

ADDIS ABABA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

PROPHYLACTIC EFFECTS OF METHANOLIC EXTRACT OF *ARTEMISIA*
ABSINTHIUM IN *TRYPANOSOMA CONGOLENSE* INFECTED MICE

BY
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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
CSA	Central Statistics Authority
DNA	Deoxyribonucleic Acid
FAO	Food and Agricultural Organization of the United Nation
FVM	Faculty of Veterinary Medicine
IC50	Inhibitory Concentration
OIE	Office International Des Epizooties
PATTEC	Pan African Tsetse and Trypanosomosis Eradication campaign
PBS	Phosphate Buffered Salin
SAT	Sequential Aerosol Technique
SIT	Sterile Insect Technique

ABSTRACT

An experiment was conducted to determine the *in vivo* anti-trypanosomal effect of methanol extracts of the aerial parts of *Artemisia absinthium* against *Trypanosoma congolense*. Thirty-six Swiss white male mice aged between 6 - 8 weeks were divided into six experimental groups each consisting of six animals. Methanol extract of the plant was prepared. *T. congolense* was isolated from cattle at Didessa valley (western Ethiopia) and propagated on mice. All groups received 1×10^5 /ml of trypanosomes in 0.2 ml of blood /PBS via intra peritoneal route of inoculation. The methanol extract of *A. absinthium* was given orally to three groups, at doses of 400 mg/kg, 800 mg/kg and 1200 mg/kg body weight for 7 consecutive days before infection. One group remained as infected non treated control and the other group received Isometamidium chloride as treated control. The last remaining group represented the non infected and non treated but received distilled water only. The effect of the extract on levels of parasitaemia, PCV, body weight, and mice survival was monitored for 70 days. No parasite was detected by wet film examination upto 21 days post infection with *T. congolense* in mice treated with methanol extracts of *A. absinthium* and Isometamidium chloride. Similarly, groups received methanol extracts of *A. absinthium* and Isometamidium chloride had significantly higher body weights than the untreated infected group ($P < 0.01$). Significant higher PCV and mice survival time were observed in groups treated with methanol extracts of the plant and Isometamidium chloride compared to the untreated infected control ($P < 0.01$). It can be concluded that the plant extract has a good prophylactic potential against pathogenic trypanosomes (*T. congolense*) of livestock importance by suppressing further multiplication of the parasite, maintaining good PCV, body weight, and survival of infected animals.

Key words: *Artemisia absinthium*, Isometamidium chloride mice, parasitaemia, PCV, survival time, *Trypanosoma congolense*, body weight

1. INTRODUCTION

African animal trypanosomosis and its vectors occur in vast areas of sub-Saharan Africa with devastating impact on livestock productivity. Its epidemiology and impact on livestock (especially cattle) production are determined largely by the prevalence and distribution of the disease and its vectors in the affected areas (PATTEC, 2001).

Trypanosomosis is a protozoan disease of both animals and humans caused by different species of the genus *Trypanosoma*. Trypanosomes are transmitted by the bite of infected tsetse flies (*Glossina* species). The mammalian species of trypanosomes fall into two groups: Stercoraria and Salivaria. The salivaria group includes the three pathogenic species and subspecies responsible for nagana disease. This group includes *T. vivax* (Duttonella subgenus), *T. congolense* (Nannomonas subgenus), *T. brucei brucei* (Trypanozoon subgenus). The trypanozoon subgenus also include the two subspecies, *T. brucei gambiense* and *T. brucei rhodesiense*, that are agents of sleeping sickness in humans (Launois *et al.*, 2004, Getachew, 2005). Biting flies such as *Tabanus*, *Haematopota* and *Stomoxys* species can transmit bloodstream trypomastigotes to mammalian hosts and this has enabled some species of pathogenic trypanosomes to spread widely beyond Africa into Asia and Latin America. This non-cyclical or mechanical means of transmission has been acquired by only two species of trypanosomes namely *T. evansi*, and *T. vivax* (Luckins, 2004).

The distribution of African trypanosomosis in domestic animals and human coincides with the distribution of the tsetse fly vectors. Tsetse flies infest wide range of habitats covering over 10 million square kilometer of fertile land that lie between latitudes 12°N and 29°S across 37 countries of the African continent, from Senegal in the North to South Africa in the South including Ethiopia (Finelle, 1980; Getachew *et al.*, 2004). Many areas that are infested with tsetse flies are the most suitable areas for livestock and crop production. In this region 50 million cattle, 230 million sheep, 40 million goats are exposed to the risk of contracting Nangana, leading every year to the death of 3 million head of cattle, the loss of 500,000 tones of meat and 1 million tones of milk. The reduction to 10% concerning animal draught power in Africa and 50% concerning livestock production prospects costs 5 billion Euros a year for the African economy (Launois *et al.*, 2004).

In countries such as Ethiopia, where livestock is an important part of the agricultural sector, trypanosomosis contributes to the direct economic losses of crop-livestock production. In the tsetse-infested areas of the country the most important trypanosome species in economic terms are *Trypanosoma congolense* and *T. vivax* (Langridge, 1976). A report by Abebe and Jobre (1996) indicated infection rates of 58.5%, 31.27% and 3.5% by *T. congolense*, *T. vivax* and *T. brucei* respectively in cattle from tsetse-infested areas of southwest Ethiopia. In tsetse-free areas, Abebe and Jobre (1996) also indicated 8.71% trypanosome prevalence rate in the highlands, out of which 99% was due to *T. vivax*.

Trypanosomiasis has direct impact on livestock productivity, reducing meat and milk off take by 20%, calving rate by 20%, increase calf mortality by 20%, decreases both lambing and kidding rates in sheep and goat and livestock management especially the number of livestock kept by farmers, the breed and species composition of the livestock herd, the way the livestock are grazed, cost of trypanocidal drugs and cost of insecticides (Swallow, 2000). It also has direct impacts on human settlement in a considerable part of sub-Saharan Africa including Ethiopia (Takele, 2006).

The control of trypanosomes mainly relies on chemotherapy and vector control. The vector control is either by ground or Aerial spray of insecticides. However, because of high cost and environmental consideration, the use of insecticide spraying becomes no more a method choice in practice (Abebe, 2005). Currently, other alternative vector control approaches are being practiced. Targets and traps have been used extensively in agricultural settings (Vale *et al.*, 1988) and considerable success has been achieved by directly applying insecticides (pour-on) on animals (Rowlands *et al.*, 2001). The sterile insect technique (SIT), which was applied in large scale tsetse eradication programs in some parts of Africa including Burkina Faso (Clair *et al.*, 1990), Northern Nigeria (Jordan, 1986), Tanzania, and Zanzibar. In Ethiopia, similar vector control strategies including fly traps, targets and pour-on insecticide applications are being practiced in different parts of tsetse infested areas. Moreover, an SIT project is being coordinated by the Ethiopian science and Technology Ministry in the Southern Nations and peoples Administrative Region (Abebe *et al.*, 2004).

All the previous attempts of tsetse and/or trypanosomosis control activities have three major characteristics: mainly aimed at fragmented small area vector and/or trypanosomosis control, and not in area wide eradication effort, mainly focus on recommended technology application,

and not in local capacity capability building and lacked serious and long term government and non-government commitment. As the result the outcome of the effort made so far was only short lasting solution, and did not allow eradication/long lasting control of vector or disease even in fragmented small area (Alemu *et al.*, 2007).

The use of trypanocidal drugs that target the trypanosomes in the host is one of second major approach in the control of trypanosomosis. Three antitrypanosomal drugs, isometamidium chloride, homidium (bromide and chloride) and diminazene aceturate- are drugs that have been on the market for over 50 years (Holmes *et al.*, 2004). It is cheaper and easier to attempt to kill the parasite than tsetse fly using this approach. The rising costs of tsetse control campaigns resulted to be reliant on the use of trypanocidal drugs for both prevention and control of the disease. At present, the most widely used trypanocidal drugs for *T.congolense* and *T.vivax* infection in Ethiopia are isometamidium and diminazine aceturate. However these drugs have limitations like toxicity, drug resistance and high cost (Doua and Yapo, 1993; Kuzoe and Schofield, 2004). Due to these limitations there are approaches towards the search of new drugs especially from plant origin. Previously trails were made on extracts of *Commiphora kerstingii* on *T.brucei brucei* (Mikail, 2009), *Azadiracta indica* on *T.b. rhodesiense* (Raphael *et al.*, 2009) and *Neurolaena lobata* on *T. cruzi* (Berger *et al.*, 2001). Trails on plant extracts against some species of trypanosomes have shown promising findings in many countries including Ethiopia (Mekonnen *et al.*, 1999; Camacho *et al.*, 2000; Sara *et al.*, 2004; Nibret and Wink, 2010, Tsegabrehan, 2011).

Therefore, this study was undertaken to investigate

- The prophylactic effect of *Artemisia absinthium* methanol extract, in *Trypanosoma congolense* infected mice after treating in three different doses.

2. LITERATURE REVIEW

2.1. The Trypanosomes

Trypanosome is single celled parasitic protozoa. It is phylogenetically classified in the Protozoa Subkingdom, Sarcomastigophore phylum, Mastigophore subphylum, Zoomastigophore class, Kinetoplastid order, Trypanosomatidae family and Trypanosoma genus. Species of trypanosomes infecting mammals are categorized into the Stercoraria group and the Salivaria group depending on their development in the vector and in the mammals (Urquhart *et al.*, 1996). The Stercoraria group contains genera in which the parasite completes its development in the "posterior station". Metatrypanosome appears in the faeces of the vector and its transmission is by contamination. In the section Salivaria, the development in the vector is completed in the "anterior station" and transmission is by inoculation of the metacyclic stage. The main pathogens in the section Salivaria fall into four subgenera: Duttonella (species: *Trypanosoma vivax*, *T. uniforme*), Nannomonas (species: *Trypanosoma congolense*, *T. simiae*), Pycnomonas (species: *T. suis*) and Trypanozoon (species: *Trypanosoma brucei*, *T. rhodesiense*, *T. gambiense*, *T. evansi*; *T. equiperdum* (Leak, 1999).

2.1.1. Nannomonas subgenus: *T. congolense* and *T. simiae*

T. congolense is monomorphic in form. It is one of the smallest trypanosomes with a length ranging from 8-24µm. There is no free flagellum. The posterior end of the body is usually rounded. The undulating membrane is inconspicuous; the medium- sized kinetoplast is usually in a marginal and sub terminal position. It moves sluggishly in fresh blood film, often apparently attached to red blood cells. *T. simiae*, the porcine trypanosome, is more pleomorphic in characteristics and its average length is 15-19µm, slightly longer than *T. congolense*. Nannomonas trypanosomes are very active in fresh blood films but do not tend to move far across the microscope field. They also demonstrate agglutinating properties by tending to adhere to host tissue in vivo. They develop in the mid gut and proboscis of tsetse, and take longer time to develop and exposed to anti trypanosomal factors in the fly gut (Uilenberg, 1998; Molyneux and Ashford, 1983).

2.1.2. Duttonella subgenus: *T. vivax*

T. vivax is monomorphic ranging from 20-26 μm . It has a long free flagellum and a large terminally placed kinetoplast, distinguishing it from the other pathogenic Salivarian trypanosomes. The undulating membrane is inconspicuous and the posterior end is broad and round. *T. vivax* is very mobile and in fresh blood film it moves rapidly across the microscope field which makes it difficult to follow its movements (Uilenberg, 1998). *T. vivax* has the simplest life cycle in tsetse. It develops in the proboscis and development is completed to the infective metatrypanosome stage in the hypopharynx. The infection rate of 100% could be achieved with *T. vivax* by repeatedly feeding flies on infected host (Leak, 1999).

2.1.3. Trypanozoon subgenus: *T. brucei*

The Trypanozoon subgenus trypanosome includes *T. b. brucie* which infects wild and domestic animals, *T. b. rhodensience* and *T. b. gambiense* which infects human (Leak 1999). *T. brucei* is pleomorphic in form and its total length measures from 11-39 μm . Pleomorphism is represented by three forms: long and slender forms, stumpy forms and intermediate forms. Long and slender forms possessing a long free flagellum and a well developed undulating membrane, elongated nucleus, sub terminal kinetoplast and narrow posterior end drawn out to a blunt point or sometimes truncated. Its average lengths are 14-39 μm . Stumpy forms are stout and usually without a free flagellum, with undulating membrane well developed kinetoplast near broadly rounded or obtusely pointed posterior end and rounded nucleus. The average length of stumpy forms is 16.6-20 μm . The intermediate forms are characterized by shorter flagellum, the posterior end blunter and the kinetoplast nearer to the extremity than in the slender forms. The kinetoplast in trypanozoon is smaller than in any of the other salivarian trypanosomes. The length of intermediate forms is 14-390 μm (Uilenberg, 1998). The cyclical development of Trypanozoon subgenus in the tsetse fly takes place in the mid gut and culminates in the metacyclic trypomastigote form in the vector's salivary glands. The metacyclic stage is infective for the mammalian host and unlike the other developmental stage in the tsetse fly, this metacyclic trypanosome possess a surface coat like that of bloodstream form (Leak, 1999).

