# Evaluation of *in vitro* and *in vivo* Antitrypanosomal activity of aqueous and methanol leaf extracts of *Clutia abyssinica* (*Euphorbiaceae)* and *Verbascum sinaiticum* (*Scrophulariaceae*) against *Trypanosoma congolense* field isolate

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This is to certify that the thesis prepared by Ermias Mergia, entitled: "Evaluation of *in vitro* and *in vivo* Antitrypanosomal activity of aqueous and methanol leaf extracts of *Clutia abyssinica* (Euphorbiaceae*)* and *Verbascum sinaiticum* (Scrophulariaceae) against *Trypanosoma congolense* field isolate" and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Pharmacology complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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## ABSTRACT

Evaluation of *in vitro* and *in vivo* Antitrypanosomal activity of aqueous and methanol leaf extracts of *Clutia abyssinica* (Euphorbiaceae*)* and *Verbascum sinaiticum* (Scrophulariaceae) against *Trypanosoma congolense* field isolate

Ermias Mergia

Addis Ababa University, 2014

Aqueous and methanol leaf extracts of *C. abyssinica* and *V. sinaiticum* were investigated for the presence of secondary metabolites, their *in vitro* and *in vivo* activity against *Trypanosoma congolence,* the main causative agent of African animal trypanosomosis in Sub-Saharan Africa and Ethiopia. The *in vitro* assay was carried out by monitoring test concentrations of 4, 2, 1, 0.4 and 0.2 mg/ml for cessation or reduction in motility of trypanosomes followed by monitoring for loss of infectivity to mice. The *in vivo* antitrypanosomal efficacy of the extracts was evaluated in Swiss albino mice infected with *T. congolense* field isolate. The leaf extracts were administered 12 days post-infection at peak parasitaemia level of ~$10^8$ trypanosomes/ml at doses of 100, 200 and 400 mg/kg by intraperitoneal injection once daily for 7 days. Parasitaemia, packed cell volume (PCV), mean survival time and change in body weight were used as indices for monitoring the efficacy of the extracts by comparing with the positive control: 28 mg/kg dose of diminazene aceturate and negative control: 2% tween 80 treated groups. Phytochemical screening revealed presence of alkaloids, anthraquinones, flavonoids, glycosides, phenolic compounds, saponins, steroids, terpenes and tannins. An appreciable *in vitro* activity was attained by the methanol extract of *C. abyssinica* at 4 mg/ml concentration which ceased motility of trypanosomes within 30 min and which caused loss of infectivity of trypanosomes to mice, which remained aparasitaemic for 21 days after the inoculation of the *in vitro* mixtures. The extracts had a lethal dose greater than 2000 mg/kg and there were no evidences of acute toxicity at the doses tested. Highly significant ($p<0.001$) reduction in pre-treatment parasitaemia by 3.91% (7.38$\pm$0.18) and increase in PCV by 1.12% (48.66$\pm$0.20) was noticed in animals treated by the methanol leaf extract of *C. abyssinica* at dose of 400 mg/kg; while body weight improvement by 1.67% (22.54$\pm$0.28) and mean survival time of 40.20 ± 0.37 days was seen in the group treated by 400 mg/kg methanol leaf extract of *V. sinaiticum.* In general, the results obtained suggest ethno-pharmacological usefulness of these plants and necessitate further studies to be carried on isolated active substances from these plants.



***Key words:*** Trypanosomosis, *Trypanosoma congolense, Clutia abyssinica, Verbascum sinaiticum, Parasitaemia, Packed cell volume, Body weight, Mean survival time*

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# ABBREVIATIONS

| | |
|---|---|
| AAT | African Animal trypanosomosis |
| ANOVA | Analysis of Variance |
| BARP | *Brucei* alanine rich protein |
| BSF | Bloodstream form |
| CDC | Center for Disease Control and prevention |
| CDER | Center for Drug Evaluation and Research |
| CESP | Congolense epimastigote specific protein |
| DA | Diminazene aceturate |
| EDTA | Ethylene diamine tetra acetic acid |
| EMF | Epimastigote form |
| FAO | Food and Agriculture Organization |
| FP | Flagellar pocket |
| GPI | Glycosyl-phosphatidyl-inositol |
| ILRI | International Livestock Research Institute |
| kDNA | kinetoplast DNA |
| MCF | Metacyclic form |
| OECD | Organization of Economic Cooperation and Development |
| PBS | Phosphate buffered saline |
| PF | Procyclic form |
| SEM | Standard error of mean |
| SPSS | Statistical Package for Social Science |
| VSG | Variant surface glycoprotein |
| WHO | World Health Organization |

# 1. INTRODUCTION

## 1.1 African animal trypanosomosis (AAT)

African animal trypanosomosis (AAT) is a serious parasitic disease caused by trypanosomes, found in the blood and other tissues of vertebrates including livestock, wildlife and people. Trypanosomes are unicellular protozoan parasites of the phylum Sarcomastigophora, order Kinetoplastida, family Trypanosomatidae, and genus Trypanosoma (Brian, 1999) (Figure 1).

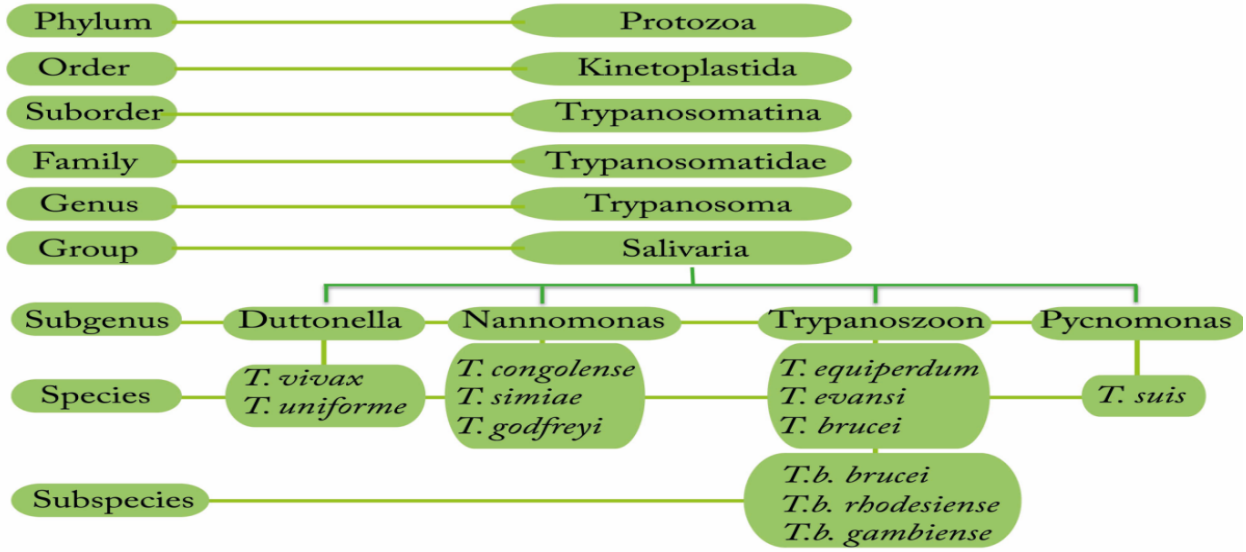



Figure 1: Classification of trypanosomes (Munender, 2013)

The *Trypanosoma brucei* species comprises three morphological identical subspecies: *T. b. brucei*, *T. b. rhodesiense*, and *T. b. gambiense*. *T. b. brucei* is pathogenic to cattle; the other subspecies cause sleeping sickness in East and West Africa (OIE, 2013). In addition the subdivisions of *T. congolense* are designated as *T. congolense* savannah type, *T. congolense tsavo* type, *T. congolense forest* type and *T. congolense kilifi* type. *T. congolense*, *T. vivax* and *T. b. brucei*, are the major pathogenic species of African cattle (Bengaly *et al.*, 2002). The other trypanosome species of economic importance are *T. evansi* of camels and *T. equiperdum* of horses (Getachew, 2005).

### 1.1.1. Transmission of AAT

#### A. Cyclical transmission

Tsetse flies (*Glossina* spp.) are found only in Africa and are responsible for the cyclical transmission of trypanosomes. They are the vectors of trypanosomes and constitute a potent and constant threat to humans and livestock over much of Sub-Saharan Africa (Abebe, 2005). The main species of tsetse flies are divided into three groups. The fusca group flies *G. austeni* (subgenus Austenina) tend to occur in the lowland rainforests of West and Central Africa. The palpalis group (subgenus Nemorhina) is found in the riverine galleries of West and Central Africa but can extend into savannah regions between river systems; *G. palpalis* and *G. tachinoides* are important AAT vectors in this group. The morsitans group (subgenus Glossina) occurs in a variety of savannah habitats lying between the forest edges and desert and includes several important vectors of AAT including *G. morsitans*, *G. pallidipes* and *G. longipennis* (Daya and Abebe, 2008). During the act of feeding the fly penetrates the skin with its proboscis (Figure 2). By the rupture of small blood vessels a pool of blood is formed in the tissues and the fly injects saliva to prevent coagulation. Infection of the host takes place at this stage, with infective metacyclic trypanosomes in the saliva (Aksoy *et al*., 2003; Leak, 1999).

#### B. Mechanical transmission

Mechanical transmission occurs by several biting flies such as horseflies (tabanids) and stable flies (*Stomoxys* species) which transmit the trypanosomes from an infected animal to another in the course of feeding. This mode of transmission has proved to be sufficiently effective to maintain *T. vivax* and *T. evansi* in South and Central America, and the latter species in North Africa and Asia as well (Desquesenes and Dia, 2004). Moreover iatrogenic transmission could also occur when using the same needle or surgical instrument on more than one animal, at

sufficiently short intervals that the blood on the needle or instrument does not dry (Desquesenes and Dia, 2003)

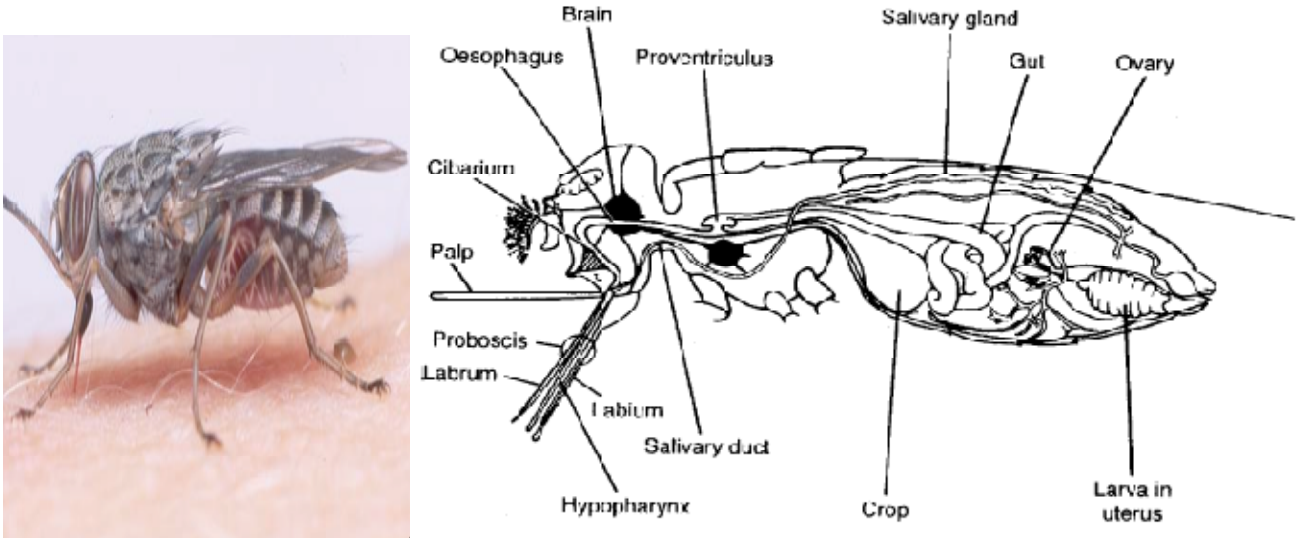



Figure 2: Diagrammatic representation of a female *Glossina* (Aksoy, 2003).

**C. Transmission by other means**

Besides the transmission of trypanosomes by tsetse and other biting flies; carnivores, vampire bats, marsupials, and leeches could transmit the disease. *Trypanosome* species (*T. cruzi*) are occasionally transmitted congenitally, from the mother to the offspring, either through the placenta while the fetus is still in the uterus, or when bleeding occurs during birth (Cardoso *et al*., 2012). Venereal transmission is the means by which dourine of equines, caused by *T. equiperdum* is transmitted directly during copulation from an infected to a healthy animal (AHT, 2011).

### 1.1.2 Epidemiology and distribution of AAT

Tsetse flies occur exclusively in Africa over an area approximately 10 million $km^2$, extending on both sides of the equator from $15^o$ N to $30^o$ S. They are of primary importance in the spread and epidemiology of this economically and socially important disease (OIE, 2013).

When dealing with the tsetse-transmitted trypanosomosis, much depends on the distribution of tsetse flies (*Glossina* species) responsible for transmission. Of the three groups of *Glossina*, the savannah and riverine are the most important since they inhabit areas suitable for grazing and watering (Emma, 2008).

Trypanosomes are able to infect a wide variety of domestic animals and more than 30 species in the wild. The pattern of the disease is mainly affected by differences in the distribution of the pathogenic trypanosomes. *T. congolense, T. vivax* and *T. brucei* are always found within tsetse infested areas. *T. congolense* is considered the most important cause of AAT in East Africa, and *T. vivax* in West Africa (Bourn *et al*., 2001).

The economic impacts of trypanosomosis in Africa are diverse and complex, with direct effects on animal production and human health, as well as indirect effects on settlement patterns, land use, animal husbandry and farming (Chanie *et al*., 2013). Trypanosomosis causes the death of over 3 million cattle annually with an estimated potential loss of 6-12 billion US dollars worldwide (Adeiza *et al*., 2010) and has a marked impact on the economy of African countries, where, approximately 20% of Africa's cattle are at risk of infection with estimated annual loss in cattle production of 1–1.2 billion US dollars (Chamond *et al*., 2010).

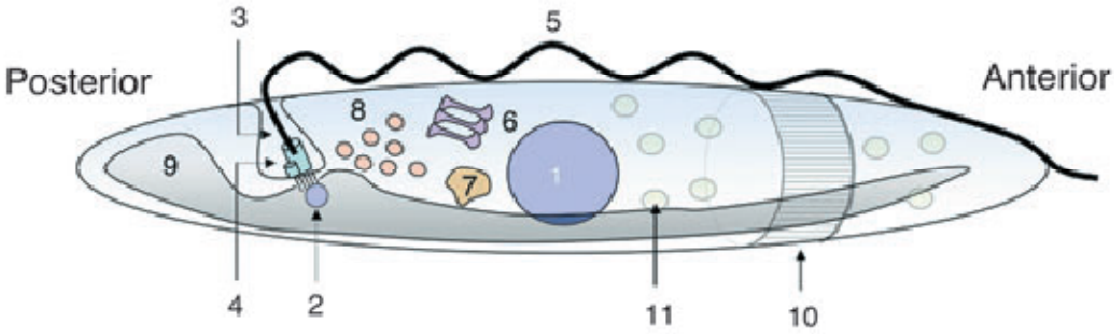
In Ethiopia, trypanososmosis is one of the most significant and costly disease hindering the effort made for food sufficiency (Abebe, 2005). In Ethiopia, about 220,000 $km^2$ in the South West and North West part of the country following the greater river basins of Abay, Omo, Ghibe and Baro, having a high potential for agricultural development are infested with tsetse flies (Nigatu and Abebe, 2009; Desta *et al*., 2013). About 10-15% of the land believed to be suitable for livestock production is affected by one or two species of the tsetse flies (NTTICC, 2004).

According to Getachew *et al*. (2004), trypanosomosis is prevalent in two main regions of Ethiopia that is, the North West and the South West regions. Similarly a number of studies have been so far undertaken in different parts of Ethiopia to determine the magnitude of this economically important disease (Adane and Gezahegne, 2007; Miruk *et al*., 2008; Abebayehu and Biniam, 2010; Abebayehu *et al*., 2011).

## 1.2. Biology of trypanosomes

### 1.2.1 Trypanosome cell architecture

The trypanosome consists of a single cell varying in size from 8 to 50 μm. The trypanosome cell is elongated and has a highly polarized microtubule cytoskeleton which defines the cell shape (Matthews, 2005). The organelles in the trypanosome cell (the flagellar pocket, flagellum, kinetoplast, mitochondrion and nucleus) are precisely positioned within the cytoskeletal corset and are concentrated between the posterior end and the centre of the cell (Gull, 2002) (Figure 3).




1. Nucleus
2. Kinetoplast
3. Flagellar pocket
4. Basal body and probasal body
5. Axoneme and paraflagellar rod
6. Golgi
7. Lysosome
8. Endosomes
9. Mitochondrion
10. Microtubule cytoskeleton
11. Glycosomes

Figure 3: Schematic representation of the principal structures of trypanosomes (Matthew, 2005)

The most posterior structure is the mouth of the flagellar pocket. This is the exit point for the flagellum, which is tethered along the exterior length of the parasite. The flagellar pocket is the site of endo- and exo-cytosis; this is important in bloodstream forms, in which the surface membrane is densely packed with variable surface glycoprotein (VSG) to protect against the alternative pathway of complement activation and to shield common antigenic determinants from immune recognition (Overath and Engstler, 2004).

The motility of the trypanosome is dependent upon its single flagellum, which has a conventional axonemal structure plus an associated paraflagellar rod. This is a semi-rigid structure found in the kinetoplastids and euglenoids that contributes to parasite motility, perhaps assisting trypanosome flagellar beat efficiency in the viscous mammalian blood stream (Vaughan and Gull, 2003).

The mitochondrion is a single elongated structure that runs from the posterior to the anterior of the cell. In bloodstream forms, the mitochondrion is a simple tubular structure devoid of cristae. This reflects the absence of mitochondrial respiration during this stage, energy generation being dependent on glycolytic reactions compartmentalized within specialized organelles termed glycosomes (Parsons, 2004). Trypanosomes are defined by the presence of a highly organized mitochondrial DNA structure, the kinetoplast (Gull, 2002).

There are distinct differences in appearance, shape and size between the various species of trypanosomes, allowing specific identification (Annex II).

**A. The subgenus *Nannomonas (*the congolense group*)***

*T. congolense* is the smallest of the pathogenic trypanosomes, with a length of 9–22 μm. The blood forms of *T. congolense* are monomorphic and lack a free flagellum. The nucleus is

centrally placed. The kinetoplast is of medium size and is usually situated at the margin of the body just in front of the posterior extremity (Delespaux *et al.*, 2008).

**B. The subgenus *Duttonella* (the vivax group)**

*T. vivax* is much larger than the other pathogenic species, and this is a distinguishing feature. It is monomorphic and has a free flagellum with large and terminal kinetoplast. The nucleus is centrally placed, but the bulk of the cytoplasm is found in the posterior part of the body as this is somewhat swollen. Its length, including the free flagellum, varies from 18 to 26 μm (Gull, 2002).

**C. The subgenus *Trypanozoon* (the brucei group)**

*T. brucei* is polymorphic, with three main forms, all of which have a small kinetoplast and a conspicuous undulating membrane: (i) *Long slender forms* (23–30 μm in length) with a free flagellum. The posterior end is pointed and the nucleus is central. The kinetoplast is placed up to 4 μm in front of the posterior extremity. (ii) *Short stumpy forms* (17–22 μm in length) normally without a free flagellum. The kinetoplast is usually sub-terminal. The nucleus is in the posterior part of the cell, sometimes so far posterior that the kinetoplast is anterior to it (so-called postero-nuclear forms). (iii) *Intermediate forms*, varying in length between the two previously mentioned types. A free flagellum, of varying length, is always present. The nucleus is centrally placed. The posterior end is somewhat variable in shape, but usually bluntly pointed. The kinetoplast is close to the posterior extremity (Mathew *et al*., 2005).

### 1.2.2. Pathogenesis and Lifecycle of AAT

The African trypanosomes have four major life cycle stages. The procyclic form (PF), epimastigote form (EMF) and metacyclic form (MCF) all develop in tsetse while the bloodstream form (BSF) is found in the mammalian host (Peacock *et al.*, 2012). Infection of the mammalian host begins when MCF trypanosomes in the saliva of an infected tsetse fly are

injected during a blood meal. The MCF trypanosomes proliferate and differentiate into BSF at the site of infection (Roditi and Liniger, 2002) (Figure 4).

The BSF parasites replicate in the interstitial fluids at the site of infection for 5-9 days before spreading to the circulatory system where they continue to replicate, forming a peak of parasitaemia (as high as $10^8$ parasites/ml of blood) (Ndung'u *et al.*, 2008). The BSF is covered with a dense surface coat of glycosyl-phosphatidylinositol (GPI) anchored variant surface glycoprotein (VSG). VSG molecules are involved in antigenic variation, allowing the trypanosome population to avoid elimination by the host immune system (Wang *et al.*, 2003).

The trypanosome life cycle continues when BSF trypanosomes are consumed by a tsetse fly during a blood meal from an infected animal. The BSF trypanosomes enter the tsetse mid-gut where most will perish (Peacock *et al.*, 2012). However, a small proportion will differentiate into the PF, a stage adapted to life in the mid-gut. Upon differentiation to PF, the VSG coat is replaced by a set of invariant insect form specific surface molecules (Vanhamme *et al.*, 2001; Roditi and Liniger, 2002).

After the PF have colonized the tsetse mid-gut, some parasites migrate to the salivary glands (*T. brucei*) or proboscis (*T. congolense*) where the parasites differentiate into adherent EMF (Oberle, *et al.*, 2010). The EMF of *T. brucei* and *T. congolense* are known to express on their surfaces, the *brucei* alanine rich protein (BARP; (Urwyler, *et al.*, 2007) and congolense epimastigote specific protein (CESP; (Sakurai, *et al.*, 2008) respectively. Both of these proteins are believed to be involved in the attachment of trypanosomes to the salivary gland epithelium (*T. brucei*) or chitinous labrum (*T. congolense*). The EMF population will grow for several days before cell division begins, giving rise to non-dividing, motile, VSG coated MCF parasites which are ready to infect a new mammalian host (Pays *et al.*, 2001).

