In Vitro and *In Vivo* Anti-trypanosomal Activity of Dichloromethane and Methanol Crude Leaf Extracts of *Dovyalis abyssinica* (*Flacourtiaceae*) against *Trypanosoma congolense* Field Isolate

Belay Tadesse

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Name	Signature	Date
Prof. Getachew Abebe (Examiner)	_____	_____
Prof. Emmanuel Onaivi (Examiner)	_____	_____
Dr. Workineh Shibeshi (Advisor)	_____	_____
Dr. Getachew Terefe (Advisor)	_____	_____
Dr. Nigatu Kebede (Advisor)	_____	_____

Dr. Workineh Shibeshi _____

(Chairman of Department)

Abstract

In vitro and *in vivo* anti-trypanosomal activity of dichloromethane and methanol crude leaf extracts of *Dovyalis abyssinica* (*Flacourtiaceae*) against *Trypanosoma congolense* field isolate

Belay Tadesse

Addis Ababa University, 2014

African Trypanosomosis is a neglected tropical disease of medical and veterinary importance. Parasite control relies on the use of few drugs whose resistance and unacceptable toxicities urged the investigation of new agents, preferably from natural sources. Though *Dovyalis abyssinica* has been reported to posses significant trypanocidal activity on *Trypanosoma brucei* in *in vitro* model, activity on trypanosome infected laboratory animals has not yet been worked out. The aim of the present study was, therefore, to investigate the *in vitro* and *in vivo* activity of *D. abyssinica* on *T. congolense* field isolate. To evaluate the effect on motility, 200 µl *T. congolense* infected blood was mixed with 50 µl of 20, 10, 2, 0.1, 0.015 mg/ml dichloromethane and methanol extracts. Reduction or cessation of motility was then microscopically monitored for 120 minutes, and the remaining *in vitro* mixtures were inoculated to healthy mice and monitored for development of infection for 21 days. Furthermore, fifty *T. congolense* infected mice were randomly grouped into ten groups of five and administered with curative doses (250, 200, 150 and 100 mg/kg) of dichloromethane and methanol and 28 mg/kg diminazene aceturate and dimethylsulfoxide. Following administration, parasitemia, packed cell volume (PCV), rectal temperature, body weight and survival time were

monitored. Suppressive doses of the extracts (250 and 200 mg/kg) were administered 24 hours post-infection and parasitemia was monitored. Dichloromethane and methanol extracts at 20, 10, 2 mg/ml concentrations ceased parasite motility within one hour and eliminated subsequent infectivity in mice for 21 days. Administration of dichloromethane and methanol extracts at 250 and 200 mg/kg reduced ($p<0.05$) parasitemia and rectal temperature, and improved ($p<0.05$) PCV, mean body weight, and mean survival time compared to DMSO treatment. In conclusion, *D. abyssinica* at higher concentrations *in vitro* and higher curative doses *in vivo* in mice posses anti-trypanosomal activity.

Keywords: Anti-trypanosomal, *D. abyssinica*, *in vitro, in vivo, T. congolense,* mice.

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Abbreviations and Acronyms

%	percentage
^{0}C	degree celsius
A.C	after Christ
ANOVA	analysis of variance
AW-IPM	area-wide integrated pest management
B.C	before Christ
BBIQ	bisbenzylisoquinoline
BDH	British drug house
BW	body weight
cm	centimeter
CSF	cerebrospinal fluid
DA	diminazene aceturate
DCM	dichloromethane
DDT	dichlorodiphenyltrichloroethane
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylene di-amine tetra acetic acid
g	gram
G3PDH	glyceraldehydes-3-phosphate dehydrogenase
h	height
HL	human promyelocytic leukemia cells

IC50	50% inhibitory concentration
IFN-γ	interferon gamma
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
IP	intraperitoneal
kg	kilogram
km^2	square kilometers
LD	lethal dose
L-G3P	l-glycerol-3-phosphate
m	meter
MBW	mean body weight
mg	milligram
mg/kg	milligram per kilogram
mg/ml	milligram per milliliter
MIC	minimum inhibitory concentration
ml	milliliter
MOH	methanol
^{o} N	degree north
^{o} S	degree south
PBS	phosphate buffered saline
PBSG	phosphate buffered saline glucose
PCV	packed cell volume

PI	post-infection
RBCs	red blood cells
RNA	ribonucleic acid
rpm	revolutions per minute
Rx	treatment
SAT	sequential aerosol technique
SEM	standard error of mean
SHAM	salicyl hydroxamine acid
SI	selectivity index
SIRS	systemic inflammatory response syndrome
SIT	sterile insect technique
SPSS	statistical package for social science
Spp	species
STEP	southern rift valley project
TAO	trypanosome alternative oxidase
UK	United Kingdom
US$	United States dollar
VSG	variable surface glycoprotein
x	magnification
β	beta
μg/mL	microgram per milliliter
µm	micro meter

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1 INTRODUCTION

1.1 Overview

African trypanosomes are single-cell, extracellular blood parasites that cause disease and death in humans and livestock. *Trypanosoma brucei gambiense* and *T. b. rhodesiense* cause sleeping sickness in humans. *T. congolense, T. b. brucei* and *T. vivax* are pathogens for cattle, sheep and goats (Abbeele and Rotureau, 2013). *T. equiperdum* and *T. evansi* cause trypanosomosis in horses and camels, respectively (Samia et al., 2012).

African trypanosomes of major economic and human health importance are mainly transmitted to mammalian hosts by tsetse flies. The occurrence of animal trypanosomosis coincides with the distribution of tsetse fly vectors which includes the regions between latitudes 14°N and 29°S. About 10 million km^2 and 37 countries are covered in this tsetse fly "belt" (OIE, 2013).

African animal trypanosomosis causes a great economic loss in the livestock industry with an estimated 3 million cattle death annually. Estimated direct production losses in cattle to African farmers are between 1-4 billion US dollars per year (Swallow, 2000).

Commonly employed control methods of trypanosomosis are chemotherapy and control of tsetse fly vectors. Vaccination is considered to be one of the best methods of controlling infections. However, continuous antigenic variation and memory β-cell destruction by trypanosomes limited the development of viable vaccines (Magez et al., 2010).

Despite the development of vector control tools and vaccine candidates over the years, the control of the disease has continued to rely heavily on the use of trypanocidal drugs. Nowadays, Chemotherapy and Chemoprophylaxis are the only means of combating the disease. For the treatment of African animal trypanosomosis three products are on market: Diminazene aceturate, isometamidium chloride and the homidium salts (Homidium bromide - ethidium and Homidium chloride - novidium). However, effectiveness of these drugs is limited by factors such as parasite resistance (Afewerk et al., 2000; Melaku and Birasa, 2013), unacceptable toxicity (Onyekwelu, 1999), treatment failures and logistics of administration. Essentially, the high cost of developing new drugs, with little hope of financial return for their investment in research and development, are serious disincentives for most pharmaceutical companies. These along with the far prospect of vaccine development (Nok, 2005), therefore, urgently necessitated the development of alternative new molecules that are safe, effective and affordable. In this respect, literature surveys and field studies have shown that plants are used in traditional medicine in Africa to treat trypanosomes in humans and animals (Freiburghaus et al., 1996; Nibret and Wink, 2011). Therefore, the current study was conducted to assess the *in vitro* and *in vivo* effects of *D. abyssinica* on *T. congolense* field isolate.

1.2 African Trypanosomes

1.2.1 Classification, Morphology and Characterization of African Trypanosomes

African trypanosomes are flagellated protozoal parasites belong to the class Zoomastigophores, order Kinetoplastida and family Trypanosomatidae. The pathogenic

trypanosomes belong to the genus *Trypanosoma.* The subgenus *Trypanosoma* is divided into two sections on the basis of their life cycle in the insect vector and the mode of transmission (Hoare, 1972).

The section salivaria consists of the trypanosomes that complete their developmental cycle in the salivary glands of their insect vector. Transmission of the parasite to the mammalian host occurs by bite of tsetse flies during a blood meal. This section consists of four subgroups; Duttonella (*T. vivax*), Nannomonas (*T. congolense, T. simiae, T. godfreyi*), Trypanozoon (*T. b. brucei, T. evansi, T. equiperdum*) and Pycnomonas (*T. suis*). They are important pathogens either to man or to domestic animals. The section stercoraria consists of groups that complete their developmental cycle in the hindgut of the insect vector. *T. cruzi* (Schizotrypanum), which causes Chagas disease in man in South and Central America, belongs to this section. Infection by such parasites occurs through contamination by the feces of wounds and damaged skins (Hughes and Piontkivska, 2003).

African trypanosomes are characterized on the basis of their size, shape, position of the nucleus, size and location of the kinetoplast, host range and geographical distribution. Generally they are elongated, spindle-shape organisms with a single flagellum. The flagellum originates from the basal body near the kinetoplast and runs the length of the trypanosome. The pellicle, the layer bordering the cytoplasm, while maintaining a definite shape, is flexible enough to permit a certain degree of body movement. The pellicle and the cytoplasm are pinched up into a thin sheet of tissue along the length of the body forming the undulating membrane (WHO, 1998).

African trypanosomes are 8-50 μm in length (WHO, 1998). *T. congolense* is a small monomorphic organism with variable length of 8-24 μm (Soltys, 1987). It has a central nucleus and a medium sized marginal kinetoplast and no flagellum. The kinetoplast has important functions in reproduction and metabolism. *T. congolense* has sluggish movements and has an undulating membrane (WHO, 1998).

1.2.2 Life Cycle

Transmission of African trypanosomes is mediated by tsetse flies (*Glossina* species). The genus *Glossina* consists of three groups called *fusca* (forest group), *palpalis* (riverine group), and *morsitans* (savannah group) (Mramba et al., 2013). These three groups have more than 30 species and subspecies and *T. congolense* is capable of developing in all of these species and subspecies. The tsetse fly acquires a trypanosomal infection during a regular blood meal from an infected mammalian host. The ingested parasites lose their variable surface glycoprotein (VSG) and undergo a series of developmental and morphological changes resulting in multiplication in the hindgut of the fly (Matthews, 2005). They migrate interiorly through the cell linings of the gut wall and up to the esophagus of the insect and to the hypopharynx where they transform into the epimastigote form. The epimastigotes migrate into the mouth pads of the proboscis and transform into metacyclic trypanosomes (Coustou et al., 2010). This transformation is associated with the regaining of the lost VSG (Fig 1). The position of the kinetoplast relative to the posterior end of the cell is the most obvious morphological difference between the different life cycle stages of the trypanosome (Matthews, 2005).