2.1.4. Pycnomonas subgenus: *Trypanosoma suis*

Trypanosoma suis is monomorphic and the total length ranges from 13-19 µm. A free flagellum is typically present. Its body is very broad and short; the posterior end usually terminates in a short point. The kinetoplast is small and situated near the posterior end and occupies a marginal position, while the voluminous nucleus lies in the anterior part of the body and the undulating membrane is conspicuous (Uilenberg, 1998).

2.2. The tsetse fly (vectors of trypanosomosis)

2.2.1. Classification and morphological features of tsetse

All tsetse flies belong to kingdom Animalia, phylum Arthropoda, class Insecta, Order Diptera, Suborder Brachycera, supersection Cyclorrhapha, superfamily families Hippoboscidae and family the Glossinidae, with a single genus the *Glossina*. The word 'tsetse' is believed to originate from the Tswana language in Botswana, meaning 'fly that kills livestock'. There are 31 members in the genus *Glossina*, 23 species and 8 sub-species. The 31 members of the Glossinidae are divided further into three sub-genera, the *Glossina* (the moristans group), *Nemorhina* (the palpalis group) and *Austenina* (the fusca group) (Kuzoe and Schofield, 2004).

The subgenus *Glossina* contains a total of seven medium size species and sub-species. Those of major economic importance are *G. morsitans* and *G. pallidipes* with *G. swynnertoni* and *G. austeni* being important in specific regions. The species of the *Glossina* sub-genus are called the savannah flies due to their preference for this environment. It is this fact that makes them the most important vectors as the African savannah is a vast area and the flies come into contact with both man, his animals and wild game animals. Flies of this sub-genus are totally dependent on wild animals for their blood meals. These animals act as reservoirs of trypanosomes (Launois *et al.*, 2004).

The sub-genus *Fusca* or *Austenina* group contains 15 large sized species and sub-species. These larger and also more primitive flies feed on small mammals in forested areas. The main exceptions are *G. longipennis* and *G. brevipalpis* which do not live in the dense equatorial forest where as *G. longipennis* lives in more arid areas and *G. brevipalpis* in secondary forest.

They have very little contact with man and his livestock due to their habitat. None of this group is a vector for sleeping sickness (Kuzoe and Schofield, 2004).

The Palpalis group contains nine small species and sub-species in this group, which mainly inhabit humid areas, local patches of dense vegetation like coffee, cocoa, mango and banana plantation, along the banks of rivers and lakes in arid country, and in dense, wet, heavily forested equatorial rain forest. Species of this group occur throughout much of western Africa. The principal hosts of these flies are reptiles, especially monitor lizards and crocodiles. They serve as vectors for pathogenic trypanosomosis that cause chronic sleeping sickness in humans and nagana disease in livestock (Launois *et al.*, 2004).

Tsetse flies are brownish-black to pale brown in colour without any metallic tinge. The body length ranges from 6 to 16 mm excluding the proboscis. Males are smaller than females. Adult body weight varies according to species. Adults of species of medical and veterinary interest, such as *G. fuscipes* and *G. morsitans*, are of medium weight (20-25mg). In general there are four characteristics which distinguish adult tsetse from other kinds of flies: tsetse have a distinct proboscis, a long thin structure attached to the bottom of the head and pointing forward, tsetse fold their wings completely one on top of the other when at rest, the cell of the wing has a characteristic hatchet shape resembling a meat cleaver or a hatchet and the antennae have arista with hairs which are themselves branched (Launois *et al.*, 2004).

2.2.2. Life cycle of tsetse flies

Tsetse has an unusual life cycle due to the fact that they are viviparous. Female tsetse flies mate shortly after emergence when females are looking for the first blood meal. Just a single copulation is enough to ensure a female's reproductive success. Spermatozoa injected by the male are preserved for life in her sperm theca. Female tsetse only fertilizes one egg at a time and retains each egg within their uterus to have the offspring develop internally during the first larval stages. During this time, the female feeds the developing offspring with a milky substance which is secreted by a modified gland in the uterus. In the third larval stage, the tsetse larva finally leaves the uterus and begins their independent life. The newly independent tsetse larva quickly burrows under the soil surface and pupates and the tsetse fly emerges 20-45 days later depending on temperature (pupal development does not succeed below 17°C and above 32°C). Thus, each female produces only one offspring at a time, and can produce up to

12 offsprings at intervals of 9-10 days during her typical adult lifespan of 2-3 months (Leak *et al.*, 2008).

2.3. Epidemiology of trypanosomosis

The epidemiology of African animal trypanosomosis is influenced by the interaction of trypanosome species, their vertebrate and invertebrate host and on herd management (Leak, 1999). The risk of infection to which cattle are exposed is closely related to the density and the species of tsetse fly present. The factors affecting susceptibility to trypanosomosis must be well understood if effective measures to control the disease are to be developed (D'iteren *et al.*, 2000).

2.3.1. The parasite

Trypanosomes are obligatory protozoan parasites that undergo their entire life cycle alternatively in arthropods like tsetse fly and vertebrate such as mammals. Some are ubiquitous, like *T. congolense*, which infect several different animal species while others are host specific (*T. equiperdum*) (Launois *et al.*, 2004). Trypanosomes are either transmitted acyclically by haematophagous flies of the genus *Tabanus* and *Stomoxys* or cyclically by the tsetse flies with the exception of *T. equiperdum* which is a venereal disease (Leak, 1999).

The transmission by tsetse flies is a complex mechanism. Trypanosome infects a young healthy tsetse fly when it first feeds on infected mammal. These trypanosomes are in the trypomastigote stage which is the infective form in the mammalian hosts. The trypanosomes then pass into the alimentary duct moving from the pharynx, through the esophagus and crop, where they are briefly stored before invading the mid gut. In the mid gut they stretch, shed their glycoprotein layer and undergo transition into non infective procyclic stage. They quickly multiply by longitudinal division into the epimastigote form in the cardia. The trypanosomes penetrate the haemocoel via mid gut epithelium and move from there to the salivary glands of the tsetse fly where they develop into the metacyclic infectious trypomastigote form which has now got its surface glycoprotein coat. An infected tsetse injects metacyclic trypanosomes from its salivary glands into the host's dermis. A chancre forms at the inoculation site which is the sign of trypanosome transition into the long slender stage and

another actively multiplying stage that invades the host's blood stream and lymphatic system (Seifert, 1996; Launois *et al.*, 2004).

The percentage of trypanosome infection successfully maturing will vary between species and is influenced by factors within population of tsetse including anti trypanosomal factors, lectins and rickettsia like organism in the tsetse mid gut. Because of the complicated development of trypanosomes within the tsetse fly, only about 0.1-0.4% of the flies is infected and remains a lifelong potential carrier trypanosome. The length of time taken for trypanosomes to complete development in tsetse is quite variable (Leak, 1999). *T. brucei brucei* has the longest and most complex life cycle, lasting around 30 days while *T. vivax* has the short simple life cycle which lasts about 10 days. The life cycle of *T. congolense* is intermediary that lasts 15 days. Trypanosomes mechanically hamper blood up take when they are located in the mouth part of tsetse flies. Blood meals thus become shorter, more frequent, and involve a greater number of hosts. This facilitates trypanosome dissemination (Launois *et al.*, 2004).

Mechanically blood sucking diptera such as *Tabanus* and *Stomoxys* can transmit bloodstream trypomastigotes to mammalian hosts and this has enabled some species of pathogenic trypanosome to spread widely beyond tsetse belt into Asia and Latin America. This non-cyclical means of transmission has been acquired by only two species of trypanosome: *T. vivax* and *T. evansi* (Luckins and Dwinger, 2004). The epidemiology of non tsetse transmitted trypanosomosis (*T. evansi*, *T. vivax*) is also influenced by many factors. There may be seasonal outbreaks, where the populations of biting flies (Tabanids, Stomoxys, etc.) are influenced by important seasonal climatic differences. The disease sometimes becomes more apparent when immunodepressive factors such the poor nutritional state of the animal diminishes its defenses, even when the initial infection occurred during the rains. The epidemiology is also greatly influenced by host preferences and diurnal behaviour patterns of the various local species of tabanids and other biting flies (e.g. whether the hours that they are active allow much contact with livestock or not) (Uilenberg, 1998).

2.3.2. The vector

Tsetse flies are confined to a belt of tropical Africa extending from southern Sahara 15° N latitude in the North to 20-30° S latitude in the South. The species are restricted to various geographical areas according to habitat within tsetse belt (Urguhart, 1996). Species of the palpalis group are more usually associated with riverine vegetation, but some also extend into

savannah regions between river systems. Species of the morsitans group are primarily associated with drier savannahs. These groupings coincide broadly with epidemiological significance (Patterson and Schofield, 2004).

Temperature and humidity as well as vegetation are limiting factors which are especially important for the pupal period of the vector. Optimum temperatures are between 20 and 28°C, with relative humidity between 50 and 80% and an annual rain fall between 635 and 1524mm (Seifert, 1996).

Adult tsetse flies feed exclusively on vertebrate blood. Each tsetse fly species has feeding preferences which it tries to fulfill. A population of savannah species feed mainly on mammalian host particularly bovids and suids while riverine species have very wide range of preferred host including reptiles and human. *G. pallidipes* and *G. longipalpis* prefer blood of cattle where as *G. morsitans* prefer blood of wildlife and cattle. However, many tsetse species feed opportunistically and hence adapt to whatever hosts are available. In the absence of suitable wild life, tsetse feed almost exclusively on livestock and humans (Clausen *et al.*, 1998).

The establishment and development of trypanosomes in the tsetse vectors, is a complex process, involving a series of vector and parasite defense and counter-defense mechanisms. Once the infection is established the flies remain infected for lifelong (Wellburn and Maudlin, 1999). The establishment of trypanosome infection in tsetse is affected by endogenous factors associated with tsetse like age, sex, etc, ecological factors (climatic factors), availability of infected host and host available for subsequent feeds, parasite and host factors such as number of parasites available to tsetse, parasite species and its infectivity to tsetse and immune state of host (Leak, 1999).

Table 1: Tsetse infested regions and river basins of Ethiopia

Region	Major River Basin	Tsetse fly
Amhara	Abay (Blue Nile)	<i>G. m. submorsitans</i> ; <i>G. tachinoides</i>
Beneshangul-Gumuz	Abay (Blue Nile)	<i>G. m. submorsitans</i> ; <i>G. tachinoides</i>
Gambella	Baro/Akobo	<i>G. m. submorsitans</i> ; <i>G. tachinoides</i> ; <i>G. pallidipes</i> ; <i>G. f. fuscipes</i>
Oromiya	Abay/Didessa, Upper Ghibe/Omo and Baro/Akobo	<i>G. m. submorsitans</i> ; <i>G. tachinoides</i> ; <i>G. pallidipes</i> ; <i>G. f. fuscipes</i>
SNNPR	Ghibe/Omo	<i>G. pallidipes</i> ; <i>G. f. fuscipes</i> ; <i>G. longipennis</i>
	Rift Valley	<i>G. pallidipes</i>

Source: Abebe, 2005.

2.3.3. The host

The epidemiology of animal trypanosomosis is extremely complicated because the disease affects quite a wide host range. It can affect cattle, sheep, goats, pigs, dogs and cats. All of these species are susceptible to the African animal trypanosomosis (AAT) and may suffer syndromes ranging from sub clinical mild or chronic infections to acute fatal disease. Besides these, there are more than 30 species of wild animals that can be infected with the pathogenic trypanosomes but many of these remain carriers of the organism. Ruminants are widely known as active reservoirs of the trypanosomes. Wild equidae, lions, leopards and wild pigs are all susceptible and can also serve as carriers of the disease. Livestock exposed to trypanosomosis risk differ in their responses depending on genetic factors such as species, breeds and even individual characters. Therefore species and breed trypanosusceptibility,

trypanotolerance or trypanoresistance are of great importance in the epidemiology of the disease (Launois *et al.*, 2004; Leak, 1999).

2.3.4. Herd management

The grazing patterns of herd and daily activity patterns of tsetse species involved are of great influence in the epidemiology of trypanosomiasis. Tsetse have innate pattern of activity. The savannah species such as *G. moristans* and *G. pallidipes* are active mostly for the first two hours and the last two hours of the days. *G. longipennis* is most active just after sun set. Many of the riverian, such as *G. fuscipus* and *G. austeni* are active in the middle of the day. These innate rhythms are influenced by temperature. Temperature less than 14°C and above 32°C suppress the activity of tsetse flies. If the herd grazes on infested site at the time of the day that the flies are most active, the transmission will be more frequent (Uilenberg, 1998; Leak, 1999).

2.4. Pathogenesis

The pathogenesis of the trypanosomes is rather complex and depends on the virulence of the infecting trypanosome species, the number of trypanosomes injected to the host and on the host's immune defenses. The real cause that leads to the death of the animal is not fully understood.