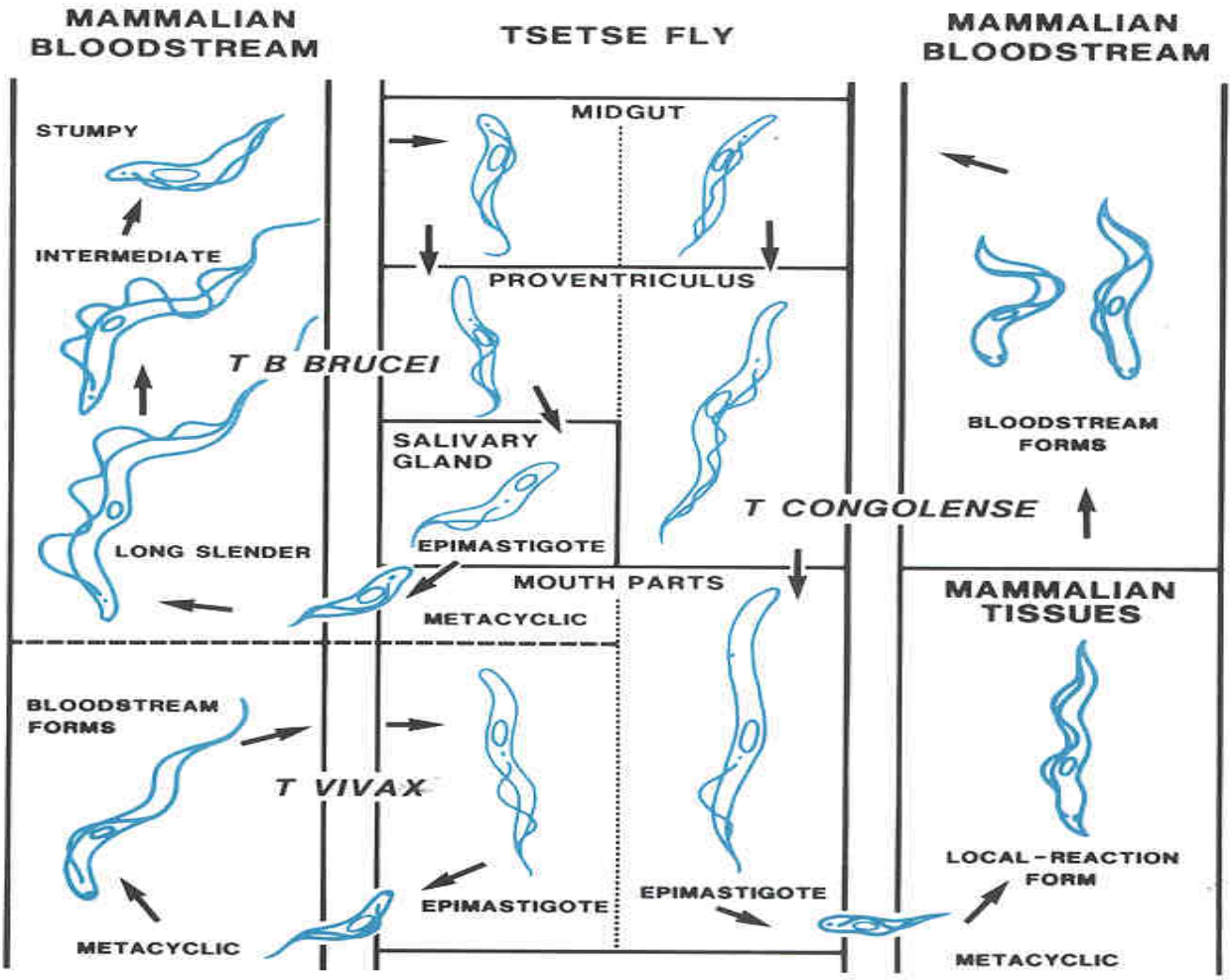



Figure 4: The life cycle of *T. b. brucei*, *T. congolense* and *T. vivax* (ILARD, 1994)

### 1.2.3 Clinical features of AAT

The trypanosomes affect firstly the bite site or in other words the inoculation site in the animal skin causing swelling and chancre. The chancre not only forms a site for the establishment of the infection but also is a focus for multiplication and persistence of trypanosomes before their dissemination into bloodstream. The parasites there after spread to the lymph nodes and blood then continue to replicate (Emma, 2008). *T. congolense* localizes in the endothelial cells of small blood vessels and capillaries. *T. b. brucei* and *T. vivax* localize in tissues like lymph nodes (Holmes *et al.*, 2000). The incubation period of the disease for *T. congolense* varies from 4 to 24

days and for *T. brucei* from 5 to 10 days. The ability of *T. congolense* to sequester in small vessels and capillaries of the brain, heart, skeletal and other tissues often leads to prolonged pre-patent period (Losos and Ikede, 1972; Maxie and Losos, 1977; Mbaya *et al*., 2007).

AAT is associated with fever, depression, weakness, anemia, salivation, lacrimation, nasal discharges, and subcutaneous edema of the mandible and prominent jugular pulse. The appetite is decreased and there is a rapid weight loss which progresses to an extreme emaciation. Often, death is related to severe anemia and circulatory collapse (Albadrani, 2012).

A significant decline in packed cell volume in response to trypanosomosis is a characteristic feature of the disease and a primary criterion for assessing its severity. Within a week of infection there is usually pronounced decrease in packed cell volume, hemoglobin, red blood cells, and white blood cells levels (Mersha *et al.*, 2012).

## 1.3 Diagnosis of AAT

The diagnosis of AAT depends on the identification of trypanosomes by examination of blood smears and occasionally, on lymph node biopsy (Mulligan, 1970). It can be accomplished through direct and/or indirect demonstration of the parasite. Direct identification of trypanosomes can generally be accomplished with a blood smear in the form of a wet film with or without concentration, e.g. by centrifugation in a haematocrit capillary (HCT), dark ground buffy coat technique (DG) or from stained blood smears as either thick or thin films (Woo, 1970; Herbert and Lumsden, 1976). Also serological tests like immune-fluorescence agglutination test (IFAT), enzyme-linked immune-sorbent assay (ELISA), card agglutination test (CAT) can be used to indicate infections with trypanosomes indirectly (Uilenberg, 1998). The use of molecular

biological tools, and in particular the Polymerase Chain Reaction (PCR), has made it possible to characterize trypanosomes both in the vectors and the hosts (Solano *et al*., 2000).

## 1.4. Prevention and Control of AAT

### 1.4.1. Vector Control

Vector control is the most reliable means of disease control since it removes the threat of trypanosomosis on a permanent basis. Many vector control methods including woody vegetation clearance to remove tsetse shelter, and large scale application of insecticides by air and ground spraying could be applied (Adamu *et al*., 2011). One of the latest methods of vector control is the Sterile Insect Technique (SIT). SIT is a genetic population suppression approach and involves sustained, systematic release of irradiated sterile male insects among the wild population. Males are sterilized by irradiation and then taken to the selected area and released by air. Releasing sterile males in high numbers over a period of 3–4 generations, after having reduced population density by other techniques (trapping, insecticide spraying, etc.), the target population can be eradicated (Rogers and Randolph, 2002; Feldman, 2004).

### 1.4.2. Use of Trypanotolerant cattle breeds

Innate resistance, or trypanotolerance, has been recognized since 1906 when the ability of indigenous taurine cattle in West Africa to survive and be productive under trypanosomosis risk was observed. Both acquired and innate resistance to African trypanosomosis can occur in cattle. It is well established that trypanotolerance has a genetic basis. The two most important trypanotolerant breeds are the *Bos taurus* subtypes, N'Dama and Baoule, whilst a degree of trypanotolerance has also been shown to occur in some *Bos indicus* zebu breeds. The use of trypanotolerant cattle had it not been limited in availability (account only for 17% of the total

cattle population of the continent), was a potential alternative strategy for coping with the problem (Vinhaes and Schofield, 2003).

## 1.5 Chemotherapy and chemoprophylaxis of AAT

The fight against the vector (tsetse fly) has not been very successful, and the chemicals used as part of the control measures pollute the environment. Immunization against trypanosomosis has not been possible because of the problem of antigenic variation. Therefore, chemotherapy continues to play a major role in the management and control of trypanosomosis. This is essential because without treatment, the outcome of African trypanosomosis is almost always fatal (Legros *et al.*, 2002).

Trypanocides are used for the control of the disease in the 37 African countries where animal trypanosomosis is endemic. Three compounds: isometamidium chloride, homidium salts (homidium bromide (Ethidium®) and homidium chloride (Novidium®) and diminazene aceturate (Bernil®, Veriben®) are used in treatment of AAT (Dolan *et al.*, 1990). Diminazene aceturate has curative properties whereas isometamidium chloride and the homidium salts have both curative and prophylactic activities. Diminazine aceturate is administered by the intravenous or intramuscular route at the doses of 3.5 -7 mg/kg body weight (Docampo and Moreno, 2002).

Chemo-prophylactic drugs such as quinapyramine derivatives, antrycide and antrycide prosalt are used to give effective protection against *T. b. brucei* infection in horses, camels, and cattle for up to 3 months (Deken *et al.*, 1989). The drug pyrithidium is useful in the prophylaxis of *T. vivax* and *T. congolense* infections in cattle, sheep, and goats and can give protection for up to 6 months. The most widely used chemo-prophylactic drug is isometamidium chloride. This drug,

is excellent for the prophylaxis of all three African animal trypanosomes, and gives protection for 3-6 months (Dolan *et al.,* 1990).

### 1.5.1 Resistance to Antitrypanosomal drugs

One of the major problems that severely limit trypanosomosis chemotherapy is the unwillingness of pharmaceutical companies to invest in development of drugs against trypanosomosis for lack of financial incentives because the disease affects largely the rural poor in Africa. Currently the treatment of animals with trypanocidal drugs still remains the most frequently applied measure to control trypanosomosis. Treatment is mainly carried out by the livestock owners themselves without any supervision by veterinary personnel. It has been observed that under-dosing occurs very frequently, which is an important risk factor for the development of drug resistance (Geerts *et al.,* 2010).

This problem of drug resistance in trypanosomes appears to be spreading geographically to many regions in which trypanosomosis occurs. So far, resistance to one or more of the three trypanocidal drugs used in cattle has been reported in at least 11 countries in sub-Saharan Africa (Burkina Faso, Chad, Côte d'Ivoire, Ethiopia, Kenya, Nigeria, Somalia, the Sudan, the United Republic of Tanzania, Uganda, and Zimbabwe) (Chitanga *et al.*, 2011).

In Ethiopia, presences of moderate to high prevalence of trypanosomea resistant to drugs were reported in different sites (Afework *et al.,* 2000; Tewolde *et al.*, 2004). Moti (2012) reported drug resistance in *T. congolense* isolates from Ghibe river basin in Ethiopia. Field isolates and laboratory stocks of diminazene resistant trypanosomes have been reported, requiring up to 45 mg/kg diminazene aceturate as the minimum required dose to achieve cure.

Chaka and Abebe (2003) reported the existence of *T. congolense* resistance from originally isolated species from cattle in the Southwest of Ethiopia, namely, Ghibe, Bedelle, Sodo and Arbaminch. Based on their report all the isolates were resistant to the usual dose of 3.5 mg/kg body weight diminazene aceturate while mice infected with Ghibe, Bedelle and Sodo isolates and treated with 7, 14 and 28 mg/kg showed no relapse throughout the experiment period. After treatment with diminazene at a dose ranging from 3.5 to 28 mg/kg body weight, trypanosomes reappeared in all mice infected with the Arbaminch isolate within 10 to 19 days (Chaka and Abebe, 2003).

More worrying, however, are the reported incidences of field stocks that have developed multiple resistances to trypanocidal drugs. Afewerk *et al*. (2000) also showed that clones of *T. congolense*, which were derived from primary isolates collected from relapsed cattle in the field after treatment with 1 mg/ kg body weight of isometamidium, were resistant to both diminazene and isometamidium when tested in mice; this indicated the appearance of a multiple drug resistant *T.congolense* population in northwestern Ethiopia.

In view of the increasing prevalence of drug resistance, it is important to know how effective tsetse control would be in alleviating trypanosomosis in livestock in situations where trypanocidal drugs alone have become ineffective. The development and spread of drug resistance to the point where drugs become ineffective over large areas of Africa is probably the greatest risk to the future use of the existing three trypanocides (Holmes *et al*., 2004).

### 1.5.2. Herbal antitrypanosomal agents

Medicinal plants provided a broad range of effective components against parasites. There are many reports about the screening of herbal extracts against *T. brucei*, *T. b. rohodensei*, *T. b. brucei, T. congolense and T .cruzi*. In the first stage, plant extracts are evaluated *in vitro* against

bloodstream forms then by *in vivo* experiments, using animal model systems (Kayser *et al*., 2003).

The activity of some medicinal plants against trypanosomosis is reviewed as follows.

Atawodi *et al*., (2003) found that extracts of *Khaya senegalensis, Piliostigma reticulatum* and *Securidaca longepedunculata* were strongly trypanocidal to *T. b. brucei* and *T. congolense* at concentrations of 4 mg/ml, 0.4 mg/ml and 0.04 mg/ml.

Bulus *et al.* (2008) have investigated *in vitro* antitrypanosomal activity of aqueous and methanol extracts of *Terminalia avicennioides.* Based on their finding, the aqueous extract of the plant immobilized trypanosomes within 20 and 50 min at 4 and 2 mg/ml respective test concentrations.

Adeyemi *et al.*, (2009) have showed that ethanol leaf extract of *Psidium guajava* has trypanocidal properties and has attributed these effects in parts to the broad antimicrobial and iron chelating activity of flavonoids and tannins.

Maikai, (2010) reported *in vitro* and *in vivo* antitrypanosomal activity of the stem bark of *Ximenia americana.* In the report aqueous and methanol stem bark extract of *X. americana* at 9 mg/ml effective concentration inhibited motility of *T. congolense* within 52 and 45 min respectively. In addition the methanol extract of the plant at 100 and 200 mg/kg dose prolonged survivability of *T. congolense* infected and treated mice for 30 days.

Atawodi and Shehu (2010) have reported antitrypanosomal activity of *Moringa oleifera*. In their report methanol extract of *M. oleifera* inhibited motility of trypanosomes within 30 min at 4 mg/ml effective concentration, while the 2 mg/ml test concentration drastically reduced motility of trypanosomes within 40 min of incubation.

Feyera *et al.* (2011) investigated the aerial parts of *Artemisia abyssinica* against *T. congolense* field isolate. The result evidenced that the dichloromethane extract had immobilized trypanosomes after 18 and 40 min at 4 and 2 mg/ml, respectively, while the hydromethanolic extract ceased the motility of the parasites after 35 min only at a concentration of 4 mg/ml. In the infectivity test, only 4 mg/ml of the dichloromethane extract caused loss of infectivity of the parasites to mice.

Ibrahim *et al*. (2012) evaluated antitrypanosomal activity of ethyl acetate extract of *Adansonia digitata* seed extract against T. *b. brucei* infected mice. They reported that mice treated with 300 and 400mg/kg dose of the extract have parasitaemia level of 11.42 and 3.59, respectively. In addition the extract prolonged the lifespan of the test animals beyond that of the untreated control by 18 days.

Based on the findings of Awulu *et al*. (2013), the methanol extract of *Peristrophe bicalyculata* at doses of 400 mg/kg body weight and 500 mg/kg body weight were found to have some curative properties and at doses of 300 mg/kg and 400 mg/kg it was found to have some suppressive properties in mice infected with *T. evansi*.

Wurochekke *et al*. (2014) investigated the trypanocidal potential of *Carrisa edulis* in male wistar rats infected with *T. congolense.* The animals treated with 100 and 200mg/kg doses of methanol extract of *C. edulis* have shown mean parasitaemia of 8.76 ± 0.01 and 8.66 ± 0.02, respectively.

The anititrypanosomal activity of the above medicinal plants is believed to be due to the various phytochemicals present in the plants. It is known from the literature that secondary plant metabolites exhibit antitrypanosomal activity (Hoet *et al*., 2004; Saeidnia *et al*., 2005; Tasdemir *et al*., 2006; Maya *et al*., 2007).

## 1.6. Review of plant species included in this study

### 1.6.1 *Clutia abyssinica (*Fam. Euphorbiaceae*)*

Euphorbiaceae is a large and fascinating family of about 300 genera and 8,000-10,000 species, mostly found in the tropics of both hemispheres. *Clutia is a* genus having about 60 species. *Clutia abyssinica* called by the Amharic name 'fyele fej' is herb 1-2 m high (Figure 5) (Edwards *et al*., 1995).

Traditionally it is used in treatment of venereal and skin diseases, chest problems, cancer (Pascaline, *et al*., 2011); Skin fungal infections (Runyoro, *et al*., 2006 and Matu, 2008); Yellow fever and malaria (Fowler, 2006); management of ear, nose and throat diseases (Njoroge *et al*., 2006); diarrhoea (Yineger *et al*., 2007); gonorrhea, cough and fever, headache, toothache, menstrual pain, burns, pneumonia, enlarged spleen and kidney, shock, abdominal problems- as a laxative and to expel intestinal worms, elephantiasis, diarrhoea and tachycardia (Matu, 2008). Moreover, maceration of the crushed leaves of *C. abyssinica* given orally has traditionally been used for the treatment of animal trypanosomosis (Fulas, 2010).

Figure 5: *Clutia abyssinica*

Ethanolic leaf, stem and root extracts of *C. abyssinica* have shown moderate antiviral activity *in vitro* against polio virus, coxsackie virus and HIV (Colegate and Molyneux, 1993; Cos *et al*., 2002), moderate antifungal activity of the extract has also been observed against *Aspergillus fumigatus* and *Fusarium culmorum* (Matu, 2008).

Phytochemical constituents in the family Euphorbiaceae include terpenoids, alkaloids, anthraquinones, polyacetates, proanthocyanidins, flavonols, ellagic acids and saponins (Mohd, 2006). Pascaline *et al*., (2011) reported the presence of alkaloids, anthraquinones, flavonoids, glycosides, phenolic compounds, terpenes and saponins in methanol extract of crude leaf extract of *C. abyssinica.* Waigh *et al*. (1990) and Jaber *et al*. (1996) have isolated the diterpenes 2"-O-glycosylisovitexin, and ent-16β,17-dihydroxykaurane from leaves of *C. abyssinica.*

### 1.5.2 V*erbascum sinaiticum* (Fam. Scrophulariaceae)

The family Scrophulariaceae is a cosmopolitic family with 300 genera and about 5400-5500 species, mainly in the tropical mountains. *Verbascum* is a genus having about 360 species. *Verbascum sinaiticum* known by the Amharic name ‘qetetina’ is a biennial plant, 60-150 cm tall (Hedberg *et al*., 2006) (Figure 6).

Figure 6: *Verbascum sinaiticum*

Traditional uses of *V. sinaiticum* include: for wound treaatment, stomachache (Teklehaymanot *et al.*, 2007); viral infection, cancer (Teklehaymanot *et al.*, 2009); sun stroke fever, abdominal colic (Weldegerima *et al.*, 2008); diarrhea, hemorrhage, anthrax (Weldegerima *et al.*, 2008); hepatitis (Yinger *et al.*, 2007). Moreover, powder of the leaves of *V. sinaiticum* mixed with water is given orally (Teklehaymanot *et al.*, 2009 and Fulas, 2010) or the filtrate is instilled into left ear and nose (Weldegerima *et al.*, 2008) for treatment of animal trypanosomosis.

Investigation of the leaves of *V. sinaiticum* has afforded two flavonolignans, hydrocarpin and the novel sinaiticin, as well as two flavones, chrysoeriol and luteolin (Afifi, *et al*., 1993).

*In vitro* studies have shown that methanol extract of *V. sinaiticum* leaves had broad spectrum antimicrobial activity against Gram (+) and Gram (-) bacteria, with strong activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Tadeg *et al*., 2005).

# 2. OBJECTIVES OF THE STUDY

## 2.1. General objective

- To conduct phytochemical screening and evaluation of the *in vitro and in vivo* antitrypanosomal activity of crude leaf extracts of *Clutia abyssinica* and *Verbascum sinaiticum*, against *Trypanosoma congolense* field isolate.

## 2.2. Specific objectives

- To undertake phytochemical screening of the leaf extracts;
- To determine the acute toxicity profile of crude leaf extracts of the two plants;
- To evaluate *in vitro* antitrypanosomal activity of crude leaf extracts of the two plants;
- To evaluate the antitrypanosomal activity of crude leaf extracts of the two plants in altering the level of parasitaemia from the circulation of mice infected with *T. congolense;*
- To evaluate the effect of the crude leaf extracts of the two plants on packed cell volume of mice infected with *T. congolense*;
- To evaluate profiles of crude leaf extracts of the two plants in improving body weight of mice infected with *T. congolense;* and
- To evaluate the effect of the crude leaf extracts of the two plants in prolonging mean survival period of mice infected with *T. congolense*

# 3. MATERIAL AND METHODS

## 3.1. Chemicals

### 3.1.1 Chemicals and Solvents

Reagents and solvents used in this work were: ammonium hydroxide (Techno. Pharm. Chem., India), benzene, dragondroff reagent, ferric sulfate, sodium hydroxide, potassium hydroxide, Wagner's reagent (ACS, Merck.), ferric chloride (FISHER Scientific Company, New Jersey), hydrochloric acid, Giemsa stain, microscopic oil, PBS tablet, tween-80 (BDH Ltd, England), 40% glucose (Pharmacure, Ethiopia), methanol, methylated spirit, lead acetate, sulfuric acid (Carlo ebra reagents, Italy) and sterile water (obtained from EPHARM).

### 3.1.2 Materials

Aluminium foils, microscopic slide, Cover slide (Vardh Man, India), mortar and pestle, digital weighing balance (Mettle-Toledo Ltd, Switzerland), diamond pencil (Krackeler Scientific Agilent), EDTA coated syringe (Bio-medical Aids Egypt), Whatman No.3 filter paper (Whatman Ltd, England), glove (Sara Health Care, India), desiccator, microhaematocrit centrifuge (Hettich haematokrit, Germany), haematocrit reader, heparinized capillary tube, oven, refrigerator, (Bebbysterilin Ltd, UK), petridish, incubator (Thermo Electro.), microscope (Olympus, Taiwan), micropipettes (Pipetman Ultra), lyophilizer, Rota Vapor (BÜCHI Rota-vapor, Switzerland), sterile lancet, syringe 1ml (Shandock Ziboshanchuan, India), thermometer, 96 wells microtiter plates (Flow laboratories, USA), amber glass vials, cristaseal, spatula, and orbital mixer.

### 3.1.3 Reference Drug

Diminazine aceturate (*Veriben*® containing 1.05 gm diminazene aceturate + 2.36 gm antipyrine, (Ceva Santé Animale, France; batch number- 719A1) a commercial trypanocidal drug was used.

### 3.1.4. Test organism

Isolation of *T. congolense* from infected cattle was done in May, 2013 from two districts (Homo loko peasant association and Sebategna kebele) of Ilu-Aba-Bora-Zone, Bedele town, Dabo Hana woreda. The area is located 145 km. from Jimma (Northwest) and 480 km. from Addis Ababa in South west direction.

The selection of the area was based on the fact that the National Tsetse and Trypanosomosis Investigation and Control Centre (NTTICC), which is the center of the National Animal Health Diagnostic center (NAHDIC) is found in the Bedele town and due to previous reports of high prevalence of animal trypanosomosis in the area (Tasew and Duguma, 2012; Desta *et al*., 2013; Moti *et al*., 2013).

Among cattle found in grazing area in the two sites a total of 100 cattle with poor physical condition, history of weakness and poor appetite were randomly screened for *T. congolense* infection (Figure 7).

Figure 7: Cattles screened for isolation of *T.congolense* infected cattle

Blood samples were collected by an expert from NTTICC from the peripheral ear vein of each animal using heparinized microhaematocrit capillary tubes that filled 3/4 of the height and sealed with cristaseal (Figure 8). The presence of *T. congolense* was detected by Microhaematocrit Buffy Coat Technique (MHBCT) (Woo, 1970), where the sealed microhaematocrit capillary

tubes were centrifuged immediately in microhaematocrit centrifuge for 5 min at 12000 rpm. After centrifugation, the capillary tube was cut by diamond pencil 1 mm below the buffy coat to include the top layer of red cells. The content of the capillary tube was expressed onto a clean microscope slide, mixed and covered with a 22 × 22 mm cover slip.


Figure 8: Blood samples collection from the peripheral ear vein of animal

Then the slide was examined for *T. congolense* based on their type of motility in the microscopic field 400X objective. Confirmations of *T. congolense* species by morphological characteristics (Annex II) was done after a thin blood smear was prepared from the buffy-coat examination and stained with Giemsa stain and examined under a microscope using oil immersion 100X objective (Murray *et al*., 1977; Uilenberg, 1998) (Figure 9)

Among the cattle screened 5 cattle from Sebategna kebele showed to be infected with *T. congolense*. Then an animal with peak parasiaemia of (~$10^8$ trypanosomes/ml) (Herbert and Lumsden, 1976) was selected and blood was collected to the ethylene diamine tetra acetic acid (EDTA) coated tube from the jugular vein of the animal and diluted with PBS.

Figure 9: Identification of *Trypanosoma congolense* in blood collected from cattle by microscope

Then 0.2 ml of the blood containing ~$10^4$ trypanosomes/ml was injected intraperitoneally to 6 laboratory mice and transported to the laboratory at Akililu Lemma Institute of Pathobiology (ALIPB), Addis Ababa University for serial passage to other mice for the experimental part of the study.

The development of trypanosomes in the infected mice was determined by wet film examination of blood obtained from the tail of the mice by buffy coat method (Woo, 1970). Following syringe challenge all the donor mice had shown parasitaemia 7 days post-infection. After establishment of infection heavily (~$10^8$ trypanosomes/ml) (21 days post- inoculation), the donor mice were then subjected to cardiac puncture and blood was collected with an EDTA coated tube and immediately diluted with phosphate buffered saline. Then about 0.2 ml of blood collected from the donor mice containing (~$10^4$ trypanosomes/ml) (Herbert and Lumsden, 1976) was injected intraperitoneally in to mice that were acclimatized to laboratory conditions and were used for *in vitro* and *in vivo* evaluation of the crude leaf extracts of *C. abyssinica* and *V. sinaiticum.*

### 3.1.5 Experimental animals

Healthy Swiss albino mice (weighing 20–30 gm and age of 8–12 weeks) were obtained from the animal house of the Ethiopian Health and Nutrition Research Institute (EHNRI) and School of Pharmacy, Addis Ababa University. Animals were housed in polypropylene cages (6–10 animals per cage), maintained under 12 hr light and 12 hr dark cycle and allowed free access to pellet diet and clean water *ad libitum*. All procedures complied with the guide for the care and use of laboratory animals (ILAR, 2000; OECD, 2001).

