaves) - Methanol.</td>
<td>250</td>
</tr>
<tr>
<td>21 Bidens pilosa, (whole plant) – Water.</td>
<td>250</td>
</tr>
<tr>
<td>22 Bidens pilosa (whole plant) - Methanol.</td>
<td>500</td>
</tr>
<tr>
<td>23 Physalis peruviana (whole plant) – Water.</td>
<td>500</td>
</tr>
<tr>
<td>24 Azadirachta indica /Neem (leaves) – Water.</td>
<td>500</td>
</tr>
<tr>
<td>25 Senna didymobotrya (stem bark) – Water.</td>
<td>500</td>
</tr>
<tr>
<td>26 Senna didymobotrya (leaves) – Water.</td>
<td>500</td>
</tr>
<tr>
<td>27 Croton megalocarpus (leaves) – Water.</td>
<td>500</td>
</tr>
</tbody>
</table>
3.2.1 Efficacy of plant extract as determined by the MIC

Tables 7, 8 and 9 show the minimum inhibition concentration of plant extracts starting with the most efficacious for the three solvents (chloroform, methanol and water).

3.2.1.1 Trypanocidal activity of chloroform extracts

The most effective chloroform extract after 24 hours was observed from *A. indica* (leaves) with MIC of 18.75 μg/ml. This was followed by extracts of *Physalis peruviana* (whole plant) with a MIC of 37.5 μg/ml. Extracts of *Azadirachta indica* (leaves) and *Physalis peruviana* (whole plant) were the most active extracts after 24 hours, while extracts of *Senna didymobotrya* (leaves) was the least effective with a MIC of 93.8 μg/ml (Table 7).

Table 7: Minimum inhibitory concentration (MIC) for chloroform extracts

<table>
<thead>
<tr>
<th>Sample</th>
<th>MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Azadirachta indica</em> /Neem (Leaves)</td>
<td>18.75</td>
</tr>
<tr>
<td><em>Physalis peruviana</em> (whole plant)</td>
<td>37.5</td>
</tr>
<tr>
<td><em>Azadirachta indica</em> /Neem (stem bark)</td>
<td>37.5</td>
</tr>
<tr>
<td><em>Prunus africana</em> (stem bark)</td>
<td>37.5</td>
</tr>
<tr>
<td><em>Azadirachta indica</em> /Neem (young plant stem bark)</td>
<td>41.8</td>
</tr>
<tr>
<td><em>Croton megalocarpus</em> (leaves)</td>
<td>52.2</td>
</tr>
<tr>
<td><em>Bidens pilosa</em> (whole plant)</td>
<td>58.3</td>
</tr>
<tr>
<td><em>Senna didymobotrya</em> (stem bark)</td>
<td>67.0</td>
</tr>
<tr>
<td><em>Senna didymobotrya</em> (leaves)</td>
<td>93.8</td>
</tr>
</tbody>
</table>

3.2.1.2 Trypanocidal activity of methanol extracts

The most effective methanol extract was *Prunus africana* (stem bark) with MIC of 37.5 μg/ml followed by extracts of *Physalis peruviana* (whole plant) which had MIC of 42.7 μg/ml. Extracts of *Bidens pilosa* (whole plant) was the least effective with MIC of 500 μg/ml (Table 8).
Table 8: Minimum inhibitory concentration (MIC) for methanol extracts

<table>
<thead>
<tr>
<th>Sample</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Prunus africana</em> (stem bark)</td>
<td>37.5</td>
</tr>
<tr>
<td><em>Physalis peruviana</em> (whole plant)</td>
<td>42.7</td>
</tr>
<tr>
<td><em>Azadirachta indica</em> /Neem (stem bark)</td>
<td>58.3</td>
</tr>
<tr>
<td><em>A. indica</em> /Neem (young plant stem bark)</td>
<td>93.7</td>
</tr>
<tr>
<td><em>Senna didymobotyra</em> (stem bark)</td>
<td>125</td>
</tr>
<tr>
<td><em>Croton megalocarpus</em> (leaves)</td>
<td>213</td>
</tr>
<tr>
<td><em>Senna didymobotyra</em> (leaves)</td>
<td>250</td>
</tr>
<tr>
<td><em>Azadirachta indica</em> /Neem (leaves)</td>
<td>250</td>
</tr>
<tr>
<td><em>Bidens pilosa</em> (whole plant)</td>
<td>500</td>
</tr>
</tbody>
</table>

3.2.1.3 Trypanocidal activity of water extracts

The most effective water extracts were *Prunus africana* (stem bark) and *A. indica* (stem bark) which had a MIC of 62.5 µg/ml, while the least effective were *Physalis peruviana* (whole plant), *Azadirachta indica* /Neem (leaves), *Senna didymobotyra* (stem bark), *Senna didymobotyra* (leaves), *Croton megalocarpus* (leaves) with a MIC of 500 µg/ml (Table 9).

Table 9: Minimum inhibitory concentration (MIC) for water extracts

<table>
<thead>
<tr>
<th>Sample</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Prunus africana</em> (stem bark)</td>
<td>62.5</td>
</tr>
<tr>
<td><em>Azadirachta indica</em> /Neem (stem bark)</td>
<td>62.5</td>
</tr>
<tr>
<td><em>Azadirachta indica</em> /Neem (young plant stem bark)</td>
<td>177</td>
</tr>
<tr>
<td><em>Bidens pilosa</em> (whole plant)</td>
<td>250</td>
</tr>
<tr>
<td><em>Physalis peruviana</em> (whole plant)</td>
<td>500</td>
</tr>
<tr>
<td><em>Azadirachta indica</em> /Neem (leaves)</td>
<td>500</td>
</tr>
<tr>
<td><em>Senna didymobotyra</em> (stem bark)</td>
<td>500</td>
</tr>
<tr>
<td><em>Senna didymobotyra</em> (leaves)</td>
<td>500</td>
</tr>
<tr>
<td><em>Croton megalocarpus</em> (leaves)</td>
<td>500</td>
</tr>
</tbody>
</table>
3.2.1.4 Trypanocidal activity of standard drugs

Table 10 shows the MIC of standard drugs. Cymelarsan had the highest trypanocidal activity among the three standard drugs tested, followed by berenil and suramin which had the least activity (Table 10).