It is believed that the parasite releases toxic substances when it is destroyed within the circulatory system, which damages the lining of the blood vessels. In some cases the sudden release of large amounts of such toxins triggers a chain of reactions, which produce a shock-like syndrome. Therefore, the damage to the host does not depend on nutrients being taken away by parasites but rather on the production of toxic substance. In general the pathogenic effect of the pathogen on the organism may be understood as a syndrome and the component includes: pancytopenia as the result of direct influence of parasite on the cells or the phagocytic defense reaction of the organism (autoimmunological reaction), mechanical cell and tissue damage caused through active mechanical invasion of the extraordinarily strong and mobile pathogens, lysosomal enzymes and the remains of autophagosomes which are excreted in the flagellar pocket that cause additional cell damage, metabolic effects of the trypanosome which withdraw essential nutrients and produce toxic metabolites, the action of the secretion that have direct pathogenic effect, a trypanosome-induced hypothyroid which

may play a role in the impairment of mitochondrial ATPase activity and depression of resistance factors during the course of the disease (Seifert, 1996; Lome *et al.*, 1995). When an animal is infected with trypanosomes, antibodies against the surface coat are produced. However, trypanosomes have multiple genes which code for different surface proteins allowing organisms with new surface glycoproteins coat to elude the immune response. This process is called antigenic variation and results in the persistence of these organisms. Antigenic variation has thus far prevented development of a vaccine and permits reinfection when animals are exposed to tsetse carrying trypanosomes with surface coat glycoproteins of a new antigenic type (Blood *et al.*, 1989).

The trypanosomes gain entry through skin when infected tsetse fly or biting fly takes blood meal. The metacyclic trypanosome deposited in the skin multiply and transform to procyclic blood stream forms. Within a few days of bite, cattle develop a hot and painful cutaneous swelling called a chancre, which is caused by the immune reaction of the host to multiplying trypanosomes. After a period of multiplication in the skin, trypanosomes begin to appear in large number in the afferent lymph that drains from the site of challenge and cause enlargement of the lymph node draining the chancre, generalized enlargement of lymph nodes and splenomegaly develop (Leak, 1999).

Distinct pathological changes may be caused by the different livestock infective trypanosome species. Pathology in tissue is associated with the relative ability of the parasite to invade extravascular spaces and organs. *T. congolense* remains confined to the vascular system whereas trypanosomes of the *Trypanozoon* group (*T. b. brucei*, *T. evansi* and *T. equiperdum*) and *T. vivax* are distributed in both the circulation and in the tissue (Taylor and Authi'e, 2004). Due to this extra vascular distribution, tissue cell damage and degeneration may be marked (Morrison *et al.*, 1981a). The heart is constantly damaged by all three species of trypanosomes. Other vital organs or systems, which are commonly affected, include the skeletal muscle, central nervous system (Whitelaw *et al.*, 1985; Morrison, *et al.*, 1983), endocrine organs (Anosa, *et al.*, 1980; Morrison *et al.*, 1981b; Getachew, 1991) and reproductive systems (Anosa and Itoun, 1980; Mutayoba, 1986).

2.5. Clinical features of trypanosomosis

Trypanosomosis develop in animals depending on the species and strains of trypanosome, the vector and the resistances of the affected breed either as an acute or chronic infection.

Trypanosomosis is more commonly seen as chronic disease in endemic area. There is no pathognomic lesion in trypanosome-infected animals. The chancre may develop within few days at the site where parasites are inoculated. The lesion appears as a swelling that is warm and painful to the animal. The early acute phase of the disease is characterized by the continuous presence of trypanosomes in the blood at detectable concentration (10^3 - 10^8 /ml). Fever is highest at the first peak of parasitemia and fluctuates thereafter with parasitemia waves. With the onset of parasitemia, anemia develops. Anemia is the most prominent features of animal trypanosomosis. The virulence of the infecting parasite population and the age, nutritional and breed of the host influence the severity of anemia. The anaemia in bovine trypanosomosis can be divided into two phases: acute and chronic phases of anaemia (Taylor and L.Authi`e, 2004; Murray and Dexter, 1988). The acute phase anaemia is characterized by progressive anaemia accompanied by parasitaemia (Coetzer *et al.*, 1994). The initial fall in packed cell volume (PCV) values is associated with the first wave of parasitemia in the blood. During this period the anaemia is extravascular and is possibly the result of increased red cell destruction by phagocytosis in the spleen, lungs, haemal nodes and bone marrow (Murray and Dexter, 1988). During chronic phase of anemia, erythrocyte synthesis is not sufficient to compensate for erythrocyte destruction. Lymph nodes and spleen are enlarged. Weakness, lethargy, loss of condition, infertility, abortion and reduced milk production are common. Calves born to infected cows are often small and weak, resulting in high rates of neonatal mortality. In the terminal stage of the disease, animals become extremely weak and unable to rise and die. Cattle infected with *T. vivax* often show sign of photophobia and excessive lacrimation. The duration of the disorders varies considerably depending on the animal species and breed and the species and strain of trypanosomes (Seifert, 1996; Taylor and Authi`e, 2004).

2.6. Diagnosis of trypanosomosis

Definite diagnosis of trypanosome infections in livestock is required for appropriate application of therapeutic and prophylactic measures, for a proper understanding of the epidemiology of the disease and to target and monitor tsetse control or eradication operations. The diagnosis of trypanosome infection is based on clinical signs and on the demonstration of the parasites by direct or indirect methods. The clinical signs of AAT are indicative but are not sufficiently pathognomonic and diagnosis must be confirmed by laboratory methods.

Therefore, the accurate diagnosis of trypanosomosis depends on the detection of the parasite through either direct demonstration of the parasite or indirectly by using serological test to indicate infection with trypanosomes or by molecular technique (Seifert, 1996; Eisler *et al.*, 2004).

2.6.1. Clinical diagnosis

The clinical picture depends on species of infecting trypanosomes, and host immune status or susceptibility. The clinical signs are non-specific and the occurrence of a number of other disease states in endemic area may result in the same clinical picture. However, in areas where trypanosomosis is an endemic problem, clinical symptoms and post mortem lesions are important considerations in diagnosing the disease especially in combination with the epidemiological history of the disease (Delespaulx *et al.*, 2003).

2.6.2. Parasitological methods

Blood film

The examination of wet blood films and giemsa-stained thick and thin fixed blood films with the aid of the light microscope is used to identify the etiological agents of trypanosomosis. With the wet blood film (fresh blood preparation), a drop of fresh blood is placed on a slide and covered with a glass slip. The preparation is examined microscopically using medium magnification. The detection limit of this technique is about 10^4 trypanosomes/ml of blood (Uilenberg, 1998).

Thick stained blood films

Two to three drops of blood are applied on a clean slide and spread with a needle or the corner of another slide, into an area about 1 cm in diameter. After drying thoroughly, the film is stained without fixation, usually with Giemsa stain. Because thick films contain much more blood per unit area the chances of finding trypanosomes are greater than in thin films. Trypanosomes are easily recognized by their morphology. The detection limit of thick blood films is up to 5.0×10^3 trypanosomes/ml of blood (Uilenberg, 1998; Luckin, 2004)

Thin stained blood films

A drop of fresh blood is dropped on the narrow edge of a microscope slide. This edge is then placed on another slide lying on a flat surface, so that the blood drop lies in the acute angle between the two slides. The inclined slide is then pushed along the recumbent one so that the blood is pulled behind it and spread in a thin layer over the stationary slide. The smear is air-dried and fixed in methanol or acetone. The fixed thin films should be stained with Giemsa's for detection of blood parasites and determine trypanosome species involved (Luckin, 2004).

Concentration technique

A practical method using centrifugation of microhaematocrit capillary tubes containing the blood sample and examination of the buffy coat/plasma junction under the microscope was described by Woo (1970). The capillary tubes are open at both ends. A blood is taken up at one end until third quarter of heparinised microhaematocrit capillary tube. The other end of the tube is then sealed by cristaseal or by heating. The sealed capillary tubes are then centrifuged for 5 minutes at 12,000 rpm in microhaemtochrit centerfuge. After the centrifugation, the tubes are removed and examined for the presence of trypanosomes either by direct examination of the buffy coat or after making smear of buffy coat. Trypanosomes are usually found in or just above the buffy coat layer.

In case of direct examination of buffy coat, the tube is placed on microscope slide in a grove formed by sticking two rectangular pieces of glass to microscope slide 1.5 mm apart, and a drop of immersion oil is placed on top of the capillary tube. Oil fills the space between the capillary tube and the two pieces of glass. This arrangement reduces the effect of light diffraction caused by the curvature of the capillary tube and facilitates the observation of the trypanosomes. The best results are observed with a special oil immersion objective lens, magnifying 40-60 times. This technique can detect up to 2.5×10^2 , 5×10^2 and 5×10^3 trypanosomes/ml of blood for *T. congolense*, *T. vivax* and *T. brucei*, respectively (Eisler *et al.*, 2004; Uilenberg, 1998).

The heamatocrit centrifuge technique was subsequently improved into the dark ground /phase contrast buffy coat technique. Following blood centrifugation at 12,000 rpm for 5 minutes in heparinised microhaematocrit capillary tubes sealed on one end, the trypanosomes are concentrated mainly in the buffy coat zone. The capillary tube is then cut using a diamond pen

1 mm below the buffy coat to include the uppermost layer of the red blood cells. The buffy coat and the upper contents of the capillary tube are extruded onto a clean slide, mixed and covered with a cover slip (22 X 22 mm). The preparation is then examined for the presence of motile trypanosomes with the dark ground or phase contrast microscope with 40x objective lens. Trypanosome species are identified by their size and movement. *T. congolense* is recognized by its small size in relation to the red blood cell diameter, its sluggish activity and its invariable attachment to the red blood cells. *T. vivax* on the other hand is seen large and strikingly apparent by the speed with which it traverses the microscopic field. *T. brucei* is distinguished by its large size and going round in circles in a particular location. The buffy coat technique is able to detect up to 2.5×10^2 trypanosomes/ml of blood (Uilenberg, 1998; Luckins, 2004).

2.6.3. Sub-inoculation methods

Sub-inoculation methods are those by which trypanosomes are demonstrated by transferring infection from the suspected case to another vertebrate host (experimental animals) or to an invertebrate host (clean susceptible vectors) (Eisler *et al.*, 2004). The sensitivity of this method varies according to the species or even the strain present and the susceptibility of the experimental animals used (Uilenberg, 1998).

Animal sub-inoculation (Use of experimental animals)

The two species of experimental animals most commonly used are laboratory rats and mice. The sub-inoculation of blood into rodents is particularly useful in revealing sub-patent infections with trypanosome parasites in animals (Uilenberg, 1998). A blood sample, with anticoagulant, from the suspected domestic animal is injected in one or several rats or mice. This is mostly done by the intraperitoneal route (in the abdominal cavity), using 1 or 2 ml for a mouse and up to 5 ml in rats. The blood of the rodent is then examined frequently by snipping off a minute piece of the tip of the tail and transferring the resulting drop of blood to a slide for microscopical examination under a cover slip. Regular examination (preferably daily/or at least twice a week) should continue for at least two months after inoculation. The

sensitivity of this method varies according to the species or even strain present and the susceptibility of the experimental animals used (Eisler *et al.*, 2004).

Xenodiagnosis

Xenodiagnosis is feeding of a clean susceptible vector species on a suspected case of trypanosomosis, after which it is either dissected and examined for the presence of infection, or allowed to feed on a clean animal which is itself examined for the development of infection. It is an extremely sensitive method. However, because of the scarcity of laboratory reared *Glossina* species in Africa, this method of diagnosis is rarely attempted for tsetse-transmitted bovine trypanosomosis (Eisler *et al.*, 2001).

2.6.4. Serological diagnosis

Diagnosis of bovine trypanosomosis by conventional parasitological techniques is often difficult since trypanosomes cannot always be detected in the peripheral blood and some species do not readily infect laboratory rodents. These conventional parasitological detection methods have their own limitation in terms of sensitivity and practicability (Eisler *et al.*, 2004). As a consequence, more sensitive diagnostic methods, including the detection of Trypanosome specific antibodies and antigens have been developed (Nantulya *et al.*, 1992). However, immunocompetence, maternal antibodies, physical exhaustion are important biological factors and it is good practice to consider age, sex and breed as potential biological factors a priori when analyzing serological data (Greiner and Böhning, 1994; Jacobson, 1998).

Serological tests are used to detect specific antibody developed by the host against the infection or, inversely, to demonstrate the occurrence of circulating parasitic antigen in the blood. Detection of antibody indicates that there has been infection, where as detection of antigen shows the live trypanosomes are present in the animals (Uilenberg, 1998). Some of serological tests used for detection of antibody or antigen in immunological diagnosis of trypanosomosis include indirect fluorescent antibody test (IFAT) and indirect enzyme linked immunosorbent assay (IELISA) (Hopkins *et al.*, 1998).