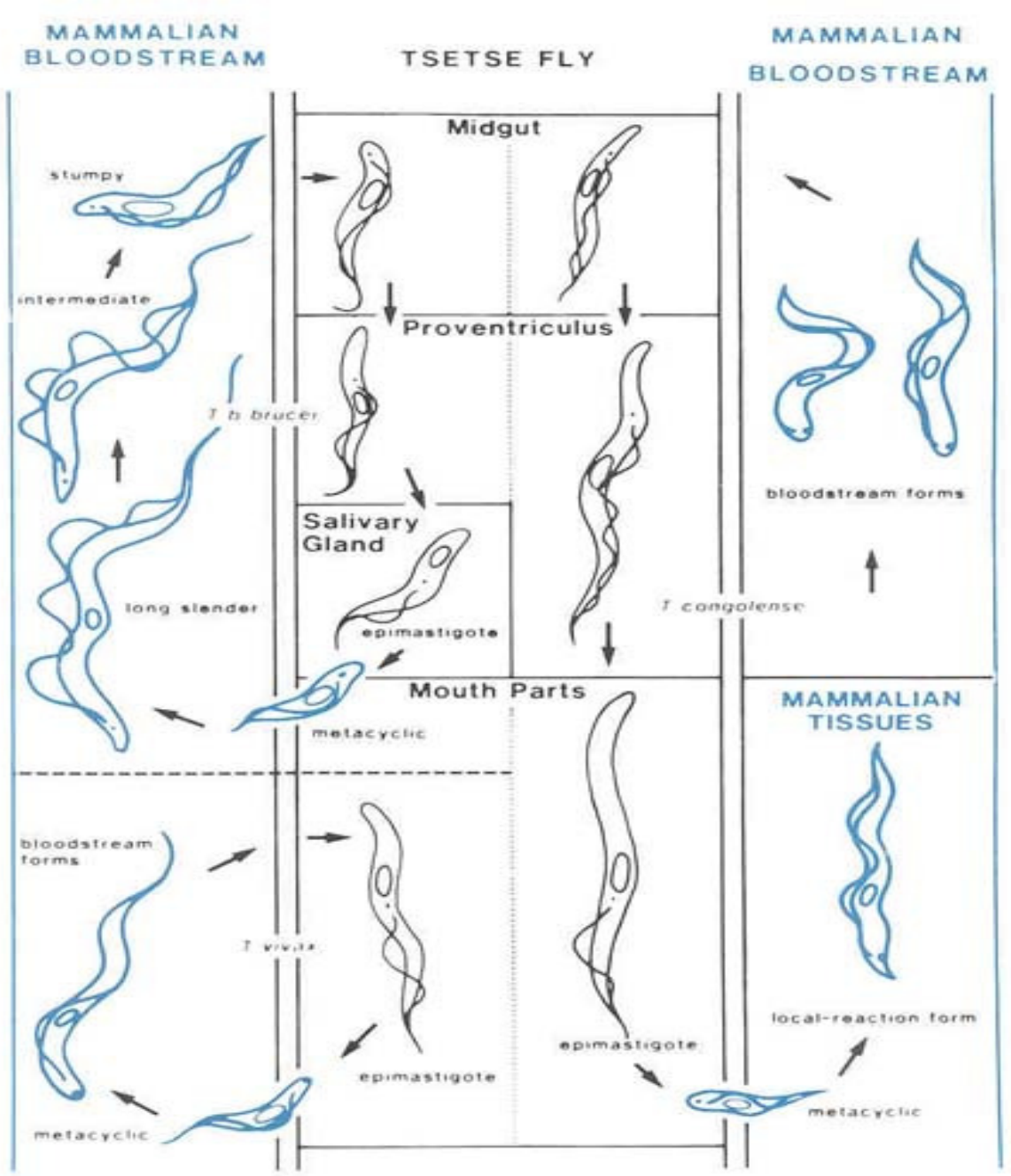


Figure 1: Life cycle of Animal *Trypanosomes* (Source: trypanosomosis - International Livestock Research Institute. http://www.ilri.org/InfoServ/Webpub/fulldocs/Ilrad88/Trypanosomosis.htm)

Infection of the mammalian host occurs when the insect vector deposits metacyclic trypanosomes within the dermal connective tissue of the animal during a blood meal. At the site of infection the trypanosomes multiply extensively as typical blood forms within

a few days, resulting in a raised cutaneous swelling called a chancre (Akol and Murray, 1982). These trypanomastigotes spread from the chancre via the blood and local lymph vessels and enter to the blood stream (WHO, 1998).

1.2.3 Pathogenesis

The mechanisms of pathogenesis of trypanosomosis are not clearly understood. Infection triggers the production of trypanosome-specific and non-specific IgM and/or IgG antibodies plus complement by cytokine activation of β-lymphocytes. Specific antibodies neutralize the corresponding trypanosomes to produce immune complexes in several tissues. Some of these antibodies are also raised against auto-antigens, corresponding to aberrant non-specific polyclonal activation of β-lymphocytes producing natural auto-antibodies and also to antigen-driven antibodies induced by molecular mimicry. Furthermore, VSG is associated with major cytokine dysfunction (Malvy and Chappuis, 2011). Highly susceptible BALB/c mice infected with *T. congolense* die of a systemic inflammatory response syndrome (SIRS) that is mediated by IFN-γ (Shi et al., 2003; Shi et al., 2005). This SIRS was associated with elevated plasma levels of IL-6, IL-12p40, IL-10, and IFN-γ. Focal liver lesions of apoptotic parenchymal cells, 5-fold enlargement of Kupffer cells, apoptosis of 10% of Kupffer cells, enlarged capillary bed, hypotension, piloerection, hypomotility and death were also associated with SIRS (Shi et al., 2006).

1.2.4 Course of Infection

Various strains of trypanosomes can cause a wide range of clinical responses ranging from acute to chronic syndromes. Acute infection of *T. congolense* in ruminants is associated with intermittent fever, depression, anemia, subcutaneous edema of the

mandible and prominent jugular pulse. The appetite is decreased and there is a rapid weight loss. Chronic syndromes often results in extreme emaciation and anemia (WHO, 1998).

Anemia is recognized as the most important clinical manifestation of animal trypanosomosis. Mice infected with *T. congolense* develops a severe anemia one week after infection which is accompanied by a marked increase in the plasma levels of acute phase proteins such as serum amyloid P component and haptoglobin (Ngure et al., 2008). Fever, a typical symptom of trypanosomosis, reflects the response to successive waves of parasitemia. The body temperature set point in the hypothalamus is then changed under the influence of pyrogenic stimuli released during infection. During the acute stage, the appetite is variable, being decreased during the fever peaks. But in the chronic stage, when the fever reactions are less pronounced, the appetite is usually normal, almost until death, even when extreme weakness prevents the animal from rising. The pronounced wasting is therefore not caused by starvation. There is consumption of the fat reserves during the recurrent bouts of fever (Pathak, 2009).

1.3 Control and Prevention of African Animal Trypanosomosis

Over the past century numerous control methods have been developed yet the disease has proved very difficult to eradicate. Current methods to control the disease, in the absence of a vaccine, rely on the use of anti-trypanosomal drugs and vector control. In animals, an additional control method is the use of trypanotolerant breeds of livestock. Unfortunately, all of these methods have disadvantages and none has proved to be ideal (Adamu et al., 2011).

1.3.1 Vector Control and Host Management Methods

Ground spraying uses residual insecticides (e.g. DDT, dieldrin, endosulfan) which target the tsetse resting sites. Because of the negative effects on the environment these persistent insecticides are largely replaced by the less toxic synthetic pyrethroids (Adamu et al., 2011). These products are also used for the sequential aerosol technique (SAT). Insecticides can also be applied on live animals by spraying or pour-on. Many African farmers use pour-on insecticides because they can be easily and rapidly applied without any sophisticated equipment (Leak et al., 1995). A large variety of traps, targets and screens (impregnated with pyrethroid insecticides) has been developed to attract and kill tsetse flies (Adamu et al., 2011).

The Sterile Insect Technique (SIT) consists of the release of irradiated sterile male flies so that up on mating with a virgin female fly, this results in no offspring because, female tsetse usually mates only once in its life (Feldman, 2004). The technique requires the release at a proportion of at least 10:1 (sterile to wild) male so that they are able to compete with the wild male flies. It is a very expensive technique. Besides, with the exception of Zanzibar, there are very few success stories of the use of SIT. Hence it is generally agreed that SIT makes only sense as part of Area-Wide Integrated Pest Management (AW-IPM) of tsetse, which aims at the sustainable removal of an entire tsetse fly population within a delimited geographical area (Feldman, 2004). Since 1997 the Ethiopian government initiated a Southern Rift Valley Project (STEP) to create a tsetse-free zone agricultural area and to develop national capacity to apply the concept of AW-IPM with SIT component to other tsetse infested parts of the country (Alemu et al., 2007).

Trypanotolerance is host-related characteristic of some livestock breeds allowing them to survive, reproduce and remain productive under trypanosomosis risk without the aid of trypanocidal drugs. Cattle in West and central Africa, mainly the N'Dama and West African Shorthorn, along with their sheep (Djallonke) and goat (Dwarf West African) counterparts constitute trypanotolerant breeds (Adamu et al., 2011). In Ethiopia four cattle breeds Abigar, Gurage, Horro and Sheko live in infected areas and could possess trypanotolerance. Among these Sheko cattle stand out as the most trypanotolerant animals; they rarely get infected by trypanosomes, and have good PCV, production and reproduction (Stein, 2011).

Currently, integrated strategies consist of vector control and treatment of existing cases has been adopted. Hence combining Viz-drugs, diagnostic test, vector and socio-economic control as package of tools permit integrated control on the vector and the disease (Adamu et al., 2011).

Recently, genetic-modification strategies are being developed to reduce the transmission of African trypanosomosis. For tsetse, a paratransgenic strategy is being considered. This approach involves modification of the commensal symbiotic bacteria *Sodalis* to express trypanosome resistance-conferring products. Modified *Sodalis* can then be driven into the tsetse population by cytoplasmic incompatibility (CI) from *Wolbachia* bacteria (Medlock et al., 2013).