**Table 10: Minimum inhibitory concentration (MIC) for the standard drugs**

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cymelarsan</td>
<td>0.034</td>
</tr>
<tr>
<td>Berenil</td>
<td>0.172</td>
</tr>
<tr>
<td>Suramin</td>
<td>250</td>
</tr>
</tbody>
</table>

The final concentration of diluent (ethanol) in the test solution was shown to have no effect on the parasite growth by including solvent controls alongside the tests which was also set to run for 24 hours period.

3.3 Preliminary acute *in vivo* toxicity of *A. indica* and *P. peruviana* extracts

3.3.1 Toxicity effects of *A. indica* extracts

Treatment of mice with 900mg/kg body weight resulted in 100% death. Administration of 700 – 800 mg/kg and 600 mg/kg led to 80% and 60% death rates respectively. On the other hand, 400 – 500 mg/kg body weight resulted in 100% survival (Table 11).
Table 11: Mortality of mice after 14 days of treatment with *A. indica* extracts

<table>
<thead>
<tr>
<th>Group</th>
<th>Dosage (mg/kg bw)</th>
<th>No. dead</th>
<th>% dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>900</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>II</td>
<td>800</td>
<td>4/5</td>
<td>80</td>
</tr>
<tr>
<td>III</td>
<td>700</td>
<td>4/5</td>
<td>80</td>
</tr>
<tr>
<td>IV</td>
<td>600</td>
<td>3/5</td>
<td>60</td>
</tr>
<tr>
<td>V</td>
<td>500</td>
<td>0/5</td>
<td>0</td>
</tr>
<tr>
<td>VI</td>
<td>400</td>
<td>0/5</td>
<td>0</td>
</tr>
<tr>
<td>VII</td>
<td>Control diluent (ethanol)</td>
<td>0/5</td>
<td>0</td>
</tr>
<tr>
<td>VIII</td>
<td>Control untreated</td>
<td>0/5</td>
<td>0</td>
</tr>
</tbody>
</table>

3.3.1.1 Clinical signs in mice after treatment with *A. indica* extracts

The most commonly observed clinical signs in mice treated with lethal doses of the extract with the best in vitro trypanocidal activity are lethargic with breathing difficulties (dyspnoea) and hair raised.

The observation on mice after injection with the most lethal dose (900 mg/kg bw) was that after the first fifteen minutes of treatment, one mouse was found to be lethargic with breathing difficulties (dyspnoea) and hair raised. By within three hours all mice showed lethargy, dyspnoea and had raised hair, and all five mice then died overnight after injection.

Mice with the moderately lethal dose (600 mg/kg bw) showed all mice had raised hair within 15 minutes. By the third hour, three mice were lethargic, with dyspnoea and hair raised, which later died after fifteen hours Two mice recovered from the clinical symptoms after one day.

The highest non lethal dose (500 mg/kg bw) showed that in the first fifteen minutes two mice had dyspnoea and hair raised. By within one hour, three mice were lethargic, with dyspnoea and
hair raised. Similar clinical signs were also observed after two hours. All mice recovered from clinical signs after twenty four hours.

Among the mice treated with ethanol, one mouse became weak and died after one and half hours. The other four mice were observed to have raised hair after the first fifteen minutes but later reverted to normal. The untreated mice were all normal.

3.3.1.2 Toxicity with extracts of *P. peruviana*

All the mice survived even after treatment with the highest dosage level of 1000mg/kg body weight of *P. peruviana* extracts (Table 12).

<table>
<thead>
<tr>
<th>Group</th>
<th>Dosage (mg/kg bw)</th>
<th>No. dead</th>
<th>% dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1,000</td>
<td>0/5</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>300</td>
<td>0/5</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>100</td>
<td>0/5</td>
<td>0</td>
</tr>
<tr>
<td>IV</td>
<td>Control diluent (ethanol)</td>
<td>0/5</td>
<td>0</td>
</tr>
<tr>
<td>V</td>
<td>Control untreated</td>
<td>0/5</td>
<td>0</td>
</tr>
</tbody>
</table>

3.3.2.1 Clinical signs in mice after treatment with *P. peruviana* extracts

The most commonly observed clinical signs in mice treated with lethal doses of the extract showing the second highest *in vitro* trypanocidal activity are lethargy and dyspnea.

Administration of 1000mg/kg body weight *P. peruviana* extract revealed that by within 4 hours two mice were lethargic, with dyspnoea and had hair raised. Observations revealed that the two
mice which had earlier expressed the clinical signs (lethargy and dyspnea) had recovered after two days and thereafter up to entire fourteen days of observation.

The dosage of 300mg/kg body weight caused one mouse to be lethargic (difficulties in breathing) by within 1 hour. Observation revealed that by within 4 hours the mouse that was lethargic had recovered.

Administration of 100mg/kg body weight caused one mouse to be lethargic and had hair raised within 30 minutes. By within 4 hours the mouse that had displayed lethargy had recovered.

3.3.2 Body weight changes in mice after treatment with plant extracts
Cumulatively, *A. indica* extracts resulted in death of large number of mice (Table 11) than *P. peruviana* extracts (Table 12) where no death was noted. The overall body weight changes in mice treated with various dosage levels is shown in figure 3.