Indirect fluorescent antibody test (IFAT)

Indirect fluorescent antibody test (IFAT) is a primary binding assay in which antibody-antigen reaction is measured indirectly. In this test, a smear of blood from infected animals with high parasitaemia are fixed and used as source of antigen to which antityrpanosomal antibody in test sera may bind specifically. In order to show that antibodies have reacted with the antigen, a commercial preparation containing antibodies (also immunoglobulins) raised in laboratory animals, usually rabbits, against bovine immunoglobulin, is applied to the smear, and washed off after being allowed to react. If these rabbit antibodies remain stuck to the slide, the test is positive, as they indicate the presence of anti-trypanosome bovine immunoglobulin which have reacted with the antigen slide. To demonstrate the presence or absence of the rabbit immunoglobulins, and thus indirectly the presence or absence of specific antibodies against trypanosomes in the bovine serum, the rabbit anti-bovine immunoglobulins in the commercial preparation are conjugated with a fluorescent dye, usually fluoresceine, which can be detected by looking at the smear with ultraviolet light. If the microscopic examination shows trypanosomes in the antigen smear to be fluorescent, the test result is positive (Eisler *et al.*, 2004; Uilenberg, 1998).

The indirect enzyme-linked immunosorbent assay (ELISA).

The principle of this test is very similar to that of the IFAT test. The binding of anti-trypanosomal antibodies to the antigen is shown by a conjugate of anti bovine (if the test serum is bovine) immunoglobulin labeled with an enzyme, which can be visualized by adding an appropriate chromogenic substrate. The interaction between enzyme and substrate will create a colour (Uilenberg, 1998).

Antigen-detecting tests (Ag-ELISA)

Demonstration of the parasite itself rather than antibodies is necessary for confirmation of active infection. Ag-ELISA was developed for the detection of circulating trypanosomal antigens at the international laboratory for research on animal disease in Nairobi, Kenya. The surface antigens of trypanosomes are variable. Only one or two of the many different variants are present in the blood at any one time, and unless one would possess mixtures of antibodies to all the possible variants, their detection is not reliable. Therefore, the tests that have been developed are based on so-called monoclonal antibodies against invariable (internal) antigens.

The principle of detecting circulating antigens using monoclonal antibodies is quite simple. The wells of a microplate are coated with a monoclonal antibody, which is specific for an invariable (non-surface) antigen of a trypanosome species. Serum of the animal to be tested is allowed to react in one of the wells, and if there is the corresponding circulating antigen, its presence in the well after washing off the test serum can be shown by finishing off the test with a conjugate of the same monoclonal antibody marked with a suitable enzyme, and the presence of the enzyme is shown by use of the suitable chromogenic substrate, as in the normal ELISA (Eisler *et al.*, 2004; Uilenberg, 1998).

2.6.5. Molecular tests

Accurate diagnosis of animal trypanosomosis and definitive identification of the causative trypanosome species are clearly useful objectives that may contribute to understand the epidemiology and ultimately lead to the long term control of the disease. These two objectives remain elusive with all the aforementioned parasitological and immunological technologies, molecular methods based on the detection and amplification of nucleic acid certainly have the technical potential to achieve them. The detection and identification of trypanosomes by molecular means should be based on stable, parasite specific genetic characteristics specific to that parasite that can withstand environmental influences exerted by either the host or the vector. The principle of molecular tests, DNA probes and PCR, is the demonstration of the occurrence of sequences of nucleotides which are specific for a trypanosome subgenus, species or even type or strain. Nucleotides are the constituents of DNA, the molecules which constitute the genes on the chromosomes in the cell nucleus. A positive result indicates active infection with the trypanosome for which the sequences are specific, as parasite DNA will not persist for long in the host after all live parasites have been eliminated. These tests are not only suitable for detecting parasites in the mammalian host, but also in the insect vector (Uilenberg, 1998; Eisler *et al.*, 2004).

2.7. Control of trypanosomosis

Various options are available for control of animal trypanosomosis: they include suppression and/or eradication of tsetse fly populations (Jordan, 1986), chemotherapy (Peregrine, 1994), the use of trypanotolerant animals or combination of these (Holmes, 1997).

2.7.1. Vector (tsetse fly) control

Tsetse control efforts have been undertaken throughout the African continent since long-time. Tsetse control has been undertaken in order to reduce the incidence of the diseases which the flies transmit in the populations living in tsetse regions and hence allowing the expansion of human settlement and agriculture into new areas. The reduction of fly numbers has generally been attempted with two different aims, either eradication which intends to completely eliminate tsetse from the area or control which aims simply to reduce the numbers. Many techniques have been used to reduce tsetse populations. Early technique to control tsetse were aimed at eliminating those basic requirements for survival of tsetse. These involve complete removal of any bush or woody vegetation which was aimed at eliminating the shaded places where tsetse rest and lays their larvae and extensive destruction of wild game animals which serve as source of blood meal for the tsetse flies. These techniques were very efficient and used with great success in certain areas of Zimbabwe, Zambia, Mozambique and Botswana. From 1946 to 1966 *G.m. submorsitans* and *G. pallidipes* were eliminated from 20,000km² in Uganda by removal of wild host. Increased environmental awareness and changing attitudes towards the preservation of biodiversity and wild life combined with an increasingly lucrative tourist industry centered on game parks and the high costs involved has made those practices unacceptable and obsolete (FAO, 2000; Vreysen, 2006, OIE, 2008).

Current vector control interventions involve the use of insecticides either through ground spraying, sequential aerosol spraying technique (SAT), insecticide-treated targets or insecticide-treated animals – live baits, the use of traps and the sterile insect technique (SIT). The discovery of cheap, persistent organochlorine insecticides (such as DDT and dieldrin) which became commercially available in 1945 marked the onset of the chemical warfare against tsetse fly and dominates most tsetse control efforts for 40 years. Initially these insecticides were applied indiscriminately from the ground as residual deposits. Later, concerns about bioaccumulation favored more selective techniques that treated only the

resting and breeding sites of the flies. The insecticide deposits have to last at least 2-3 months and have their maximum effectiveness during dry season. Over 200,000 square kilometer of tsetse infested land was cleared by ground spraying in West Africa, mainly in Nigeria between 1955 and 1978 (Vreysen, 2006). Until the mid-1970s the insecticides used were almost exclusively organochlorine compound, including DDT, Dieldrin and Endosulfan. Over the years, the negative impact of these insecticides on beneficial non target insects and natural enemies of the tsetse (like birds, small mammals etc), the potential development of resistance to the insecticide, the possibility of outbreaks of pests due to the elimination of predators, the general pollution of environment and the health hazard it poses to the staff of the spraying team were considered (Vreysen, 2001).

In the 1970s and 1980s, the application of persistent insecticides was gradually replaced by the more environmental friendly sequential aerosol technique (SAT) that uses aerial spraying of non residual ultra-low volumes of insecticide with fixed wing aircraft or helicopters. The goal is to kill adult tsetse in the first spraying cycle by contact and kill the emerging flies in five subsequent application cycles before the emerged flies can deposit a larva. The SAT was used successfully in Kenya, Zambia, Botswana, and in Zimbabwe, where 48,000 square kilometer of savanna area were cleared from tsetse between 1981 and 1989 (Vreysen, 2006).

The strategic thinking with respect to tsetse control shifted and more emphasis was placed on the use of stationary and live bait techniques that could be used by the farmer community in the 1990s. Because of the relative low cost of its components and the perception of being 'unsophisticated' the use of traps and targets has been promoted in the last decades as the most sustainable method, especially in the context of community participation (Vreysen, 2006). Trapping techniques have been greatly enhanced by development of designs that mimic the fly's perception of vertebrate hosts. These generally use blue and black cloth in a shape that attracts the flies and then funnels them upwards into a netting trap usually in the form of a monoconical (pyramidal) or biconical shape. For tsetse control, a simpler and cheaper device involves a suspended screen of blue and black cloth (often known as a tsetse target) impregnated with a biodegradable pyrethroid insecticide such as deltamethrin. Flies are attracted by the blue segments and land on the black segment, quickly succumbing to the insecticide. The effectiveness of traps and targets can be greatly enhanced by addition of an appropriate odour-bait. Such odour-baits are usually short-chain aromatic compounds such as acetone or octenol (Kuzoe and Schofield, 2004). The efficiency of the bait technology is

affected by various sociological factors (e.g theft, sustaining the interest of farmers, willingness to contribute etc.), inaccessibility of riparian during the rainy season, destruction by fire, the absence of baits for some species and the very high target density required per square kilometer in dense forest ecosystem. However, this technique is effective temporarily alleviating the burden of trypanosomosis over small areas (Vreysen, 2006).

Similar to the concept of traps and targets, the live bait technique involves treating cattle with appropriate persistent insecticide formulations, usually by means of cattle dips, or as pour-on, spot-on, or spray-on veterinary formulations in those areas with high cattle density and adequate expert support (Vreysen, 2001). These are highly effective against tsetse, and have the additional advantage of controlling other flies and cattle ticks (Kuzoe and Schofield, 2004). The technique has several advantages over traps and targets in terms of theft and maintenance, but several important issues like the required cattle density, the required proportion of the herd to be treated, host preference of different tsetse species, etc. require further research. Disadvantages are the high treatment frequency, the high cost of insecticides and the development of resistance to the insecticide (Vreysen, 2001).

The Sterile Insect Technique (SIT) is another approach to reduce/eradicate tsetse populations. This technique is based on the fact that females mate only once in their lifetime, thus any mating with a sterile male will prevent females from giving birth to any offspring. SIT consists in rearing a large numbers of laboratory male tsetse flies which are irradiated and subsequently released in the wild to compete with wild (naturally occurring) males so that females inseminated by them produce no off-spring. The technique has been effectively used for eradication of tsetse (*G. austeni*) from Unguja island (Zanzibar, United Republic of Tanzania), where some 1 600 square kilometer were cleared of tsetse flies (*G. austeni*). The project was carried out from 1994 to 1997 in Zanzibar. Effective suppression using conventional methods is a pre-requisite before this environmentally safe species-specific technique can be used to eradicate residual populations. Ecological islands are needed for the success of this technique or else re-invasion is bound to occur (Ulenberg, 1998). SIT is more efficient at lower fly densities, and is ideally suited to the final phase of local tsetse eradication (Kuzoe and Schofield, 2004).

2.7.2. Control of the causal agent (the trypanosome)

Targeting trypanosomes: Chemotherapy and chemoprophylaxis

The use of drugs for the prevention and treatment of trypanosomosis has been important for many decades, but the rapidity with which the trypanosomes have developed resistance to each drug introduced has tremendously complicated this approach to control the disease. Treatment and prevention of animal trypanosomosis relies on three compounds: Homidium, diminazene aceturate and isometamidium chloride. All the three compounds have been on the market for over 40 years. Chemoprophylaxis against bovine trypanosomosis has been in widespread use in tropical Africa for many years. Isometamidium has been marketed since 1961 as a prophylactic and therapeutic drug and can provide up to six months protection against tsetse challenge whilst homidium has limited prophylactic properties. It is primarily used as a therapeutic agent. Diminazine has only therapeutic properties. Prophylaxis can be useful under high tsetse challenge situations and enables cattle to remain productive (Holmes *et al.*, 2004). The incidence of resistance to these drugs is apparently increasing and the main means of controlling the disease is therefore under threat. Drug resistance is reported in Burkina Faso, Chad, Côte d'Ivoire, Ethiopia, Kenya, Nigeria, Somalia, the Sudan, the United Republic of Tanzania, Uganda, and Zimbabwe (Peregrine, 1994), the Central African Republic (Congo) (Finelle and Yvove, 1962) and Zambia (Mubanga and Sinyangwe, 1997). Drug resistance in trypanosomes is accelerated as a result of their exposure to a sub-lethal level of the compound used. It is, therefore, clear that such an event, so often the result of carelessness use of drugs, must be avoided (D'Ieteren, 1999). Trypanosomes are usually not resistant to both diminazene and Isometamidium at the same time. Thus, these two compounds have been termed as sanative pair and in instances of resistance to one drug the application of the other one will usually control the disease (Peregrine, 1994).

Alternatives to chemotherapy: traditional medicinal plants

Herbal Medicine sometimes referred to as Herbalism or Botanical Medicine is used for its therapeutic or medicinal value. Herb is a plant or plant part valued for its medicinal, aromatic or savory qualities. Herb plants produce and contain a variety of chemical substances that act upon the body.

Herbalists use the leaves, flowers, stems, berries, and roots of plants to prevent, relieve, and treat illness. From a "scientific" perspective, many herbal treatments are considered experimental. The reality is, however, that herbal medicine has a long and respected history. Many familiar medications of the twentieth century were developed from ancient healing traditions that treated health problems with specific plants. Today, science has isolated the medicinal properties of a large number of botanicals, and their healing components have been extracted and analyzed. Many plant components are now synthesized in large laboratories for use in pharmaceutical preparations. For example, vincristine (an antitumor drug), digitalis (a heart regulator), and ephedrine (a bronchodilator used to decrease respiratory congestion) were all originally discovered through research on plants.