1.3.2 Anti-trypanosomal Vaccines

Vaccination is considered to be one of the best methods of controlling infections. It is widely believed that the ability of African trypanosomes to continually express

antigenically distinct VSG genes (Magez et al., 2010) reduces the likelihood of the development of an effective VSG-based vaccine. Therefore, invariant parasite antigens were being considered as vaccine candidates but only partial and minimal cross-protection has been reported so far (Mkunza et al., 1995). While infections of cattle with *T. congolense* would probably benefit from an IgG vaccine memory response, the data available to date from mice infected with *T. brucei* suggests that an efficient anti-trypanosome vaccine should most likely be based on the induction of a high affinity IgM memory response. In both models, however, the maintenance of high circulating anti-trypanosome antibody titres in the absence of parasite antigen might allow the immediate elimination of metacyclic parasite upon entry in the body, thereby avoiding the potential initiation of active β-lymphocytes memory destruction by living and dividing parasites (Magez et al., 2010). In particular, this last requirement appears extremely hard to achieve and hence the 28 year-old conclusion of Cornellissen and colleagues (1985) that 'if the interpretation of the data is correct, then vaccination prospects are not good', remains up-to date.

1.4 Chemotherapy of African Animal Trypanosomosis

1.4.1 Drugs Approved for the Treatment of African Animal Trypanosomosis

Diminazene, an aromatic diamidine derived from surfen C, is marketed as the diaceturate salt and consists of two amidinophenyl moieties linked by a triazene bridge p,p-diamidinodiazo-aminbenzene diaceturate tetrahydrateand N-1,3-diamidinophenyltriazine diaceturate tetrahydrate. Diminazene has subsequently become the most commonly used therapeutic agent for trypanosomosis in domestic livestock. It is highly effective against Babessia spp., *T. congolense* and *T. vivax*, but less effective against *T. b. brucei* and *T.*

evansi infections. Trypanosomes resistant to other drugs (except quinapramine) are commonly susceptible to diminazene (Peregrine and Mamman, 1993). Diminazne interferes with nucleic acid synthesis and binds to DNA *in vitro* (particularly to kinetoplast DNA) by a non-inercalative mechanism, thereby blocking DNA and RNA synthesis (Mehlhom, 2008). It appears to enter *T. b. brucei* group parasites via the high affinity pentamidine P2 nucleoside transporter that is also capable of transporting other diamidines and melamine-based arsenicals (Ibrahim et al., 2011).

Isometamidium (Samorin, Trypamidium) and **Homidium** (chloride salt; Novidium; bromide salt or ethidium bromide: Ethidium) are phenanthridinium compounds. Isomethamidium differs from homidium by an additional moiety of m-amidiophenyl-azo-amine which in fact is part of the diminazene molecule. Both isomethamidium and homidium are effective against *T. congolense* and *T. vivax*. Additionally, isometamidium is also of value against infections caused by *T. b. brucei* and *T. evansi* infections in donkeys, horses and camels. Homidium was extensively used in the 1960s and 1970s but its usefulness has been greatly reduced due to widespread resistance (Scott and Pegram, 1974). The primary mode of action of phenanthridinium drugs is blockade of nucleic acid synthesis through interaction between DNA and RNA (Lantz and Van Dyke, 1972). The mechanism of resistance to isomethamidium is associated with reduced accumulation of the drug in the parasite (Sutherland and Holmes, 1993).

1.4.2 Problems of Current Chemotherapy and Constraints to Develop New Drugs

Drug toxicity and drug resistance presented themselves as problems right from the time of discovery of trypanocidal drugs in the early part of the last century (Maser et al.,

2003). Diminazene acetuate attacks the central nervous system, whereby tremor, nystagmus, ataxia, shaking cramps, vomiting and even death can occur. The main adverse drug reactions seen in animals treated with diminazene aceturate are severe cerebral haemorrages (Holmes et al., 2004). One of the major adverse properties of phenanthridium drugs, particularly isometamidium, is tissue damage at injection site (Kinabo and Bogan, 1988). Although ethidium is mutagenic and should be withdrawn from the market, it is still widely used in East Africa (Geerts et al., 2010).

Treatment of affected animals with trypanocidal drugs 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 (Delespaux et al., 2002). Trypanocidal drug resistance is increasingly reported all over Africa and is now present in 21 sub-Saharan countries (Geerts et al., 2010; Chitanga et al., 2011). Previous studies have shown the prevalence of drug resistant trypanosomes in cattle herd of Ethiopia. Scott and Pegram (1974) described the occurrence of homidium- resistant population of *T. congolense* in Didessa and Angar valleys in Wollega province. Afewerk (1998) reported that *T. congolense* field isolates from Metekel region, expressed resistance to both isometamidium chloride and diminazene aceturate in mice. Meanwhile, Mesfin Ademe and Getachew Abebe (2000) identified population of trypanosomes in North Omo, which express resistance to both isometamidium chloride and diminazene aceturate. Afewerk et al. (2000) has confirmed multiple drug resistance in cloned *T. congolense.* However, farmers continue to use the drugs because alternative products are not available (Delespaux et al., 2010; Chitanga et al., 2011).

The disease represents a major public health problem in regions of the world least able to deal with the associated economic burden. The infection is transmitted only in rural Africa and does not have a profitable market that would encourage drug development because the purchasing power of the affected consumers is poor and rapidly deteriorating (Kabayo and Boussaha, 2002; Moore, 2005). Therefore, the high cost of developing new drugs, with little hope of a reasonable financial return for their investment in research and development, are serious disincentives for most pharmaceutical companies. The research for new trypanocidal drugs, with most research and development occurring in academic settings, has been extended to indigenous medicinal plants (Mbaya and Ibrahim, 2011). However, these investigations are in their infant stages.

1.5 Herbal Products in African Animal Trypanosomosis

1.5.1 Herbal Drugs: Historical, Current and Future Perspectives

The history of herbal medicine is old and dates back to the time when the early man became conscious of his environment. Since then, medicinal plants have been used virtually in all cultures as a source of medicine (Lanfranco, 1999).

Many conventional drugs were obtained from plants which were initially used in crude form in folk healing practice (Mourice et al., 1999). Beginning with the discovery of pure compounds from plants, the art of exploiting drugs from natural products became part of the molecular sciences. Fats and oils, nitrogen compounds, terpenoids and other plant secondary metabolites were among active compounds discovered and exhibited diverse bioactivities such as antioxidant, antinflammatory, antitumor, antimutagenic, anticarcinogenic, antiprotozoal, antifungal, antibacterial or antiviral. Some of the drugs

included aspirin (from willow bark), digoxin (from foxglove), quinine (from cinchona bark), taxol (from Pacific yew tree) and morphine (from opium poppy) (Vickers and Zollman, 1999). Generally, natural products (their derivatives and analogs) represent over 50% of all drugs in clinical use (Pan et al., 2013), with higher plant derived natural products representing 25% of them (Chin et al., 2006). About 250,000 to 500,000 species of higher plants are estimated to be present on earth. But only small proportions (1-10%) of these are used either as foods or medicine both by humans and other animals. Only few (<10%) of these has been systematically investigated for the presence of bioactive compounds (Cowan, 1999). Diversity in class of compounds that plants contain still makes them indispensable sources of novel and effective drugs (Paterson and Anderson, 2005).

In Ethiopia about 80% of human and 90% of livestock depend on traditional herbal medicine for treatment of various ailments (Birhan et al., 2011). The Ethiopian flora is also estimated to contain between 6,500 and 7,000 species of higher plants of which about 12% are endemic (Regassa, 2013). Therefore scientific evaluation of these plants may provide modern medicine with lead compounds for the development of new drugs.

1.5.2 Herbal Anti-trypanosomal Agents

Several researches were made on anti-trypanosomal activity of crude or fractionated extracts derived from plants *in vitro* against *T. congolense, T. brucei,* or *in vivo* against one of these trypanosomes infected experimental animals. Here some of the works are reviewed.

Methanol extracts of *Khaya senegalensis, Piliostigma reticulatum, Securidaca longepedunculata* and *Terminalia avicennoides* were strongly trypanocidal *in vitro* at concentrations of 4, 0.4 and 0.04 mg/ml to both *T. b. brucei* or *T. congolense* while extracts of *Anchomanes difformis, Cassytha spp, Lannea kerstingii, Parkia clappertioniana, Striga spp, Adansonia digitata* and *Prosopis africana* were trypanocidal to either of the organisms (Atawodi et al., 2003).

Among the pyrrolizidine alkaloid-producing plants of Ethiopia the dichloromethane extract of *Solanecio angulatus* (flowers) and *Crotalaria phillipsiae* (twigs), with IC50 values of 12.17 mg/ml and 12.67 mg/ml on *T. b. brucei in vitro*, were shown to posses activity and proposed as sources of novel trypanocidal compounds (Nibret et al., 2009).

The *in vitro* and *in vivo* anti-trypanosomal activity of aqueous and methanol extracts of bark of *Ximenia americana* on *T. congolense* were evaluated. Results showed that *in vitro* incubation of infected blood with methanol extract immobilized the parasites at 9 mg/ml. *In vivo* study showed the aqueous extract at 50, 100, 200 and 300mg/kg treated groups were parasite free for 1, 5, 7 and 14 days, respectively (Maikai, 2010).

Methanol extract of the leaves, stem bark and root bark of *Boswellia dalzielii* at 20 mg/ml caused *T. b. brucei* motility to cease after 35, 20 and 20 min, respectively. Chloroform extract of root bark of the plant ceased motility after 40 min at 20 mg/ml. *In vivo* activity of methanol extract of the plant on *T. b. brucei* infected mice showed consistent parasitemic suppressions at 300 mg/kg with extract from the leaves displaying highest activity (Atawodi et al., 2011).

Methanol extract of stem bark of *Ximennia americana* were partitioned to obtain their major phytochemical fractions and evaluated for their *in vitro* and *in vivo* anti-trypanosomal activities against *T. congolense*. The flavonoid fraction inhibited the motility of the blood stream forms at 10mg/ml. When the infected mice were treated with 5, 10 and 25mg/ml of the flavonoid fractions, suppression of the growth of the parasites was dose dependent (Maikai, 2011).

Trypanocidal and cytotoxic effects of dichloromethane and methanol extracts of traditionally used 30 Ethiopian medicinal plants species were evaluated on both bloodstream forms of *T. b. brucei* and human leukaemia HL-60 cells. Of all, dichloromethane extracts from five plants showed trypanocidal activity: *Dovyalis abyssinica (Flacourtiaceae); Albizia schimperiana (Fabaceae); Ocimum urticifolium (Lamiaceae); Acokanthera schimperi (Apocynaceae); and Chenopodium ambrosioides (Chenopodiaceae);* with IC50 of 1.4, 7.2, 14.0, 16.6 and 17.1 μg/ml, respectively. The researchers speculated that *D. abyssinica* might be a promising candidate for phytotherapy of trypanosomosis (Nibret and Wink, 2011).

Crude methanol extract of *Combretum racemosum* leaves exhibited *in vitro* activity by immobilizing *T. b. brucei* and rendering them uninfective to mice at 125 to 0.2559 mg/ml. The extract also reduced parasitemia and improved PCV in infected mice at dose of 50, 100 and 200 mg/kg body weight (Eze et al., 2012).