## 3.2 Experimental part

### 3.2.1 Collection of Plant specimens

The leaves of *V. sinaiticum* were collected in the month of March 2013, from Entoto-mountain about 10 km north of the center of Addis Ababa, while the leaves of *C. abyssinica* were collected in the month of April 2013 from Debre Libanos 'gedam', a Monastery in Amhara regional state, Ethiopia. The fresh leaves were wrapped by plastic sheets during transportation. Taxonomic identification was done and a voucher specimen was deposited (Collection EM/001 and EM/002) at the National Herbarium, College of Natural sciences, Addis Ababa University.

The leaves were thoroughly washed with distilled water to remove dirt, soil and any other foreign materials and left to drain off. The they were then spread on laboratory bench and dried under shade. The dried leaves were pulverized using mortar and pestle at medicinal plants laboratory of ALIPB.

### 3.2.2 Preparation and storage of extracts

#### 3.2.2.1 Preparation of aqueous extract

200g of dried leaf powder of each of *C. abyssinica* and *V. sinaiticum* were macerated with 1000 ml of distilled water for 48 hours with frequent agitation in orbital shaker and the resulting liquid was filtered using Whatman No. 3 filter paper (Whatman Ltd., England). Extraction was repeated three times and the filtrates of all portions were pooled in one vessel. The extract was then placed in a Petridish and lyophilized for one week to yield a solid residue. The resulting dried mass was then powdered, weighed and packed into a glass vial and stored in a desiccator over silica gel until use.

#### 3.2.2.2 Preparation of methanol extract

Similarly 200g of dried leaf powder of each of *C. abyssinica* and *V. sinaiticum* were macerated with 1000 ml of absolute methanol. Maceration was continued for 48 h with frequent agitation in orbital shaker and the resulting liquid was filtered using Whatman No. 3 filter paper (Whatman Ltd., England). Extraction was repeated three times and the filtrates of all portions were pooled in one vessel. The extract was concentrated using Rota vapor (BÜCHI Rota-vapor, Switzerland) at not more than $40^{o}$C in order to obtain dry extracts. The resulting dried mass was then powdered, weighed and packed into a glass vial and stored in a desiccator over silica gel until use.

The percentage yield was calculated as:

$$\text{Percentage yield} = \frac{\text{Amount of extract obtained}}{\text{Amount of initial sample}} X\ 100$$

### 3.2.3 Phytochemical screening

Aqueous and methanol extracts of the two plants were screened for the presence of active principles such as alkaloids, anthraquinones, flavonoids, glycosides, phenolic compounds, saponins, steroids, tannins and terpenes.

#### 3.2.3.1 Test for alkaloids

This was carried out as described by Rafauf (1970) and Sofowora (1982)

a. Dragendorff's test: 1 ml of hydrochloric acid (HCl) and 3 drops of Dragendorff's reagent were added to the extract solution. The formation of orange precipitate indicated the presence of alkaloids.
b. Wagner's test*:* 1 ml of HCl and 3 drops of Wagner's reagent were added to the extract solution. The formation of a brown precipitate indicated the presence of alkaloids.

#### 3.2.3.2 Test for anthraquinones

This was carried out as described by Tyler *et al.* (1988):

a. Free anthraquinones: 5 gm of each plant extract was shaken with 10 ml of benzene and filtered. A 10% ammonium hydroxide solution (5 ml) was added to the filtrate, and the mixture was shaken. The presence of a pink, red or violet color in the ammonia phase was taken as an indication of the presence of anthraquinones.
b. Combined anthraquinones: 5 gm of plant extract was boiled with 10 ml of 1% HCl and filtered while hot. The filtrate was shaken with 5 ml of benzene. The benzene layer was removed and 10% ammonium hydroxide (equal to half the volume of benzene) was added to it. A pink, red or violet color in the ammonia phase indicated the presence of anthraquinone derivatives

#### 3.2.3.3 Test for flavonoids

This was carried out as described by Dermarderosian and Liberti (1988):

a. Ferric chloride test: Few drops of ferric chloride were added to the extract solution. Formation of blackish red color indicated the presence of flavonoids.
b. Alkaline reagent test: 3 ml of 10% sodium hydroxide (NaOH) was added to the extract test solution followed by 3 ml of 10% HCl. The formation of a yellow color on addition of NaOH, which disappeared on addition of the HCl, indicated the presence of flavonoids.
c. Lead acetate solution Test: Formation of yellow precipitate after addition of few drops of lead acetate (10%) solution to the extract solution indicated the presence of flavonoids.

#### 3.2.3.4 Test for glycosides

This was carried out as described by Evans (1996):

a. Keller Killiani test: the extract test solution was treated with few drops of glacial acetic acid and ferric chloride solution and mixed. Concentrated sulphuric acid was added, and observed for the formation of two layers. Formation of lower reddish brown layer and upper acetic acid layer which turns bluish green was taken as an indication for presence of glycosides.
b. Bromine water test: the extract test solution was dissolved in bromine water and observed for the formation of yellow precipitate to show a positive result for the presence of glycosides.

#### 3.2.3.5 Test for saponins

Foam test: To 1 ml of each extract, 3 ml of water was added and shaken and observed for the formation of froth, which is stable for 15 min for a positive result (Evans, 1996).

#### 3.2.3.6 Test for steroids and terpenes

Liebermann Burchard test: extract solution was mixed with few drops of acetic anhydride, boiled and cooled. Concentrated sulphuric acid was then added from the sides of the test tube and observed for the formation of a brown ring at the junction of two layers. Green coloration of the upper layer indicated the presence of steroids while the formation of deep red color in the lower layer indicated a positive test for terpenes (Briggs, 1970).

#### 3.2.3.7 Test for tannins

Gelatin test: to 1 ml of the extract solution, 5 ml of 1% gelatin containing sodium chloride (NaCl) were added. Formation of a yellow precipitate denoted the presence of tannins (Evans, 1996).

#### 3.2.3.8 Test for phenolic compounds

To test for presence phenolic compounds, few drops of ferric sulfate were added to each extract solution. Formation of dark-violet color indicated the presence of phenolic compounds (Sofowara, 1982).

### 3.2.4. Acute toxicity study

The acute toxicity study was conducted in accordance with the Lorke's (1983) method. The study was conducted for each extract in two phases using female swiss albino mice after 7 days of adaptation. In the first phase, nine mice were divided into 3 groups of 3 mice each. Each group was given 10, 100, and 1000 mg/kg body weight of the test substance respectively. In the second phase, further specific doses (1600, 2900, and 5000 mg/kg) of each extract were administered to nine mice (three mice per dose) to further determine the correct lethal dose ($LD_{50}$) value. In addition, a fourth group of six mice was set up as control group and received the reconstituting solvent 2% tween 80 in sterile water.

The extract was dissolved in 2% tween 80 in sterile water and given through intraperitoneal route. All animals were kept under strict observation for behavioral, neurological, autonomic or physical changes such as alertness, motor activity, restlessness, convulsions, coma, diarrhea and lacrimation for 24 h, with special attention during the first 4 h. These observations continued for further 14 days for any signs of overt toxicity. Then the lowest dose which killed one mouse (minimum toxic dose) and the highest dose which had not killed any mouse (maximum tolerated dose) were noted, and the geometric mean of these two doses gave $LD_{50}$.

The $LD_{50}$ was computed using the formula:

$$LD50 = \sqrt{\text{minimum toxic dose X maximum tolerated dose}}$$

### 3.2.5 *In vitro* antitrypanosomal activity and blood incubation infectivity test

#### 3.2.5.1 *In vitro* antitrypanosomal activity test

The *in vitro* test was performed in triplicates in 96 well micro-titter plates (Flow laboratories Inc.). Infected blood obtained by cardiac puncture of mice at peak parasitaemia (~$10^8$ trypanosomes/ml) (Herbert and Lumsden, 1976) was put into EDTA tube. Stock solutions of the aqueous and methanol leaf extracts of *C. abyssinica* and *V. sinaiticum* were first prepared in 2% tween 80 in phosphate buffered saline glucose (PBSG) as used by Endeshaw *et al*. (1997), Martin *et al*. (2006), Ene *et al*. (2009), Johnson *et al*. (2011) and Feyera *et al*. (2011). Aliquot of 50 µl of crude extracts solution of 20.0 mg/ml, 10.0 mg/ml, 5 mg/ml, 2.0 mg/ml, and 1 mg/ml were separately mixed with 200µl of blood containing about 20-25 trypanosomes/field (~$10^8$ trypanosomes/ml) in micro-titter plates to produce effective test concentrations of 4 mg/ml, 2 mg/ml, 1 mg/ml, 0.4 mg/ml, and 0.2 mg/ml, respectively.

To ensure that the effect monitored was that of the extract alone, negative and positive controls were included which contained the parasite (200 µl of infected blood) suspended in 50µl of 2% tween 80 in PBSG and similar effective test concentrations of diminazene aceurate, respectively (Atawodi *et al*., 2003; Maikai *et al*., 2007; Atawodi and Ogunbusola, 2009; Ene *et al*., 2009).

After 5 min incubation in covered micro-titter plates maintained at 37$^{o}$C, a drop of the test mixture was placed on separate microscope slide covered with cover slip and the motility of the trypanosomes was observed under the microscope (400X) at 10 min interval for 2 h by two independent laboratory technicians. The procedure was carried out separately for the aqueous and methanol extracts of each plant in triplicates.

Cessation or drop in motility of the trypanosomes in extract-treated blood compared to that of parasite-loaded control blood without extract was taken as a measure of antitrypannosomal activity. Time (minute) after which motility ceased or reduced drastically was recorded for comparison. The movement of the parasite are grouped as; actively motile (motile parasite in $\leq 5$ microscopic fields), drastically reduced motility (motile parasite in the range of 10-20 microscopic fields), ceased (no motile parasite in 10-20 microscopic fields). The shorter the time of cessation of motility of the parasite, the more active the extract was considered to be. Under this *in vitro* system, parasites survived for about 4 h when no extract was present (Wurochekke and Nok, 2004; Feyera *et al*., 2011).

**3.2.5.2 Blood incubation infectivity test**

For the validation of the *in vitro* antitrypanosomal activity, concentrations of extracts that ceased or drastically reduced motility of trypanosomes in the *in vitro* study were assessed for blood incubation infectivity test. Parasite suspension was incubated in the presence of the aqueous/methanol leaf extract of *C. abyssinica* or *V. sinaiticum*, as described in the *in vitro* study

then contents of the *in vitro* mixtures in the micro-titter plates were injected intraperitoneally into five healthy mice and the level of parasiteamia was assessed every other day by collecting blood from tail of each mouse and checked for the presence of trypanosomes using the wet blood film by Microhaematocrit Buffy Coat Technique (MHBCT) (Woo, 1970). The loss of infectivity of the trypanosomes to mice was concluded if no trypanosome was detectable within 21 days as described in the works of Maikai, (2011), Atawodi *et al*. (2003), Wurochekke and Nok (2004), and Abu *et al* (2009). In addition effect of the extracts in prolongation of establishment of infection was monitored by comparing with the negative control.

### 3.2.6 *In vivo* antitrypanosomal activity test

#### 3.2.6.1 Parasite inoculation and extract administration

70 healthy Swiss albino mice that are infected intraperitoneally with 0.2 ml of *T. congolense* infected blood (~$10^4$ trypanosomes/ml) collected by cardiac puncture from donor mice as mentioned in section 3.1.4 were divided into fourteen groups *C. abyssinica* aqueous extract (CAAE 100, CAAE 200, CAAE 400), *C. abyssinica* methanol extract (CAME 100, CAME 200, CAME 400), *V. sinaiticum* aqueous extract (VSAE100, VSAE 200, VSAE 400), *V. sinaiticum* methanol extract (VSME 100, VSME 200, VSME400), Diminazine aceturate (DA28), and 2% tween 80 (TW80) each comprising of 5 mice.

Treatment with the extracts began on the $12^{th}$ day post-infection (day 0 of treatment), when the infected mice show peak parasitaemia of (~$10^8$ trypanosomes/ml). On each day of drug administration, the aqueous and methanol extracts of *C. abyssinica* and *V. sinaiticum* were freshly prepared by solublising in 2% Tween-80 in sterile water for injection and administered intraperitoneally (Figure 10) daily at 9 a.m for seven days at the doses of (100, 200 and 400

mg/kg). The doses were selected based on the acute toxicity study. The middle dose was one tenth of the lethal dose (~2000 mg/kg) which was 200 mg/kg. Higher dose was calculated as twice the middle dose, which was 400 mg/kg. The lower dose level was calculated by taking half of the middle dose, which was 100 mg/kg. Volume administered was determined based on the organization of Economic Co-operation and Development (OECD) guideline that states 2 ml/100 gm of body weight of the animal (OECD, 2001).

For the positive control, diminazine aceturate (Veriben®) (DA28), dissolved in sterile water as recommended by the manufacturer (Ceva Santé Animale, France) was administered at the dose of 28 mg/kg intraperitoneally based on previous reports of Moti *et al.* (2012), Feyera *et al*. (2011), and Chaka and Abebe (2003), while for the negative control, 2% tween 80 in sterile water (TW80), was administered intraperitoneally.

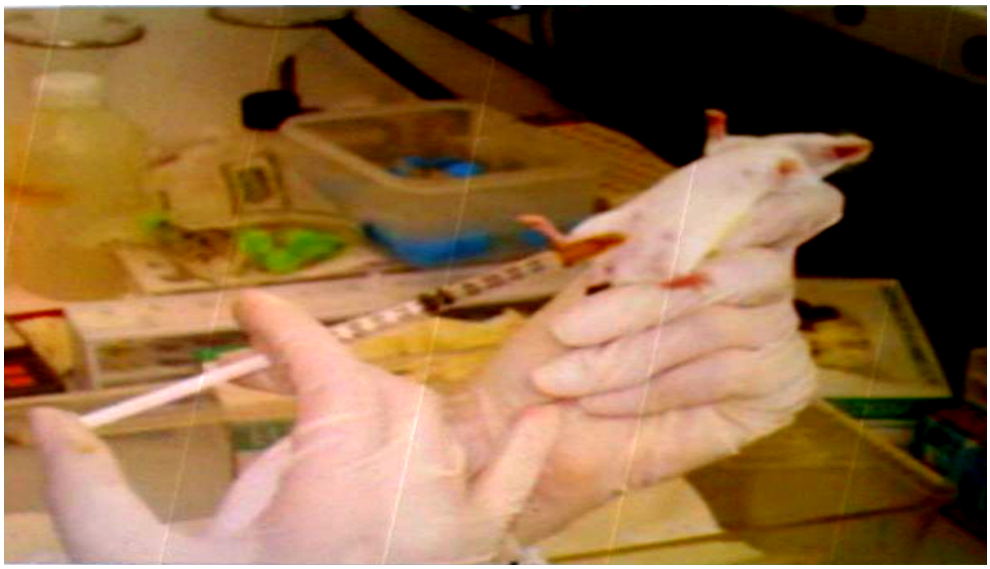


Figure 10: Photo during intraperitoneal administration of extract to mice

### 3.2.6.2 Determination of parasitaemia

Parasitaemia was monitored every other day by microscopic examination of blood obtained from the tail of each mouse that was pre-sterilized with methylated spirit. The tail tip was cut to extrude blood and drop of blood was placed on microscope slide and covering with a cover-slide (22 × 22 mm) (OIE, 2013). The blood was examined microscopically at 400X total magnification. The degree of parasitaemia was determined using the "Rapid Matching" method of Herbert and Lumsden (1976). Wet smear were prepared in triplicates from each animal and the mean value of slide counts were taken per sample examined microscopically. Briefly, when higher levels of infection, parasite levels was measured by matching microscopic fields of a wet blood film against charts and when fewer parasites were present, by counting the number of trypanosomes in 5, 10 or 20 such microscope fields. Logarithm values of these counts were obtained by matching with the table given by Herbert and Lumsden (1976) (Annex III).

The treatment continued daily for seven days with continuous monitoring of parasitaemia every other day (on Day 0, Day 2, Day 4, Day 6, Day 8, Day 10, Day 12 and Day 14) until the 14$^{th}$ day. For the assessment of antitrypanosomal effect of the extracts, the level of parasitaemia in the treated animals was compared to that of the control animals

$$\%\ \text{Change in parasitemia DAY } 14-0 \;=\; \frac{\text{Mean parasitemia on DAY } 14 - \text{Mean Parasitemia on DAY } 0}{\text{Mean Parasitemia on DAY } 0} \times 100$$

### 3.2.6.3 Determination of packed cell volume (PCV)

PCV was measured using Wintrobe's method (Wintrobe and Landsberg, 1935) to predict the effectiveness of the test extracts in preventing hemolysis resulting from increasing parasitaemia associated with trypanosomosis. It was monitored before infection and three times till the 14$^{th}$ day (on Day 0, Day 7 and Day 14). Briefly, blood was collected from tail of each mouse in

heparinized microhaematocrit capillary tubes filled up to $3/4^{th}$ of their length. The tubes were then sealed immediately by cristalseal and centrifuged in a microhaematocrit centrifuge (Hettich Haematokrit, Germany) for 5 min at 12,000 rpm. After centrifugation, the height of the red blood cell column were measured by use of haematocrit reader and compared to the total height of the column of the whole blood (Wernery *et al.*, 2001). The effect of extracts in improving PCV of treated animals was compared with the controls.

$$\% \text{ PCV change day 0 to 7} = \frac{\text{Mean PCV on DAY 7} - \text{Mean PCV on DAY 0}}{\text{Mean PCV on DAY 0}} \text{ X 100}$$

$$\% \text{ PCV change on day 7 to 14} = \frac{\text{Mean PCV on DAY 14} - \text{Mean PCV on DAY 7}}{\text{Mean PCV on DAY 7}} \text{ X 100}$$

### 3.2.6.4 Determination of body weight

The body weight (in gram) of each mouse in all groups was measured before infection, on the day treatment commenced (day 0) and every other day (on Day 2, Day 4, Day 6, Day 8, Day 10, Day 12 and Day 14) up to day 14.

$$\% \text{ Change in body weight Day 14} - 0 = \frac{\text{Mean body weight on Day 14} - \text{Mean body weight Day 0}}{\text{Mean body weight on Day 0}} \text{ X 100}$$

### 3.2.6.5 Determination of mean survival time

Mortality was monitored daily and the number of days from the time of inoculation of the parasite up to death was recorded for each mouse in the treatment and control groups throughout the follow up period for six weeks. The mean survival time (MST) for each group was calculated as;

$$\text{Mean survival time} = \frac{\text{Sum of survival time of all mice in a group (days)}}{\text{Total number of mice in that group}}$$

## 3.3 Statistical analysis

Values of the data obtained from the study were summarized and expressed as mean ± standard error of mean (SEM). Data analysis was performed using Statistical Package for Social Science (SPSS), version 17.0. To compare the results obtained from different groups, one way ANOVA followed by Tukey's multiple comparison tests were performed to determine statistical significance. P values less than 0.05 were considered significant.

# 4. RESULTS

## 4.1 Yield for plant extraction

As shown in Table 1, maximum and minimum yields were obtained from the leaves of *V. sinaiticum* and *C. abyssinica*, respectively. Among the solvents used for extraction methanol gave a maximum percentage yield from the two plants as compared to the aqueous extract.

Table 1: Percentage yields of aqueous and methanol leaf extracts of *Clutia abyssinica* and *Verbascum sinaiticum*

| Plant species | Part extracted | Solvent | Percentage yield (%w/w) |
|---|---|---|---|
| *C. abyssinica* | Leaf | Distilled water | 12.92% |
| | | Methanol | 17.21% |
| *V. sinaiticum* | Leaf | Distilled water | 13.09% |
| | | Methanol | 18.13% |

## 4.2. Phytochemical screening

Phytochemical screening of the aqueous and methanol leaf extracts of *C. abyssinica* and *V. sinaiticum* had revealed the presence of different secondary metabolites (Table 2).

Table 2: Phytochemical screening results for the aqueous and methanol leaf extracts of *Clutia abyssinica* and *Verbascum sinaiticum*

| Constituents | *C. abyssinica* | | *V. sinaiticum* | |
|---|---|---|---|---|
| | Aqueous extract | Methanol extract | Aqueous extract | Methanol extract |
| Alkaloids | - | + | + | + |
| Anthraquinones | + | + | - | - |
| Flavonoids | - | + | - | + |
| Glycoside | - | + | - | + |
| Saponins | + | - | + | - |
| Steroids | - | + | - | + |
| Phenolic compounds | + | + | - | + |
| Tannins | - | + | - | + |
| Terpenes | - | + | - | - |

+ = present, - = absent

## 4.3. Acute toxicity test

The acute toxicity bioassay had shown that the lethal dosage ($LD_{50}$) of the aqueous and methanol leaf extracts of *C. abyssinica and V. sinaiticum* was above 2000 mg/kg and there were no evidences of acute toxicity at the doses tested indicating good safety margin (Annex I).

## 4.4. *In vitro* antitrypanosomal activity and blood incubation infectivity test

### 4.4.1. *In vitro* antitrypanosomal activity

As shown in Table 3, the methanol leaf extract of C. *abyssinica* had ceased motility of the parasites within 30 and 40 min at 4 and 2 mg/ml test concentrations respectively. Similarly the methanol extracts of *V. sinaiticum* had shown *in vitro* antitrypanosomal activity by ceasing motility of the trypanosomes within 50 and 80 min at 4 and 2 mg/ml concentration respectively. The aqueous extract of *V. sinaiticum* had shown similar effect within 60 min only at the 4 mg/ml concentration.

Drastic reduction in motility of trypanosomes was observed after 50 and 70 min at 4mg/ml aqueous extract of *C. abyssinica* and 2 mg/ml aqueous extract of *V. sinaiticum*, respectively.

However the positive control diminazine aceturate immobilized motility of trypanosomes within 20, 30 and 60 min at 4, 2, and 1 mg/ml test concentrations, respectively, whereas the negative control 2% tween 80 and lower test concentrations (1, 0.4 and 0.2 mg/ml) of both extracts neither immobilized nor reduced motility of trypanosomes.

Table 3: *In vitro* antitrypanosomal effect of the aqueous and methanol leaf extracts of *Clutia abyssinica* and *Verbascum sinaiticum* on motility of *Trypanosoma congolense.*

| Treatment | Extract | Time (min) of cessation or drastic reduction in motility Test concentrations | | | | |
|---|---|---|---|---|---|---|
| | | 4 mg/ml | 2 mg/ml | 1 mg/ml | 0.4 mg/ml | 0.2 mg/ml |
| *C. abyssinica* | Aqueous | 50** | NE | NE | NE | NE |
| | Methanol | 30* | 40* | NE | NE | NE |
| *V. sinaiticum* | Aqueous | 60* | 70** | NE | NE | NE |
| | Methanol | 50* | 80* | NE | NE | NE |
| Positive control | Diminazine aceturate | 20* | 30* | 60* | NE | NE |
| Negative control | 2 % Tween 80 in PBSG | NE | | | | |

*= ceased motility;**drastically reduced motility, NE= no noticeable effect on motility

### 4.4.2. Blood incubation infectivity test

The mice which received the test concentrations containing 4 mg/ml of methanol extract of *C. abyssinica* and diminazine aceturate were found to be aparasitaemic after 21 days monitoring period. In addition, the mice which received the test concentrations containing 2 mg/ml methanol extract of *C. abyssinica,* 4 and 2 mg/ml methanol extract of *V. sinaiticum* and 1 and 2 mg/ml diminazine aceturate lost infectivity to some of the animals and had prolonged establishment of infection as compared to the negative control (Table 4).

Table 4: The effect of aqueous and methanol leaf extract of *Clutia abyssinica* and *Verbascum sinaiticum* on blood incubation infectivity test.

| Plant | Extract | Test Concentration | Number of mice which developed infection | Infection interval in days (Mean±SEM) |
|---|---|---|---|---|
| *C. abyssinica* | Methanol | 4 mg/ml | 0/5 | Ni |
| | | 2 mg/ml | 3/5 | 16.66 ± 0.66 |
| | Aqueous | 4 mg/ml | 5/5 | 14.80 ± 0.48 |
| *V. sinaiticum* | Methanol | 4 mg/ml | 2/5 | 16.00 ± 0.00 |
| | | 2 mg/ml | 4/5 | 14.50 ± 0.50 |
| | Aqueous | 4 mg/ml | 5/5 | 13.20 ± 0.48 |
| | | 2 mg/ml | 5/5 | 12.80 ± 0.48 |
| Positive control | DA | 4 mg/ml | 0/5 | Ni |
| | | 2 mg/ml | 3/5 | 18.66 ± 0.66 |
| | | 1 mgml | 4/5 | 16.50 ± 0.50 |
| Negative control | 2% Tween 80 in PBSG | | 5/5 | 11.80 ± 0.37 |

Values are Mean ± SEM; N= 5; Ni=No infection, DA= Diminazine aceturate

## 4.5. *In vivo antitrypanosomal* activity

### 4.5.1. Effect of crude extracts on parasitaemia

#### 4.5.1.1. Effect of crude leaf extracts of *Clutia abyssinica* on parasitaemia of *Trypanosoma congolense* infected mice

Mice treated with aqueous extract of *C. abyssinica* at 400 mg/kg dose had low parasitaemia on day 6 ($p<0.01$), day 8, 10 and 14 ($p<0.001$) of treatment as compared to the negative control group on day 14 of treatment. The finding also revealed that mice treated with 100 and 200 mg/kg doses of the aqueous extract of *C. abyssinica* had low level of parasitaemia on days 8 and 10 of treatment when compared to the negative control group ($p<0.001$) (Table 5).