The use of plants as medicine is older than recorded history. As mute witness to this fact marshmallow root, hyacinth, and yarrow have been found carefully tucked around the bones of a Stone Age man in Iraq. These three medicinal herbs continue to be used today. Drug discovery efforts against trypanosomatid diseases are limited, either because infected persons in underdeveloped areas cannot afford therapy, or because the infected population is too small to justify the required research expenditures. Different parts of plants have been used as antiprotozoal agents based on the species of the plant; these are roots, bark, leaves and seeds (Wurochekke and Nok, 2004). Table 2 shows some plant species from different countries (Ethiopia, Nigeria, Saudi Arabia etc) that have been tested to have activity on different species of trypanosomes and some other protozoan parasites of public and economic importance.

Table 2: List of some plant species reported to have activity on trypanosomes and others

Plant species	Part used	Parasite tested	Countries	Reference
<i>Sclerocarya birrea</i>	Stem bark	<i>T. b. brucei</i>	Nigeria	Mikail, 2009
<i>Commiphora kerstingii</i>	Stem bark	<i>T. b. brucei</i>		Wurochekke and Nok, 2004
<i>Khaya senegalensis</i>	Stem bark	<i>T. b. brucei</i>		
<i>Morinda morindiodes</i>	Stem bark	<i>T. b. brucei</i>	Nigeria	Olukunle <i>et al.</i> , 2010
<i>Azadiracta indica</i>	Stem bark	<i>T. b. rhodesiense</i>	Kenya	Raphael <i>et al.</i> , 2009
<i>Ximenia americana</i>	Stem bark	<i>T. congolense</i>	Nigeria	Maikai <i>et al.</i> , 2008
<i>Combretum molle</i>	Stem bark	<i>T. b. rhodesiense</i>	Ethiopia	Asres <i>et al.</i> , 2001
		<i>P. falciparum</i>		
<i>Argemone ochroleuca</i>	Not indicated	<i>P. falciparum</i>	Saudi Arabia	Abdel-Sattar <i>et al.</i> , 2010
		<i>T. cruzi</i>		
<i>Moringa stenopetala</i>	Leaf, root	<i>T. b. brucei</i>	Ethiopia	Mekonnen <i>et al.</i> , 1999
<i>Hymenocardia acida</i>	Stem bark	<i>T. b. brucei</i>	Nigeria	Abu <i>et al.</i> , 2009
<i>Neurolaena lobata</i>	Leaf	<i>L. mexicana</i>	Austria	Berger <i>et al.</i> , 2001
		<i>T. cruzi</i>		
<i>Jatropha grossidentata</i>	Root	<i>L. amazonensis</i>	Nigeria	Schmeda-Hirschmann and Razmilic, 1996
		<i>T. cruzi</i>		
<i>Artemisia spp</i>	Aerial part	<i>T. b. brucei</i>	Ethiopia	Nibret and Wink, 2010
<i>Moringa stenopetala</i>	Leave extracts	<i>T. congolense</i>	Ethiopia	Tsegabrehan, 2011
<i>Artemisia absinthium</i>				

Natural products active against African trypanosomes

So far a number of various compounds exhibiting different activities on trypanosomes (such as *T. brucei*, *T. congolense* and *T. vivax*) were isolated from natural sources (alkaloids, phenolic derivatives, quinones, and terpenes) (Sara *et al.*, 2004).

Alkaloids

The efficacy of different alkaloids was tested on trypanosomes by using *in vitro* procedures. However, certain alkaloids were found, in general, to be inactive. For instance, among others isoquinoline and quinoline alkaloids were noticed to have a significant trypanocidal activity. The possible mechanism of action could be explained as DNA intercalation in combination with the inhibition of protein synthesis exerting trypanocidal and cytotoxic effects by the alkaloids (Merschjohann *et al.*, 2001). Accordingly, findings revealed that *T. congolense* to be less susceptible to these compounds as opposed to *T. b. brucei*. The trypanocidal activity of quinoline alkaloids from Cinchona bark (Rubiaceae) (quinidine, cinchonine etc) was found to be significant with IC₅₀ values of 0.8, 1.2, 4.9, 7.1 μm , respectively, on *T. b. brucei*. The selectivity indices for quinidine and cinchonine were greater than 200, suggesting the potential of these alkaloids for further trypanocidal drug development. Specifically the so called Emetine, an isoquinoline alkaloid belonging to *Cephaelis ipecacuanha* (Rubiaceae) has been used in the treatment of amoebiasis, was also effective against trypanosomes (IC₅₀ = 0.039 μm and 0.43 μm for *T. b. brucei* and *T. congolense*) but without any selectivity.

Phenolic derivatives

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

suppresses a glycerol-producing anaerobic pathway by mass action, inhibitors of the TAO become trypanocidal due to a total block of the energy production of bloodstream forms (Nihei *et al.*, 2002).

Quinones

Quinones, especially 1,4-naphthoquinones such as plumbagin, can induce oxidative stress in trypanosomes (*T. congolense* and *T. cruzi*). This may be explained by their reduction to semi-quinone radicals by enzymes such as those present in the mitochondrial electron transport chain and the trypanothione reductase, a key enzyme of the trypanosomal antioxidant thiol metabolism (Cenas *et al.*, 1994; Salmon-Chemin *et al.*, 2001). Bioactivity-guided fractionation of an extract from the aerial parts of *Stephania dinklagei* (Menispermaceae) led to the isolation of one active compound on *T. b. brucei* bloodstream forms, aloe-emodin, an anthraquinone, along with inactive aporphinoid alkaloids. This compound possessed a weak but rather selective antitrypanosomal activity. It has been showed that an ethanolic leaf extract of *Mitracarpus scaber* (Rubiaceae) cured mice infected with *T. congolense* at a dosage of 50 mg/kg given intraperitoneally for five days. Azaanthraquinone, the active component isolated from this extract, inhibited the motility and induced lysis of *T. congolense* bloodstream forms *in vitro* and induced a complete lysis of the parasites in one hour. The addition of glycerol to the medium rendered the compound more trypanocidal (Sara *et al.*, 2004).

Terpenes

These compounds have antitrypanosomal activity. The mechanism of action of these molecules towards trypanosomes might depend on interference with trypanothione metabolism (for example trypanothione reductase) leading to increased oxidative stress in the parasite (Schmidt *et al.*, 2002). Mikus *et al.* (2000) evaluated the effect of several mono- and sesquiterpenes, which are frequently present in essential oils, on the viability of *T. b. brucei* bloodstream forms. The only active monoterpene was terpinen-4-ol, a monoterpene alcohol, which exhibited a high antitrypanosomal activity. A diastereoisomer of the diterpene kolavenol, was isolated from a root bark extract of *Entada abyssinica* (Leguminosae), a plant traditionally used in Uganda to treat sleeping sickness. It showed a trypanocidal activity with an IC₅₀ value of 8.6 μ m against *T. b. rhodesiense* bloodstream trypomastigotes (Freiburghaus *et al.*, 1998).

2.7.3. Plants tested as trypanocides in Ethiopia

A number of plant species used by traditional healers have been documented but very few of them are tested against trypanosomes. Examples are *Verbascum sinaiticum*, *Rumex nepalensis*, *Momordica fotida* and *Gloriosa superba* reported from North Ethiopia (Dek Island) (Teklehymanot, 2009). *M. stenopetala*, and different species of *Artemisia* have been reported to have activity against different species of trypanosomes in Ethiopia (Mekonnen *et al.*, 1999; Asres *et al.*, 2001; Nibret and Wink, 2010). Brief descriptions of these two plants are given below.

Moringa stenopetala

Moringa stenopetala (Figure 1) a species that grows widely in southern parts of Ethiopia, also known as *aleko* or *shiferaw* among local communities has a variety of uses, many of them medicinal. The local people cook the leaves of the *M. stenopetala* tree and eat them with their traditional *kurkufa* (a cereal dish made with maize and sorghum). In contrast, the people of Konso use the tree not only for food but also as a medicine and they cultivate it in large areas around their villages. There are claims that the leaves, boiled in water, can cure malaria, hypertension and stomach pain, whereas the roots, chopped and mixed with water, are also used for treating severe cases of malaria. *M. stenopetala* is used as an herbal medicine in areas where visceral leishmaniasis or kala-azar (caused by the *Leishmania* parasite) prevails. In addition, there are reported cases of *M. stenopetala* leaves being used to expel the retained placenta in women who have just given birth, and the seeds are used to clear muddy water. Leaf and root extracts of *M. stenopetala* were also tested against the infective stages of *T. brucei* (a parasite that causes a sleeping-sickness) showed an activity against the parasite (Mekonnen *et al.*, 1999). The different parts: leaves and roots are used as traditional medication.



Figure 1: Photograph of *Moringa stenopetala* (Source: www/help.plant-trees.org)

Artemisia absinthium

Artemisia absinthium (Figure 2) is one of the many traditional medicinal plants in Ethiopia used for the treatment of infectious and non-infectious health problems. The genus *Artemisia* contains more than 400 species and most of its known species are found predominantly in Asia, Europe and North America (Mucciarelli and Maffei, 2002). *Artemisia* species are most commonly used in the traditional medicine, particularly in treatment of malaria. The genus is known for the production of various types of sesquiterpene lactones, including artemisinin which is the best known antimalarial compound from *A. annua* (Kelsey and Shafizadeh, 1979). *A. absinthium* (known as ‘Ariti’ in Ethiopia) is an erect, perennial herb, 30-60 cm high. It is widely grown especially in the northern and central parts of Ethiopia for its aroma and is widely used in rituals (Tadesse, 2004). It is also used as a vermifuge in addition to malaria (Jansen, 1981). In combination with other herbs it is used for treating wounds of animals (Yineger *et al.*, 2007). Aerial parts of this plant were reported to be active against *T. b. brucei* (Nibret and Wink, 2010).

(Table 3). Nowadays, this plant is being cultivated on experimental basis in Ethiopia (Jansen, 2006).

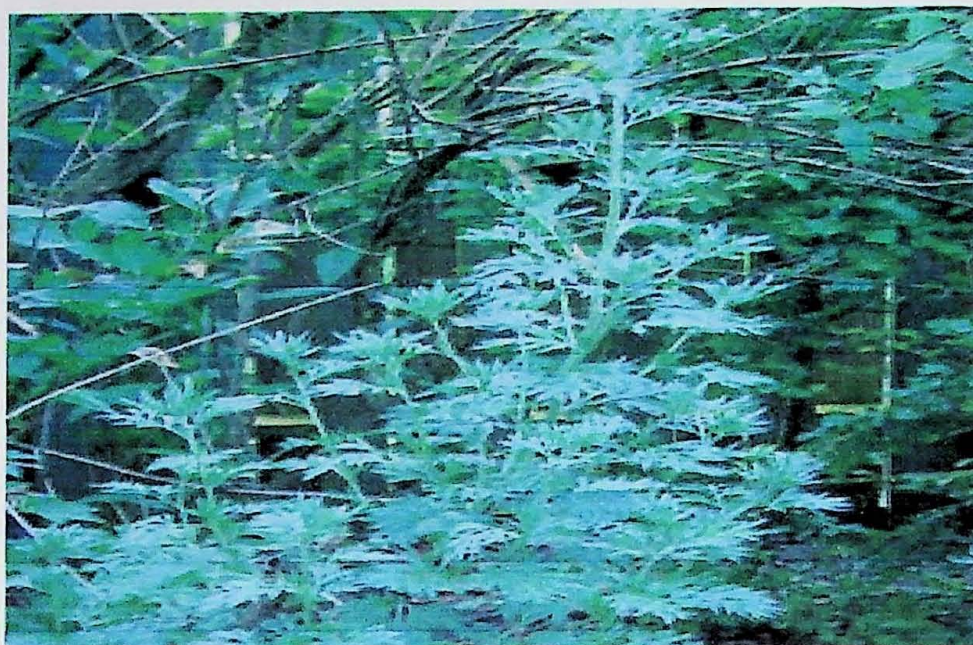


Figure 2: Photograph of *Artemisia absinthium* (Source: www/help.plant-trees.org)

Table 3: Trypanocidal activities of artemisinin and crude extracts from different *Artemisia* species.