Mice treated with *A. indica* leaves chloroform extracts had a significant (P= 0.001) decline in mean body weight while those treated with *P. peruviana* extracts did not show any significant change statistically in mean body weight (Figure 3).
Figure 3: Change in mean body weight of mice treated with chloroform extracts of *A. indica* and *P. peruviana*.

3.3.3 Histopathology

*P. peruviana* extract (Bc) caused no pathology in all the tissues even at its highest concentration of 1000 mg/kg body weight. *A. indica* extract (Ec) showed pathological changes in kidney, liver and lungs.
Chloroform extract of *A. indica* leaves caused distension of pulmonary alveoli which were occasionally ruptured in some foci. This emphysema (Figure 4) was prominently observed at the highest concentration of 900 mg/kg. Dosage of 600 mg/kg body weight caused mild emphysema. Comparison of emphysematous and normal lungs (shown here at the same magnification) demonstrates the marked increase in alveolar volume and consequent marked reduction in area of alveolar wall available for gaseous exchange in emphysema (Figure 4).

In the highest dosage level, 900 mg/kg, of *A. indica* chloroform extract, showed marked congestion of the renal cortex and medulla (Figure 6). Renal congestion also occurred at dosage level 600 mg/kg but decreased in intensity at dosage level 500 mg/kg body weight. Congestion of the liver also occurred after treatment with the *A. indica* chloroform extract at a dosage level of 900 mg/kg body weight (Figure 8) but was absent at lower dosage levels. Dark granular deposits occurred in kupffer cells at this dose.
Figure 4: Photomicrograph of a mouse lung tissue after administration of *A. indica* chloroform extract.

Figure 5: Photomicrograph of a normal mouse lung alveoli.
Figure 6: Photomicrograph of a mouse kidney tissue after administration of *A. indica* chloroform extract.

Figure 7: Photomicrograph showing control mouse kidney tissue.
Figure 8: Photomicrograph of a mouse liver tissue after treatment with chloroform extract of *A. indica*.

Figure 9: Photomicrograph of control mouse liver tissue.
3.4. *In vivo* evaluation of efficacy of plant extracts against *T. evansi*

3.4.1 Prepatent period

Results on duration from inoculation to the appearance of parasites in the blood (prepatent period) showed that a part from the four outlying observations, variation within drug groups was low as shown by the tabulated ranges. Those assigned 250mg/kg dosage of *A. indica* extract and ethanol were treated after 6 - 8 days post inoculation and the rest after 10 to 17 days (Table 13).

**Table 13: Prepatent period in days**

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Mouse No.</th>
<th>Range</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bc 250mg/kg</td>
<td>18 15</td>
<td>3</td>
<td>16.50</td>
</tr>
<tr>
<td>Bc 500mg/kg</td>
<td>16 18</td>
<td>(2) 13</td>
<td>(17) 21.00</td>
</tr>
<tr>
<td>Bc 1000mg/kg</td>
<td>15 15 17 14</td>
<td>3</td>
<td>15.25</td>
</tr>
<tr>
<td>Ec 125mg/kg</td>
<td>32 18 15 16</td>
<td>(3) 17</td>
<td>(16.3) 20.25</td>
</tr>
<tr>
<td>Ec 250mg/kg</td>
<td>7 7 7 7 7</td>
<td>0</td>
<td>7.00</td>
</tr>
<tr>
<td>Ec 500mg/kg</td>
<td>23 16</td>
<td>( ) 7</td>
<td>(16) 19.50</td>
</tr>
<tr>
<td>Berenil 7mg/kg</td>
<td>10 10 10 10 10</td>
<td>0</td>
<td>10.00</td>
</tr>
<tr>
<td>Cymelarsan 5mg/kg</td>
<td>11 21 11 11 11</td>
<td>(0) 10</td>
<td>(11) 13.00</td>
</tr>
<tr>
<td>Suramin 5mg/kg</td>
<td>10 10 10 10</td>
<td>0</td>
<td>10.00</td>
</tr>
<tr>
<td>Control</td>
<td>16 15 15 1</td>
<td>1</td>
<td>15.33</td>
</tr>
<tr>
<td>Ethanol control</td>
<td>7 6 8 8 2</td>
<td>2</td>
<td>7.25</td>
</tr>
</tbody>
</table>

**Key**

Bc - *P. Peruviana* chloroform extract

Ec - *A. indica* leaves chloroform extract

Bold face indicates outlier.
Values in the bracket indicates value ignoring outlier.

### 3.4.2 Level of parasitaemia after treatment of mice with different extracts

Figures, 10, 11, 12, 13, 14 and 15 show mean parasitaemia levels of infected mice treated with plant extracts and standard drugs against controls (treated with ethanol and untreated) over a period of 42 days post treatment. The values of parasitaemia indicated are in logarithms. To get the actual number of parasites per ml, antilog was worked out using a scientific calculator.

e.g. 8.4 inv. Log = 2.5 x 10^8 tryps/ml.

#### 3.4.2.1 Treatment response with *A. indica* extracts

At a dosage level of 500 mg/kg *A. indica* chloroform leaves extract reduced the number of parasites to undetectable levels by the end of day twelve (after the first peak of parasitaemia). The parasites reappeared at reduced levels (7.94 tryps/ml) on day 21 after which they fell to undetectable levels from day 24 to the end of the experiment. Dosage level 250 mg/kg bw also reduced the parasite load from 5.0 x 10^6 tryps/ml at day zero to undetectable levels on day 12. The parasitaemia relapsed by day 15 and parasite remained detectable up to day 24. Between days 27 and 36, the parasites remained undetectable. On day 39, parasitaemia relapsed again and remained detectable up to the end of the experiment. Dosage level of 125 mg/ kg bw reduced the number of parasites from 1.26 x 10^6 tryps/ml at day zero to undetectable levels from day 24 to day 33 after which parasites were then noted in mice on day 36 and day 42, though at reduced numbers of 3.98 tryps/ ml and 2.5 tryps/ml respectively (Figure 10).
Figure 10: Mean parasitaemia levels of infected mice after treatment with *A. indica*.