Feyera and his colleagues (2012) reported on the anti-trypanosomal activity of crude dichloromethane and methanol aerial part extracts of *Artemisia abyssinica.* According to their findings, the plant reduced motility *in vitro,* and eliminated infectivity and mitigated

infection upon *in vivo* administration of the extracts to mice infected with *T. congolense* field isolates.

Effect of methanol extract of *Vernonia amygdalina* leaf was investigated in acute *T. b. brucei* infection. Although none of the extracts tested cured infected mice, the methanol extract of *V. amygdalina* leaf at a dose of 300 mg/kg/day increased the maximum survival days of the mice to 24 days compared to 8 days for infected control, and also temporarily cleared the parasites from circulation for up to 72 h before relapse occurred (Yusuf et al., 2012).

Methanol extract of *E. camaldulensis* (leaf) administered to mice infected with *T. b. brucei* produced complete cure for the animals in the different dose groups and survived as long as those treated with the diminazene aceturate, although the clearance time was faster for the standard drug. Sub inoculation of healthy mice with the blood and CSF of the cured mice did not result in infection, thus indicating a complete and permanent cure (Kabiru et al., 2013).

1.5.3 Natural Products Active against African Trypanosomes

a) Alkaloids

The quinoline alkaloids from cinchona bark; emetine, an isoquinoline alkaloid from *Cephaelis ipecacuanha (Rubiaceae)*; Berberine and sanguinarine, two quaternary benzylisoquinoline alkaloids, and berbamine, a bisbenzylisoquinoline (BBIQ) were found to be trypanocidal. DNA intercalation in combination with the inhibition of protein synthesis could be responsible for the observed effects of these alkaloids. Dioncophyllines, monomeric Naphthylisoquinoline alkaloids; three aporphines alkaloids,

actinodaphnine, cassythine and dicentrine, isolated from *Cassytha filiformis (Lauraceae)* are also active trypanocidal. The activity of these aporphines, which intercalate into DNA, seemed to be related to their ability to stabilize the DNA helix against heat denaturation and to inhibit the catalytic activity of topoisomerase I. Lepadins isolated from a tunicate species from the genus *Didemnum*, possess an unusual decahydroquinoline skeleton and show significant anti-trypanosomal activity (Hoet et al., 2004).

b) Phenolic Compounds

Ascofuranone, a prenylated phenol antibiotic isolated from *Ascochyta visiae*, is a potent and specific inhibitor of the glycerol-3-phosphate-dependent mitochondrial oxygen consumption of trypanosomes. The mechanism of action is attributed to its binding at the coenzyme Q site of the ubiquinol oxidase, thus blocking the trypanosome alternative oxidase (TAO). It has been demonstrated that in combination with glycerol, which suppresses a glycerol-producing anaerobic pathway by mass action, inhibitors of the TAO become trypanocidal due to a total block of the energy production of bloodstream forms. Gallic acid, a well-known component of hydrolysable tannins, is equally active on the bloodstream and procyclic forms of *T. b. brucei.* Gallic acid-induced trypanocidal activity might be due to the formation of reactive oxygen species (such as the superoxide anion), acting as a pro-oxidant (Hoet et al., 2004).

c) Flavonoids

Flavonoids may target the replicating forms of trypanosomes which are totally dependent on glycolysis for energy production (Opperdoes, 1987). The mitochondrial respiration of

the long slender forms in the mammalian host is limited to oxidizing L-G3P mediated electron transport system composed of Glyceraldehyde 3 phosphate dehydrogenase (G3PDH) and cyanide insensitive and Salicyl hydroxamine acid (SHAM) – sensitive trypanosome alternative oxidase ubiquinol oxygen oxidoreductase (Hannae and Mitchel 1994). Trypanothione may also be targeted by flavonoids, especially sesquiterpenes containing ϒ-lactons (Saeidenia et al., 2013).

1.6 *Dovyalis abyssinica*: The Experimental Plant

Dovyalis abyssinica (A. Rich), commonly called African gooseberry which is native to Africa (Veridiana and Adriana, 2007) and locally known as "**Koshim**" in Amharic, belongs to the small genus *Dovyalis (Flacourtiaceae),* comprising 16 species (Rasmussen et al., 2006). *D. abyssinica* occur naturally from Ethiopia, Eritrea and Somalia in the North through Kenya and Tanzania to Malawi in the South and grows in upland rainforest, dry evergreen forest, on riverbanks and sometimes in more open woodland (Kiamba et al., 2009).

Dovyalis abyssinica (Fig 2) is a spiny evergreen shrub or tree, up to 5m height, with a rounded crown. The bark is ash grey, almost always supporting lichens. Branches armed with stout spines, up to 1½ cm long. The branchlets are covered with numerous dotted pores (lenticels). Leaves are oval to obovate, up to 5-7 cm long and 3 cm wide with a rounded tip, edges unevenly rounded. It is shiny, dark green, with reddish stalks and veins. Flowers are unisexual, yellow-green or greenish without petals, 5-7 mm long. Female flowers are single or in 2-3 flowered fascicles. Male flowers occur in clusters, with 40-60 stamens (Kiamba et al., 2009).

Figure 2: Picture of *Dovyalis abyssinica*

Phytochemical information on the genus is sparse and mostly related to its role as a source of food. Studies reported on cyclopentenyl fatty acids (Rehfeldt et al., 1980) and tannins (Saleh et al., 1969) constituents of the genus. Fruits of *D. caffra* have been investigated for their composition of pectin and amino acids (Abdel-Fattah et al., 1975), and for the antioxidant activity of the polyphenols present in the fruit juice (Loots et al., 2006). Although alkaloids are generally uncommon in this family, two alkaloids have been identified in *D. caffra* by Sayed et al. (2000). Moreover, Stærk et al. (2003) showed the presence of a new class of spermidine-type alkaloids, dovyalicins A-D, in the leaves of *D. macrocalyx*, with dovyalicin A as the main alkaloid. Rasmussen et al. (2006) investigated the photochemistry of *D. macrocalyx*, *D. abyssinica*, and *D. hebecarpa* and reported the presence of two new dovyalicin-type alkaloids, dovyalicin E and dovyalicin F, along with the previously described Dovyalicin A. In addition, a new phenol glucoside, 4-hydroxytremulacin, and the new 1,2-cyclohexanediol glucoside 9, as well as the known compounds methyl 1-hydroxy-6-oxocyclohex-2-enecarboxylate and tremulacin, were isolated and reported by the same authors (Rasmussen et al., 2006). Ten

anthocyanins and 26 carotenoids were found in ripe fruit of *D. abyssinica* (Veridiana and Adriana, 2007). Jan and coworkers (2010) identified Itoside A and 4-hydroxytremulacin from leaves of *D. caffra* and stem bark of *D. zeyheri*.

Data on the pharmacological evaluation of *Dovyalis* species is limited (Rasmusssen et al., 2006). The extracts of fruits, leaves, stems, and roots have shown antibacterial activity (Zaki, 1975; Basile et al., 1997). The roots and thorns are used in African traditional medicine to treat amenorrhea and chest pain (Cumes et al., 2008), and *D. caffra* and other *Dovyalis* species are used by the Zulu to treat pain in rheumatic fever and rheumatism (Bryant, 1966). Geyid et al. (2005) reported on antibacterial and antifungal activity of extracts of *D. abyssinica* leaves. Aside from report of Nibret and Wink (2011) which indicated that *D. abyssinica* had *in vitro* anti-trypanosomal activity against *T. b. brucei* there are no other reports on the anti-trypanosomal properties of the plant especially on *T. congolenese*. Hence this work was undertaken to evaluate the *in vitro* and *in vivo* anti-trypanosomal effects of crude dichloromethane and methanol leaf extracts of *D. abyssinica* on the most pathogenic East African animal trypanosome*, T. congolense*.

2 OBJECTIVES

2.1 General objective

This study aimed at evaluating the *in vitro* and *in vivo* anti-trypanosomal activity of dichloromethane and methanol crude leaf extracts of *Dovyalis abyssinica* against *Trypanosoma congolense* field isolate.

2.2 Specific objectives

- To determine the preliminary acute toxicity profile of the crude extracts of *Dovyalis abyssinica* in mice;
- To do preliminary test on the crude plant extracts for their phytochemicals;
- To assess the *in vitro* anti-trypanosomal activity of the crude plant extracts on motility of *T. congolense* and effect on infectivity of the parasites to mice;
- To assess the *in vivo* anti-trypanosomal activity of the crude extracts in established infection to cure mice infected with *T. congolense* by measuring parasitemia, packed cell volume, rectal temperature, body weight, and mean survival time;
- To evaluate the *in vivo* anti-trypanosomal activity of the crude plant extracts in early infection to suppress the development of parasitemia in *T. congolense* infected mice;

3 MATERIALS AND METHODS

3.1 Collection and Preparation of Plant Specimens

The fresh leaves of *D. abyssinica* were collected in February, 2013, from Debrezeit, about 47.9 kilometers Southeast of Addis Ababa, and botanically identified by a taxonomist in Addis Ababa University. The voucher number, BT001/13, of the authenticated plant sample was then preserved in the national herbarium at Addis Ababa University.

3.2 Preparation and Storage of Extracts

The fresh plant material was rapidly washed under running tap water, shade air dried at room temperature until brittle, and grounded to coarse powder using laboratory pestle and mortar. The powdered plant material was extracted using two solvents; methanol (CH_3OH) and dichloromethane (CH_2Cl_2) as used by Rasmussen et al. (2006) and Nibret and Wink (2011). To obtain dichloromethane extracts, two hundred (200) grams were macerated in 2 Liters of dichloromethane in conical flask for three days. Similarly, to obtain methanol extract 200 g of powder were macerated in 2 Liters of methanol. The residue left after maceration was successively extracted twice with the same medium separately. Each extracts of both solvents combined in a separate flask were kept undisturbed and filtered through a sterile filter paper (wattman No. 1) into a clean conical flask. The filtrate was dried by evaporating the solvents using oven. The dried extracts were weighed and placed in refrigerator at 4 ^{0}C until needed.

3.3 Experimental Animals

Swiss albino mice (20-35 g) of either sex were obtained from the breeding colony of school of pharmacy, Addis Ababa University. The mice were housed in polypropylene cages and maintained under room temperature and 12 h light and dark cycle. The mice were provided with commercial pelleted ration and clean water *ad libitum*. The animals were left under controlled conditions at least for one week to acclimatize them before conducting any experimental procedure. Usage of mice in experiment was in accordance with institute for laboratory animals' resources (ILAR) guideline (1996).