Plant species	Plant part	Extract type	IC ₅₀ (µg/ml)
			<i>T. b. brucei</i>
<i>Artemisia absinthium</i> (Asteraceae)	Aerial part	MeOH	27.90
<i>Artemisia abyssinica</i>	Aerial part	CH ₂ Cl ₂	27.05
	Leaves	MeOH	41.76
<i>Artemisia afra</i>	Leaves	CH ₂ Cl ₂	19.13
Artemisinin	Leaves	MeOH	77.54

2.7.4. Trypanotolerant breeds

The term 'trypanotolerance' means reduced susceptibility to trypanosomosis and denotes an inherited biological property allowing animals to live, breed, grow and survive in a naturally infected environment without exhibiting clinical signs of trypanosomosis after harboring pathogenic trypanosomes. Innate resistance or trypanotolerance have been observed early when the ability of indigenous taurine cattle in West Africa to survive and produce under trypanosomosis risk was observed. Both acquired and innate resistance to AAT can occur in cattle. The two most important trypanotolerant breeds are *B. Taurus* subtypes *N'Dama* and *Baoulé*, while a degree of trypanotolerance has also been shown to occur in some *B. indicus* zebu and with breeds, for example the Orma Boran and Maasi zebu (Akol *et al.*, 1982). The N'Dama only accounts for 5% of the total cattle population of the sub-Saharan Africa and there is now considerable interest in their conservation. At least two characteristics contribute to the resistance of trypanosome: the ability to regulate parasites population expansion and the capacity to resist anaemia. Murray (1988) had investigated the genetic basis for trypanotolerance and have shown that the ability to control parasitaemia and the capacity to resist anaemia during infection in trypanotolerant breeds.

2.7.5. Integrated control program

Integrating several control methods seems to be the most effective way of tsetse intervention (Veryson, 2006). The economic and feasibilities of employing various control methods must be compared for any given tsetse infested area. In many African countries, reducing the risk of trypanosomosis by employing more effective control methods may well increase both livestock and crop production. Successful strategies for controlling animal trypanosomosis must be based on accurate appraisals of the impacts of the disease constraints on village farming system and the development of cost-effective sustainable disease control packages which can be adapted by producers (ILRAD, 1993).

3. MATERIALS AND METHODS

3.1. Experimental mice

Swiss white male mice aged between 6-8 weeks, weighing 30-50gm body obtained from the Debre-Zeit National Veterinary Institute were used. The mice were kept in a room where the temperature was maintained through ventilation under a 12 hrs light and 12hrs dark cycle. They were provided with a commercial pellet ration and water *ad-libitum* throughout the experimental period including the 15 days of acclimatization in the experimental room. The 36 mice were randomly divided into six experimental groups: NIC (none infected control, treated with distilled water), INC (infected with *T. congolense* isolates and non-treated control), IMT (infected and treated with a single dose of Isomethadium chloride [ISOMIDIUM-1g, Genevet Pvt, Korea] at the rate of 4 mg/kg). The other three groups received 400 mg/kg, 800 mg/kg and 1200 mg/kg of methanolic extract of *Artemisia absinthium* as prophylaxis for seven days, respectively. On the eighth day all except the negative controls (NIC) were infected with 1×10^5 parasites in 0.2 ml blood/PBS solution intraperitoneally (Eisler *et al.*, 2001). All animals were handled by respecting standard protocols in accordance with the Good Laboratory Practice regulations.

Table 4: Experimental design of infection and treatment schedule

Groups	Infection dose	Treatment
NIC	Non infected	Distilled water (7 days)
INC	1×10^5 /ml	Non treated infected control
IMT	1×10^5 /ml	Isomethamidium chloride 4mg/kg (single dose)
AP 400 mg/kg	1×10^5 /ml	Methanol extract of <i>A. absinthium</i> (for 7 days)
AP 800 mg/kg	1×10^5 /ml	Methanol extract of <i>A. absinthium</i> (for 7 days)
AP 1200 mg/kg	1×10^5 /ml	Methanol extract of <i>A. absinthium</i> (for 7 days)

3.2. Plant material collection and extraction

Artemisia absinthium was used for this experiment. Areal part of *A. absinthium* was collected from the field around Addis Ababa. The collected plants of *A. absinthium* were washed under running tap water to remove dust and any other foreign materials and left to drain off. The plants were then spread on laboratory benches and left to air dry for four weeks. The leaves were powdered using mortar and pestle. The powdered material of the plant was sieved and taken for extraction. The extraction was done at Hawassa University of Agriculture, department of photochemistry.

3.3. Preparation of methanol extract of the plant

The powdered plant material was extracted using the maceration method. In maceration method of extraction the powdered plant material is placed in stoppered container with the solvent and allowed to stand at room temperature for a period of at least three days with frequent agitation until the soluble matter has dissolved. The mixture then is strained, the marc (the damp solid material) is pressed and the combined liquids are clarified by vacuum suction filtration using Whatmann 42 filter paper. The marc is then transferred to small bottles, sealed and dried in a lyophilizer overnight.

3.4. Test organism: *Trypanosoma congolense*

The study was conducted by inoculating *Trypanosoma congolense* isolates from upper Diddesa valley of southwestern Ethiopia to these experimental mice at Parasitology laboratory of the College of Veterinary Medicine and Agriculture in Debre Zeit.

3.5. In vivo toxicity test

Before the beginning of the experimental treatment with plant extract three mice were orally drenched with 400mg/kg and 800mg/kg (experimental doses) and 2000mg/kg of the crude extract respectively for seven days for sub acute toxicity test (OECD, 2001). Changes in behavior, clinical symptoms and signs of pain and stress were observed for 10-15 minutes following administration of the test drug for signs of acute and sub acute toxicity, including the hypotensive response.

3.6. Measurements

3.6.1. Level of parasitaemia

The level of parasitaemia was measured from the blood collected from the tail every other day for 28 days and every week from day 28 onwards until the end of the experiment (D70) by the 'rapid matching' method. A drop of blood (wet film) was placed and covered with a cover slip and pressed until it is fully spread under the cover slip. The prepared slides were examined under a binocular microscope using 40X objective and parasites were counted. The number of parasites counted was then converted to logarithmic values to the base 10. The values obtained were taken as the number of parasites per milliliter of blood (Herbert and Lumsden, 1976). When no parasite was counted in 20 fields of the, then the sample was taken as negative.

3.6.2. Determination of PCV

Packed cell volume (PCV) is the percentage of red blood cell (RBC) obtained by centrifuging whole blood. EDTA coated capillary tubes were used to collect blood from the tail vein of mice every other day for 28 days after infection and every week from day 28 onwards (until day 70, experimental period) The capillary tube was filled to $\frac{3}{4}$, sealed on one end and centrifuged in a micro-centrifuge for 5 min at 12000 rpm. PCV was measured by using

haematocrit reader and values were taken as percentage of cell (RBC) volume to total volume of blood.

3.6.3. Body weight measurement

The weight of mice was measured every other day using a weighing balance. Body weight was measured every other day from day zero before infection until day 28 after infection from day 28 onwards every seven days until the end of the experiment for those survived.

3.6.4. Survival of mice

The survival rate was measured by recording the number of days the mice has lived with or without treatment, from the day of treatment to 70 days after treatment. The percentage of mice surviving on the 70th day was also calculated for each group.

3.7. Statistical analysis

Data on parasitemia, body weight and packed cell volume were analyzed using Windows SPSS Version 15. T - test was used to compare results among and within groups for differences between initial and final results. All data were analyzed at a 95% confidence interval ($\alpha = 0.05$).

4. RESULTS

4.1. Toxicity test in uninfected mice

Methanolic extracts of *A. absinthium*, given at doses of 400 mg/kg, 800 mg/kg and 2000 mg/kg to the uninfected mice did not significantly affect the levels of PCV (Figure 3) and body weight (Figure 4) of the mice during the toxicity test period when compared to those of water-treated mice (NIC). In all mice, PCV and body weight gradually increased during the toxicity test period and no sign of acute and sub acute toxicity was observed. Thus the extract was considered to be safe to proceed with the experiment.

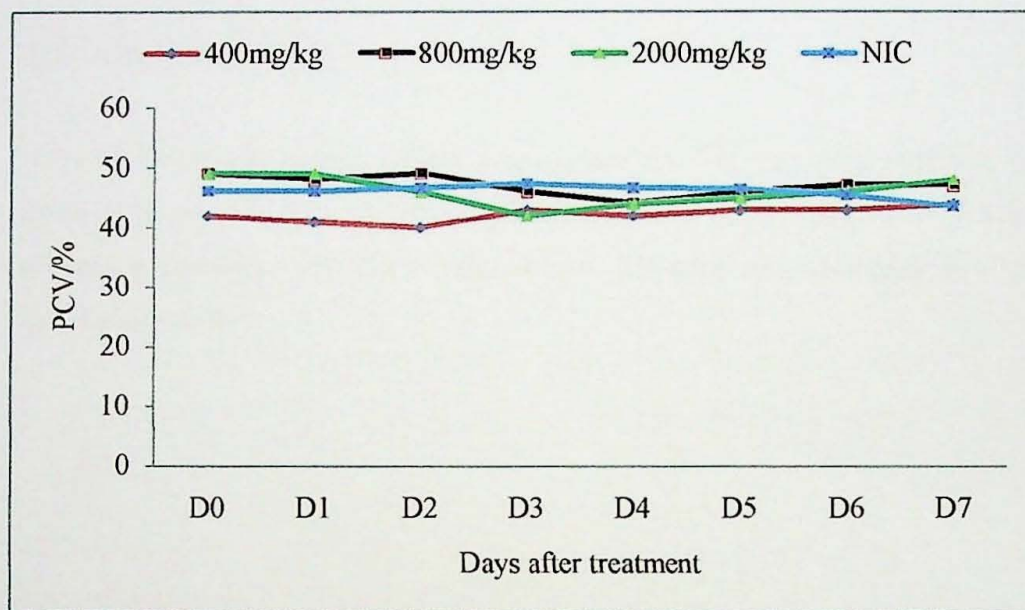


Figure 3: Mean PCV of uninfected mice during acute and subacute toxicity testing (Uninfected mice were treated with either water or methanolic extracts of *A. absinthium*).

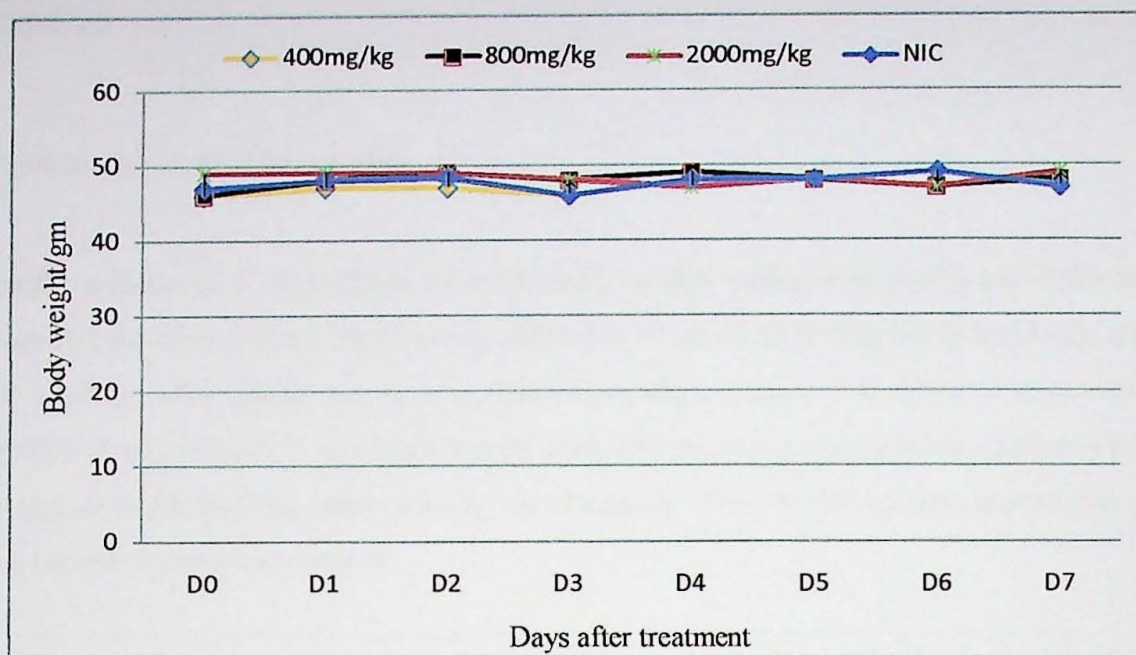


Figure 4: Mean body weights of uninfected mice during subacute toxicity testing (The mice were given either water or methanol extracts of *A. absinthium*)

4.2. Impact of treatment on parasitaemia

Infected-none treated mice showed the presence of parasites 17 days after infection (DAI) with all the mice being positive by day 21 (DAI). There was variation in appearance of parasitaemia in infected mice with the different doses of plant extracts and Isometamidium chloride which dose was dependent. The first days where parasitemia became evident was as follows: 400 mg/kg treatment group (positive at 21 DAI); 800 mg/kg treatment group (positive at 24 DAI; 1200 mg/kg treatment group (positive at 28 DAI). Comparison of the level of parasitaemia in various treatment groups specifically at 28 and 42 DAI showed that mice from the group infected-non treated (INT) had the highest level. The higher extract concentrations showed a dose-dependent effect on the level of parasitemia and survival time. Indeed, the 1200 mg/kg extract treatment was comparable to that of Isometamidium chloride group in controlling parasitaemia levels (Figure 5).