As shown in Table 6, animals treated with the methanol extract of *C. abyssinica* at 200 and 400 mg/kg dose had statistically significant low parasitaemia on day 6, 8, 10 and 14 ($p<0.001$) of treatment as compared to the negative control group.

In addition, the methanol extract of *C. abyssinica* at dose of 400 mg/kg had continually reduced parasitaemia level from day 2 of treatment to day 10 which was further kept on average at lowest level up to the end of the monitoring period.

Animals treated with the methanol extract of *C. abyssinica* at 400 mg/kg dose had significantly ($p<0.001$) lower mean parasitaemia ($7.38 \pm 0.18$) as compared to the negative control and other treatment groups on day 14 of treatment. The lowest mean parasitaemia value of $5.94 \pm 0.24$ was also observed in this group on day 8 of treatment which was highly significant ($p<0.001$) when compared to the 100 and 200 mg/kg dose.

Comparison of the percentage change in parasitaemia on day 14 of treatment had shown that only the 400 mg/kg dose of the methanol extract reduced pre-treatment parasitaemia level by 3.91% as compared to the 18.55% increment in the negative control group (Table 6).

Table 5: The effect of aqueous leaf extract of *Clutia abyssinica* on parasitaemia level of *Trypanosoma congolense* infected mice

| Days | Parasitaemia (log number/ml) | | | | |
|---|---|---|---|---|---|
| | DA28 | TW80 | CAAE100 | CAAE200 | CAAE400 |
| Day0 | 7.68 ±0.18 | 7.44 ± 0.17 | 7.38 ±0.07 | 7.68 ±0.18 | 7.33 ±0.14 |
| Day2 | 0.00 ±0.00[*3] | 7.74 ±0.17 | 7.62 ±0.07 | 7.92 ±0.15 | 7.56 ±0.11 |
| Day4 | 0.00 ±0.00[*3] | 7.86 ± 0.11 | 7.86 ±0.11 | 7.98 ±0.07 | 7.68 ±0.18 |
| Day6 | 0.00 ±0.00[*3] | 8.16 ± 0.11 | 7.68 ±0.12 | 7.80 ±0.13 | 7.44 ±0.19[b2] |
| Day8 | 0.00 ±0.00[*3] | 8.28 ± 0.07[*3] | 7.44 ±0.11[b3] | 7.62 ±0.07[b3] | 6.96±0.17[b3,c1,d2] |
| Day10 | 0.00 ±0.00[*3] | 8.52 ± 0.12[*3] | 7.80 ±0.13[b3] | 7.86 ±0.06 [b3] | 7.26±0.14 [b3,c1,d2] |
| Day12 | 2.16 ±1.32[*3] | 8.64 ± 0.06 | 8.22 ±0.07 | 8.04 ±0.06 | 7.74 ±0.11 |
| Day14 | 5.52 ±0.07[*3] | 8.82 ± 0.12 | 8.52 ±0.07 | 8.28 ±0.07 | 8.04 ±0.06 [b3,c2] |
| % Change in Parasitaemia (Day 0-14) | -27.3 | 18.55 | 15.45 | 7.81 | 9.68 |

Values are expressed in Mean±S.E.M (n=5) performed with ANOVA followed by Tukey's Post hoc multiple comparison test; DA28= diminazine aceturate 28 mg/kg-the positive control, [b]compared to TW80= 2% tween 80-the negative control; [c]compared to CAAE100= *C. abyssinica* aqueous extract 100 mg/kg, [d]compared to CAAE200= *C. abyssinica* aqueous extract 200 mg/kg, [e]compared to CAAE400= *C. abyssinica* aqueous extract 400 mg/kg, [*]compared with all groups; [1]$p < 0.05$, [2]$p < 0.01$ and [3]$p < 0.001$

Table 6: The effect of methanol leaf extract of *Clutia abyssinica* on parasitaemia of *Trypanosoma congolense* infected mice

| Days | Parasitaemia (log number/ml) | | | | |
|---|---|---|---|---|---|
| | DA28 | TW80 | CAME100 | CAME200 | CAME400 |
| Day0 | 7.68 ±0.18 | 7.44 ± 0.17 | 7.56 ± 0.15 | 7.32 ± 0.12 | 7.68 ± 0.18 |
| Day2 | 0.00 ±0.00$^{*3}$ | 7.74 ±0.17 | 7.68 ± 0.18 | 7.50 ± 0.13 | 7.62 ± 0.20 |
| Day4 | 0.00 ±0.00$^{*3}$ | 7.86 ± 0.11 | 7.92 ± 0.24 | 7.38 ± 0.07 | 7.32 ± 0.22 |
| Day6 | 0.00 ±0.00$^{*3}$ | 8.16 ± 0.11 | 7.68 ± 0.18 | 7.26 ± 0.22$^{b3}$ | 6.54±0.11$^{bc3, d1}$ |
| Day8 | 0.00 ±0.00$^{*3}$ | 8.28 ± 0.07 | 7.92 ± 0.18 | 6.78 ± 0.07 $^{bc3}$ | 5.94 ± 0.24$^{*3}$ |
| Day10 | 0.00 ±0.00$^{*3}$ | 8.52 ± 0.12 | 8.16 ± 0.17 | 7.32 ± 0.15 $^{b3,c2}$ | 6.24 ± 0.22 $^{*3}$ |
| Day12 | 2.16 ±1.32$^{*3}$ | 8.64 ± 0.06 | 8.28 ± 0.15 | 7.74 ± 0.17 | 7.26 ±0.11 |
| Day14 | 5.52 ±0.07$^{*3}$ | 8.82 ± 0.12 | 8.52 ± 0.15 | 7.92 ± 0.15 $^{b3,c1}$ | 7.38 ± 0.18 $^{bc3}$ |
| % Change in Parasitaemia (Day 0-14) | -27.3 | 18.55 | 12.69 | 8.19 | -3.91 |

Values are expressed in Mean±S.E.M (n=5) performed with ANOVA followed by Tukey's Post hoc multiple comparison test; DA28= diminazine aceturate 28 mg/kg-the positive control, $^{b}$compared to TW80= 2% tween 80-the negative control; $^{c}$compared to CAME100= *C. abyssinica* methanol extract 100 mg/kg, $^{d}$compared to CAME200= *C. abyssinica* methanol extract 200 mg/kg, $^{e}$compared to CAME400= *C. abyssinica* methanol extract 400 mg/kg, $^{*}$compared with all groups; $^{1}p < 0.05$, $^{2}p < 0.01$ and $^{3}p < 0.001$

#### 4.5.1.2 Effect of crude leaf extracts of *Verbascum sinaiticum* on parasitaemia of *Trypanosoma congolence* infected mice

The aqueous extract of *V. sinaiticum* had significantly ($p<0.001$) reduced parasitaemia of treated animals on days 6, 8 and 10 as compared to the negative control group; while early reduction in parasitaemia ($p<0.01$) was noted by the 400 mg/kg dose of the aqueous extract on day 4 of treatment.Similarly these group of animals had significantly ($p<0.001$) low level of parasitaemia on days 8 and 10 when compared with other doses of the aqueous extract treated mice (Table 7).

As shown in Table 8, the methanol leaf extract of *V. sinaiticum* had kept parasitaemia at a significantly low level on day 4 ($p<0.01$) on day 6, 8, 10 and on day 14 ($p<0.001$) as compared with the negative control. Animals treated with the methanol extract of *V. sinaiticum* at 400 mg/kg dose had significantly ($p<0.001$) lower mean parasitaemia ($7.20 \pm 0.16$) as compared to the negative control group ($8.82 \pm 0.12$) on day 14 of treatment. Comparison of the percentage change in parasitaemia on day 14 of treatment from pretreatment value showed that only the 400 mg/kg dose of the methanol extract significantly ($P<0.05$) reduced parasitemia by 1.64% (Table 8). Comparative analysis had shown that animals treated with 400 mg/kg dose of the methanol extract of *C. abyssinica* had significant ($P<0.05$) lower level of parasitaemia as compared to those treated by the same dose of methanol extract of *V. sinaiticum* on days 6, 8 and 10 with a higher reduction of the pre-treatment parasitaemia by 3.91% on day 14 of treatment.

The mean parasitaemia level on day 14 shows that the 400 mg/kg methanol extract of *V. sinaiticum* exerted lower parasitaemia level (7.20 +0.16) as compared with the same dose of methanol extract of *C. abyssinica* ($7.38 \pm 0.18$). But change in percentage parasitaemia from day 14-0 indicated that the methanol extract of *V. sinaiticum* had lower reduction of parasitaemia by 1.94 % as compared to the 3.91% reduction in parasitaemia by 400 mg/kg dose of methanol extract *C. abyssinica*.

Table 7: The effect of aqueous leaf extract of *Verbascum sinaiticum* on parasitaemia level of *Trypanosoma congolense* infected mice

| DAYS | Parasitaemia level (log number/ml) | | | | |
|---|---|---|---|---|---|
| | DA28 | TW80 | VSAE100 | VSAE200 | VSAE400 |
| Day0 | 7.68 +0.18 | 7.44 + 0.17 | 7.32 +0.07 | 7.08 +0.07 | 7.31 +0.11 |
| Day2 | 0.00+0.00[*3] | 7.74 +0.17 | 7.56 +0.11 | 7.44 +0.11 | 7.56 +0.11 |
| Day4 | 0.00+0.00[*3] | 7.86 + 0.11 | 7.50 +0.09 | 7.80 +0.13 | 7.32+0.07[b2,d1] |
| Day6 | 0.00+0.00[*3] | 8.16 + 0.11 [*3] | 7.32 +0.00[b3] | 7.44 +0.17 [b3] | 7.14 +0.11[b3] |
| Day8 | 0.00+0.00[*3] | 8.28 + 0.07[*3] | 7.44 +0.06[b3] | 7.38 +0.07 [b3] | 6.78 +0.12[*3] |
| Day10 | 0.00+0.00[*3] | 8.52 + 0.12 [*3] | 7.74 +0.11[b3] | 7.62 +0.07 [b3] | 7.08 +0.12[*3] |
| Day12 | 2.16 +1.32[*3] | 8.64 + 0.06 | 8.04 +0.11 | 7.92 +0.12 | 7.56 +0.11 |
| Day14 | 5.52 +0.07[*3] | 8.82 + 0.12 | 8.40 +0.09 | 8.16 +0.11 [b3] | 8.04 +0.11 [b3] |
| % Change in Parasitaemia (Day 0-14) | -27.3 | 18.55 | 14.75 | 15.25 | 9.98 |

Values are expressed in Mean±S.E.M (n=5) performed with ANOVA followed by Tukey's Post hoc multiple comparison test; DA28= diminazine aceturate 28 mg/kg-the positive control, VSAE100= *V. sinaiticum* aqueous extract 100 mg/kg, VSAE400= *V. sinaiticum* aqueous extract 400 mg/kg; [b]compared to TW80= 2% tween 80-the negative control, [d]compared to VSAE200= *V. sinaiticum* aqueous extract 200 mg/kg, [*]compared with all groups; [1]$p < 0.05$, [2]$p < 0.01$ and [3]$p < 0.001$

Table 8: The effect of methanol leaf extract of *Verbascum sinaiticum* on parasitaemia level of *Trypanosoma congolense* infected mice

| DAYS | Parasitaemia level (log number/ml) | | | | |
|---|---|---|---|---|---|
| | DA28 | TW80 | VSME100 | VSME200 | VSME400 |
| Day0 | 7.68 +0.18 | 7.44 + 0.17 | 7.44 +0.11 | 7.08 +0.15 | 7.32 +0.12 |
| Day2 | 0.00 +0.00$^{*3}$ | 7.74 +0.17 | 7.68 +0.12 | 7.56 +0.11 | 7.62 +0.12 |
| Day4 | 0.00 +0.00$^{*3}$ | 7.86 + 0.11 | 7.50 +0.16 | 7.32 +0.07$^{b1}$ | 7.26 +0.11$^{b2}$ |
| Day6 | 0.00 +0.00$^{*3}$ | 8.16 + 0.11 $^{*3}$ | 7.44+0.11$^{b3}$ | 7.02 +0.07 $^{b3,c1}$ | 6.96 +0.11 $^{b3,c1}$ |
| Day8 | 0.00 +0.00$^{*3}$ | 8.28 + 0.07$^{*3}$ | 7.68 +0.12 $^{b3}$ | 6.66 +0.06 $^{bc3}$ | 6.54 +0.11 $^{bc3}$ |
| Day10 | 0.00 +0.00$^{*3}$ | 8.52 + 0.12 $^{*3}$ | 7.86 +0.11 $^{b3}$ | 7.14 +0.11 $^{bc3}$ | 6.36 +0.17 $^{bcd3}$ |
| Day12 | 2.16 +1.32$^{*3}$ | 8.64 + 0.06 | 7.98 +0.07 | 7.44 +0.11 | 6.72 +0.20 |
| Day14 | 5.52 +0.07$^{*3}$ | 8.82 + 0.12 | 8.28 +0.07 $^{b1}$ | 7.74 +0.11 $^{b3,c1}$ | 7.20 +0.16 $^{bc3, d1}$ |
| % Change in Parasitaemia (Day 0-14) | -27.3 | 18.55 | 11.29 | 9.32 | -1.64 |

Values are expressed in Mean±S.E.M (n=5) performed with ANOVA followed by Tukey's Post hoc multiple comparison test; DA28= diminazine aceturate 28 mg/kg-the positive control, VSME400= *V. sinaiticum* methanol extract 400 mg/kg; $^{b}$compared to TW80= 2% tween 80-the negative control; $^{c}$compared to VSME100= *V. sinaiticum* methanol extract 100 mg/kg, $^{d}$compared to VSME200= *V. sinaiticum* methanol extract 200 mg/kg, $^{*}$compared with all groups; $^{1}p < 0.05$, $^{2}p < 0.01$ and $^{3}p < 0.001$

Figure 11 shows that animals treated with 400mg/kg doses of methanol extract of *C. abyssinica* had lower parasitaemia on day 8 of treatment. While animals treated with 400 mg/kg dose of methanol extract of *V. sinaiticum* had lower parasitaemia on day 12 and 14 of treatment.

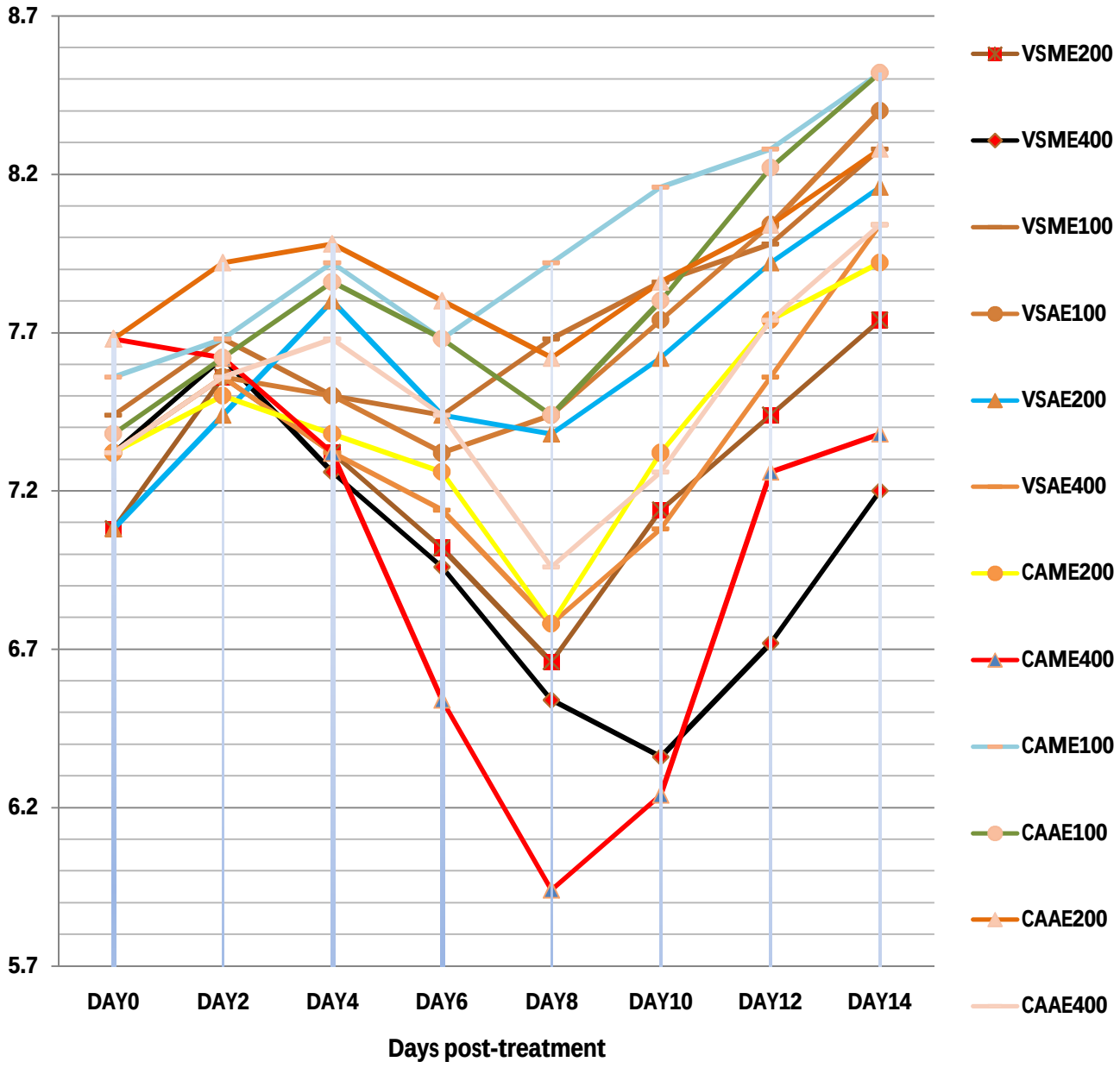



Figure 11: Comparison of effect of the aqueous and methanol leaf extracts of *Clutia abyssinica* and *Verbascum sinaiticum* on parasitaemia of *Trypanosoma congolense* infected mice

### 4.5.2. Effect of crude extracts on packed cell volume

#### 4.5.2.1 Effect of *Clutia abyssinica* leaf extract on packed cell volume of *Trypanosoma congolence* infected mice

The PCV value (45.04 ± 0.31) of animals treated with 400 mg/kg of the aqueous extract of *C. abyssinica* was higher ($p<0.001$) as compared to the negative control groups (40.58 ± 0.28) on day 14 of treatment (Table 9).

Animals treated with 200 and 400 mg/kg dose of the methanol extract of *C. abyssinica* had a statistically significant ($p<0.001$) higher PCV value (46.82 ± 0.34 and 48.66 ± 0.20 respectively) as compared to the negative control groups (40.58 ± 0.27) on day 14 of treatment (Table 10). This finding is consistent with the result seen on parasitaemia (Figure 13).

Analysis of change in percentage of PCV from day 7 to day 14 of treatment also showed that the methanol extracts at 200 and 400 mg/kg doses had significantly increased PCV value of treated animals by 1.29 and 1.12%, respectively as compared to the negative control groups which had a drop in PCV by 9.38 % from day 7 to 14 of treatment (Table 10).

#### 4.5.2.2 Effect of *Verbascum sinaiticum* leaf extracts on packed cell volume of *Trypanosoma congolence* infected mice

As shown on Table 11 animals treated with higher dose (400 mg/kg) of the aqueous extract of *V. sinaiticum* had a statistically significant ($p<0.001$) higher PCV value (47.14 ± 0.25) as compared to the negative control group (40.58 ± 0.28) on day 14 of treatment. Analysis of change in percentage PCV from day 7 to day 14 of treatment also showed that the aqueous extract at 200 and 400 mg/kg dose had prevented a drop in PCV associated by trypanosomes as compared to the negative control group (Table 11).

In consistence with the results seen in parasitaemia (Figure 14), animals treated with the methanol extract of *V. sinaiticum* had higher PCV value ($p<0.001$) as compared to the negative control groups at the end of the observation period (Table 12).

Table 9: The effect of the aqueous leaf extract of *Clutia abyssinica* on packed cell volume of *Trypanosoma congolense* infected mice

| Days | PCV Values | | | | |
|---|---|---|---|---|---|
| | DA28 | TW80 | CAAE100 | CAAE200 | CAAE400 |
| Pre-infection | 51.18 ± 0.37 | 50.88 ± 0.14 | 51.40 ±0.22 | 51.02 ±0.22 | 50.66 ±0.23 |
| Day 0 | 49.40 ±0.23 | 49.04±0.29 | 49.24±0.28 | 49.10 ±0.24 | 49.16 ±0.24 |
| Day 7 | 48.88±0.25$^{*3}$ | 44.78±0.37 | 44.04±0.12 | 45.34±0.42$^{c1}$ | 45.48±0.28$^{c1}$ |
| %change PCV day 7-0 | -1.05 | -8.69 | -10.56 | -7.65 | -7.48 |
| Day 14 | 50.08±0.15$^{*3}$ | 40.58±0.27$^{*3}$ | 43.36±0.24$^{b3}$ | 44.70±0.28$^{b3,c1}$ | 45.04±0.31$^{b3,c2}$ |
| %change PCVday 7-14 | 2.45 | -9.38 | -1.54 | -1.41 | -0.96 |

Values are expressed in Mean±S.E.M (n=5) performed with ANOVA followed by Tukey's Post hoc multiple comparison test; DA28= diminazine aceturate 28 mg/kg-the positive control, CAAE200= *C. abyssinica* aqueous extract 200 mg/kg, CAAE400= *C. abyssinica* aqueous extract 400 mg/kg; $^{b}$compared to TW80= 2% tween 80-the negative control, $^{c}$compared to CAAE100= *C. abyssinica* aqueous extract 100 mg/kg, $^{*}$compared with all groups; $^{1}p < 0.05$, $^{2}p < 0.01$ and $^{3}p < 0.001$

Table 10: The effect of the *methanol* leaf extract of *Clutia abyssinica* on packed cell volume of *Trypanosoma congolense* infected mice

| Days | PCV Values | | | | |
|---|---|---|---|---|---|
| | DA28 | TW80 | CAME100 | CAME200 | CAME400 |
| Pre-infection | 51.18 ± 0.37 | 50.88 ± 0.14 | 51.10 ±0.26 | 51.24 ±0.20 | 51.56 ±0.08 |
| Day 0 | 49.40 ±0.23 | 49.04±0.29 | 49.16±0.24 | 49.08 ±0.29 | 49.04 ±0.23 |
| Day 7 | 48.88±0.25$^{*3}$ | 44.78±0.37 | 46.06±0.22$^{b1}$ | 46.22 ±0.32$^{b2}$ | 48.12±0.15$^{bcd3}$ |
| %change PCV day 7-0 | -1.05 | -8.69 | -6.30 | -5.82 | -1.87 |
| Day 14 | 50.08±0.15$^{*3}$ | 40.58±0.27$^{*3}$ | 45.66±0.22$^{b3}$ | 46.82 ±0.34$^{b3}$ | 48.66±0.20$^{b3,c1,d2}$ |
| %change PCV day7-14 | 2.45 | -9.38 | -0.86 | 1.29 | 1.12 |

Values are expressed in Mean±S.E.M (n=5) performed with ANOVA followed by Tukey's Post hoc multiple comparison test; DA28= diminazine aceturate 28 mg/kg-the positive control, CAME400= *C. abyssinica* methanol extract 400 mg/kg; $^{b}$compared to TW80= 2% tween 80-the negative control, $^{c}$compared to CAME100= *C. abyssinica* methanol extract 100 mg/kg, $^{d}$compared to CAME200= *C. abyssinica* methanol extract 200 mg/kg; $^{*}$compared with all groups; $^{1}p < 0.05$, $^{2}p < 0.01$ and $^{3}p < 0.001$