3.4 Acute Toxicity Study

Acute toxicity test was carried out according to the Organization for Economic Co-operation and Development (OECD) guidelines for Testing of Chemicals number 420 (OECD, 2001) and Lork (1983). The test was initiated with a sighting study comprised a female mice dosed in a stepwise procedure using the fixed doses 300 and 2000 mg/kg. Starting with 300 mg/kg body weight, the test extract was administered orally to one mouse. The mouse was then observed for toxic effect for the first 30 minutes followed by hourly for 8 h for the first 24 hours. Then, after 24 h, another mouse was dosed with the next dose (2000 mg/kg BW) and a similar procedure carried out.

Median lethal dose was estimated using twenty (20) adult female mice grouped in to eight groups performed in two phases. The first phase consisted six groups of three mice each dosed IP with 10, 100, and 1000; while the second phase consisted two groups one mouse each dosed with IP 1,600 mg/kg of both extracts (Lork, 1983). The experimental animals were then observed for 30 minutes after treatment, followed by observation

hourly for 8 h and once daily for the next 13 days for any sign of toxicity such as loss of appetite, hair erection, lacrimation, tremors, convulsions, salivation, diarrhea, mortality and other signs of toxicity manifestation (OECD, 2001). So the lethal dose (LD_{50}) of the plant extracts was calculated using the Lork's formula:

$$\boldsymbol{LD50 = \sqrt{aXb}}$$

(where a = least dose that killed a mice, while b = highest dose that did not kill any mice).

3.5 Phytochemical Screening

Tests for Phytochemical constituents of the medicinal plant were performed by testing independent samples from both dichloromethane and methanol leaf extracts of *D. abyssinica* using standard methods (Trease and Evans, 1989).

Test for Alkaloids: To 1ml of test solution, few drops of Dragendrof‟s and Mayer‟s reagent were added and formation of precipitate indicates a positive result.

Test for Saponins: About 0.5 g of the extract was dissolved in 10ml of distilled water in a test tube. The test tube was stoppered and shaken vigorously for 30 seconds and allowed to stand in a vertical position and observed over 30 minutes. Formation of a "honey comb" froth over the surface of liquid and persistence after 30 minutes indicates presence of saponins.

Test for Tannins: To 2 ml of water diluted sample with 3 drops of 10% ferric chloride was added. Formation of bluish-black color denotes the presence of tannins.

Test for Sterols and Terpenes: To 1ml of test solution, few drops of concentrated sulphuric acid in a slant position was added and left standing for an hour. The formation of brown ring at interphase indicates the presence of sterols and terpenes

Test for Flavonoids: To 1ml of the test solution, 3mls of 10% sodium hydroxide was added followed by 3mls of 10% HCl. The formation of a yellow color on addition of sodium hydroxide, which disappeared on addition of the HCl, indicates the presence of flavonoids.

Test for Steroids: The test for steroids was done by the Liberman acid test. A portion of the extract was treated with drops of acetic anhydride. Concentrated H2SO4 was carefully added to the side of the test tube. The presence of a brown ring at the boundary of the mixture was taken as positive result.

Test for Phenolic Compounds: To 2 ml of filtered solution 1ml of 1% ferric chloride ($FeCl_3$ and 1 ml of potassium fero cyanate ($KFe(CN)_3$) were added. Formation of bluish green color indicated the presence of phenolic compounds.

3.6 Isolation of *T. congolense*

T. congolense field isolate was collected from Illibabur Zone, Dabu woreda, sebategna district, about 523 km Southwest of Addis Ababa. Cattle were screened for the presence of *T. congolense* using the buffy coat technique or Murray method (Murray et al., 1977). Briefly, fresh ear vein blood from cattle was collected into heparinised capillary tubes (75 × 1.5 mm). One end of the capillary tube was sealed with cristaseal and the sealed capillary tubes were placed in a microhaematocrit centrifuge with the sealed ends

pointing towards the outside. To ensure good balance, the tubes were loaded symmetrically. The rotary cover was screwed on and the centrifuge lid was closed. The capillary tubes were centrifuged at 11,000 rpm for 5 minutes.

Then the capillary tube was cut, with a diamond tipped pencil, 1 mm below the buffy coat, to include the top layer of RBCs. The buffy coat and the uppermost layer RBCs extruded on to a clean microscope slide and covered with a cover-slip (22 x 22 mm) were examined for the presence of motile trypanosomes with microscope with x10 and then x40 objective lens. Presence of *T. congolense* was identified based on motility and further confirmed by National Tsetse and Trypanosomosis Control Centre (NTTCC) in Bedelle following thin blood smear preparation. They were fixed by methanol and stained with Giemsa stain and read using an oil immersion objective (40–50x for scanning, 100x for identification of trypanosomes). The morphology of the trypanosome in the stained field was compared with that of reference species (WHO, 1998).

Blood was then drawn from the jugular vein of the positive cattle using heparinized vacutainer tube and injected (0.5 ml in two divided doses) intraperitonealy to apparently healthy mice and rats and travelled to Aklilu Lemma Institute of Pathobiology (AL-IPB), Addis Ababa University. After establishment of infection, the organisms were maintained by serial passages in mice until required as described by Atawodi et al. (2011).

3.7 *In Vitro* Motility and *In Vivo* Infectivity Studies

Extract solutions were prepared just before use. About 10 mg of each dried solvent extracts of the plant was weighed separately into Eppendorf tubes and first dissolved in 100 µl of 10% dimethylsulfoxide (DMSO) in Phosphate buffered saline glucose (PBSG)

according to Atawodi et al. (2011). PBSG (300 µl) was then added to produce extract solutions of 25.0 mg/ml (stock). Another five extract concentrations (20, 10, 2, 0.1 and 0.015 mg/ml) (Atawodi et al., 2003; Nibret and Wink, 2011) were prepared from the first extract solution by appropriate dilution with PBSG.

Assessment of in vitro anti-trypanosomal activity was performed in triplicates in 96 wells micro titer plates (flat bottom). Two hundred (200) µl of blood containing $1.58x10^7$ trypanosomes/ml obtained as described by Herbert and Lumsden (1976) was mixed with 50 µl of extract solution of 20, 10, 2, 0.1 and 0.015 mg/ml to produce effective test concentrations of 4, 2, 0.4, 0.02 and 0.003 mg/ml, respectively. Two hundred (200) µl infected blood was incubated with similar concentrations (20, 10, 2, 0.1, and 0.015 mg/ml) of diminazene acetuarte to produce 4, 2, 0.4, 0.02 and 0.003 mg/ml, respectively, effective concentrations as a positive control. A set of negative control was included containing the parasite (200 µl of infected blood) suspended in 50 µl 10% DMSO only.

After 5 minutes incubation in micro titer plates maintained at 37 ^{0}C, a drop of test mixtures was placed on separate microscope slides and covered with cover slips, where the parasites were observed every 5 minutes for death/motility using X40 objective for a total duration of 2 hours. Cessation or drop in motility of the parasites in extract-treated blood compared to that of parasite-loaded control blood without extract was taken as a measure of trypanocidal activity (Atawodi et al., 2003). Time after which motility ceased, reduced drastically, or reduced slightly with different effective concentrations (mg/ml) of extracts was recorded for comparison (Atawodi et al., 2003; Atawodi et al., 2011). Data were reported from two independent laboratory technicians.

Immediately after the completion of *in vitro* assay, the *in vitro* mixtures (0.20 ml) were inoculated intraperitoneally into 80 (5 animals per concentration) apparently healthy mice which were then monitored for 21 days post-parasite inoculation for development of infection (parasitemia) as described by Eze et al. (2012). The *in vitro* mixtures contained the remaining incubation mixture from each well of the micro titer plate left after samples were drawn for microscopic parasite motility examination in the *in vitro* motility test. Parasitemia was checked daily according to the method of Herbert and Lumsden (1976).

3.8 Curative *In Vivo* Test

3.8.1 Parasite Inoculation and Extract Administration

A total of fifty (50) apparently healthy mice of both sex were randomly grouped into ten groups (A-I, A-II, A-III, A-IV, B-I, B-II, B-III, B-IV, C, and D) of five mice each. Infected blood containing $2x10^6$ trypanosomes/ml was collected from donor mice by cardiac puncture under ethyl ether anesthesia and diluted with PBS to contain 10^3 trypanosomes/ml. All groups of mice were then infected intraperitoneally with 0.2 ml of infected blood/PBS (Atawodi et al., 2011). The animals were left to develop parasitemia and 12 days post parasite challenge (Eze et al., 2012), when the parasitmia level reached $1.58x10^7$ trypanosomes/ml, Groups A-I, A-II, A-III and A-IV were administered with doses 250, 200, 150, and 100 mg/kg BW, respectively, of the dichloromethane extract, while groups B-I, B-II, B-III and B-IV received respective methanol extracts of 250, 200, 150, 100mg/kg BW. Dose selection of the extracts for each group was made after undertaking acute toxicity study to estimate the best therapeutic range of concentrations.

The extracts were separately dissolved and reconstituted in 10% DMSO and administered to these groups intraperitoneally once daily for seven days. Infected mice in group C received 28 mg/kg of standard drug, diminazene aceturate (Inabo and Fathuddin, 2011). Group D, infected with the parasite that were administered with the solvent used for reconstitution (DMSO) but not treated with the extracts, was considered as negative control.

3.8.2 Determination of Parasitemia

Parasitemia was monitored by examination of blood drawn from the tail of mice microscopically at X400 magnification using the "Rapid Matching" method of Herbert and Lumsden (1976). Briefly, the method involves microscopic counting of parasites per field in pure blood. Logarithm values of these counts were obtained by matching with the table of Herbert and Lumsden (1976). Monitoring of parasitemia (average taken from two independent laboratory technicians' data) was performed every other day until the 14^{th} day post-treatment initiation. For the assessment of anti-trypanosomal effect of the extracts, the level of parasitemia in the treated animals was compared to that of the control animals (Maikai, 2011).

3.8.3 Determination of Packed Cell Volume

Packed Cell Volume was determined using microhaematocrit centrifuge and microhaematocrit tube reader. Microhaematocrit centrifuge, fitted with head capable for carrying 24 capillary tubes and a revolution of 11, 000 rpm for five minutes was used. The heparinized capillary tube was ¾ filled with blood samples obtained from the tail vein of the mice. The end of the tube was sealed and excess cleared off using cotton

wool. The filled tubes were placed in a slot in the centrifuge head with sealed end outward. A special scale, the microhaematocrit reader was used to obtain the PCV percentage and the reading recorded (Ngulde et al., 2013). PCV was monitored on day of parasite inoculation, treatment initiation, and 7^{th} and 14^{th} post-treatment initiation.