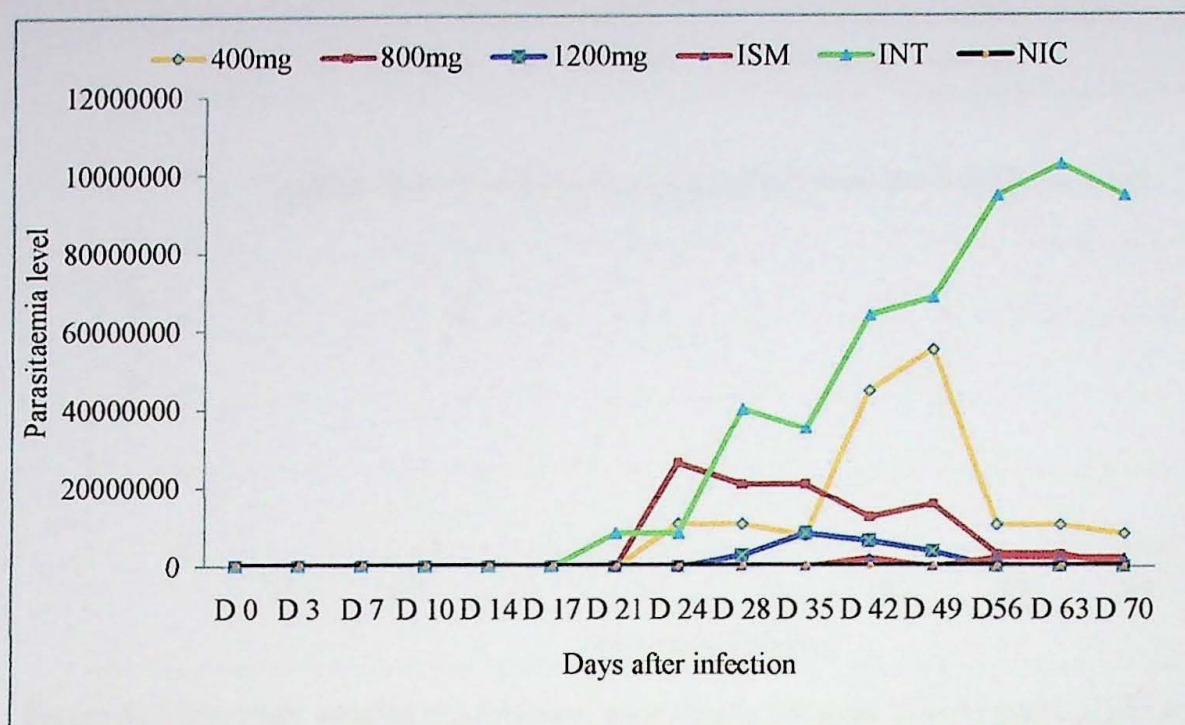


Figure 5: Parasitaemia of *T. congolense* infection in mice treated with either water or methanol extract of *A. absinthium* and Isometamidium chloride only

Pair wise analysis of the impact of treatment on parasitemia of the mice is indicated in the table below (Table 5). Accordingly, there was a statistically significant difference ($P < 0.05$) in the mean parasitemia levels of animals treated with the three doses of AP 400 mg/kg, 800 mg/kg and 1200 mg/kg and infected non treated group (INT). Mean while, the level of parasitaemia observed in groups of mice treated with the highest dose of *A. absinthium* (1200 mg/kg) and isomethadium chloride treated group was very similar indicating the suppressive effect of the plant extract on circulating trypanosomes compared with infected non-treated group (INT). The mean parasitemia level of mice in groups with methanol extracts of *A. absinthium* 800 mg/kg and 1200 mg/kg and Isomethamedium chloride showed no statistically significant difference ($P > 0.01$).

Table 5. Analysis of Paired Samples t- Test for mean Parasitemia.

Paired treatment groups	Paired differences					t	df	P value
	Mean	Std Deviation	Std Error Mean	95% confidence interval of the difference				
				Lower	Upper			
400 mg and 800 mg	10578319.6	41344725.5	14617567.9	45143375.1	23986735.9	.72	7	0.5
800 mg and 1200 mg	28270449.0	54270718.6	19187596.6	17101007.2	73641905.2	1.7	7	0.2
400 mg and 1200 mg	17692129.4	20300619.4	7177352.8	720386.9	34663871.9	2.5	7	0.04
400 mg and ISMM	1091311.9	9559523.8	3379802.1	6900650.0	9083273.8	.33	7	0.6
800 mg and ISMM	11669631.5	43839034.5	15499439.3	24980718.5	48319981.5	.75	7	0.5
1200 mg and ISMM	9486181.43	14742544.9	3940110.9	17998273.4	974089.4	2.4	13	0.1
400 mg and INT	43537216.6	15655361.6	6391274.6	59966511.0	27107922.3	6.8	5	0.001
800 mg and INT	26916423.9	52355408.5	21374006.0	81860055.6	28027207.7	1.3	5	0.001
1200 mg and INT	49514172.7	36313062.5	12838606.4	79872652.7	19155692.7	3.9	7	0.006

4.3. Impact of treatment on PCV

There was a gradual fall in the mean PCV levels following infection in infected non treated mice (INT) starting from 14 DAI. In the isomethamedium chloride treated group, the level of mean PCV was consistently higher as opposed to all the remaining treatment and control groups. Methanolic extract of *A. absinthium* treated mice with the doses of 400 mg/kg particularly showed a gradual decrease in mean PCV from 24 - 49 DAI and showed an increase from 56 DAI until the end of the experimental (Figure 7). All mice treated with methanol extract of *A. absinthium* with the doses of 800 mg/kg and 1200 mg/kg showed a relatively higher and increasing levels of PCV like the trends in Isomethamidium chloride and NIC groups. On day 28 for INT group and D42 DAI for mice treated with 400 mg/kg *A. absinthium* observed sharp decrease in mean PCV levels compared to other experimental groups. There was no statistically significant difference ($P>0.01$) in mean PCV levels between groups treated with methanol extracts of *A. absinthium* (800 mg/kg and 12000 mg/kg) and Isomethamedium chloride treated mice.

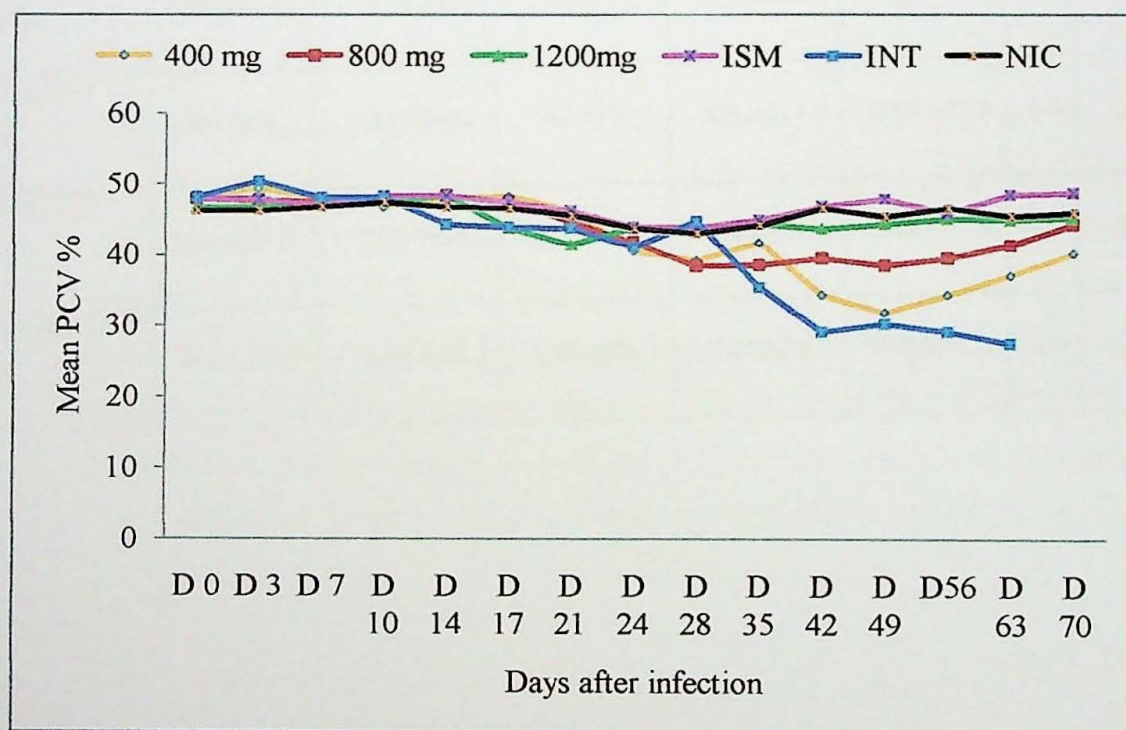


Figure 6: Mean PCV levels in *T. congolense* infected mice treated with either water or extract of *A. absinthium* and Isomethamedium chloride only

Pair wise analysis of the impact of treatment on mean PCV of the mice is illustrated in the table below (Table 7). Accordingly, there was a statistically significant difference ($P < 0.05$) in the mean PCV levels of animals treated with AP 400 mg/kg, 800 mg/kg and 1200 mg/kg and INT. The mean PCV value of mice in groups with methanol extracts of *A. absinthium* (800 mg/kg and 1200 mg/kg) and Isomethamedium chloride revealed no statistically significant difference ($P > 0.01$) (Table 7).

Table 6. Analysis of Paired Samples t-Test for mean PCV.

Paired treatment groups	Paired differences					t	df	P value
	Mean	Std Deviation	Std Error Mean	95% confidence interval of the difference				
				Lower	Upper			
400 mg and 800 mg	.97	2.9	.78	2.64	.70	1.3	13	0.23
800 mg and 1200 mg	.70	3.4	.90	2.65	1.25	.79	13	0.4
400 mg and 1200 mg	1.7	5.2	1.4	4.67	1.3	1.2	13	0.2
400 mg and ISMM	.39	2.4	.64	.98	1.8	.62	13	0.5
800 mg and ISMM	1.4	3.4	.91	.60	3.3	1.8	13	0.2
1200 mg and ISMM	2.1	4.3	1.14	.39	4.5	1.8	13	0.09
400 mg and INT	1.9	3.9	1.09	.40	4.4	1.8	12	0.0
800 mg and INT	2.7	5.5	1.53	.62	6.02	1.7	12	0.0
1200 mg and INT	3.5	7.0	1.94	.77	7.7	1.8	12	0.0
400 mg and NIC	2.0	5.4	1.6	5.5	1.4	1.3	11	0.2
800 mg and NIC	1.6	3.4	.98	3.8	.58	1.6	11	0.1
1200 mg and NIC	1.0	2.0	.58	2.3	.29	1.7	11	0.1
ISMM and NIC	2.42	4.6	1.33	5.3	.50	1.8	11	0.09

Body weight measurements were made every other day until the end of the experiment. The weight in the untreated infected mice group (INT) started to decline after 14 DAI. However, all the mice in this group (INT) died before the end of the experiment. All mice treated with methanolic extract of *A. absinthium* generally showed a gradual increase in mean body weight during the experimental period as compared to groups (INT) (Figure 6). On 49th day post infection, groups receiving methanol extracts of *A. absinthium* and Isomethamedium chloride had significantly higher weights ($P<0.01$) than the untreated infected group (INT). Though, all the mice in groups with methanol extracts of *A. absinthium*, 400 mg/kg, 800 mg/kg and 1200 mg/kg had relatively higher body weights than the Isomethamedium chloride groups, there was no statistically significant difference ($P>0.01$) in mean body weights between groups treated with methanol extracts of *A. absinthium* and Isomethamedium chloride (Table 6).