### 3.4.2.2 Treatment response with *P. peruviana* extracts

The three dosage levels of *P. peruviana* chloroform extracts reduced parasite load to undetectable levels at different days. Although the parasites were later noted in mice the numbers were reduced compared to initial levels before treatment. 500 mg/kg bw dose reduced the number of parasites from $4.0 \times 10^5$ tryps/ml at day zero to undetectable levels by day 9. Then on day 12, 15.8 tryps/ml were then noted again until day 18 after which they were reduced to undetectable levels up to the end of the 42 days observation period (with exception of day 27 when 15.8 tryps/ml were noted) (Figure 11).
3.4.2.3 Treatment response with standard drugs

Cymelarsan reduced the parasites level from $4.0 \times 10^5$ tryps/ml at day zero to undetectable levels by day 3. No parasite was detected in mice thereafter up to the end of 42 day observation period. Treatment of infected mice with berenil (at parasite load of $1.0 \times 10^6$ tryps/ml) caused a reduction in number of parasite to undetectable levels by day 3. The parasites were again observed in mice at reduced levels of 6.3 tryps/ml on day 21 and 12.3 tryps/ml on day 24. Parasites then remained undetectable from day 27 up to day 39 after which reduced numbers 3.98 tryps/ml were noted again on day 42. Though suramin was able to bring down the number of parasites in mice, it didn’t reduce to undetectable levels at any one time during the 42 days observation period (Figure 12).

Figure 11: Mean parasitaemia levels of infected mice after treatment with *P. peruviana*.
Cymelarsan was the most effective drug in eliminating parasites from infected animals. Berenil which was the second after cymelarsan was closely followed by *A. indica* leaves chloroform extract at dosage level of 500 mg/kg body weight. Suramin had the least antitrypanosomal activity which was significantly lower than the rest at P = 0.001 (Figure 13).
Figure 13: Mean parasitaemia levels of infected mice after treatment with the most effective dosage of *A. indica*, and standard drugs.

500 mg/kg bw of *P. peruviana* chloroform extract performed better than suramin. However, cymelarsan was the most effective antitrypanosomal agent followed by berenil (Figure 14).
Figure 14: Mean parasitaemia levels of infected mice after treatment with the most effective dosage of *P. peruviana* and standard drugs.

Chloroform extract of *A. indica* leaves was found to be more effective than that of *P. peruviana* whole plant, both at similar dose of 500 mg/kg body weight (Figure 15).
Figure 15: Mean parasitaemia levels of infected mice after treatment with the most effective two dosages each of *A. indica* and *P. peruviana*.

### 3.4.3 Parasite relapse after treatment of infected mice

Out of the 18 mice treated with the plant extracts and with available data, only 3 did not relapse, one after treatment with *P. peruviana* and two after treatment with *A. indica*. Extract of *A. indica* performed better in reducing the number of parasites than *P. peruviana* and both were better than the standard drugs suramin and berenil where all mice relapsed. Cymelarsan had no relapsing parasitaemia.
3.4.4 Mortality of treated infected mice

Table 14 shows the levels of parasitaemia last recorded immediately before the deaths of treated (with test extracts, standard drugs and diluent) infected (with *T. evansi*) mice. All mice that were treated with *A. indica* extract (125 mg/kg bw), cymelarsan and berenil survived the entire period of treatment. Treatment with suramin resulted in death of one of the five treated mice. Three mice died after treatment with diluent. Treatment with each of the three dosage levels of *P. peruviana* caused death of one out of the five mice.

**Table 14: Last recorded levels of parasitaemia (logarithms) of dead infected mice**

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Mouse No.</th>
<th>Total</th>
<th>Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>Bc</strong> 250mg/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bc</strong> 500mg/kg</td>
<td></td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td><strong>Bc</strong> 1000mg/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ec</strong> 125mg/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ec</strong> 250mg/kg</td>
<td></td>
<td>8.1</td>
<td>9.0</td>
</tr>
<tr>
<td><strong>Ec</strong> 500mg/kg</td>
<td></td>
<td>7.2</td>
<td>6.3</td>
</tr>
<tr>
<td>Berenil (DA) 7mg/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cymelarsan 5mg/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suramin 5mg/kg</td>
<td></td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol control</td>
<td></td>
<td>5.4</td>
<td>9.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Key
Bc - *P. peruviana* chloroform extract
Ec - *A. indica* leaves chloroform extract
3.4.5 Change in body weight over a duration of 60 days

Treatment with chloroform extracts of *A. indica* 500mg/kg showed no significant (*P* = 0.05) change in mean body weight of infected mice during the 60 days observation period (Figure 16).

![Graph showing body weight changes over 60 days](image)

**Figure 16:** Mean body weight of infected mice treated with chloroform extract of *A. indica*.

Infected mice treated with *P. peruviana* extracts showed an increase in mean body weight with dosage level 250 mg/kg body weight having the highest body weight increase from 23.5 g at day zero to 25.6g on day 60 was statistically significant (*P* = 0.001) (Figure 17).
Figure 17: Mean body weight of infected mice treated with *P. peruviana*. 
A slight but significant \((P = 0.01)\) increase in mean body weight was noted after treatment of infected mice with standard drugs particularly cymelarsan (Figure 18).