3.8.4 Determination of Rectal Temperature

Rectal temperature was measured by digital rectal thermometer per rectum on the day of parasite inoculation, day of treatment commencement and every other day thereafter for two weeks (Nweze et al., 2011).

3.8.5 Determination of Body Weight

The body weight of each mouse in all groups was recorded following the period of fasting on the day of parasite challenge, day of treatment initiation, and every other day up to day 14 by a sensitive digital weighing balance (Nweze et al., 2011).

3.8.6 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 (Feyera et al., 201).

3.9 Suppressive *In Vivo* Test

The crude extracts were tested for their anti-trypanosomal activity to suppress the growth of *T. congolense* according to Awulu et al. (2013). This involves treatment with the extracts 24 hours post-parasite inoculation of the mice (early infection). Thirty (30) mice

of both sexes were divided into six groups of five each. The passage mouse infected with the *T. congolense* was anaesthetized with diethyl ether and its blood containing about 1.59 x 10^7 trypanosomes/ml collected by cardiac puncture with sterilized syringe and needle and diluted using PBS to contain 10^3 trypanosomes/ml. Each of the thirty mice was inoculated intraperitoneally with 0.2 ml diluted blood. The dichloromethane extracts at dose levels of 250 (A-I), 200 (A-II) mg/kg body weight and methanol extract at dose levels of 250 (B-I) and 200 (B-II) mg/kg BW doses were administered IP once daily for twelve days. A parallel test with 28 mg/kg diaminazine aceturate (C) in the fifth group served as positive control. The last group (D) was given DMSO and served as negative control. Wet mount, thin blood films were made at two days interval post-treatment termination by cutting the tail tip and rapid matching method for estimating host parasitemia at x400 magnification was employed (Herbert and Lumsden, 1976).

3.10 Statistical Analysis

All data generated during the course of the research were expressed as mean ± standard error of mean (SEM) and analyzed statistically by one way ANOVA followed by Tukey's multiple comparison tests to determine statistical significance. The level of significance for the differences between means within a group was computed by student's t test. Data Analysis was performed using Statistical Package for Social Science (SPSS) version 16. P values less than 0.05 were considered statistically significant.

4 RESULTS

4.1 Yield for Plant Extraction

Yields of the crude leaf extracts of *D. abyssinica* are presented in Table 1. The percentage yield of dichloromethane and methanol extracts, respectively, was 4.5 and 6.5 % w/w, with a better yield obtained with methanol extraction. Both DCM and MOH extracts were green semisolids.

Table 1: Yields of crude dichloromethane and methanol leaf extracts of *D. abyssinica.*

Solvent	Dry powder (g)	Volume of solvent (ml)	Yield (g)	Yield (%)
Dichloromethane	200	2000	9	4.5
Methanol	200	2000	13	6.5

4.2 Acute Toxicity Test

In the sighting study, administration of oral 300 mg/kg of both extracts into mouse did not cause any signs of toxicity throughout the monitoring period. However, single 2000 mg/kg oral dose of each extracts produced death of a mouse. To determine the appropriate dose for the *in vivo* study that was carried out per IP, Lork's (1983) method was used and the results of which are depicted in Table 2. At 10, 100, and 1000 mg/kg IP there were no noticeable signs of toxicity. However administration of 1600 mg/kg dichloromethane and methanol extracts caused the death of a mouse.

So the lethal dose (LD_{50}) of the plant extracts was calculated to be 1,265 mg/kg body weight. Based on this result four doses (250, 200, 150, and 100 mg/kg) were selected for the *in vivo* studies.

Table 2: Acute intraperitoneal toxicity of dichloromethane and methanol leaf extracts of *D. abyssinica* (Lorke's method) in swiss albino mice.

Phase	Extract	Number of mice	Dose (mg/kg)	Clinical signs	Mortality
1	DCM	3	10	None	Zero
1	DCM	3	100	None	Zero
1	DCM	3	1000	None	Zero
2	DCM	1	1600	-	One
1	MOH	3	10	None	Zero
1	MOH	3	100	None	Zero
1	MOH	3	1000	None	Zero
2	MOH	1	1600	-	One

DCM= dichloromethane extract; MOH= methanol extract.

4.3 Phytochemical Constituents of *D. abyssinica*

Phytochemical screening tests performed on the leaf extracts of *D. abyssinica* revealed the presence of alkaloids, sapponins, phenolic compounds, flavonoids, and steroids (Table 3).

Table 3: Phytochemicals of crude dichloromethane and methanol leaf extracts of *D. abyssinica.*

Phytochemical constituents	Dichloromethane extract	Methanol extract
Alkaloids	+	+
Sapponins	+	+
Tannins	-	-
Phenolic compounds	+	-
Flavonoids	+	+
Sterols and terpenes	-	-
Steroids	-	+

Key: + = Present; - = Absent

4.4 *In Vitro* Motility Test

Dichloromethane and methanol crude leaf extracts of *D. abyssinica* were analyzed for their *in vitro* trypanocidal activity against *T. congolense* motility at effective concentrations of 4, 2, 0.4, 0.02, 0.003 mg/ml. All effective test concentrations, except 0.02 mg/ml and 0.003 mg/ml of both extracts, appreciably either eliminated or reduced motility of *T. congolense* within two hours when the mixtures were viewed under microscope. Compared to the DMSO-treated control which showed active and motile blood stream forms of *T. congolense* after two hours, both dichloromethane and methanol extracts completely eliminated motility of *T. congolense* within one hour at effective concentrations of 4.0, 2.0, 0.4 mg/ml. Compared to the methanol extract, dichloromethane extract exhibited higher *in vitro* activity (Table 4).

Diminazene aceturate, except at the lowest concentration tested (0.003mg/ml), eliminated trypanosomal motility within one hour, such that after 30 - 55 min of incubation no motility was visible with drug concentrations of 4.0, 2.0, 0.4 and 0.02 mg/ml (Table 4).

Table 4: *In vitro* anti-trypanosomal activity of crude dichloromethane and methanol leaf extracts of *D. abyssinica* on motility of *T. congolense.*

Rx group	Rx	Time (min) at which motility was reduced or ceased				
		4 mg/ml	2 mg/ml	0.4 mg/ml	0.02 mg/ml	0.003 mg/ml
D. abyssinica leaf extract	DCM Extract	20[a]	20[a]	25[a]	30[a]	NE
		25[b]	30[b]	35[b]	60[b]	
		35[c]	35[c]	40[c]		
	MOH Extract	30[a]	30[a]	40[a]	50[a]	NE
		40[b]	50[b]	55[b]	65[b]	
		50[c]	60[c]	60[c]		
Positive control	DA	10[a]	10[a]	15[a]	30[a]	45[a]
		20[b]	20[b]	30[b]	40[b]	120[b]
		30[c]	30[c]	35[c]	55[c]	
Negative control	DMSO	NE	NE	NE	NE	NE

Rx= treatment; [a] slight reduction in motility; [b] drastic reduction in motility, [c] cessation of parasite motility; NE= no noticeable change in motility; DCM= dichloromethane extract; MOH= methanol extract; DA= diminazene aceturate; DMSO= dimethylsulfoxide.

4.5 *In Vivo* Infectivity Test

Results (Table 5) obtained from the infectivity study revealed that only higher concentrations of *in vitro* mixtures (4.0, 2.0, and 0.4 mg/ml) of both DCM and MOH extracts and all concentrations of diminazene aceturate rendered mice aparasitemic for the follow up period of 21 days.

Compared to the DMSO treated control group, lowest concentrations (0.02 and 0.003 mg/ml) of both extracts significantly ($p<0.05$) prolonged the 6.40±0.40 days pre-patent period in the control mice to 9.40±0.40 to 12.60±0.40 days post-infection, though still parasitaemic. At these lowest concentrations, 0.02 mg/ml of DCM and MOH extracts

significantly ($p<0.05$) prolonged pre-patent period compared to their respective lowest concentration (0.003 mg/ml). However, there was no significant ($p>0.05$) difference between similar concentrations of the two extracts.

Table 5: *In vivo* anti-trypanosomal activity of crude dichloromethane and methanol leaf extracts of *D. abyssinica* on infectivity on mice administered with *in vitro* mixtures.

Treatment	Concentration of *in vitro* mixture (mg/ml)	Number of mice which developed infection	Infection intervals in days (mean ± SEM)
DCM	4	0/5	NI
	2	0/5	NI
	0.4	0/5	NI
	0.02	5/5	12.60 ± 0.40^{ab}
	0.003	5/5	10.60 ± 0.40^{a}
MOH	4	0/5	NI
	2	0/5	NI
	0.4	0/5	NI
	0.02	5/5	11.60 ± 0.24^{ab}
	0.003	5/5	9.40 ± 0.40^{a}
DA	4	0/5	NI
	2	0/5	NI
	0.4	0/5	NI
	0.02	0/5	NI
	0.003	0/5	NI
DMSO	0.05 DMSO + 0.2 ml blood	5/5	6.40 ± 0.40

Values are Mean±SEM; N=5; [a] significant compared to DMSO treated group; [b] compared to 0.003 mg/ml treated groups; DCM=dichloromethane extract; MOH=methanol extract; DA=diminazene aceturate; DMSO=dimethylsulfoxide; NI=no infection in the observation period of 21 days.

4.6 Curative *In Vivo* Anti-trypanosomal Effect

4.6.1 Effect of Extracts on Parasitemia

Pre-treatment mean parasite count for all treatment and control infected groups was $8.9x10^6$ trypanosomes/ml. Treatment with the extracts resulted in reduction in the level of parasitemia generally between days 2-10 of treatment compared to the negative control, in which there was a progressive rise in parasitemia throughout the two weeks monitoring period.

Higher doses of dichloromethane extract (250 and 200 mg/kg) significantly ($p<0.05$) reduced the parasite load between days 2 and 8 of treatment initiation, compared to the lowest doses (DCM150 and DCM100) and the DMSO-treated control. Especially, DCM250 was shown to reduce parasitemia level to approximately 12 ($10^{1.08}$) trypanosomes/ml at day 8 post-treatment initiation, though failed to totally clear the trypanosomes from the blood, and displayed comparable activity to diminazene aceturate ($p>0.05$) from days 6 to 10. Compared to DCM200, DCM250 displayed higher activity which failed to reach statistical significance ($p>0.05$), except at day 4 post-treatment initiation ($p<0.05$). Even though the activity was lower than ($p<0.05$) the two higher doses, DCM150 significantly ($p<0.05$) controlled the progression of parasitemia compared to the lowest dose (100 mg/kg) and infected DMSO-treated groups. Still DCM100-treated was statistically superior ($p<0.05$) to DMSO-treated group in reducing parasitemia (Table 6a).