Table 11: Effect of the aqueous leaf extracts of *Verbascum sinaiticum* on packed cell volume of *Trypanosoma congolense* infected mice

| Days | PCV Values | | | | |
|---|---|---|---|---|---|
| | DA28 | TW80 | VSAE100 | VSAE200 | VSAE400 |
| Pre-infection | 51.18 ± 0.37 | 50.88 ± 0.14 | 51.38 ±0.18 | 51.18±0.24 | 51.44±0.12 |
| Day 0 | 49.40 ±0.23 | 49.04±0.29 | 49.10 ±0.19 | 49.18±0.18 | 49.24±0.16 |
| Day 7 | 48.88±0.25$^{*3}$ | 44.78±0.37 | 44.78±0.36 | 46.08±0.32$^{b1,c1}$ | 48.24±0.18$^{bcd3}$ |
| %change PCV day 7-0 | -1.05 | -8.69 | -8.79 | -6.30 | -2.03 |
| Day 14 | 50.08±0.15$^{*3}$ | 40.58±0.27$^{*3}$ | 43.72±0.39$^{b3}$ | 45.32±0.37$^{b3,c1}$ | 47.14±0.25$^{*3}$ |
| % change PCV day 7-14 | 2.45 | -9.38 | -2.36 | -1.65 | -2.28 |

Values are expressed in Mean±S.E.M (n=5) performed with ANOVA followed by Tukey's Post hoc multiple comparison test; DA28= diminazine aceturate 28 mg/kg-the positive control, VSAE400= *V. sinaiticum* aqueous extract 400 mg/kg; $^{b}$compared to TW80= 2% tween 80-the negative control,; $^{c}$compared to VSAE100= *V. sinaiticum* aqueous extract 100 mg/kg, $^{d}$compared to VSAE200= *V. sinaiticum* aqueous extract 200 mg/kg, $^{*}$compared with all groups; $^{1}p < 0.05$ and $^{3}p < 0.001$

Table 12: The effect of the methanol leaf extract of *Verbascum sinaiticum* on packed cell volume of *Trypanosoma congolense* infected mice

| Days | PCV Values | | | | |
|---|---|---|---|---|---|
| | DA28 | TW80 | VSME100 | VSME200 | VSME400 |
| Pre-infection | 51.18 ± 0.37 | 50.88 ± 0.14 | 51.22 ±0.19 | 51.30 ±0.27 | 51.48 ±0.23 |
| Day 0 | 49.40 ±0.23 | 49.04±0.29 | 49.20 ±0.22 | 49.24 ±0.17 | 48.88 ±0.20 |
| Day 7 | 48.88±0.25$^{bcd3,e1}$ | 44.78±0.37 | 44.76±0.27 | 47.40±0.41$^{bc3}$ | 47.56±0.16$^{bc3}$ |
| % change PCV day 7-0 | -1.05 | -8.69 | -9.02 | -3.73 | -2.70 |
| Day 14 | 50.08±0.15$^{*3}$ | 40.58±0.27$^{*3}$ | 44.30±0.32$^{b3}$ | 46.88 ±0.35$^{bc3}$ | 46.80±0.18$^{bc3}$ |
| % change PCV day 7-14 | 2.45 | -9.38 | -1.02 | -1.09 | -1.59 |

Values are expressed in Mean±S.E.M (n=5) performed with ANOVA followed by Tukey's Post hoc multiple comparison test; DA28= diminazine aceturate 28 mg/kg-the positive control, VSME400= *V. sinaiticum* methanol extract 400 mg/kg; $^{b}$compared to TW80= 2% tween 80-the negative control, $^{c}$compared to VSME100= *V. sinaiticum* methanol extract 100 mg/kg, $^{d}$compared to VSME200= *V. sinaiticum* methanol extract 200 mg/kg, $^{*}$compared with all groups; $^{1}p < 0.05$ and $^{3}p<0.001$

Comparative analysis among various doses of the aqueous and methanol extracts of *C. abyssinica* and *V. sinaiticum* had shown that animals treated with 400 mg/kg dose of aqueous extract of *V. sinaiticum* extract had higher PCV value (47.14 ± 0.25) than the same dose of

aqueous extract of *C. abyssinica* extract (45.04 ± 0.31) on day 14 of treatment, whereas, animals treated with 400mg/kg dose of methanol extract of C. *abyssinica* had statistically significant (P<0.001) higher PCV value (48.66 ± 0.20) than the animals treated with the same dose *V. sinaiticum* of methanol extract of (46.80 ± 0.18) on day 14 of treatment. Among animals treated with either of the two plants, only those treated with methanol extract of C. *abyssinica* at 200 and 400 mg/kg dose had shown an increase in PCV values by 1.29 and 1.12%, respectively in days 7-14 (Figure 12).

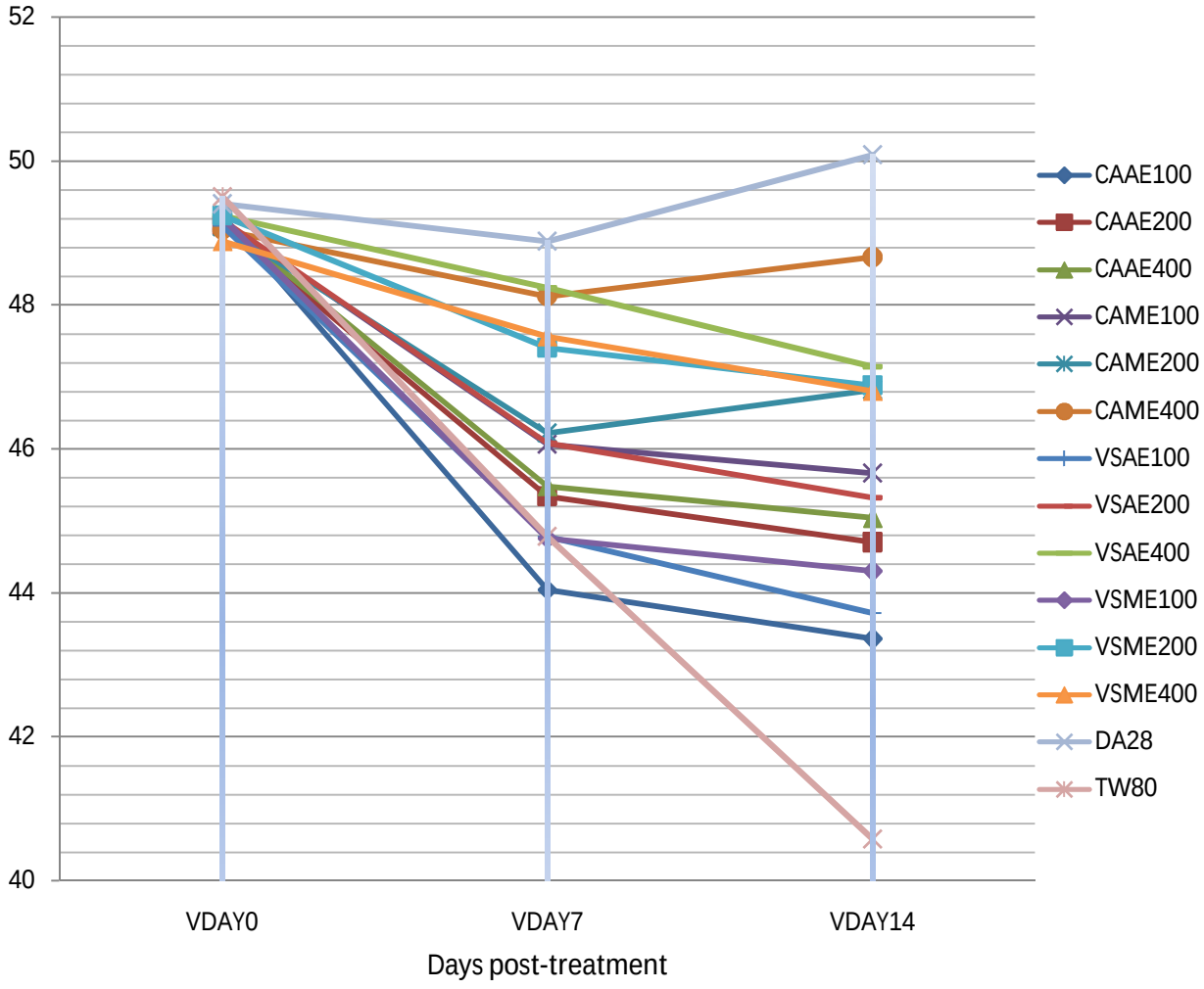



Figure 12: Comparison of the effect of aqueous and methanol leaf extracts of *Clutia abyssinica* and *Verbascum sinaiticum* on packed cell volume of *Trypanosoma congolense* infected mice

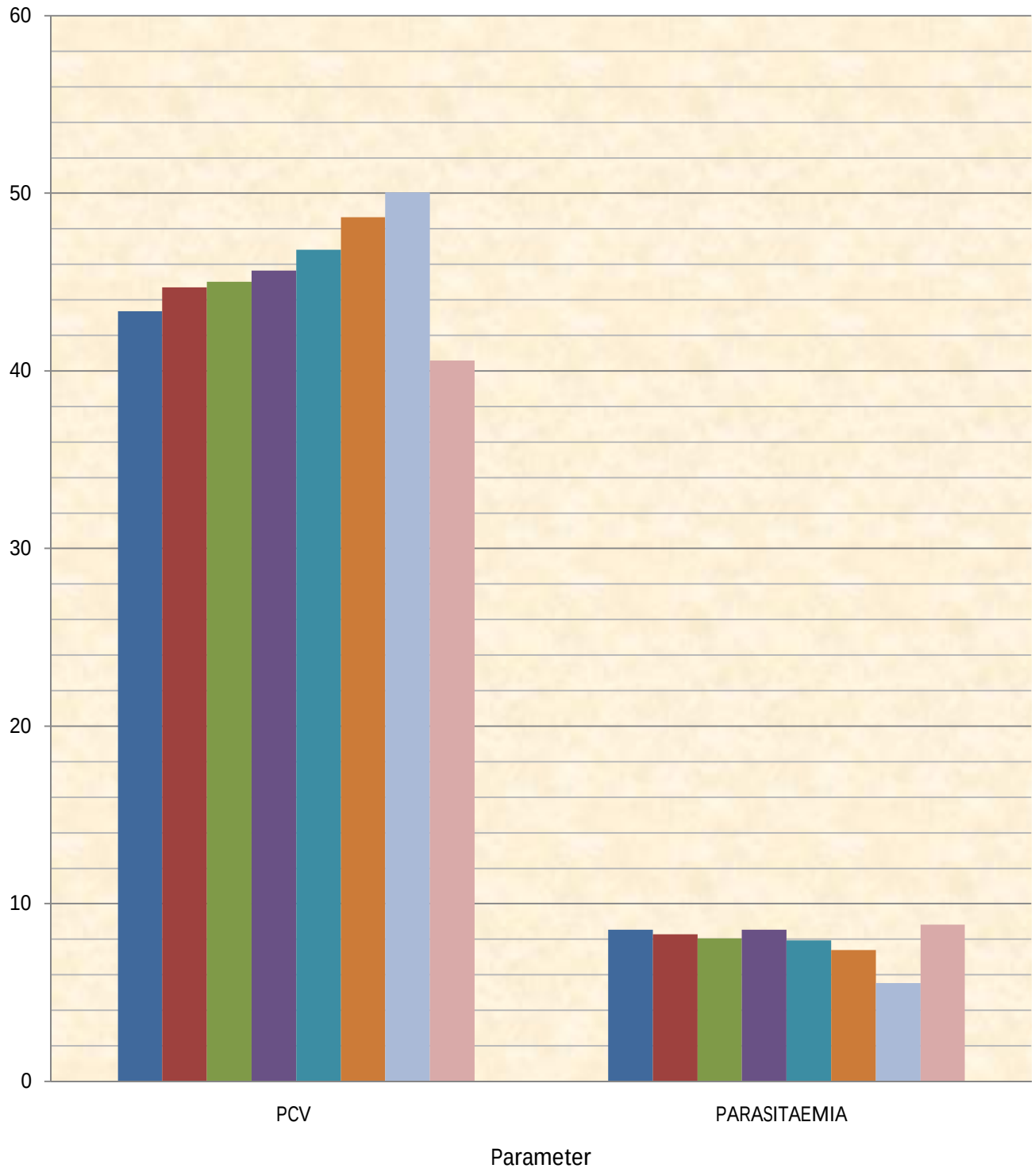



Figure 13: Comparison of the effect of aqueous and methanol leaf extracts of *Clutia abyssinica* on packed cell volume and parasitaemia

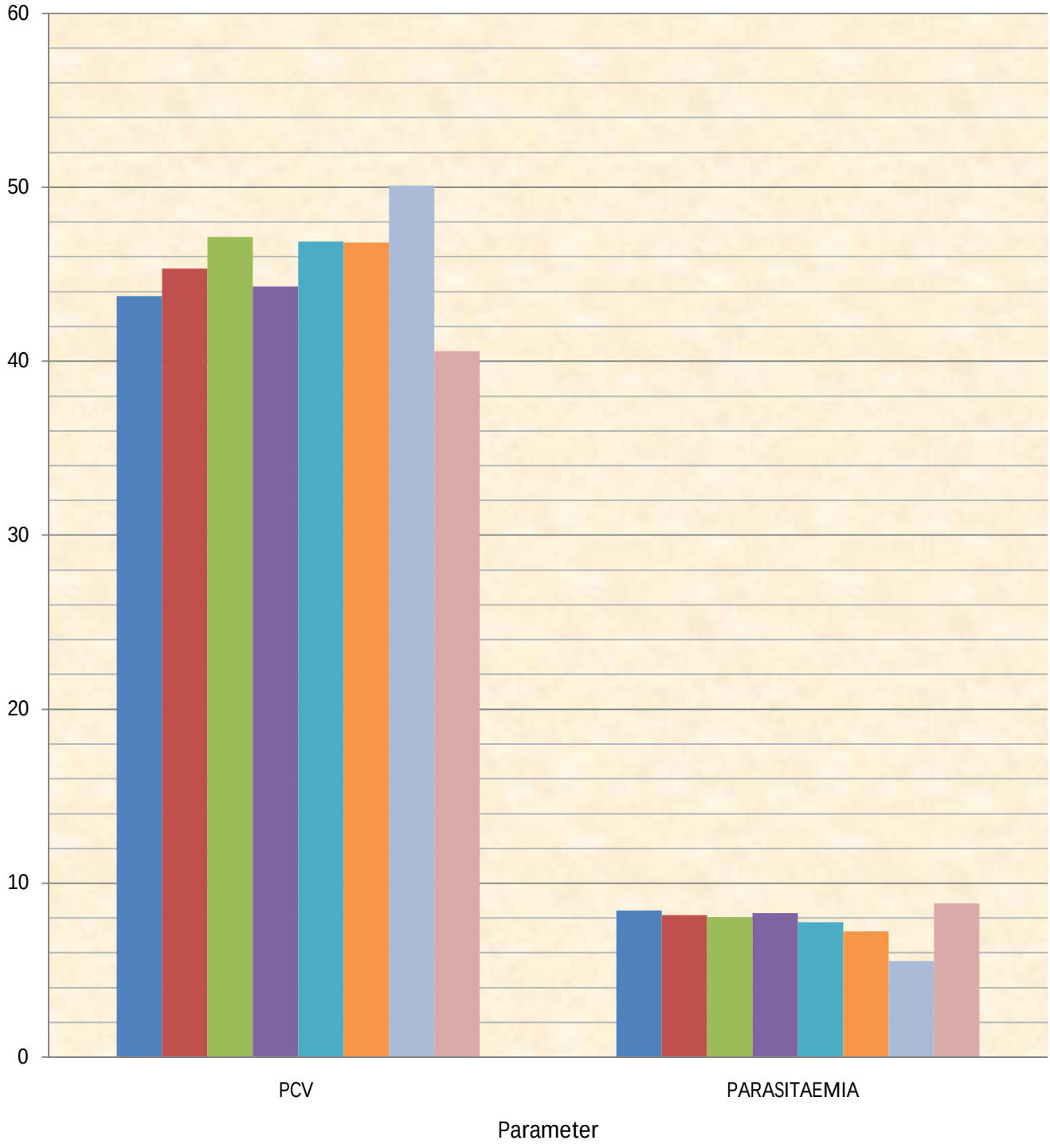



Figure 14: Comparison of the effect of aqueous and methanol leaf extracts of *Verbascum sinaiticum* on packed cell volume and parasitaemia

### 4.5.3. Effect of crude extracts on body weight

#### 4.5.3.1 Effect of crude leaf extracts of *Clutia abyssinica on* body weight of *Trypanosoma congolence* infected mice

Animals treated with 400 mg/kg dose of the aqueous extract of *C. abyssinica* had shown significant improvement on their body weight on day 6 ($p<0.01$), 8-14 ($p<0.001$) of treatment; while animals treated with 100 or 200 mg/kg dose of the aqueous extract had higher body weight on day 12 and 14 ($p<0.001$) as compared to the negative control group (Table 13).

The methanol extract of *C. abyssinica* at 400 mg/kg dose had improved body weight of treated animals ($P<0.001$) as compared to the negative control groups and lower dose (100 and 200 mg/kg) treated groups at $p<0.05$. In addition animals treated with 400 mg/kg dose of the aqueous and methanol extracts had shown an improvement in their body weight by 0.77 and 1.36%, respectively (Table 14).

#### 4.5.2.2 Effect of crude extracts of *Verbascum sinaiticum* on body weight of *Trypanosoma congolence* infected mice

The aqueous extracts of *V. sinaiticum* were capable to improve body weight of treated animals on days 8-14 when compared to the negative control group at $p<0.001$. Animals treated with 400 mg/kg dose of the aqueous extract of *V. sinaiticum* had a significantly ($p<0.001$) higher body weight ($22.20 \pm 0.25$) as compared to the negative control group ($19.09 \pm 0.34$) on day 14 of treatment (Table 15). Significant ($p<0.001$) improvement in body weight was seen on days 8-14 in animals treated with the methanol extract of *V. sinaiticum*. Animals treated with 400 mg/kg dose of the methanol extract of *V. sinaiticum* had a significantly ($p<0.001$) higher body weight ($22.54 \pm 0.28$) as compared to the negative control groups ($p<0.001$) and lower dose (100 and 200mg/kg) ($p<0.05$) treated groups on day 14 of treatment (Table 16).

Table 13: The effect of the aqueous leaf extract of *Clutia abyssinica* on body weight of *Trypanosoma congolense* infected mice

| Days | Body Weight (in grams) | | | | |
|---|---|---|---|---|---|
| | DA28 | TW80 | CAAE100 | CAAE200 | CAAE400 |
| Pre-infection | 22.15 ±0.20 | 22.04 ±0.21 | 22.18 ±0.19 | 22.18 ±0.12 | 22.59 ±0.21 |
| Day0 | 21.63±0.19 | 21.46±0.16 | 21.63±0.18 | 21.65±0.11 | 22.01±0.20 |
| Day2 | 21.31±0.17 | 21.00±0.14 | 21.34±0.14 | 21.33±0.12 | 21.65±0.22 |
| Day4 | 21.77±0.17$^{b1}$ | 20.66±0.20 | 21.01±0.15 | 20.98±0.10 | 21.39±0.25 |
| Day6 | 21.96±0.17$^{b3,c2,d1}$ | 20.33±0.13 | 21.10±0.12 | 20.95±0.13 | 21.42±0.26$^{b2}$ |
| Day8 | 22.31±0.17$^{bcd3}$ | 20.19±0.10 | 20.98±0.16 | 20.86±0.16 | 21.60±0.21$^{b3}$ |
| Day10 | 22.57±0.17$^{bcd3, e1}$ | 20.03±0.11 | 21.06±0.20$^{b2}$ | 20.98±0.11$^{b1}$ | 21.77±0.24$^{b3}$ |
| Day12 | 22.72±0.17$^{bcd3}$ | 19.92±0.15$^{*3}$ | 21.17±0.21 $^{b3}$ | 21.13±0.13$^{b3}$ | 21.99±0.23$^{b3}$ |
| Day14 | 22.61±0.17$^{bcd3}$ | 19.09±0.34$^{*3}$ | 21.25±0.20 $^{b3}$ | 21.25±0.18$^{b3}$ | 22.18±0.22$^{b3}$ |
| % change Body weight (Day 0-14) | 4.52 | -11.03 | -1.74 | -1.83 | 0.77 |

Values are expressed in Mean±S.E.M (n=5) performed with ANOVA followed by Tukey's Post hoc multiple comparison test; DA28= diminazine aceturate 28 mg/kg-the positive control, CAAE400= *C. abyssinica* aqueous extract 400 mg/kg; $^{b}$compared to TW80= 2% tween 80-the negative control, $^{c}$compared to CAAE100= *C. abyssinica* aqueous extract 100 mg/kg, $^{d}$compared to CAAE200= *C. abyssinica* aqueous extract 200 mg/kg, $^{*}$compared with all groups; $^{1}p < 0.05$, $^{2}p < 0.01$ and $^{3}p < 0.001$

Table 14: Effect of the methanol crude extracts of leaves of *Clutia abyssinica* on body weight of *Trypanosoma congolense* infected mice

| Days | Body Weight (in grams) | | | | |
|---|---|---|---|---|---|
| | DA28 | TW80 | CAME100 | CAME200 | CAME400 |
| Pre-infection | 22.15 ±0.20 | 22.04 ±0.21 | 22.18 ±0.19 | 22.18±0.12 | 22.59 ±0.25 |
| Day0 | 21.63±0.19 | 21.46±0.16 | 21.62±0.20 | 21.72 ±0.14 | 22.04±0.26 |
| Day2 | 21.31±0.17 | 21.00±0.14 | 21.29±0.22 | 21.28 ±0.10 | 21.23 ±0.2 |
| Day4 | 21.77±0.17$^{b2}$ | 20.66±0.20 | 21.02±0.21 | 21.19 ±0.18 | 21.58±0.09 $^{b1}$ |
| Day6 | 21.96±0.17$^{b3,c1,d2}$ | 20.33±0.13 | 21.02±0.17 | 20.91 ±0.13 | 21.49±0.26 $^{b2}$ |
| Day8 | 22.31±0.17$^{b3,c2,d3}$ | 20.19±0.10 | 21.13±0.19 $^{b1}$ | 20.89 ±0.14 | 21.64±0.26 $^{b3}$ |
| Day10 | 22.57±0.17$^{bcd3}$ | 20.03±0.11$^{*3}$ | 21.10±0.15 $^{b3}$ | 20.99 ±0.12 $^{b3}$ | 21.98±0.18$^{b3cd2}$ |
| Day12 | 22.72±0.17$^{bcd3}$ | 19.92±0.15$^{*3}$ | 21.16±0.20 $^{b3}$ | 21.25 ±0.11 $^{b3}$ | 22.09±0.19 $^{b3c2,d1}$ |
| Day14 | 22.61±0.17$^{bcd3}$ | 19.09±0.34$^{*3}$ | 21.28±0.21 $^{b3}$ | 21.31 ±0.14 $^{b3}$ | 22.34±0.27 $^{b3cd1}$ |
| % change Body weight (Day 14-0) | 4.52 | -11.03 | -1.59 | -1.86 | 1.36 |

Values are expressed in Mean±S.E.M (n=5) performed with ANOVA followed by Tukey's Post hoc multiple comparison test; DA28= diminazine aceturate 28 mg/kg-the positive control, CAME400=*C. abyssinica* methanol extract 400 mg/kg; $^{b}$compared to TW80= 2% tween 80-the negative control, , $^{c}$compared to CAME100= *C. abyssinica* methanol extract 100 mg/kg, $^{d}$compared to CAME200= *C. abyssinica* methanol extract 200 mg/kg, $^{*}$compared with all groups; $^{1}p < 0.05$, $^{2}p < 0.01$ and $^{3}p < 0.001$

There was a statistically significant ($P<0.05$) body weight improvement in groups treated with the crude extracts of *V. sinaiticum* and diminazine aceturate, where the highest increment being caused by diminazine aceturate 28 mg/kg (22.61 $\pm$ 0.17) followed by 400 mg/kg methanol extract (22.54 $\pm$ 0.28) and 400 mg/kg aqueous extract (22.20 $\pm$ 0.25) on day 14 of treatment when compared with the pre-treatment value. The result of the study also showed that 400 mg/kg dose of the aqueous and methanol extracts of *V. sinaiticum* had improved body weight of treated animals by 1.08 and 1.67 %, respectively.

Comparative analysis among the 400 mg/kg doses of the methanol extract of the two plants showed that they had no statistically significant different effect on body weight of treated animals during the observation period, while a better body weight increment was achieved by the 400 mg/kg dose of *V. sinaiticum* methanol extract which increased the body weight of treated animals by 1.67% as compared to the animals treated with same dose of methanol extract of C. *abyssinica*. Compared with the negative control groups that have lost 11% their body weight, animals treated with lower doses (100 and 200 mg/kg) of the aqueous and methanol extracts of *V. sinaiticum* had lost 1.27-2.49 % of their body weight, which was slightly lower than the *C. abysinica* treated groups which had lost 1.74-1.86% of their body weight (Figure 15).