Figure 18: Mean body weight of infected mice treated with standard drugs.
CHAPTER FOUR

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 Discussion

Methanol yielded the highest percentage of crude extracts followed by water and the chloroform. This is consistent with experiments on plant extracts by Sara et al. (2004). This observation can be accounted for in terms of solubility, implying that the active principles present in the various plant extracts were mostly soluble in methanol than in either water or chloroform.

*In vitro* studies showed that among the three extracts tested, chloroform extracts had the best trypanocidal effects. The results correspond well with findings of Sara et al. (2004) where methylene chloride extracts were reported to be more active on trypanosomes (*T. b. brucei*) than the water and methanol extracts. *A. indica* leaves chloroform extract emerged the best among the twenty seven samples tested with MIC of 18.75 μg/ml after 24 hours. Similar antitrypanosomal activity were observed by Sara et al. (2004) with *Trichilia emetica* (Meliaceae), the same family with Neem, against *T. b. rhodesiense*. Chloroform extracts of mature plant stem bark of *A. indica* had similar MIC levels (37.5 μg/ml) after 24 hours with two other extracts *P. peruviana*, whole plant chloroform extract, and *P. africana* stem bark methanoic extract. Water extract of Neem, stem bark, had a MIC of 62.5 μg/ml. The same results were achieved in a study carried out by Gathumbi et al. (2007) where aqueous extracts of *Azadirachta indica* (Neem) stem bark inhibited growth of *T. brucei* at *in vitro* concentrations of 62.5 μg/ml.

Results showed that chloroform extracts, from older (mature) Neem plants gave a lower MIC (18.75 μg/ml) than extract from younger plants with MIC of 41.8μ g/ml. This suggest that the difference in antitrypanosomal could be influenced by the age of the plant (Table 6 and 7). Older
plants are known to concentrate their active components as the quantity of plant secondary metabolites increases with maturity (Trease and Evans, 2000).

The MIC of chloroform extract of *A. indica* leaves decreased from 18.75 μg/ml after 24 hours to 7.88 μg/ml after 48 hours. Those extracts that were active after 24 hours also retained activity after 48 hours. This suggests that an agent with trypanocidal activity show its effect at the outset. The six best extracts after 48 hours of bioassay were all chloroformic, suggesting that chloroform was a better solvent of active principle with antitrypanosomal activity, and the activity increased with time. This is as expected that the exposure time of parasites to the plant extracts should significantly improve the biological activity of the extract. The two standard drugs, cymelarsan and berenil performed better than any of the plant extracts tested. Their cytotoxicity effects notwithstanding most of the extracts performed better than suramin (Table 10). The results agree with those of Sara *et al.* (2004) who observed that the IC_{50} values for the active plant extracts were high compared to the values obtained for commonly used drugs such as suramin.

These results corresponded well with studies by Atindehou *et al.* (2003) where *Trichilia emetica* (Meliaceae) was the best crude ethanol extracts tested for antitrypanosomal activity against *T. b. rhodesiense*. Leave extracts from other plants, *Strychnos spinosa* and *Hymenocardia acida* have also shown *in vitro* activity against *T.b. rhodesiense* (Freiburghaus *et al.*, 1996). These plants (*S. spinosa* (Loganiaceae), *H. acida* (Euphorbiaceae) and *A. indica* (Meliaceae)) antitrypanosomal activity could be due to alkaloids which are common in the three families or other compounds having antiprotozoal activity *in vitro* (Ashutosh, 2007). Although this study did not carry out structure elucidation, it is documented that extracts with potent trypanocidal activity have been reported to contain alkaloids, flavonoids, phenolics, and/or terpenes (Hopp *et al.*, 1976).
The results of Freiburghaus et al. (1996) indicated that different solvent extracts of the same plant may exhibit different trypanocidal activity just as extracts of different parts of the same plants. This corresponds well with results of this study (Table 6). Such differences between results in this study and those of other authors may be due to variation in the chemical composition of plants from different geographical areas, age and the time or season of plant collection (Trease and Evans, 2000). Plant constituents are affected by environmental factors such as altitude, temperature, moisture, light, soil type and genetic factors, together with handling after harvest. In many alkaloids- yielding plants (e.g. *Datura* metel), the alkaloid content peaks in the dry season and is lowest in the rainy season (Padua et al., 1999).

Alkaloids such as berberine have been reported to be rapidly active against amoebae and the trophozoites of *Entamoeba histolytica* and also effective against the pathogens of leishmaniosis (Hans, 1996). In cancer chemotherapy, however, the two binary indole alkaloids, vincristine and vinblastine present in *Catharanthus roseus* (Apocynaceae) are well known for their antitumor activity (Padua et al., 1999). Two compounds found in Neem leaves gedunin, a limonoid, and quercetin, a flavonoid are at least as effective as quinine and chloroquine against malaria. Irodin A also from Neem leaves was found effective against resistant strains of malaria (http://freedomantiviral.addr.com/feedback.htm-19/09/2007). The antitrypanosomal activity of *A. indica* can also be attributed to presence of saponins as reported by Igweh et al. (1989) on the chemotherapeutic effects of *Annona senegalensis* in *Trypanosoma brucei brucei*.

Preliminary phytochemical analysis of *P. peruviana* has shown presence of various components in the crude extract such as flavonoids, phenols, tocopherols, fatty acids, β- carotene, vitamin K (Arun, 2006). Trypanocidal activity of *P. peruviana* extracts can further be attributed to presence of phenols. Presence of phenolic compound in plant extracts have been reported to exhibit trypanocidal activity (Hopp et al., 1976). Physalins an ergostane type steroidal derivative
extracted from *P. angulata* (solanaceae) same family with *P. peruviana* was found useful in the treatment of infections caused by protozoans. The physalins also have prophylactic and therapeutic properties for treating auto-immune diseases in mammals (http://freepatentsonline.com/20020103386.html-20/09/2007).