On the other hand, only MOH250 and MOH200 were shown to significantly reduce parasitemia level from days 2 to 8 post-treatment initiation compared to the lowest two

doses (150 and 100 mg/kg) and DMSO-treated groups. Within this period (days 2-8) compared to MOH200 the parasitemia reduction was shown to be higher in the MOH250-treated group, though failed to reach statistical significance ($p>0.05$) except at day 8 ($p<0.05$). MOH250 displayed statistically similar ($p>0.05$) parasitemia reduction to diminazene aceturate from days 6 to 10 post-treatment initiation. Unfortunately, all the methanol extracts were not able to totally clear *T. congolense* from the blood of infected mice. Even, the lowest two doses (MOH150 and MOH100) was statistically indifferent from DMSO ($p>0.05$) (Table 6b).

However, treatment with 28 mg/kg diminazene aceturate temporarily cleared *T. congolense* from the blood within two days post-treatment initiation which reached statistical significance ($p<0.05$) compared to all extracts-, except DCM250 and MOH250-, and DMSO treated groups. Relapse in this group was recorded in all mice after day 12 post-treatment initiation.

Table 6a: *In vivo* anti-trypanosomal activity of dichloromethane crude leaf extract of *D. abyssinica* on parasitemia of *T. congolense* infected mice.

Rx	D(mg/kg)	Parasitemia level (log number/ml)							
		D0	D2	D4	D6	D8	D10	D12	D14
DCM	250	6.96±0.11	6.66±0.17^{bcd}	5.70±0.16^{a}	2.16±1.32^{bcd}	1.08±1.08^{bcd}	2.16±1.32^{bcd}	3.24±1.3^{a}	4.32±1.08
	200	6.66±0.26	6.48±.24^{cd}	6.12±.20^{cd}	4.5±1.13	3.24±1.32^{cd}	3.24±1.32^{cd}	5.52±.07	5.88±.07
	150	6.66±.22	6.72±.12^{cd}	6.54±.15^{cd}	6.12±.18	5.88±.15	5.70±.13	5.94±.17	6.06±.17
	100	6.90±.21	7.14±.26	7.32±.28	7.32±.28	7.20±.27	7.86±.22	8.22±.18	8.46±.11
DA	28	7.08±.24	.00±.00	.00±.00	.00±.00	.00±.00	.00±.00	1.08±1.0	2.16±1.32
DMSO	-	7.08±.07	7.26±.11	7.48±.14	7.74±.11	7.74±.06	8.04±.11	8.04±.11	8.46±.11

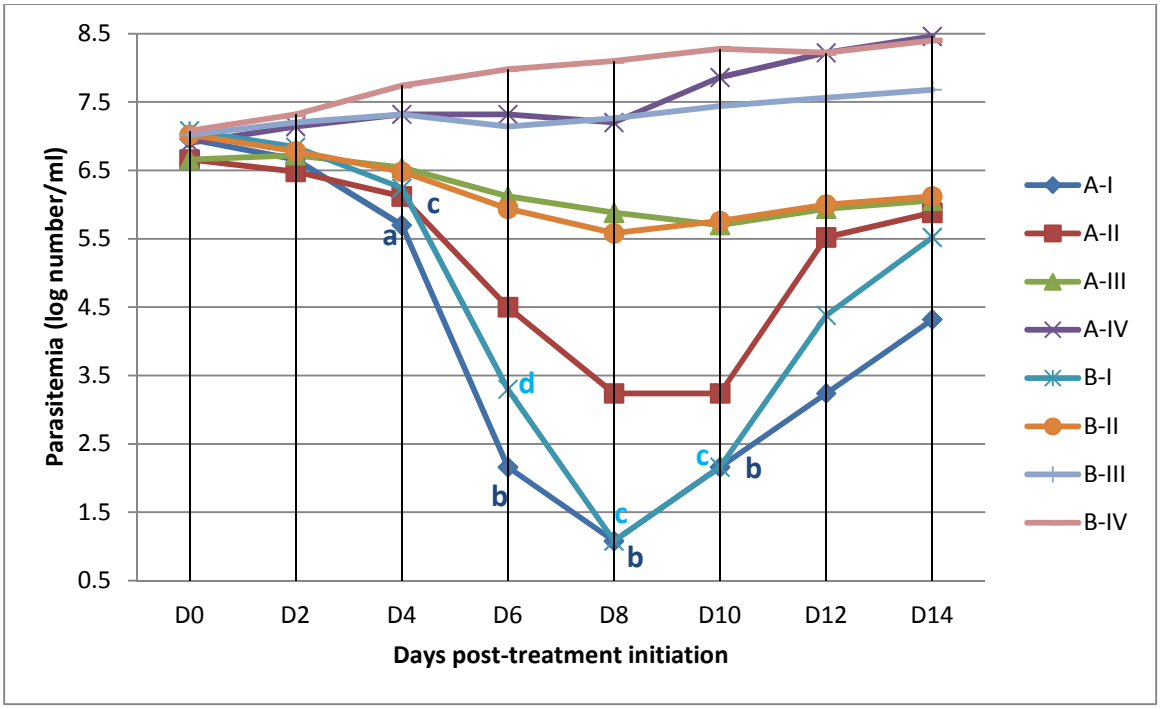
Values are Mean±SEM; $p<0.05$; N=5; [a] compared to all but diminazene aceturate; [b] compared to DCM 150 mg/kg; [c] compared to DCM 100 mg/kg; [d] compared to DMSO; Rx= treatment; D= dose; DCM= dichloromethane extract; DA= diminazene aceturate; DMSO= dimethylsulfoxide; D= day; D0= day of treatment initiation.

Table 6b: Anti-trypanosomal activity of crude methanol leaf extract of *D. abyssinica* on parasitemia of mice infected with *T. congolense*.

Rx	D(mg/kg)	Parasitemia level (log number/ml)							
		D0	D2	D4	D6	D8	D10	D12	D14
MOH	250	7.08±.07	6.84±.11[bcd]	6.24±.11[bcd]	3.30±1.35[bcd]	1.08±1.08[a]	2.16±1.32[a]	4.38±1.10[bcd]	5.52±.07
	200	7.02±.07	6.78±.07[bcd]	6.48±.07[bcd]	5.94±.06	5.58±.07	5.76±.11	6.00±.09	6.12±.153
	150	7.02±.12	7.20±.16	7.32±.12	7.14±.11	7.26±.11	7.44±.11	7.56±.11	7.68±.15
	100	7.08±.07	7.32±.07	7.74±.11	7.98±.15	8.10±.16	8.28±.15	8.22±.12	8.40±.09
DA	28	7.08±.24	.00±.00	.00±.00	.00±.00	.00±.00	.00±.00	1.08±1.0	2.16±1.32
DMSO	-	7.08±.07	7.26±.11	7.48±.14	7.74±.11	7.74±.06	8.04±.11	8.04±.11	8.46±.11

Values are mean±SEM; N= 5; $p<0.05$; [a] compared to all but DA; [b] compared to 150 mg/kg; [c] compared to 100 mg/kg; [d] compared to DMSO; MOH= methanol extract; Rx= treatment; D= dose; DA= diminzene aceturate; DMSO= dimethylsulfoxide; D= day; D0= day of treatment initiation.

The comparative analysis in Figure 3 revealed that dichloromethane extract appeared to be superior to the methanol extract in reducing parasite burden. Mice treated with DCM250 had continually reduced parasitemia level from days 2 to 8 post-treatment initiation which was further kept on average at lowest level up to the end of monitoring period. This was statistically significant ($p<0.05$) compared to MOH250 (on day 4) and MOH200 (on days 4, 6, 8, and 10). MOH250 was significantly superior ($p<0.05$) in reducing parasitemia compared to DCM150 (day 4, 8 and 10) and DCM100 (days 4 to 10). Though high parasitemia reduction was observed with dichloromethane extract compared to similar dose of methanol extract, the difference failed to reach statistical significance ($p>0.05$), except on day 4 post-treatment.



Values are Mean±SEM; N=5; $p<0.05$; [a] compared against all; [b] compared against all but MOH 250 mg/kg; [c] compared against DCM 150 and 100 mg/kg; [d] compared against DCM 100 mg/kg; A= dichloromethane extract; B= methanol extract; I= 250 mg/kg; II= 200 mg/kg; III= 150 mg/kg; IV= IV; D= days; D0= day of parasite inoculation.

Figure 3: Comparison of effect of crude dichloromethane and methanol extracts of leaf of *D. abyssinica* on parasitemia of mice infected by *T. congolense*.

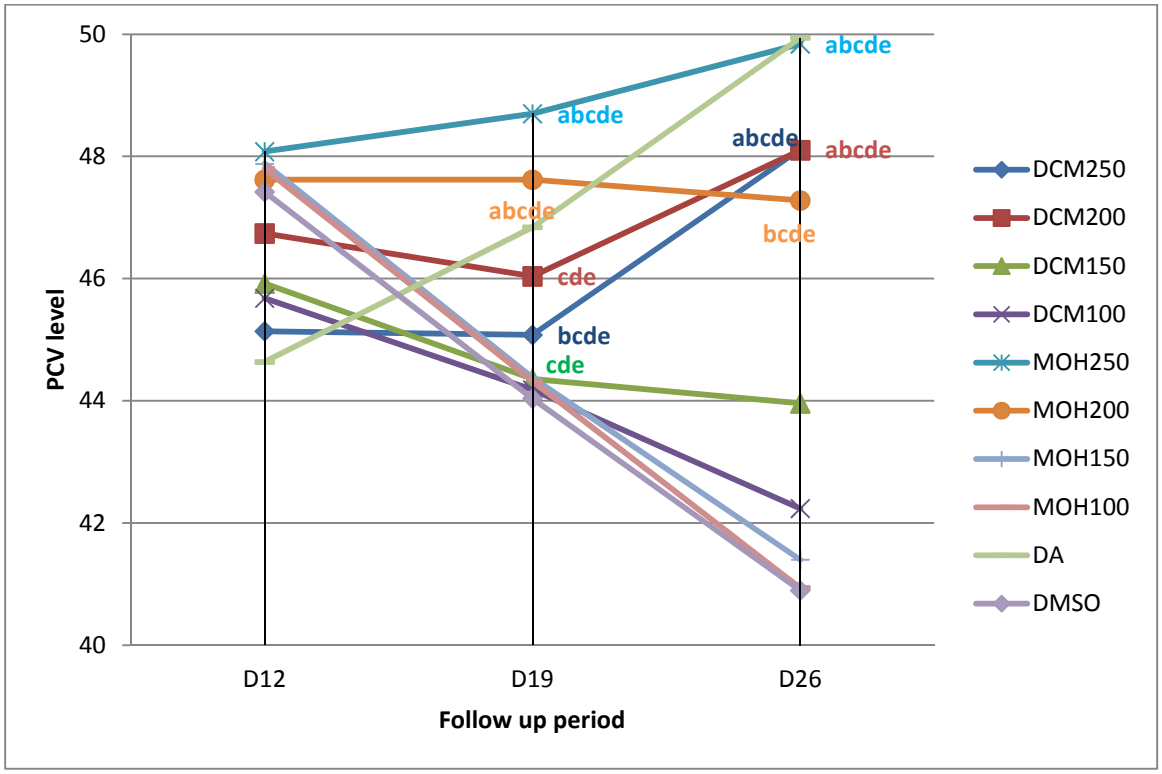
4.6.2 Effect of Extracts on Packed Cell Volume

Figure 4 shows that *T. congolense* caused a significant PCV reduction ($p<0.05$) in infected mice as the average value was found to drop from 49.04±0.30 to 50.50±0.50 on the day of parasite inoculation to 44.64±0.42 to 48.08±0.14 on the day of treatment initiation.