Table 15: The effect of the aqueous leaf extract of *Verbascum sinaiticum* on body weight of *Trypanosoma congolense* infected mice

| Days | Body Weight (In grams) | | | | |
|---|---|---|---|---|---|
| | DA28 | TW80 | VSAE100 | VSAE200 | VSAE400 |
| Pre-infection | 22.15 ±0.20 | 22.04 ±0.21 | 22.19 ±0.13 | 22.22 ±0.15 | 22.43 ±0.24 |
| Day0 | 21.63 ±0.19 | 21.46 ±0.16 | 21.55 ±0.18 | 21.73 ±0.12 | 21.96 ±0.25 |
| Day2 | 21.31 ±0.17 | 21.00 ±0.14 | 21.38 ±0.20 | 21.25 ±0.17 | 21.55 ±0.23 |
| Day4 | 21.77 ±0.17 | 20.66 ±0.20 | 21.04 ±0.14 | 21.09 ±0.20 | 21.38 ±0.25 |
| Day6 | 21.96±0.17$^{bcd2}$ | 20.33 ±0.13 | 21.06 ±0.23 | 20.88 ±0.11 | 21.53 ±0.26$^{b2}$ |
| Day8 | 22.31±0.17$^{bcd3}$ | 20.19 ±0.10 | 20.91 ±0.23 | 20.87 ±0.15 | 21.64 ±0.27$^{b3}$ |
| Day10 | 22.57±0.17$^{bcd3}$ | 20.03±0.11$^{*3}$ | 20.98 ±0.15 | 21.02 ±0.14 | 22.00±0.10$^{bcd3}$ |
| Day12 | 22.72±0.17$^{bcd3}$ | 19.92±0.15$^{*3}$ | 21.20 ±0.21 | 21.15 ±0.09 | 22.06±0.27$^{b3,cd1}$ |
| Day14 | 22.61±0.17$^{bcd3}$ | 19.09±0.34$^{*3}$ | 21.21 ±0.20 | 21.27 ±0.15 | 22.20 ±0.25$^{b3}$ |
| % change Body weight (Day 0-14) | 4.52 | -11.03 | -1.27 | -2.14 | 1.08 |

Values are expressed in Mean±S.E.M (n=5) performed with ANOVA followed by Tukey's Post hoc multiple comparison test; DA28= diminazine aceturate 28 mg/kg-the positive control, $^{b}$compared to TW80= 2% tween 80-the negative control, ,$^{c}$compared to VSAE100= *V. sinaiticum* aqueous extract 100 mg/kg, $^{d}$compared to VSAE200= *V. sinaiticum* aqueous extract 200 mg/kg, $^{e}$compared to VSAE400= *V. sinaiticum* aqueous extract 400 mg/kg, $^{*}$compared with all groups; $^{1}p < 0.05$, $^{2}p < 0.01$ and $^{3}p < 0.001$

Table 16: The effect of the methanol leaf extract of *Verbascum sinaiticum* on body weight of *Trypanosoma congolense* infected mice

| Days | Body Weight (In grams) | | | | |
|---|---|---|---|---|---|
| | DA28 | TW80 | VSME100 | VSME200 | VSME400 |
| Pre-infection | 22.15 ±0.20 | 22.04 ±0.21 | 22.08 ±0.20 | 22.30 ±0.13 | 22.31 ±0.17 |
| Day0 | 21.63 ±0.19 | 21.46 ±0.16 | 21.76 ±0.25 | 21.83 ±0.14 | 22.17±0.24 |
| Day2 | 21.31 ±0.17 | 21.00 ±0.14 | 21.27 ±0.23 | 21.41 ±0.08 | 21.22±0.22 |
| Day4 | 21.77±0.17$^{b2}$ | 20.66±0.20 | 21.03 ±0.24 | 21.25±0.17 | 21.63 ±0.08$^{b1}$ |
| Day6 | 21.96±0.17$^{b3d2}$ | 20.33±0.13 | 21.20 ±0.30 | 20.88±0.12$^{b1}$ | 21.65 ±0.18$^{b2}$ |
| Day8 | 22.31±0.17$^{bcd3}$ | 20.19±0.10 | 20.96 ±0.15 | 21.00±0.14$^{b1}$ | 21.94 ±0.16$^{b3,cd2}$ |
| Day10 | 22.57 ±0.17$^{bcd3}$ | 20.03±0.11$^{*3}$ | 21.21 ±0.15 | 21.02±0.14 | 22.05±0.28$^{b3,d3}$ |
| Day12 | 22.72±0.17$^{bcd3}$ | 19.92±0.15$^{*3}$ | 21.20 ±0.15 | 21.27±0.13 | 22.26±0.21$^{b3,c1,d2}$ |
| Day14 | 22.61±0.17$^{b3cd1}$ | 19.09±0.34$^{*3}$ | 21.22 ±0.25 | 21.41±0.14 | 22.54±0.28$^{b3,cd1}$ |
| % change body weight (Day 0-14) | 4.52 | -11.03 | -2.49 | -1.90 | 1.67 |

Values are expressed in Mean±S.E.M (n=5) performed with ANOVA followed by Tukey's Post hoc multiple comparison test; DA28= diminazine aceturate 28 mg/kg-the positive control, VSME400= *V. sinaiticum* methanol extract 400 mg/kg; $^{b}$compared to TW80= 2% tween 80-the negative control, $^{c}$compared to VSME100= *V. sinaiticum* methanol extract 100 mg/kg, $^{d}$compared to VSME200= *V. sinaiticum* methanol extract 200 mg/kg, $^{*}$compared with all groups; $^{1}p < 0.05$, $^{2}p < 0.01$ and $^{3}p < 0.001$

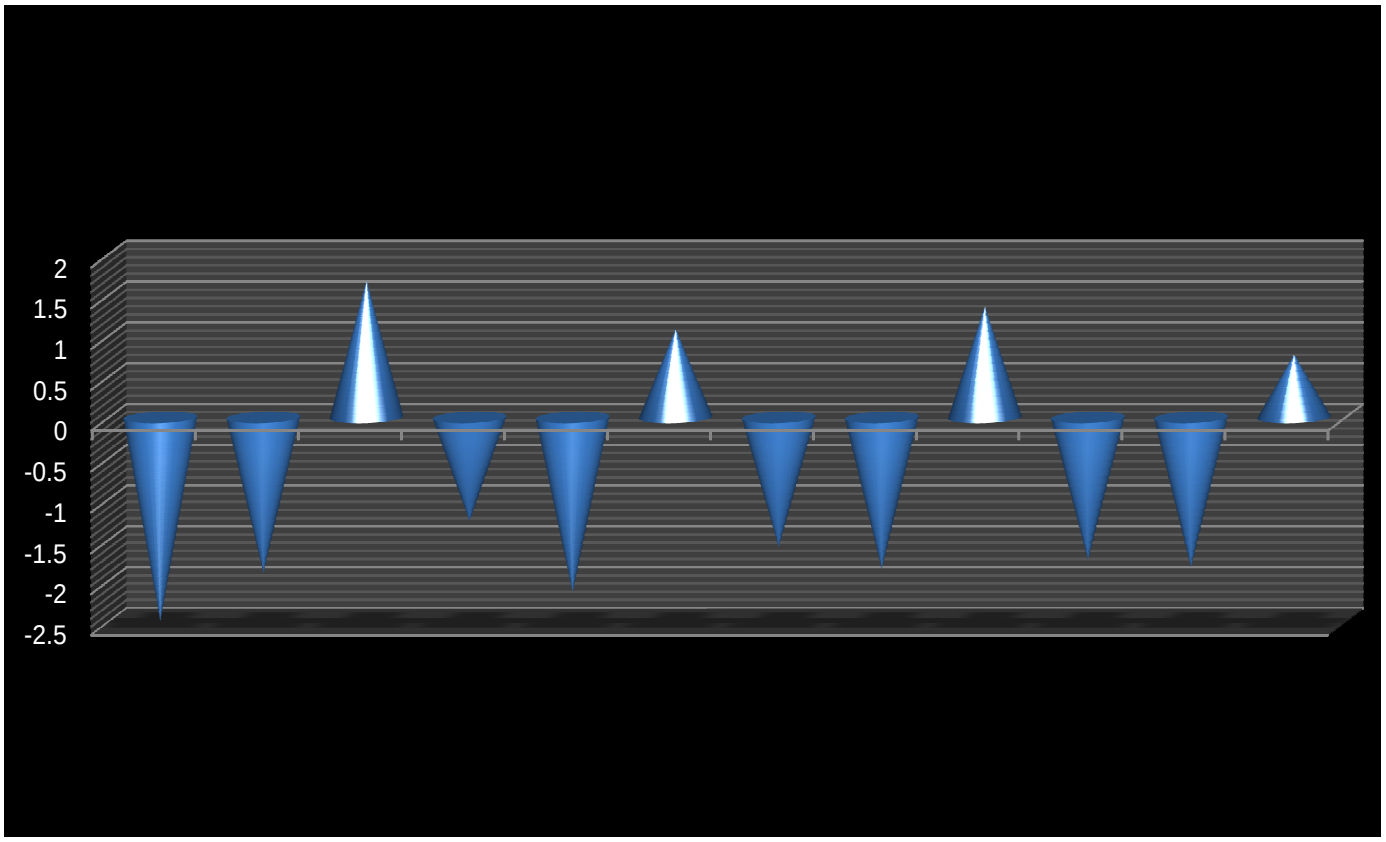



Figure 15: Comparison of the effect of aqueous and methanol leaf extracts of *Clutia abyssinica* and *Verbascum sinaiticum* on body weight of *Trypanosoma congolense* infected mice

### 4.5.4. Effect of crude extracts on mean survival time

Animals treated with 400 mg/kg of the methanol extract of *C. abysinica* had shown higher mean survival time while lower survival time was noticed by the aqueous extract of *V. sinaiticum* (Figure 16). Animals treated with 400 mg/kg of the methanol extract of *V. sinaiticum* and *C. abyssinica* had highest mean survival time of 40.20 $\pm$ 0.31 and 39.20 $\pm$ 0.37 days respectively as compared to the negative control group (25.40 $\pm$ 0.43), while animals that received the positive control diminazine aceturate had mean survival time of 44.00$\pm$0.63 days (Table 17). This finding also correlate with the effect shown by these extracts on parasitaemia level of *T. congolense* infected mice (Figures 17 and 18)

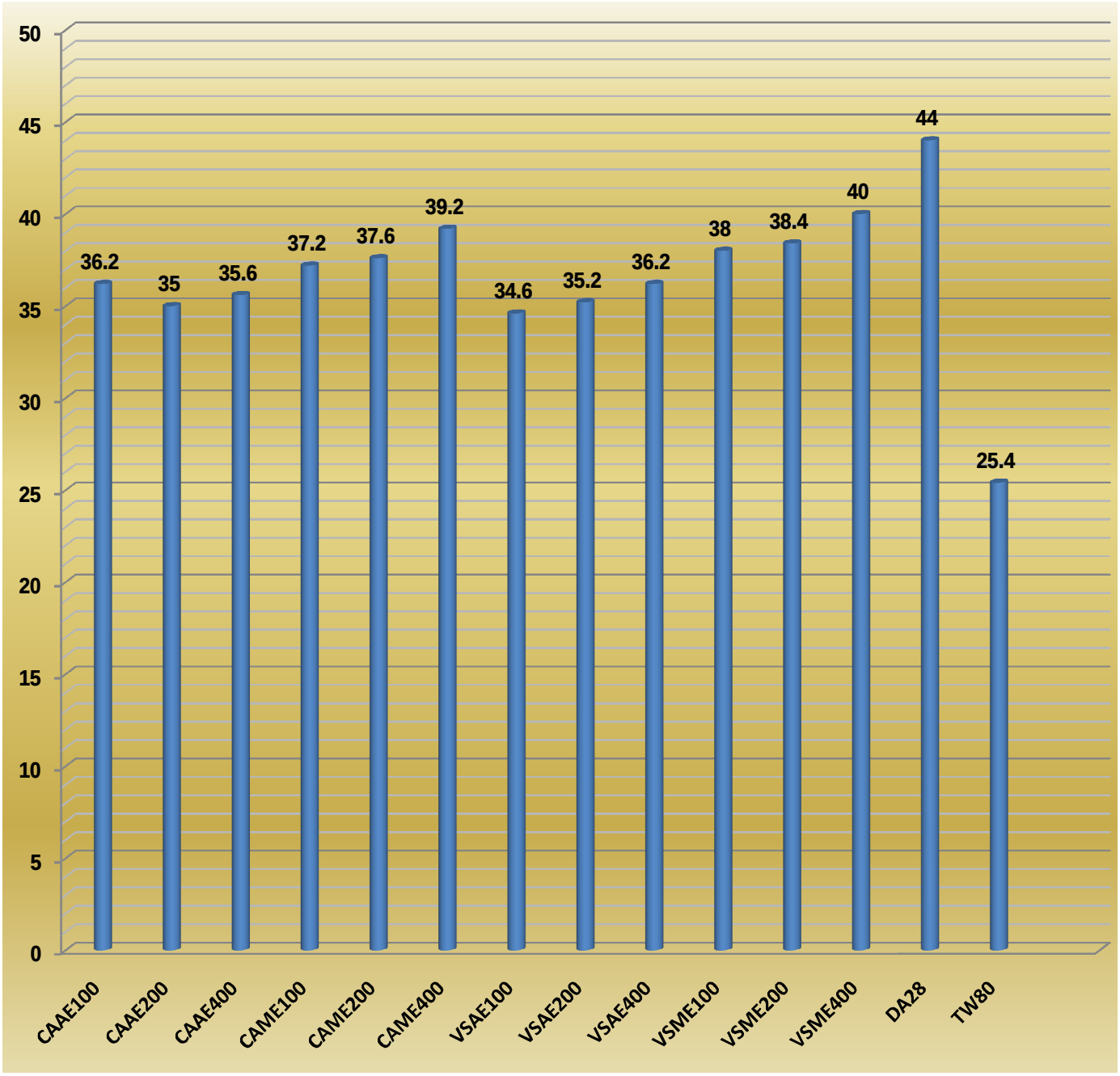



Figure 16: Survivality of *Trypanosoma congolense* infected mice treated with aqueous and methanol crude extracts of leaves of *Clutia abyssinica* and *Verbascum sinaiticum*

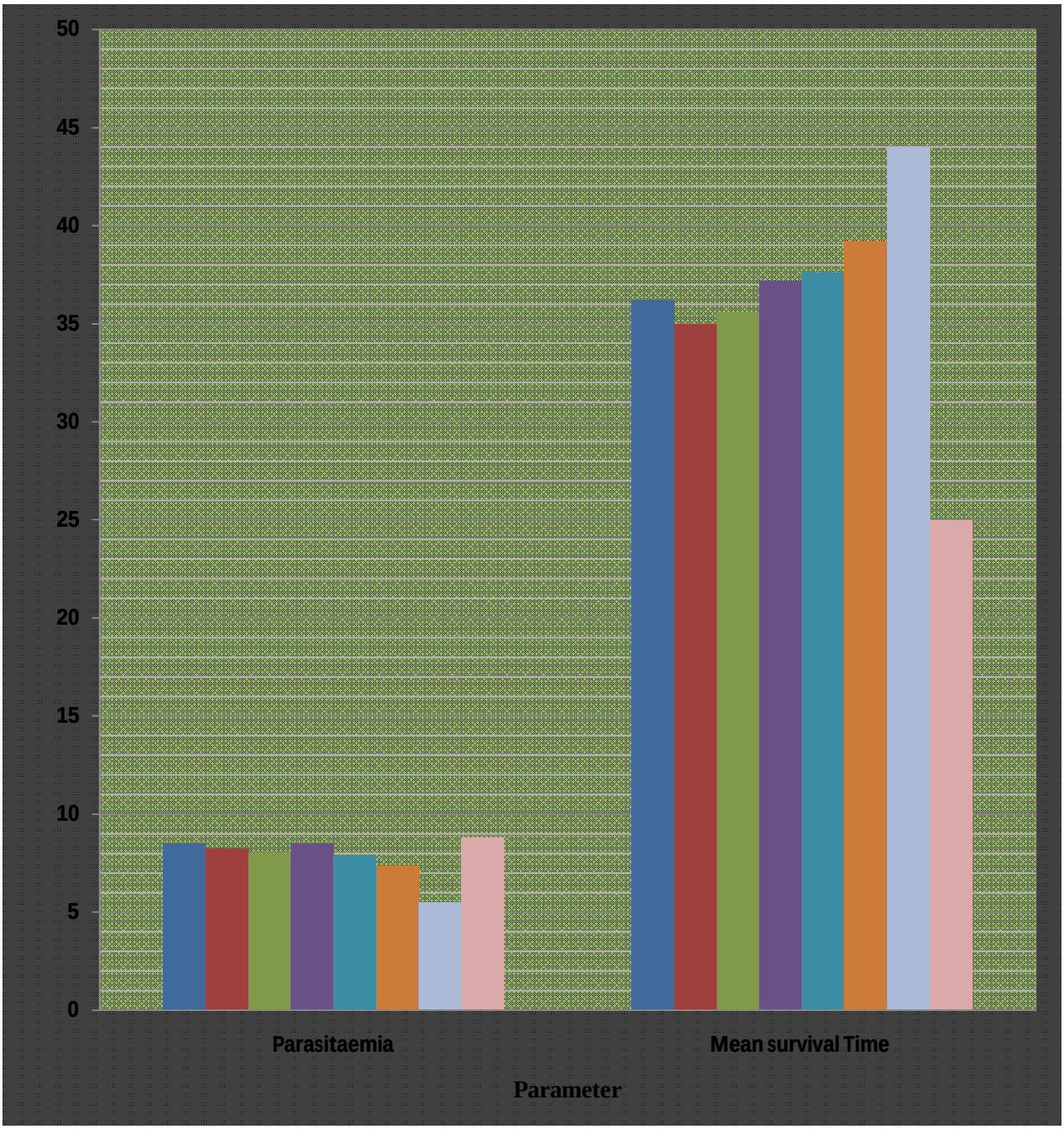



Figure 17: Comparison of the effect of aqueous and methanol leaf extracts of *Clutia abyssinica* on parasitaemia and mean survival time of *Trypanosoma congolense* infected mice

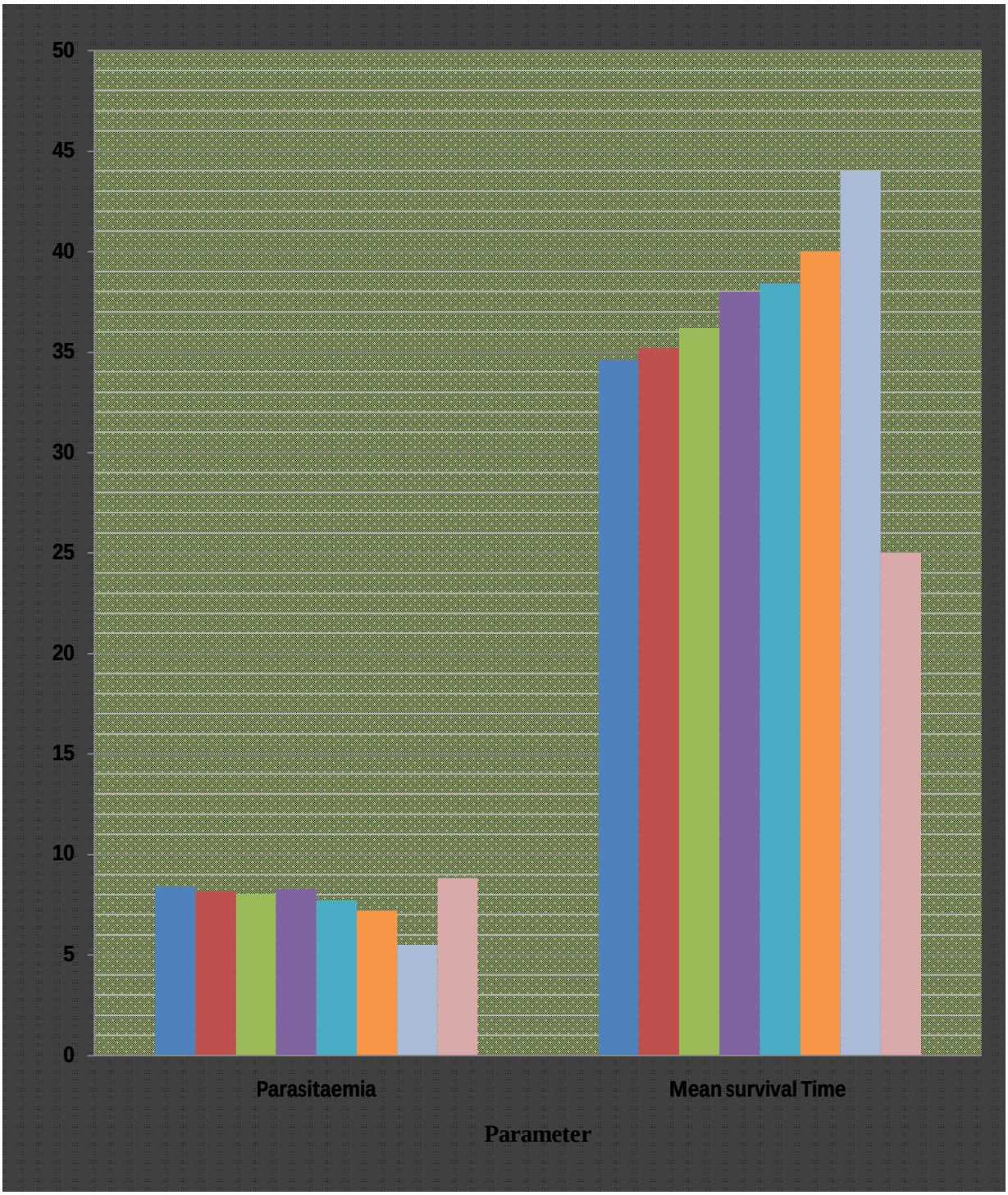



Figure 18:Comparison of the effect of aqueous and methanol leaf extracts of *Verbascum sinaiticum* on parasitaemia and mean survival time of *Trypanosoma congolense* infected mice

Table 17: Comparison of the effect of aqueous and methanol extract of *Clutia abyssinica* and *Verbascum sinaiticum* on parasitaemia, packed cell volume, body weight, and survival time of *Trypanosoma congolense* infected mice at the end of the experimental study

| Plant | Extract | Dose (mg/kg) | Mean Parasitaemia | Mean PCV value | Mean Body weight | Mean survival time |
|---|---|---|---|---|---|---|
| *C. abyssinica* | Aqueous | 100 | 8.52 ±0.07 | 43.36±0.24 | 21.25±0.20 | 36.20±0.66 |
| | | 200 | 8.28 ±0.07 | 44.70±0.28 | 21.25±0.18 | 35.00±0.31 |
| | | 400 | 8.04 ±0.06 | 45.04±0.31 | 22.18±0.22 | 35.60±0.40 |
| | Methanol | 100 | 8.52 ± 0.15 | 45.66±0.22 | 21.28±0.21 | 37.20±0.66 |
| | | 200 | 7.92 ± 0.15 | 46.82 ±0.34 | 21.31±0.14 | 37.60±0.50 |
| | | 400 | 7.38 ± 0.18 | 48.66±0.20 | 22.34±0.27 | 39.20±0.37 |
| *V. sinaiticum* | Aqueous | 100 | 8.40 +0.09 | 43.72±0.39 | 21.21±0.20 | 34.60±0.50 |
| | | 200 | 8.16 +0.11 | 45.32±0.37 | 21.27±0.15 | 35.20±0.37 |
| | | 400 | 8.04 +0.11 | 47.14±0.25 | 22.20±0.25 | 36.20±0.20 |
| | Methanol | 100 | 8.28 +0.07 | 44.30±0.32 | 21.22±0.25 | 38.00±0.70 |
| | | 200 | 7.74 +0.11 | 46.88 ±0.35 | 21.41±0.14 | 38.40±0.40 |
| | | 400 | 7.20 +0.16 | 46.80±0.18 | 22.54±0.28 | 40.00±0.31 |
| Positive control | Diminazine aceturate | 28 | 5.52 +0.07 | 50.64±0.15 | 22.61±0.17 | 44.00±0.63 |
| Negative control | 2% Tween 80 | 2ml/100gm | 8.82 + 0.12 | 40.58±0.27 | 19.09±0.34 | 25.40±0.43 |

# 5. DISCUSSION

For several decades, trypanosomosis has continued to contribute adversely to the economic and social well-being of sub-Saharan Africans (WHO, 2006). Despite the enormity of the health and economic implication of African trypanosomosis, current chemotherapeutic options are very limited and far from ideal for both human and livestock (Legros *et al.*, 2002; Mattioli *et al*., 2004). Therefore, the need for safer, cheaper and readily available sources of medicaments cannot be over-emphasized**.**

Literature surveys and field studies have shown that plants are used in traditional medicine in Africa to treat trypanosomes in humans and animals (Mbaya and Ibrahim, 2011). In this regard the objective of this study was to screen and evaluate the *in vitro* and *in vivo* antitrypanosomal activity of aqueous and methanol extracts of *C. abyssinica* and *V. sinaiticum* leaves which have medicinal uses in the treatment of infectious diseases including trypanosomosis (Teklehaymanot, 2009 and Fulas, 2010).