It is difficult to speculate the mechanism by which the plants active principles exhibit their trypanocidal action. However, accumulated evidence (Sepulveda- Boza and Cassles, 1996) suggest that many natural products exhibit their trypanocidal activity by virtue of their interference with the redox balance of the parasites acting either on the respiratory chain or on the cellular defenses against oxidative stress. This is because natural products possess structures capable of generating radicals that may cause peroxidative damage to trypanothione reductase that is very sensitive to alterations in redox balance. It is also known that some agents act by binding with the kinetoplast DNA of the parasite (Sepulveda- Boza and Cassles, 1996).

Toxicity results revealed high doses (900 mg/kg body weight) of Neem leaves chloroform extract expressed clinical signs of lethargy, dyspnoea and raised hair within the first fifteen minutes and that all the mice died within 24 hours. Similar results were observed with extract of *Xanthium strumarium* leaves (compositae) at 1000 mg/kg in a study carried out by Talakal *et al.* (1995). This implies that both plants although belonging to different families may be highly toxic. Histopathology of lungs showed pulmonary emphysema at this dosage level (Figure 4). Similar to the clinical sign of dyspnoea which was observed in most animals before death. The histopathology results showed normal lungs. This implied that a single dose of 500 mg/kg was not toxic to the experimental animals. However, sub acute to chronic toxicity study should be determined in order to assess the safety of this extract.

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The findings on liver histopathology (Figure 9) also agree with observations of (Hans, 1996) where autopsy of a rabbit poisoned with berberine an alkaloid, showed liver congestion. Results obtained on clinical signs of lethargy and dyspnoea also corresponded with those of (Hans, 1996) on rabbit poisoned with berberine. The effect of Neem extract administration on healthy mice produced congestion in the liver and kidney (Figures 7 and 9) which corresponded well with severe toxic manifestation and histologically degenerative changes and congestion in both liver and kidney upon administration of nuciferine an alkaloid into the experimental animals (Hans, 1996). Furthermore, the findings on nephrotoxicity can be attributed to the presence of saponins in the Neem leaves extract, which have been shown to cause contraction of the vascular system in the kidney (Diwan et al., 2000). In addition, intense congestion of blood vessels in the kidney tissues and damage to epithelial cortical cells were noted due to presence of saponins in extracts of Catha edulis and Erythrina abyssinica (Piero, 2006).

Previous histopathological examination of rat liver sections after administration of saponins isolated from fruit extracts of Citrullus colocynthis showed that saponins cause minor haemorrhages in many lobules and congestion of central veins and liver sinusoids. Moreover, destruction of the liver architecture due to the necrosis of liver cells was also observed (Diwan et al., 2000). In addition, hepatotoxicity could have been due to the presence of terpenoids in the Neem leaves extracts. In their study, Zeinsteger et al. (2003) found that triterpenes caused congestion in the sinusoids with centrilobular hydropic and fatty degeneration as well as hepatonecrosis.

There was no toxicity by P. peruviana extract. Similar results were achieved by Hoet et al. (2004) with extract of Strychnos spinosa leaves. Literature from other studies on medicinal plants revealed both extracts contain flavonoids, adverse reactions to flavonoids in human is rare (Elliot et al., 2000). Toxicity of alkaloid can be supported by the fact that the famous European
Plant hemlock (*Conium maculatum*, Apiaceae) produces highly poisonous piperidine alkaloid called coniine was used to execute the Greek philosopher Socrates who was found guilty of treason and forced to drink a preparation of this plant (Heinrich *et al.*, 2004). Furthermore, British missionaries working in the Calabar Coast area of West Africa (Nigeria and Cameroon) reported that criminal trials were conducted using the Calabar bean (*Physostigma venenosum*, Fabaceae) which contain indole alkaloids. The accused individual was forced to consume an extract of bean, which is highly toxic with side effects of neurotoxicity and cytotoxicity (Heinrich *et al.*, 2004). The toxicity of alkaloids in plants is attributed to the fact that alkaloids are strategically located poisonous agents in plants thereby protecting them either against herbivorous animals or insects (Ashutosh, 2007). This concept is further supported by the fact that alkaloids are by-products of various detoxification reactions representing a metabolic locking-up of compounds otherwise harmful or detrimental to the plant (Ashutosh, 2007).

Examination of the vital organs in normal mice after administration of the highest dose of 1000 mg/kg body weight of *Physalis peruviana* chloroform extract displayed a normal picture. In view of the fact that only 2 out of 5 mice expressed mild clinical signs of lethargy, dyspnoea and hair raised after one and a half hours, disappearing after 2 days up to the end of 14 days period was indicative of non-toxic nature of the classes of phytochemicals in the plant extract. This implies that this extract was not acutely toxic to the host and was safe for *in vivo* tests. But more comprehensive sub acute to chronic toxicity studies were not done in this study. These findings corresponded with those of Arun *et al.* (2006) on evaluation of *P. peruviana* extracts for antihepatotoxicity and the acute toxicity which revealed that histological changes induced by carbon tetrachloride (*CCL*$_4$) on evaluation of *P. peruviana* for the acute toxicity were significantly reduced by the extract. In addition the extract was found to be devoid of any conspicuous acute toxicity.
*In vivo* results showed that extract of *A. indica* at the three different dosage levels expressed antitrypanosomal activity, with dosage level of 500 mg/kg body weight being the most active extract (Figure 10). This is in accordance with the strength of dosage administered.