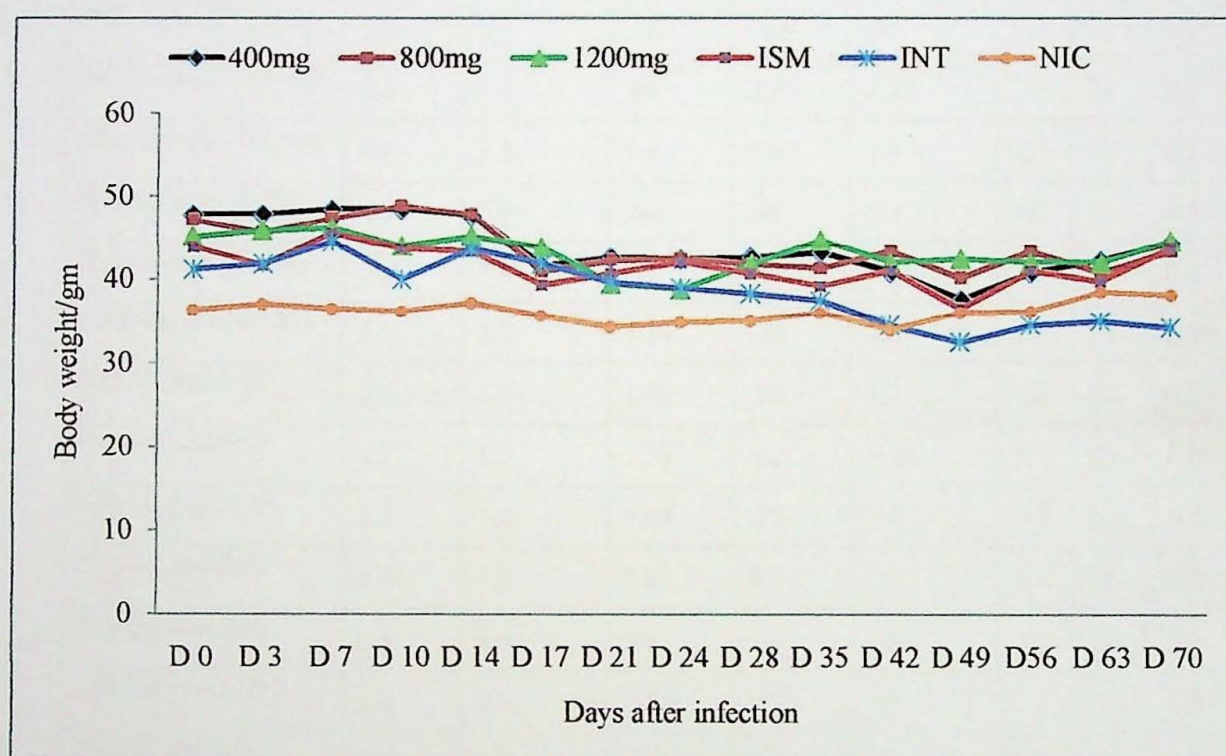


Figure 7: Body weights of *T. congolense* infected mice treated with either water or extract of *A. absinthium* and Isomethamedium chloride only.

Table 7. Analysis of Paired Samples t- Test for mean body weight.

Paired treatment groups	Paired differences					t	df	P value
	Mean	Std Deviation	Std Error Mean	95% confidence interval of the difference				
				Lower	Upper			
400 mg and 800 mg	0.3	1.7	0.4	0.9	0.9	0.1	13	0.9
800 mg and 1200 mg	6.1	7.3	1.9	1.8	10.3	3.1	13	0.008
400 mg and 1200 mg	6.0	7.6	2.0	1.6	10.4	2.9	13	0.01
400 mg and ISMM	0.5	1.8	0.5	0.7	0.8	0.01	13.	0.7
800 mg and ISMM	0.9	1.1	0.2	0.5	0.4	0.2	13	0.5
1200 mg and ISMM	0.7	1.0	0.01	0.7	0.51	0.2	13	0.9
400 mg and INT	5.0	3.3	0.9	3.0	7.0	5.6	12	0.000
800 mg and INT	5.1	3.4	.93	3.1	7.2	5.5	12	0.0
1200 mg and INT	5.2	3.0	0.5	3.0	7.0	5.6	12	0.001
400 mg and NIC	8.2	3.3	0.9	6.1	10.3	8.7	11	0.0
800 mg and NIC	8.4	2.6	0.8	6.7	10.0	11.0	11	0.0
1200 mg and NIC	8.3	2.5	0.6	6.2	10.0	11.7	11	0.002
ISMM and NIC	3.3	7.4	2.2	5.9	1.6	1.6	11	0.2

4.5. Survival periods of trypanosome infected mice

There were only slight differences between the three treatment groups treated with the three different doses of the extracts i.e 400 mg/kg, 800 mg/kg and 1200 mg/kg on the survival time of mice following infection. Death started to occur in INT group from 49 DAI and at the end of the experiment all mice were dead (0% survived at the end of the experiment). In the group INT, the establishment of parasitaemia was faster than the arthemisia treated groups. In the Isomethamedium treated group no death occurred (100% survived at the end of the experiment) with a similar trend in groups of 1200 mg/kg. However, most of the mice treated with 400 mg/kg and 800 mg/kg of methanol extract of *A. absinthium* survived until the end of the experiment (Table 5).

Table 8. Survival rate of experimental mice

Treatment groups	No of mice survived
400 mg/kg	4/6
800 mg/kg	4/6
1200 mg/kg	6/6
ISMM	6/6
INT	0/6
NIC	6/6

5. DISCUSSION

5.1. General

This experimental study was planned to see the prophylactic effect methanolic extract of *Artemisia absinthium* in three different doses against *T.congolense* isolates in experimentally infected male swiss mice. *A. absinthium* is grown especially in the northern and central parts of Ethiopia for its aroma and is widely applied in rituals (Tadesse, 2004). The prophylactic effect of this medicinal plant extract has not been investigated.

5.2. Impact of crude extracts on parasite survival in mice

Death started to occur in INT group from 49 DAI and at the end of the experiment all mice were died (0% survived at the end of the experiment). In the group INT, the establishment of parasitaemia was faster than the arthemisia treated group (D17 after infection). In the Isomethamedium treated group no death occurred (100% survived at the end of the experiment) with a similar trend in groups of 1200 mg/kg. The *in vivo* antitrypanosomal activity exhibited by the methanolic extract of the *A. absinthium* was in agreement with the findings of previous worker such as Tsegabrehan (2011) who demonstrated curative effect of this extract on *T.congolense*, and other species of trypanosomes *T. cruzi*, *T. b. brucei* and *T. b. rhodesiense* who have shown that extracts of different plants may exhibit *in vivo* antitrypanosomal activity (Ene *et al.*, 2009; Ngure *et al.*, 2009).

The result of the current study indicates that the antitrypanosomal prophylactic effect was observed in mice obtaining 800mg/kg and 1200mg/kg. This effect was observed gradually, it was comparable with mice infected with *T.congolense* and treated with a single prophylactic dose of isomethamidium chloride. Prophylactic activity manifested by the methanolic extract of *A. absinthium* might be due to the presence of some phytochemicals like flavonoids, triterpenes, terpenoids, tannins alkaloids and other constituents. (Hoet *et al.*, 2004). The *in vivo* antitrypanosomal effect of the *A. absinthium* can equally be attributed to the presence of the above secondary metabolites present as reported by (Mekonnen *et al* (1999); Nibret and Wink (2010). However, further study may be needed to identify the exact molecule(s) that

has/have played a major role in the killing and/or suppression of *T. congolense* infection in mice. Nibret and Wink (2010) working on *A. absinthium* have shown that extracts of the herbs had an effect on the growth inhibition of *T. brucei* in vitro. They suggested that this activity of the extracts might be due to the presence of major compound camphor and two other major sesquiterpene lactones, absinthin, artabsin and glucosinolates which are known to occur in the plants.

The findings of this study indicated that the plant extract (*A. absinthium*) in the three doses of methanol extract showed moderate to high *in vivo* antitrypanosomal activity. There is a slight difference in the survival of mice between the doses. Highest survival rate is observed in 1200mg/kg. The prolongation of lives of treated animals may therefore be associated with the ability of this extract to improve the PCV possibly by controlling further multiplication of the parasite or inactivating the toxic metabolites produced by trypanosomes. The consistent suppression of parasitaemia combined with prolonged survival time of mice as shown in *in vivo* results may be linked to the prophylactic ability of the extract against the parasite.

The appearance of the parasitaemia was also dose dependant where parasitaemia appeared in mice obtained higher doses of *A. absinthium* compared to infected but not treated control ones. Several researchers made similar observations on reduction in parasitaemia and concluded that high parasite load could mask the efficacy of crude extracts (Ekanem *et al.*, 2008; Abu and Uchendu, 2011; Tsegabrehan, 2011). Moreover, the crudeness of the extracts and the oral route of administration (to avoid toxicity if given through other routes) might have also reduced the availability of sufficient active ingredients.

5.3. Effect of crude extracts on performance parameters of mice (survival, BW, PCV)

The positive effect of plant extracts can further be deduced from weight measurements of the experimental animals. Animals treated with the plant extracts on average maintained their body weight while the animals in the untreated infected group showed progressive reduction in body weights. This finding supports the reports of Tsegabrehan (2011) and similar studies elsewhere (Peter *et al.*, 2009). Moreover, animals treated with extract of the plants generally had comparable weights to those treated with Isomethamedium chloride in the maintenance of close to normal body weights throughout the experimental period.

It has been shown that the measurement of anemia gives a reliable indication of the disease status and productive performance of trypanosome infected animals (Ekanem *et al.*, 2006). Severity of anemia usually reflects the intensity and duration of parasitaemia. Several reports (Ogunsanmi and Taiwo, 2001; Umar *et al.*, 2007; Ekanem *et al.*, 2008; Saleh *et al.*, 2009) have also ascribed acute anemia in trypanosomosis to proliferating parasites. The result of this study showed that this plant extract has comparable potential to Isomethamedium chlorid since it is able to control anemia, especially at the later stages, by minimizing sudden drops in PCV values. In untreated mice, the parasite count increased and the packed cell volume (PCV) decreased markedly from day to day until the death of the animals, which was also observed in previous studies by Abubakar *et al.* (2005) and Ngure *et al.* (2009).

Treatment with different doses of *A. absinthium* has shown extended survival of mice in the treatment groups compared to the non-treated control group. The prolongation of survival time in oral administration of crude extract before infection agrees with previous reports (Abubakar *et al.*, 2005; Tsegabrehan, 2011, Abu and Uchendu, 2011) who have applied extracts of *Momordica balsamina* and *Hymenocardia acida* on *T. brucei*. Of special interest are the methanolic extracts of our test plant; *A. absinthium* which clearly demonstrated an interesting antitrypanosomal profile; they maintained the animals above 70 days despite the presence of parasites in circulation.

6. CONCLUSION AND RECOMMENDATIONS

The use and misuse of drugs has contributed to the development of drug resistance in trypanosomes. An urgent need arises therefore to develop newer drugs to counter trypanocidal resistance. This study showed that methanol extract of the aerial parts of *Artemisia absinthium* have shown prophylactic effect on *T. congolense* infected Swiss white mice and resulted in the improvement of PCV and body weight. The extract also prolonged the lives of mice to comparable level to that of commercial drug Isomethamedium chloride (Trypamidium). Thus, it can be concluded that the plant has excellent prophylactic potential against pathogenic trypanosomes (*T. congolense*) of livestock importance.

Therefore, the following recommendations are forwarded for further study and future exploitation of this potential.

- Further study is required to refine the active components in the crude extract of this plant, standardize the dosage and mode of application and make field trial on livestock species.
- There is a large plant biodiversity in Ethiopia that the medicinal plant potential is still not reached by scientific studies. Therefore, those already known plant species of medicinal importance and those yet to be identified should be given enough attention for trials on major diseases of livestock importance.
- The livestock rearing community has tremendous rich knowledge on different ethno veterinary practices and herbal medications. This should be systematically gathered and exploited in the selection of effective plants against various diseases including trypanosomosis.

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8. ANNEXES

Annex I. Calculation of treatment dose of Isometamedium chlorid

The maximum dosage used for : Isometamedium chlorid treatment groups in the present study was 4mg/kg b.w.

For 1kg (1000g) b.w $\rightarrow$ 4mg/kg b.w Isometamedium chloride is given

The weight of the mice is 50gm $\frac{50\text{gm} \times 4\text{mg/kg b.w}}{1000\text{g}} = 0.2\text{mg}$ Isometamedium chloride

Therefore for a mouse weighing 50 gm 0.2mg Isometamedium chloride is needed

1% solution was prepared

Annex II: Maceration Method of Extraction *A. absinthium*

- A. In maceration method of extraction, the whole or coarsely powdered plant material is placed in a stoppered container with the solvent and allowed to stand at room temperature for a period of at least 3 days with frequent agitation until the soluble matter has dissolved.
- B. The mixture then is strained, the marc (the damp solid material) is pressed, and the combined liquids are clarified by Vacuum suction filtration using whatmann 42 filter paper.

Maceration procedure employed to extract the plant sample according to the method imploied by Emmert et al (2004).

1. The powdered plant material was prepared.
2. The powdered plant material was placed in a closed conical flask
3. The selected solvent system (80%) Methanol was added.
4. The plant material with the solvent of choice was allowed to stand for a day (24hrs) with continuous shaking using shaker (Grant GLS400, England).

5. Then the menstruum was filtered using whatmann 42 filter paper under vacuum suction filtration.
6. The solid residue (marc) was pressed to recover as much as occluded solution.
7. The occluded and pressed filtrates were mixed together to scale up the amount of crude extract.
8. The mixed filtrates were further clarified by following the same procedure indicated at step 5 above.
9. Then, filtrates free from any insoluble matter were concentrated using rotary evaporator (Laborota 4003 control, made by Heidolph company, Germany)
10. The concentrated 80% methanol crude extract was dried in hood at room temperature to a constant mass.
11. The dried crude extract was subjected to bioassay activity evaluation.