## 5.1 Extraction and percentage yield

The aqueous extracts of the two plants were prepared by macerating the dried leaves in distilled water in order to simulate the way they are traditionally used (Teklehaymanot, 2009 and Fulas, 2010). With the assumption that some of the active ingredients responsible for the claimed antitrypanosomal activity might not be soluble in water adequately; the methanol leaf extract of the plants was also included in the study. The choice of absolute methanol was based on the review on extraction methods of plants with antitrypanosomal activity by Mbaya and Ibrahim (2011) who stated that, in most situations, where air dried materials were powdered into small particles, and extraction was most productive with 100% methanol or ethanol.

Methanol extraction of *C. abyssinica* yielded a higher percentage (17.21%) than the aqueous extract (12.92%). This result was comparable with Pascaline *et al*., 2011 who obtained 23.6% and 12.8%, respective yields from methanol and aqueous extracts of the same plant collected from south Nandi district of kenya. The difference in the percentage yield of the methanol extract could be attributed to the difference in geographic source of the plants (Evans, 1996).

Extraction of *V. sinaiticum* with water and methanol yielded 13.09% and 18.13%, respectively. Eventhough there is no prior report on percentage yield from aqueous extract of the plant; Tadeg *et al*. (2005) have obtained 24.6% yield from 80% methanol extract of *V. sinaiticum*.

The yield obtained from the methanol extracts of the two plants was found to be higher as compared to the aqueous extracts which could be an indication of the extracting power of the solvent which was also noticed in the phytochemical screening. This yield, if the extracts are found to be active and promising for further development, can add advantage to the commercial production of these plants.

**5.2. Acute toxicity study**

Before pharmacological evaluation of plant preparation, investigation of the possible toxic effects, which are the main concern of indigenous therapeutic preparations, in order to ascertain its safety for subsequent efficacy experiments is no doubt required. One method for evaluation of herbal toxicity is the acute toxicity test in which the herbal preparation is given intraperitoneally as a single and very high dose to laboratory animals like Swiss albino mice (Lorke, 1983). In this study aqueous and methanol extracts of the leaves of *C. abyssinica and V. sinaiticum* were tested for their toxicity against Swiss albino mice. According to the Center for Drug Evaluation and Research CDER (1996), acute toxicity is a toxicity produced by a pharmaceutical when administered in one or more doses within a period not exceeding 24 hours.

Based on this, the plant extracts had shown $LD_{50}$ greater than 2000 mg/kg (Annex I). Thus, since *C. abyssinica* and *V. sinaiticum* are believed to have several traditional medicinal uses by different traditional healers, the experimental determination of this good safety margin would justify that the plants are safe at the dose levels (100, 200 and 400 mg/kg) used in the study which is an additional proof for the medicinal value of these plants in folk medicine.

### 5.3. Phytochemical screening

Recognition of the biological properties of myriad natural products has fueled the current focus of this field, namely, the search for new drugs against trypanosomosis. One of the objective for evaluating plants for biological activity is to isolate one or more biologically active compounds that may be potentially useful in treating certain disease conditions or serve as a structural analogue (template) from which better synthetic modifications can be derived.

Chemical characterization and compositional analysis of traditional medicines provide the necessary scientific basis for the discovery and development of new drugs of natural origin. Should isolation of the active ingredients be unnecessary as is the case in the current trends of herbal therapy, knowledge of the type of chemical constituents found in a given herbal drug can be very helpful for standardization and quality control purposes. To this end, phytochemical screening can be a valuable aid.

Therefore, the leaves of *C. abyssinica* and *V. sinaiticum* were screened for the presence of different phytochemicals of therapeutic interest using chemical method with the objective of finding out the possible class of compounds present in the respective plants (Table 2).

According to the results of the phytochemical screening study, both species were found to show a positive test for the presence of saponins, steroids, phenols, alkaloids, glycosides, flavonoids

and tannins, while *C. abyssinica* showed positive test for the presence of anthraquinones and terpenes, *V. sinaiticum* showed a negative result for the same test.

Numerous *in vitro* and *in vivo* studies conducted on the antitrypanosomal activities of the class of compounds listed above reported the potential of each class of compounds in killing or inhibiting the growth of wide ranges of trypanosomes.

Phytochemical screening on the two plants had shown that all extracts except the aqueous extract of *V. sinaiticum* had positive test for the presence of phenolic compounds. Phenolics and polyphenols have been reported in the literature to have antitrypanosomal potential. For instance, ascofuranone, phenol antibiotic isolated from a phytopathogenic fungus, *Ascochyta visiae*, was found to be effective against *T.b.brucei*. Inhibition of the trypanosome alternative oxidase (TAO) enzyme was thought to be responsible for antitrypanosomal activity of phenolic compounds (Yabu *et al*., 2003).

Phytochemical screening of *C. abyssinica* had shown presence of anthraquinones in the crude aqueous and methanol leaf extracts (Table 2). Pascaline *et al*. (2011) reported that anthraquinones are the main constituents in *C. abyssinica* leaves. Cenas *et al*. (1994) and Chemin *et al*. (2001) have reported that quinones, especially 1,4-naphthoquinones can induce oxidative stress in trypanosomes (*T. congolense* and *T. cruzi*). This may be explained by their reduction to semiquinone radicals by enzymes such as those present in the mitochondrial electron transport chain and the trypanothione reductase (Nok, 2002). As a result, there is a great potential for quinones to serve as antitrypanosomal agents (Eze *et al*., 2012; Maikai *et al*., 2007). Azaanthraquinone, (an anthraquinone isolated from *Mitracarpus scaber,* Nok, 2002) and aloe-emodin, another anthraquinone isolated from *Stephania dinklagei* (Camacho *et al*., 2000) were

also reported to have antitrypanosomal activities against *T. congolense* and *T. b. brucei* respectively.

As shown in Table 2, methanol leaf extracts of *C. abyssinica* and *V. sinaticum* had a positive test for flavonoids, which is in agreement with the report of Abdel (1986), who reported that the family Euphorbiaceae is rich in flavonoids, particularly flavones and flavonols and Afifi *et al*. (1993), who reported that *V. sinaiticum* contain two flavonolignans, hydrocarpin and sinaiticin, as well as two flavones, chrysoeriol and luteolin.

Eventhough the antitrypanosomal activity of the isolated flavonoids from *C. abyssinica* and *V. sinaiticum* are not yet reported; flavonoids and flavonoid-derived plant natural products have long been known to function as free radical scavengers and metal chelators: they inhibit lipid peroxidation and exhibit various physiological activities, including antihypertensive and anti-arthritic activities (Harborne and Williams 2000). Different studies have shown that flavonoids are effective antitrypanosomal substances against the different trypanosome species. To mention a few, the flavonoids, chrysosplenetin and chrysosplenol (isolated from *Ehretia amoena)*; luteolin (isolated from *Cymbopogon citratus*) have been shown to exert antitrypanosomal activity against trypanosome species (Hoet, 2003).

Phytochemical screening results had shown that methanol leaf extracts of both plants contain tanins. It has long been known that consumption of tannin-containing beverages, especially green teas and red wines cure or prevent a variety of ills including wide ranges of infections (Haslaam, 1996). Gallic acid, a well-known component of hydrolysable tannins, is active against *T. b. brucei* (Koide *et al*., 1998). One of the molecular actions of tannins is by complexing proteins

through the so-called nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation (Taylor, 2000).

The findings of the phytochemical tests on the two plants had shown that only the methanol extract of *C. abyssinica* contain terpenes. This is in agreement with the report of Waigh *et al*. (1990) and Jaber *et al*. (1996) who isolated the diterpenes 2"-O-glycosylisovitexin, and the C-glycoside ent-16β,17-dihydroxykaurane from leaves of *C. abyssinica*. Similarly, Muhammad *et al*. (1999) have isolated chemical compounds from aerial parts of C*lutia richardiana* (family Euphobriacea) and have shown presence of diterpenoids like 6,7-secolabdanes, including saudin, richardianidin , tiglianes, daphnanes and ingenanes.; modified labdane diterpenoids: the 6(7), 9(10)-biseco-6(11), 1(19) bicyclolabdanes, cluytenes A and C and saudinolide, dihydrosaudinolide, 5β-hydroxyrichardianidines and cluytenes.

Eventhough we cannot for sure tell that the observed activity by *C. abyssinica* was due to the diterpene constituents, it will be logical to speculate their contribution as there are promising terpenes isolated from plant products which exhibit selective, potent antitrypanosomal activity like; α-eudesmol, hinesol, nardosinone and 4-peroxy-1,2,4,5-tetrahydro- α –santonin (Otoguro *et al., 2011*)..

The phytochemical test for the presence of essential oil constituents in *C. abyssinica* and *V. sinaiticum* was not addressed in this study. But, review of the literature has indicated the presence of essential oils like β-ionone, α-farnesene and farnesylacetone in *C. abyssinica* (Zerihun *et al*., 1987). Herewith, essential oils of promising antitrypannosomal effect were screened by Nibret and Wink (2010). Based on their finding the essential oil Octa-3,5-diene-2,7-dione, 4,5-dihydroxy, found in Ethiopian *Artemisia* species has promising antitrypanosomal

activity. The proposed mechanism for antitrypanosomal activity of essential oils include: by formation of aldehyde-thiol adducts with sulphur containing components thereby decreasing the buffering agents which can create oxidative stress in cells (Nibret and Wink, 2010); by oxidation of glutathione, pyruvic and alpha-ketoglutaric acids and the oxidative decarboxylation of pyruvic acid by hydroperoxy group which makes them toxic (Saeidnia *et al*., 2004).

The presence of alkaloids in *C. abyssinica* concurs with the findings of Abdel (1986), who reported various alkaloids in certain genera of the family Euphorbiaceae. In addition Samia *et al*, (2007), reported the presence of alkaloids in *Verbascum* species.

Similarly, previous studies have shown that the quinoline alkaloids from Cinchona bark (quinidine, cinchonine, quinine, cinchonidine) and emetine, an isoquinoline alkaloid from *Cephaelis ipecacuanha*, have significant trypanocidal activity against *T. b. brucei* and *T. congolense* (Merschjohann *et al*., 2001).

The finding of glycosides in the methanol extract of *C. abyssinica* and *V. sinaiticum* in this study coincide with the report of Pascaline *et al*. (2011) and Elgindi *et al*. (2000). Elgindi *et al*. (2000) have reported the presence of phenylethanoid glycoside, verbascoside (acteoside) in *V. sinaiticum*. Yalcin *et al*. (2003) have shown free radical scavenging activity of verbascoside (acteoside).

Although the antitrypanosomal activity of some herbs is attributed to a specific chemical compound, labeling the activity, especially those commonly used in traditional therapy, to a single compound is a difficult undertaking and it is very unlikely that the activity is due to a single compound only. The possible explanation one can propose for their effectiveness in treating various infectious diseases is that each class of compounds might act synergistically

contributing their own share to the total activity of the herbal drug (Abdelhady *et al*., 2002; Pelttari *et al*., 2002).

Therefore, the observed antitrypanosomal activity of *C. abyssinica* and *V. sinaiticum* might be attributed to either the individual class of compounds present in each herb, or to the synergistic effect that each class of compounds exert to give the observed biological activity. Hence, further in-depth investigations should be carried out to resolve this issue.

**5.4 *In vitro* activity test**

Parasites motility constitutes a relatively reliable indicator of viability of most trypanosomes (Kaminskey *et al*., 1996) and a complete elimination or reduction in motility of trypanosomes when compared to the control could be taken as index of trypanocidal activity (Atawodi *et al*., 2009).

*C. abyssinica* and *V. sinaiticum* crude leaf extracts had shown appreciable *in vitro* antitrypanosomal activity, with the methanol extracts exhibiting the highest activity. The *in vitro* activity of the methanol extract of *C.abyssinica* which immobilized motility of the trypanosomes within 30 min at 4 mg/ml concentration is comparable with the diminazine aceturates's effect which had shown similar effect within 20 min of incubation. In addition the activity of methanol extract of *C. abyssinica* at 2 and 4 mg/ml concentration which ceased motility of the trypanosomes within 40 and 30 min, respectively (Table 3) is higher as compared with the *in vitro* antitrypanosomal activity of methanol extracts of *Ximenia americana* (Maikai, 2010). Methanol extracts of *X. americana* at 9 mg/ml effective concentration inhibited motility of *T. congolense* within 45 min of incubation (Maikai, 2010).

Steroidal compounds and/or terpenes in *C. abyssinica* might be responsible for the observed *in vitro* antitrypanosomal activity. This speculation originates from the findings of Freiburghaus *et al*. (1998) who reported the *in vitro* antitrypanosomal activity of the diterpene kolavenol, isolated from a rootbark extract of *Entada abyssinica*. The proposed mechanism for antitrypanosomal activity of terpenes include: alterations of membrane permeability by formation of covalent bonds with amino residues of proteins and inactivate most of the proteins that are likely to affect numerous cellular activities (Santoro *et al*., 2007).

The *in vitro* antitrypanosomal activity of the methanol extract of *V. sinaiticum* at 4 mg/ml concentration which ceased motility of parasites within 50 min of incubation is in agreement with *in vitro* antitrypanosomal activity of methanol extract of *Pseudocedrella kotschi* which similarly immobilized motility of *T. congolense* at 4 mg/ml concentration within 55 min of incubation (Atawodi *et al*., 2003).

It is not known why 4 mg/ml aqueous extracts of *C. abyssinica* and 2 mg/ml aqueous extract *V. sinaiticum* reduced trypanosome motility (within 50 and 70 min) but could not completely eliminate motility. But similar observation was made by Atawodi and Shehu (2010) where methanol and chloroform leaf extract of *Moringa oleifera* drastically reduced motility of trypanosomes within 40 min at 2 mg/ml concentration. However, it appears reasonable to speculate that these extracts may belong to the group that acts by static action affecting growth and multiplication of trypanosomes rather than eliminating them altogether.

The aqueous leaf extract of *C. abyssinica* at 2 mg/ml had shown no effect on motility of trypanosomes as compared to the same concentration and solvent extract of *V. sinaiticum* which reduced motility of trypanosomes within 70 min of incubation at 2 mg/ml. The differences

observed are partly attributable to variation in the types of phytocemicals in these plants as revealed by the results of the phytochemical screening (Table 2). The major observed differences between the phytochemical pictures of the aqueous extracts of the two plants are with respect to the alkaloids which were present only in the aqueous extract of *V. sinaiticum*. The DNA intercalation in combination with protein biosynthesis inhibition is reported to be the mechanism of action of alkaloids (Merschjohann *et al*., 2001).

The aqueous leaf extracts of both plants at lower concentration (1 mg/ml) had no effect on the motility of the parasites. This finding is not in agreement with Bulus *et al.* (2008) where aqueous extract of *Terminalia avicennioides* at 1 mg/ml test concentration reduced motility of rypanosomes within 50 min of incubation.

The mechanism by which the extracts immobilized or reduced motility of the trypanosomes is not known at this stage of the work. However, accumulated evidences suggested that many natural products exhibited their antitrypanosomal activity by virtue of their interference with the redox balance of the parasites acting either on the respiratory chain or on the cellular defenses against oxidative stress (Antia *et al*., 2009; Atawodi *et al*., 2003; Joseph *et al*., 2011). Respiration of trypanosomes is obligatory for their motility as well as for managing the energy reserve required for the synthesis of the variable surface glycoproteins. The inhibition of cellular and mitochondrial respiration by any chemotherapeutic agent will obviously compromise all the energy dependent processes. This was confirmed by the microscopy of the trypanosomes, which showed a cessation or reduction in motility after incubation with different concentrations of extracts (Nok, 2002).

The positive control diminazine aceturate immobilized trypanosomes within 20, 30 and 60 min at test concentrations of 4, 2, 1 mg/ml, respectively. This finding is not in agreement with Maikai (2010), in which even lower concentration 0.1 mg/ml of diminazine aceturate ceased motility of the trypanosomes within 20 min. The difference might be due to the *T. congolense* isolate that could have developed resistance to the drug as reported by Chaka and Abebe (2003).

Comparison analysis revealed that the standard drug exhibited superior *in vitro* antitrypanosomal activity even at lower concentration (2 mg/ml) when compared to the extracts. This is consistent with several reports made on other medicinal plant extracts (Atawodi *et al.,* 2003; Ene *et al.*, 2009; Hoet *et al*., 2004; Maikai and Kobo, 2008; Maikai *et al.,* 2011).

**5.5. Blood incubation infectivity test**

The observation that incubation of trypanosomes with the 4 mg/ml methanol extract of *C. abyssinica* inhibited healthy mice from developing infection in the observation period agrees with earlier reports which showed that plant extracts can cause trypanosomes to lose their infectivity to rodents (Maikai *et al.,* 2011; Yusuf *et al.,* 2012).

However the *in vitro* antitrypanosomal activities of other concentrations were not confirmed by blood incubation infectivity. Yusuf *et al.* (2012) suggested that complete immobility of the parasites *in vitro* may not necessarily indicate that the parasites were dead, but rather the parasites may have lost their infectivity. This may be due to the respective concentration might have only immobilized, but not killed the parasite by causing unfavorable conditions. The parasites might have recovered and become infective in contact with suitable physiological conditions.

Prolongation of the pre patent period (time from inoculation to presense of motile parasites in blood) of animals inoculated with the *in vitro* mixtures containing lower test concentrations of methanol extract of *C. abyssinica* (2 mg/ml) and *V.sinaiticum* (2 and 4 mg/ml) is in agreement with the findings of Feyera *et al*. (2011) and Yusuf *et al.* (2012).

It may appear to contemplate that the highest (4 mg/ml) concentration level either killed the parasites or caused them to lose their infectivity coupled to cease in motility of the trypanosomes *in vitro*. Loss of infectivity may be by abrogating some vital metabolic processes in the parasites or could be due to some morphological changes on the parasites induced by the extract at this concentration that render them more susceptible to the mice immune defense systems.

### *5.6 In vivo* antitrypanosomal activity test

#### 5.6.1 Effect of crude extracts on parasitaemia and Survivability

According to Atawodi *et al*. (2003), plants found to be active *in vitro* must be tested *in vivo* before a definite statement can be made on their antitrypanosomal potentials. *In vivo* assays usually provide relatively reliable information of determining the extracts sensitivity on trypanosome isolates and the use of laboratory mice has the advantage of being relatively inexpensive with respect to cost of animals, housing and maintenance. However, mouse assays are considered to provide a broad indication of the level of sensitivity of a trypanosome population (Sones *et al*., 1988).

The mean parasitaemia (7.38$\pm$ 0.18) of animals treated with 400 mg/kg dose of methanol extract of *C. abyssinica* on day 14 post-treatment is lower as compared to 8.66 ± 0.02 parasitaemia in animals (infected with *T.congolense*) treated with *Carrisa edulis* (Wurochekke *et al*., 2014).

In addition the result of this study had shown that the two plants did not completely eliminate parasites from the blood stream of infected mice, but only reduced the level of parasitaemia. Several researchers made similar observations on reduction in parasitaemia (Ibrahim *et al*., 2012; Ogbadoyi et al., 2011; Wurochekle *et al*., 2014) and concluded that high parasite load could mask the efficacy of crude extract (Ekanem *et al*., 2008). Also, the reduced efficacy of the crude extracts in clearing trypanosomes from blood circulation could be due to enzymatic inactivation of active compounds and impaired absorption from the site of administration (Mann *et al*., 2009). In addition failing to reach these target organs in sufficient concentration and duration to effect a cure; short half life of the constituents making them unable to stay long enough to exert pronounced effect on the parasites (William *et al*., 2005) could also be attributed to the failure of the extracts to clear the blood of infected mice from trypanosomes.

The remarkable trypanostatic effect of the methanol extract of *C. abyssinica* might resemble the type of antiviral activity reported by Colegate and Molyneux, (1993) and Cos *et al*., (2002). In that report, it was shown that the ethanolic leaf, stem and root extracts of *C. abyssinica* showed moderate antiviral activity against polio virus and Coxsackie virus (Colegate and Molyneux, 1993), while the leaf extracts exhibited anti-HIV-1 activity (Cos *et al*., 2002). Although we do not yet know the mechanism by which the extract exerts this remarkable trypanostatic effect, our speculation is that the extract may be interfering with cell cycle progression in the parasite, possibly causing cell cycle arrest and thereby halting cell proliferation, which is a similar mechanism for currently available antitrypanosomal agents.. It is also possible that the extract might be exerting its trypanostatic effect through the modulation of the animal's immune system, which in turn enables the animal to withstand the ravaging parasites for a long time. It may well be the interplay of both effects that resulted in the observed tremendous trypanostatic effect.

Even though the mechanism by which methanol extracts of *C. abyssinica* at doses of 400 mg/kg reduced parasitaemia of treated animals is not known, the activity could be attributed to the individual or combined effect of terpenes and other metabolites found in the leaf extract. This speculation was based on the fact that previous isolation studies have shown presence of diterpenes like 2"-O-glycosylisovitexin and the C-glycoside ent-16β,17-dihydroxykaurane in the leaf extract of *C. abyssinica* (Waigh *et al*.,1990; Jaber *et al*, 1996). Kaurenoic acid, a chemically related compound with ent-16β,17-dihydroxykaurane has shown antibacterial, larvicidal and trypanocidal activity (Vieira *et al*., 2002).

The fact that both plants contain saponins might contribute to their antitrypanosomal activity due to their the surface active property which contribute to the activity by reducing surface tension and facilitating the penetration of another active agent into the protoplasm.

In the group of mice treated with diminazine aceturate, there was no parasite development from day 2 to 10. Although relapse occurred in all mice approximately on days 12-14 of treatment. Similar observations were made by Ibrahim *et al*. (2012). The relapse of parasitaemia might be due to the ability of *T. congolense* to sequester in small vessels and capillaries of the heart, skeletal and other tissues which often leads to prolonged pre-patent period (Losos, and Ikede, 1972; Maxie, and Losos, 1977; Mbaya *et al*., 2007).

However the relapse seen in mice treated with the drug was not surprising as trypanocidal drug resistance has been wide spread in several African countries, including Ethiopia (Afewerk *et al*., 2000; Assefa and Abebe, 2001; Delespaux *et al*., 2008; Miruk *et al*., 2008).

Moreover the relapse seen in animals treated with 28 mg/kg dose of diminazine aceturate was not in agreement with previous finding of Chaka and Abebe (2003). Unlike our observation,

relapse of parasitaemia was not seen in mice infected with *T. congolence* (field isolates from Bedele town) and treated with 28 mg/kg of diminazine aceturate (Chaka and Abebe (2003). The difference could be attributed to the development of drug-resistant strains of *T. congolense* 10 years after the report of Chaka and Abebe (2003).

The findings of this study on the *in vitro* and *in vivo* activity of diminazine aceturate support the notion on the development of drug-resistant strains of *T. congolense* in the area from which the parasites are isolated, that might be associated with the use of the same drug- diminazine aceturate for treatment of animal trypanosomosis in the community over long period. This could also necessitate the search of new effective antitrypanosomal drugs, assessment drug-resistant strains of *T. congolense* and their sensitivity to different doses of the drug.

### 5.6.2. Effect of extracts on PCV

The study on packed cell volume (PCV) gave results that were fairly consistent with the observations made on parasitaemia (Figures 10 and 11). Infection caused significant drop in PCV in the negative control groups approximately by day 25 post infection (14th day of treatment) with mean value being below the reference values (42-52%).

The low PCV value in the infected groups may be due to acute hemolysis and is a result of the growing infection. In addition infection with trypanosomes results in increased susceptibility of red blood cell membrane to oxidative damage. Reactive oxygen species generated by trypanosomes can also attack red blood cells' membranes, induce oxidation and subsequently hemolysis (Karori *et al*., 2008). This phenomenon subjects RBC to massive erythrophagocytosis by an expanded and active mononuclear phagocytic system (MPS) of the host resulting in

anemia (Albert and Hussein, 2012) Thus, scavenging the trypanosome associated free radicals may ameliorate anemia.