Figure 11 showed that plant extract of *P. peruviana* at the three different dosage levels reduced the number of parasites in comparison to controls where the number of parasites remained high.

Figure 12 showed that cymelarsan eliminated the parasites \(3.98 \times 10^{-5}\) tryps/ml completely from day 3 post treatment to the end of 42 days period of the observation. Cymelarsan is a standard drug for treatment of *T. evansi* infection which is able to cross the blood brain barrier and eliminate parasites in the central nervous system, which is not the case with berenil and suramin.
4.2 Conclusions

In conclusion, chloroform extracts of *A. indica* (Neem) leaves was found to have the highest activity against *T. evansi* both *in vitro* and *in vivo* among the twenty seven plant extracts. Although cytotoxicity studies were not done *in vitro* and conclusive toxicological studies have not been done *in vivo*, Neem extracts were found to be more effective than standard drug suramin in both *in vitro* and *in vivo* trials conducted. Chloroform was the best solvent for extraction of the active principles with antitrypanosomal activities from Neem leaves. Chloroform extract of *P. peruviana* whole plant was also found to possess antitrypanosomal activity both *in vitro* and *in vivo*. The efficacy of plant extract was influenced by the type of solvent used in extraction of active ingredients and age of the plant.

Treatment of healthy mice with 500 mg/kg Neem leaves chloroform extract revealed only mild clinical manifestation of acute toxicity which disappeared after 24 hours. The histopathology results showed normal lungs. Therefore, 500 mg/kg was the highest therapeutic trial dose for *in vivo* bioassay but its safety levels have not been comprehensively studied. The organs affected by Neem extract at dosage levels above 500 mg/kg were kidneys, lungs and liver. The clinical manifestation of toxicity of Neem extract expressed on mice upon treatment included dyspnoea, lethargy and raised hair. Chloroform extract of *P. peruviana* which did not cause mortality, severe toxic manifestation or histopathology on mice even after treatment with the highest dose of 1000 mg/kg body weight was considered non-toxic.

Finally, this study has demonstrated the trypanocidal potential of some Kenyan medicinal plants.
4.3 Recommendations

1. A further study should be carried out to identify the nature and structure of phytochemical components of Neem leaves chloroform extract and Physalis peruviana whole plant chloroform extract.

2. The antitrypanosomal activity performance of the plant extracts when administered via the other routes of administration should be assessed.

3. *In vitro* tests should be conducted for a longer period of time perhaps for 36 hours to monitor further the trend of the plant extracts efficacy.

4. Assessment of the toxicity of plant extracts need to be investigated further biochemically and hematologically to establish the undesirable side effects on the host.

5. Combination therapy study of the two most active plant extracts should be carried out to determine if the results can create a rationale for a combination therapy in elimination of trypanosomes.

6. Plant extracts to be tested on human infective parasites.
REFERENCES


Websites


http://www.emedicine.com/EMERG/topic173.htm-16/6/06.


APPENDICES

Appendix 1:

Materials/Apparatus / Equipment's for herbal extraction

Grinding mill
Water bath
Measuring cylinder
Whatman’s No.1 filters papers
Rotary evaporator
Freeze drier
Pipettes (10mls)
Round bottomed flasks
Polythene bags
Newspapers
Sample bottles
Conical flasks
Aluminium foil
Separating funnel
Spatula
Knives /Matchet
Analytical balance

Chemicals/Reagents

Distilled water
Chloroform
Methanol
Nitrogen gas

Biological materials

Azadirachta indica (Neem) (leaves, stem bark)
Prunus africana (stem bark)
Bidens pilosa (whole plant)
Physalis peruviana (whole plant)
Senna didymobotrya (stem bark, leaves)
Croton megalocarpus (leaves)
Appendix 2:

Materials for in vivo and in vitro testing

Apparatus / Equipment

Tissue culture bottles
96-well microtiter plate
Pipettes (individually packed) 1 – 5mls, 5-20mls, 20-50mls
Yellow tips – 1 μl-5μl, 5-10 μl, 20-100μl, 50-200 μl and 200 – 1000μl
Microscope- inverted
Haemocytometer
Weighing balance
Weighing tray
DE-52 column
Filter syringes
Multichannel pipette
Modular chamber
Laminar flow hood
Incubator (37°C)
Centrifuge
Bijou bottles

Chemical/Reagents

Culture media- MEM with Earle’s salts + foetal bovine serum.
Ethanol, 70%
Carbon dioxide (5%)
Antibiotic- Gentamycin
Sodium chloride
Glucose
Ethylene – diamine tetra acetic acid (EDTA)
EDTA-Saline glucose (ESG)
Phosphate saline glucose (PSG)
Distilled water
Methylated spirit
Experimental Animals
Swiss white mice

The parasite
Trypanosoma evansi

Drugs
Berenil (Diminazene aceturate)
Cymelarsan
Suramin
Herbal extracts

Others
Mice cages
Wood shavings
Labels
Marking tar
Mice pellets
Water bottles
Syringes (1ml)
Sterile needles
Bijou bottles
Microscope slides
Cover slips
A pair of scissors
Cotton wool
Aluminium foil
Appendix 3:

96 well micro titer plate.

\[
\text{MIC} = \frac{C}{D \times 2^n}
\]

Where

- \( \text{MIC} \) = 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.
- \( C \) = Concentration of drug solution.
Appendix 4:

Phosphate Buffered Saline Glucose (PSG) PH 8.0

To make PS (phosphate saline) stock solution.
Anhydrous disodium hydrogen phosphate – 14.38g.
Sodium dihydrogen phosphate – 0.78g.
Sodium Chloride – 4.25g.
Add distilled water up to – 1000ml.

To make PSG (Phosphate Saline Glucose)
PS stock 6 parts
Distilled water 4 parts
Add 1% Glucose to give 1% w/v.
Appendix 5:

Materials for acute toxicity testing

Apparatus / Equipment

Histokinette (tissue processing machine)  
Microtome  
Specimen tube  
Embedding moulds  
Microscope and oil immersion  
Cover slips  
Dessicator  
Dissecting board  
Pins  
Sterile syringes and needles  
Disposable blades  
Haemocytometer  
Scalpel holder  
Racks  
Weighing balance  
Cages  
Cage labels  
Surgical gloves  
Spatula  
Conical flasks  
Marker pen  
Microtome knife  
Wax dispenser  
Wooden blocks  
Microscope slides  
Water bath  
Dissecting kit  
Mounting needles  
Sterile forceps, Scissors and scalpel  
Sterile pipettes 1ml, 5ml, 10ml  
Marking tar  
Scalpel  
Forceps  
Wood shavings  
Weighing tray  
Water bottles  
Bijou bottles  
Nose masks  
Measuring cylinder  
Labels

Chemical and Reagents

10% formalin  
Ethanol 70%, 90%, 100%  
phenol crystals  
Iso propanol  
Xylene  
Propanol  
Moutant (DPX)
Haematoxylin stain
Distilled water
Eosin stain
Sodium chloride
Formaldehyde 40%
Benzyl alcohol
Methanol
Surgical spirit
Carbon dioxide

Experimental Animal
Swiss white mice

Drugs
Berenil (Diminazene aceturate)
Cymelarsan
Suramin
Herbal extracts

Others
Cotton wool
Mice pellets
Wood shavings
Appendix 6:

Standard hemocytometer chamber

Technical Information

DIAGRAM I
STANDARD HEMOCYTOMETER CHAMBER

The circle indicates the approximate area covered at 100× microscope magnification (10× ocular and 10× objective). Include cells on top and left touching middle line (O). Do not count cells touching middle line at bottom and right (O). Count 4 corner squares and middle square in both chambers (one chamber represented here).

DIAGRAM II
CORNER SQUARE (ENLARGEMENT)

Count cells on top and left touching middle line (O). Do not count cells touching middle line at bottom and right (O).
USE OF TRYPAN BLUE STAIN AND THE HEMOCYTOMETER TO DETERMINE TOTAL CELL COUNTS AND VIABLE CELL NUMBER

Trypan Blue is one of several stains recommended for use in dye exclusion procedures for viable cell counting. This method is based on the principle that live (viable) cells do not take up certain dyes, whereas dead (non-viable) cells do. Staining facilitates the visualization of cell morphology.

NOTE: Trypan Blue has a greater affinity for serum proteins than for cellular protein. If the background is too dark, cells should be pelleted and resuspended in protein-free medium or salt solution prior to counting.

1) Prepare a cell suspension in a balanced salt solution (e.g., Hank's Balanced Salts [HBSS], Product No. H 2513).
2) Transfer 0.5 ml of 0.4% Trypan Blue solution (w/v) to a test tube. Add 0.3 ml of HBSS and 0.2 ml of the cell suspension (dilution factor = 5) and mix thoroughly. Allow to stand for 5 to 15 minutes.

NOTE: If cells are exposed to Trypan Blue for extended periods of time, viable cells, as well as non-viable cells, may begin to take up dye.

3) With the cover-slip in place, use a Pasteur pipette or other suitable device to transfer a small amount of Trypan Blue-cell suspension mixture to both chambers of the hemocytometer. Carefully touch the edge of the cover-slip with the pipette tip and allow each chamber to fill by capillary action. Do not overfill or underfill the chambers.

4) Starting with chamber 1 of the hemocytometer, count all the cells in the 1 mm center square and four 1 mm corner squares (see Diagram I on page 202). Non-viable cells will stain blue. Keep a separate count of viable and non-viable cells.

NOTE: Count cells on top and left touching middle line of the perimeter of each square. Do not count cells touching the middle line at bottom and right sides (see Diagram II on page 202).

5) Repeat this procedure for chamber 2.

NOTE: If greater than 10% of the cells appear clustered, repeat entire procedure making sure the cells are dispersed by vigorous pipetting in the original cell suspension as well as the Trypan Blue-cell suspension mixture. If less than 200 or greater than 500 cells (i.e., 20-50 cells/square) are observed in the 10 squares, repeat the procedure adjusting to an appropriate dilution factor.

6) Withdraw a second sample and repeat count procedure to ensure accuracy.

7) CELL COUNTS—Each square of the hemocytometer, with cover-slip in place, represents a total volume of 0.1 mm³ or 10⁻³ cm³. Since 1 cm³ is equivalent to approximately 1 ml, the subsequent cell concentration per ml (and the total number of cells) will be determined using the following calculations:

\[ \text{CELLS per ml} = \text{average count per square} \times \text{dilution factor} \times 10^4 \text{ (count 10 squares)} \]

Ex: If the average count per square is 45 cells x 5 x 10⁴ = 2.25 x 10⁶ cells/ml.

TOTAL CELLS = cells per ml x the original volume of fluid from which cell sample was removed.

Ex: 2.25 x 10⁶ (cells/ml) x 10 ml (original volume) = 2.25 x 10⁷ total cells.

8) CELL VIABILITY (%) = total viable cells (unstained) ÷ total cells (stained and unstained) x 100.

Ex: If the average count per square of unstained (viable) cells is 37.5, the total viable cells = [37.5 x 5 x 10⁴] viable cells/ml x 10 ml (original volume) = 1.875 x 10⁶ viable cells. Cell viability (%) = 1.875 x 10⁶ (viable cells) ÷ 2.25 x 10⁷ (total cells) x 100 = 83% viability.