The effect of extracts in ameliolating anemia is possibly by reducing the parasite load, neutralizing the toxic metabolites produced by trypanosomes or scavenging the trypanosome associated free radicals which could be attributed to the secondary metabolites present in the extracts (Ekanem *et al*., 2008; Mpiana *et al*., 2007; Ogoti *et al.,* 2009).

The findings of this study suggest that the extracts considerably improved PCV or prevented PCV reduction associated with parasiteamia and thus ameliorated anemia. This was particularly noticed in the day 14 of treatment, in groups with significantly reduced parasitaemia, where the final PCV values were more or less similar to the pre-treatment values. Percentage PCV reduction was maximal in groups treated with the aqueous extracts, where parasitaemia was not comparatively kept lower (Figures 10 and 11). In the negative control and lower doses of aqueous extract treated mice, the PCV decreased markedly from day to day until the death of the animals as a result of higher parasitaemia level, which was also observed in a similar study by Abubakar *et al*., (2005).

The observed higher PCV value of animals treated with 200 and 400 mg/kg doses of the methanol extract of *V. sinaiticum* as compared to the negative control could be attributed to the potential antioxidant activity of the flavonoids and glycosides present in the leaves (Table 2) which was also confirmed by similar studies previously done on *V. sinaiticum* and related plant species from the same family (Akdemir *et al*., 2003; Akdemir *et al*., 2004).

Previous studies have shown the presence of the flavonoids: apigenin-7-glucoside, chrysoeriol-7-lucoside (Kawashty, 1997), luteolin-7- glucoside , sinaiticin, hydrocarpin (Afifi *et al*.,1993) and

the phenylethanoid glycoside: verbascoside (acteoside) (Elgindi *et al*., 2000; Tatli *et al*., 2004) in *V. sinaiticum*. Akdemir *et al*. (2003) and Akdemir *et al*. (2004) have shown antioxidant properties of the above compounds isolated from various fractions of the methanolic extract of *Verbascum macrurum (*Family Scrophulariaceae). In addition Hussain *et al*., 2009; Kumar and Tyagi, 2013 have accounted free radicals scavenging property of flavonoids and verbascosides (Yalcin *et al*., 2003)

The infected mice treated with the diminazine aceturate showed significant improvement in PCV. This is because the drug was able to eliminate parasites from the blood to levels detectable by microscopy on days 2-10 (Figures 10 and 11). This is in harmony with previous reports (Inabo and Fathuddin, 2011; Umar *et al*., 2010)

**5.6.3. Effect of extracts on body weight**

Based on our findings, animals in the negative control group lost 11.03% of their body weight which might be due to the significant drop in PCV associated with high level of parasitaemia in this group. The majority of trials provide evidence of the negative effect of trypanosomosis on body weight. During the high levels of parasitaemia the appetite is decreased and the animal losses condition as a result there is wasting. There is consumption of the fat reserves but there are also severe degenerative changes of the muscle cells and other tissue cells, and there is an increased breakdown of protein in muscles and elsewhere, leading to atrophic degeneration. The decreased supply of oxygen because of the anemia is also an important factor (Holmes, 2000; Yusuf *et al*., 2012).

The trypanosuppresive effect of the extracts against trypanosome infection can further be infered from the weight status of the treated animals. At day 14 post-treatment, animals that received

400 mg/kg dose of the aqueous and methanol extracts of *C. abyssinica* gained weight by 0.77% and 1.36%, respectively (Tables 13 and 14), while animals which received similar solvent extracts of *V. sinaiticum* at the same dose gained weight by 1.07% and 1.67 % respectively (Tables 15 and 16) which was satatistically significant ($p<0.001$) as compard to the negative control groups. This shows that as a result of reduction in parasitaemia and prevention of drop in PCV by the extracts physical status of the treated mice improved. They were therefore more able to resist weight loss that is usually associated with trypanosomosis. Similar observations have been made by other researchers (Abubakar *et al*., 2005; Adamu *et al.*, 2008; Ekanem *et al.*, 2008; Alli *et al.*, 2011; Inabo and Fathuddin, 2011; Umar *et al.*, 2010).

### 5.6.4. Effect of extracts on mean survival time

The results obtained during the monitoring period had shown that the higher dose (400mg/kg) of the methanol extracts of C. *abyssinica* and *V. sinaiticum* exhibited appreciable antitrypanosomal activity by reducing the level of parasitaemia and prolonging the lifespan of the test animals (37-40 days) beyond that of the negative control ($25.40 \pm 0.43$) by more than 10 days (Figures 13 and 14). This effect of the methanol extract of both plants on mean survival time of treated animals was superior as compared to the effect of the methanol extract of *Ximenia americana* at 100 and 200 mg/kg dose which prolonged survivability of *T. congolense* infected and treated mice for 30 days *(*Maikai, 2010). Prolongation in mean survival time may be attributed to the trypanosuppressive effect of the extracts.

# 6. CONCLUSION

The search for an active trypanocides from medicinal plants is one of the concern for many researchers. This study gave indications of *in vitro* and *in vivo* antitrypanosomal activity of methanol crude leaf extracts of *C. abyssinica* and *V. sinaiticum* against *T. congolence* field isolate. The work demonstrated good safety margin of the crude extracts of both plants in mice suggesting their ethnopharmacological usefulness.

The aqueous extracts of the two plants exhibited lower *in vitro* and *in vivo* antitrypanosomal activity, while the methanol extracts have shown better activity at higher concentration. However, both of the extracts were not effective enough to eradicate the parasites completely.

*In vitro*, the higher concentration (4 mg/ml) of the methanol extract of *C. abyssinica* showed superior activity by immobilizing trypanosome motility within 30 min and abrogated infectivity of trypanosomes to mice which remained aparasitaemic for 21 days after the inoculation. *In vivo* study in *T. congolense* infected swiss albino mice revealed that methanol leaf extract of *C. abyssinica* has promising effect by reducing pre-treatment parasitemia of infected mice by 3.91% (7.38$\pm$0.18), increasing PCV by 1.12% (48.66$\pm$0.20) on day 7-14 of treatment, while the methanol leaf extract of *V. sinaiticum* had showed potential activity by increasing pretreatment body weight of infected animals by 1.36% (22.34+0.27) and prolonging survival time of mice by 40.00$\pm$0.31 days.

Generally, the current study established that leaves of *C. abyssinica* and *V. sinaiticum* could have potential antitrypanosomal activity which can be considered as a potential source for the search of new drugs against Africal animal trypanosomosis.

# 7. RECOMMENDATION

The results of this study indicate that scientific studies carried out on medicinal plants having traditional claims of effectiveness might warrant fruitful results. Further studies on the following directions are recommended;

- Although the extracts of the plant appear to be safe under the preliminary study, further toxicological studies needs to be done to confirm absence of toxicity in repeated administration of the extracts.
- Further investigations aimed at isolation and identification of active substances from C. *abyssinica* and *V. sinaiticum* and evaluating their activity against trypanosomes
- The effect of the extracts on serum biochemical changes, hematological parameters other than PCV, total plasma protein of the experimental animals need to be investigated.

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# ANNEX I: Acute toxicity study: $LD_{50}$ values

## $LD_{50}$ for aqueous leaf extract of *Clutia abyssinica*

The first phase of the lethal dosage ($LD_{50}$) test (mortality expressed in %) for aqueous leaf extract of *Clutia abyssinica* using mice as test animal.

| 10 mg/kg body weight (%) | 100 mg/kg body weight (%) | 1000 mg/kg body weight (%) |
|---|---|---|
| 0/3(0%) | 0/3(0%) | 0/3(0%) |

**NB:** The numerator denotes number of animal which died while the denominator is the number of animals used.

The second phase of lethal dosage (LD50) test (mortality expressed in %) for aqueous leaf extract of *Clutia abyssinica* using mice as test animal.

| 1600 mg/kg body weight (%) | 2900 mg/kg body weight (%) | 5000 mg/kg body weight (%) |
|---|---|---|
| 0/3(0%) | 1/3(33.3%) | 2/3 (66.7%%) |

**NB:** The numerator denotes number of animal which died while the denominator is the number of animals used.

Lowest dose which killed one mouse (minimum toxic dose) =2900mg/kg

The highest dose which had not killed any mouse (maximum tolerated dose) = 1600mg/kg

$LD_{50} = \sqrt{(2900 X 1600)}$

***$LD_{50}$*=2154.065 mg/kg**

## $LD_{50}$ for Methanol leaf extract of *Clutia abyssinica*

The first phase of the lethal dosage ($LD_{50}$) test (mortality expressed in %) for methanol extract of *Clutia abyssinica* using mice as test animal.

| 10 mg/kg body weight (%) | 100 mg/kg body weight (%) | 1000 mg/kg body weight (%) |
|---|---|---|
| 0/3(0%) | 0/3(0%) | 0/3(0%) |

**NB:** The numerator denotes number of animal which died while the denominator is the number of animals used

The second phase of lethal dosage (LD50) test (mortality expressed in %) for methanol extract of *Clutia abyssinica* using mice as test animal

| 1600 mg/kg body weight (%) | 2900 mg/kg body weight (%) | 5000 mg/kg body weight (%) |
|---|---|---|
| 0/3(0%) | 0/3(0%) | 1/3(33.3%%) |

**NB:** The numerator denotes number of animal which died while the denominator is the number of animals used

Lowest dose which killed one mouse (minimum toxic dose) =5000mg/kg

The highest dose which had not killed any mouse (maximum tolerated dose) = 2900mg/kg

$LD_{50} = \sqrt{(5000 X 2900)}$

***$LD_{50}$*=3807.886 mg/kg**

**$LD_{50}$ for aqueous leaf extract of *Verbascum sinaiticum***

The first phase of the lethal dosage ($LD_{50}$) test (mortality expressed in %) for aqueous leaf extract of *Verbascum sinaiticum* using mice as test animal.

| 10 mg/kg body weight (%) | 100 mg/kg body weight (%) | 1000 mg/kg body weight (%) |
|---|---|---|
| 0/3(0%) | 0/3(0%) | 0/3(0%) |

**NB:** The numerator denotes number of animal which died while the denominator is the number of animals used.

The second phase of lethal dosage (LD50) test (mortality expressed in %) for aqueous leaf extract of *Verbascum sinaiticum* using mice as test animal.

| 1600 mg/kg body weight (%) | 2900 mg/kg body weight (%) | 5000 mg/kg body weight (%) |
|---|---|---|
| 0/3(0%) | 0/3(0%) | 1/3(33.3%) |

**NB:** The numerator denotes number of animal which died while the denominator is the number of animals used.

Lowest dose which killed one mouse (minimum toxic dose) =5000mg/kg

The highest dose which had not killed any mouse (maximum tolerated dose) = 2900mg/kg

$$LD_{50} = \sqrt{(5000 X 2900)}$$

***$LD_{50}$=3807.886* mg/kg**

**$LD_{50}$ for methanol leaf extract of *Verbascum sinaiticum***

The first phase of the lethal dosage ($LD_{50}$) test (mortality expressed in %) for methanol extract of *Verbascum sinaiticum* using mice as test animal.

| 10 mg/kg body weight (%) | 100 mg/kg body weight (%) | 1000 mg/kg body weight (%) |
|---|---|---|
| 0/3(0%) | 0/3(0%) | 0/3(0%) |

**NB:** The numerator denotes number of animal which died while the denominator is the number of animals used

The second phase of lethal dosage ($LD_{50}$) test (mortality expressed in %) for methanol extract of *Verbascum sinaiticum* using mice as test animal

| 1600 mg/kg body weight (%) | 2900 mg/kg body weight (%) | 5000 mg/kg body weight (%) |
|---|---|---|
| 0/3(0%) | 1/3(33.3%) | 1/3(33.3%%) |

**NB:** The numerator denotes number of animal which died while the denominator is the number of animals used

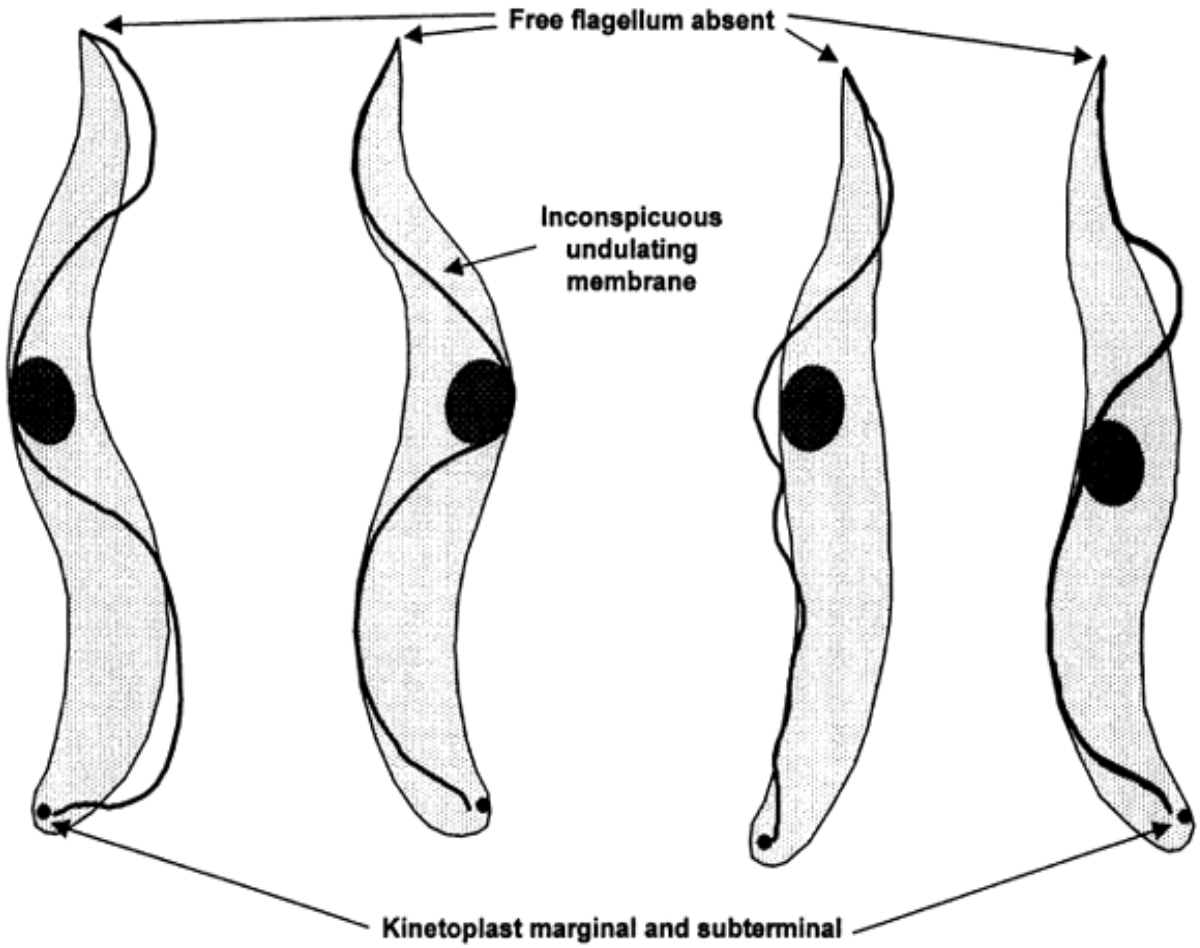
Lowest dose which killed one mouse (minimum toxic dose) =2900mg/kg

The highest dose which had not killed any mouse (maximum tolerated dose) = 1600mg/kg

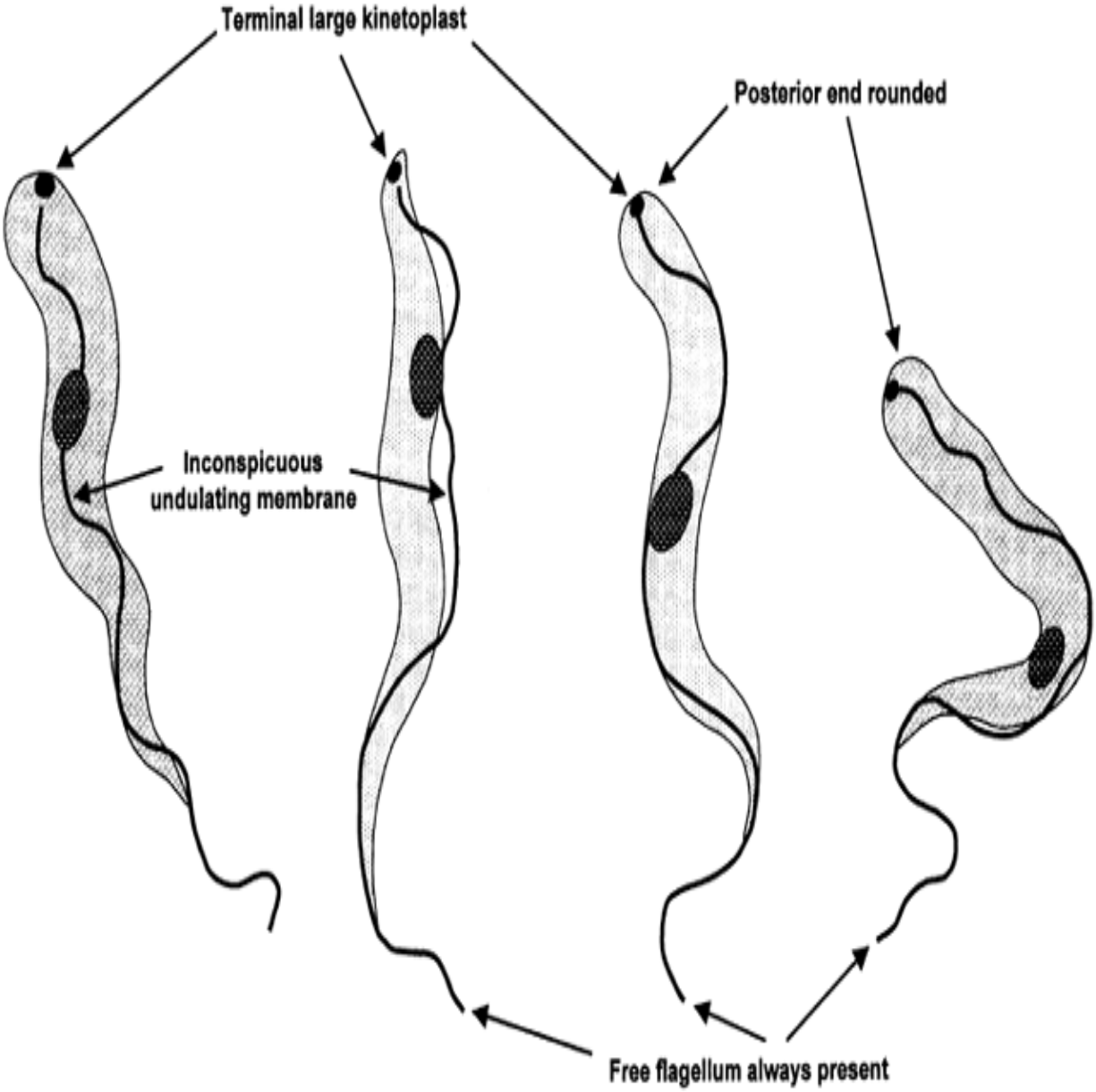
$$LD_{50} = \sqrt{(1600 X 2900)}$$

***$LD_{50}$*=2154.065 mg/kg**

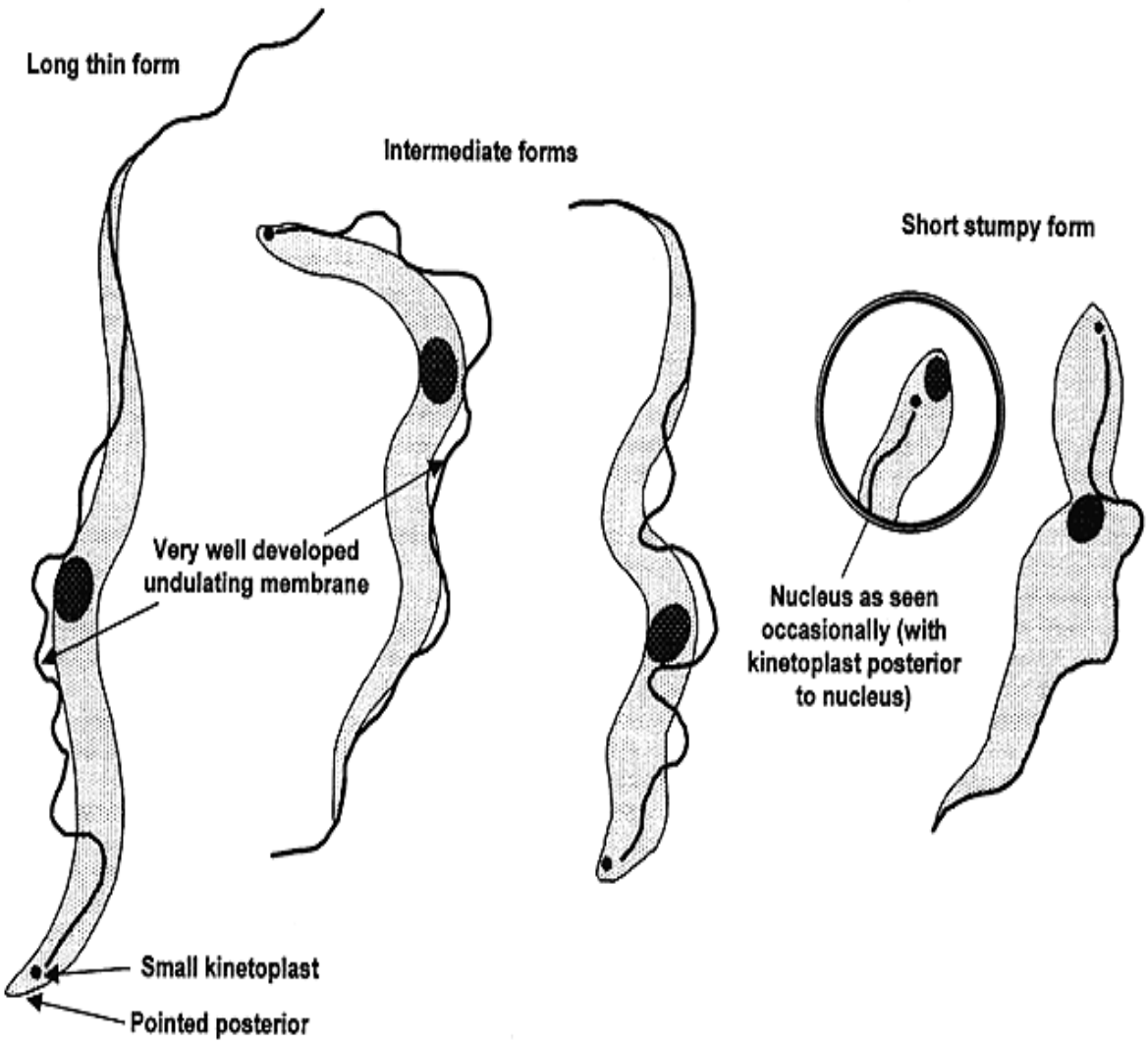
## ANNEX II: Differential morphology of trypanosomes as seen in a stained blood smear




*A. Trypanosoma congolense* blood stream forms




*B. Trypanosoma vivax* blood stream forms




C. *Trypanosoma brucei* blood stream forms

## Annex III Rapid Matching method of Herbert and Lumsden (1976)

Chart showing rationale of computation of values for wet films of blood from mice infected with trypanosomes viewed under X400 Magnification (Herbert and Lumsden, 1976)

| Organisms per field | | Equivalent log number of organisms per milliliter of blood | |
|---|---|---|---|
| | >256 | >9.0 | |
| M | 256 | 9.0 | |
| A | 128 | 8.7 | |
| T | 64 | 8.4 | |
| C | 32 | 8.1 | Reference point |
| H | 16 | 7.8 | |
| I | 8 | 7.5 | |
| N | 4 | 7.2 | |
| G | 2 | 6.9 | |

| Organisms in | | | | | |
|---|---|---|---|---|---|
| 20 fields | 10 fields | 5 fields | | | |
| | | | C | | |
| | | 4–5 | O | 1 | 6.6 |
| | | 2–3 | U | 0.5 | 6.3 |
| | 2–3 | | N | 0.25 | 6.0 |
| 2–3 | | | T | 0.125 | 5.7 |
| 1 | | | I | 0.0625 | 5.4 |
| 0 | | | N | <0.0625 | <5.4 |
| | | | G | | |