Seven days post-treatment initiation the PCV level significantly ($p<0.01$) improved in those groups which received DCM250 compared to those treated with the DCM150 and DCM100 and the negative control. Similarly, compared to the negative control, DCM 200 mg/kg-treated mice had improved PCV ($p<0.01$). However, mice treated with DCM150 and DCM100 doses had a consistently reduced PCV values, though statistically indifferent ($p>0.05$) from those treated with DCM200. The PCV improvement brought by DCM250 and DCM200 were statistically similar ($p>0.05$) to each other. On day 14^{th}, PCV was further improved significanty ($p<0.01$) in mice treated with DCM250 and DCM200 compared to those treated with DCM150 and DCM100 and the negative control (Fig 4).

Similarly, seven days post-treatent initiation MOH250 and MOH200 treated animals showed improved PCV level ($p<0.01$) compared to those treated with MOH150, MOH100, and negative control whose PCV declined continuously. This effect of MOH250 and MOH200 was significantly ceiled ($p<0.01$) to high PCV level compared to MOH150, MOH150, and negative control during the end of the monitoring day (day 14^{th} post-treatment intiation), though higher PCV increament ($p<0.05$) was obtained from MOH250 than MOH200 (Fig 4).

Comparative analysis (Fig 4) revealed that comparable doses of dichloromethane extracts were superior to methanol extracts on day 7th and vice versa on day 14th post-treatment initiation, but the difference failed to reach statistical significance ($p>0.05$). Despite the significant enhancement in mean PCV level at these highest doses, it was significantly lower compared to that brought by diminazene aceturate ($p<0.05$) on days 7th and 14th post-treatmet initiation.

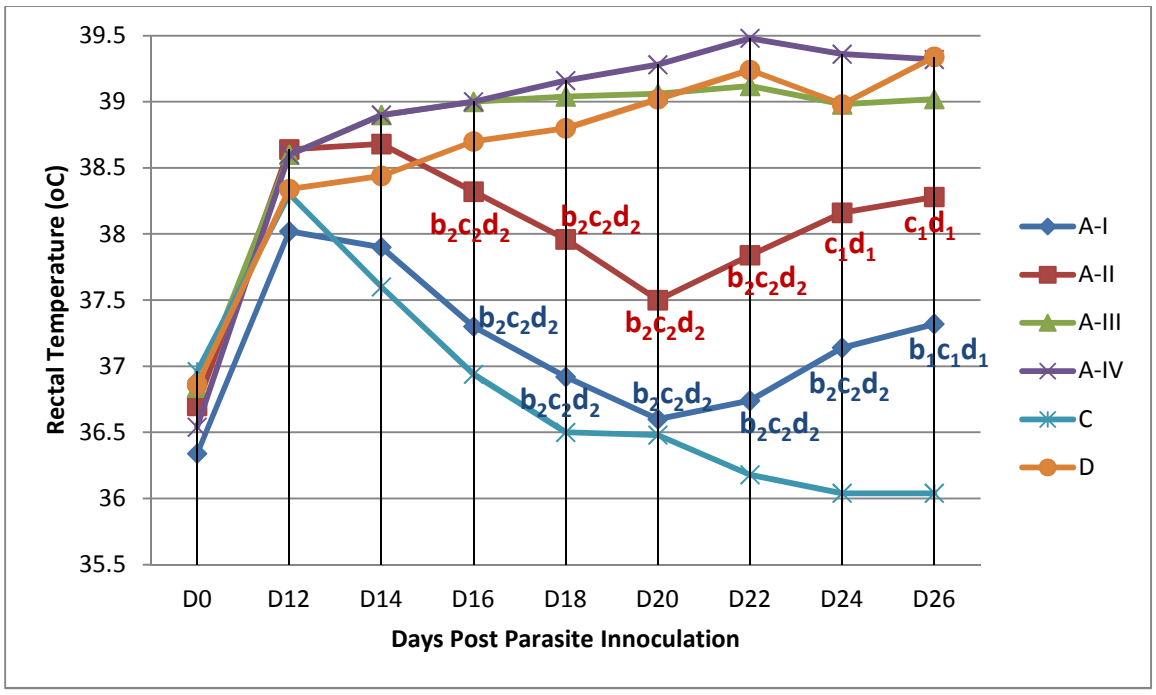


Values are Mean±SEM; N=5; $p<0.01$; [a] compared to DCM150; [b] compared to DCM100; [c] compared to MOH150; [d] compared to MOH100; [e] compared to DMSO; DCM= dichloromethane extract; MOH= methanol extract; DA= diminazene aceturate; DMSO= dimethylsulfoxide; PCV= packed cell volume; D= day; D12= day of treatment initiation.

Figure 4: *In vivo* anti-trypanosomal activity of crude dichloromethane and methanol leaf extracts of *D. abyssinica* on packed cell volume of mice infected with *T. congolense.*

4.6.3 Effect of Extracts on Rectal Temperature

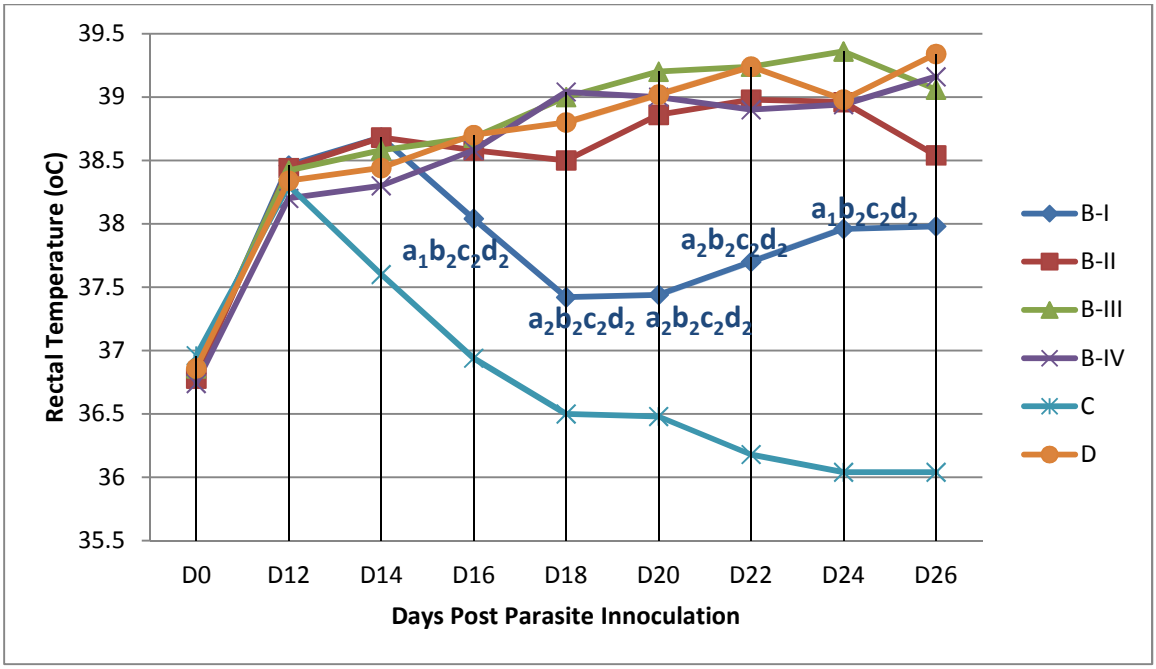
The mean rectal temperature at the day of infection (36.34±0.25 to 36.96±0.10) was elevated following infection (38.02±.24 to 38.64±0.33), which indicated fever was manifested ($p<0.05$) in mice infected with *T. congolense* (Fig 5a and 5b). Mice treated with DCM250 and DCM200 produced pronounced fever reduction ($p<0.05$) from 4th day post-treatment until the end of monitoring period compared to those treated with DCM150, DCM100, and DMSO. Whereas the reduction in rectal temperature caused by these two doses was comparable ($p>0.05$), DCM250 was superior to DCM200 from days 4 to 14 post-treatment initiation. Despite this, the rectal temperature was shown to slowly rise after 10th day post-treatment (Fig 5a).



Values are Mean±SEM; N=5; [1] $p<0.05$; [2] $p<0.01$; [b] against 150 mg/kg; [c] against 100 mg/kg; [d] against D; A= dichloromethane extract; I=250 mg/kg; II=200 mg/kg; III= 150 mg/kg; IV=100 mg/kg; C= diminazene aceturate; D= dimethylsulfoxide; D0= day of parasite inoculation.

Figure 5a: Rectal temperature change of *T. congolense* infected mice treated with crude dichloromethane leaf extract of *D. abyssinica*.

By contrast, MOH200 was unable to significantly reduce rectal temperature ($p>0.05$) compared to DMSO. Only MOH250 ameliorated temperature elevation from day 4th to the final monitoring day which reached statistical significance ($p<0.05$) compared to all doses of MOH and the negative control. However, rectal temperature started to rise on day 10th post-infection (Fig 5b).



Values are Mean±SEM; N=5; [1] $p<0.05$; [2] $p<0.01$; B=Methanol extract; I=250 mg/kg; II=200 mg/kg; III= 150 mg/kg; IV=100 mg/kg; C= diminazene aceturate; D= DMSO; [a] against 200 mg/kg; [b] against 150 mg/kg; [c] against 100 mg/kg; [d] against DMSO; D0= day of parasite inoculation.

Figure 5b: Rectal temperature change of *T. congolense* infected mice treated with crude methanol leaf extract of *D. abyssinica*.

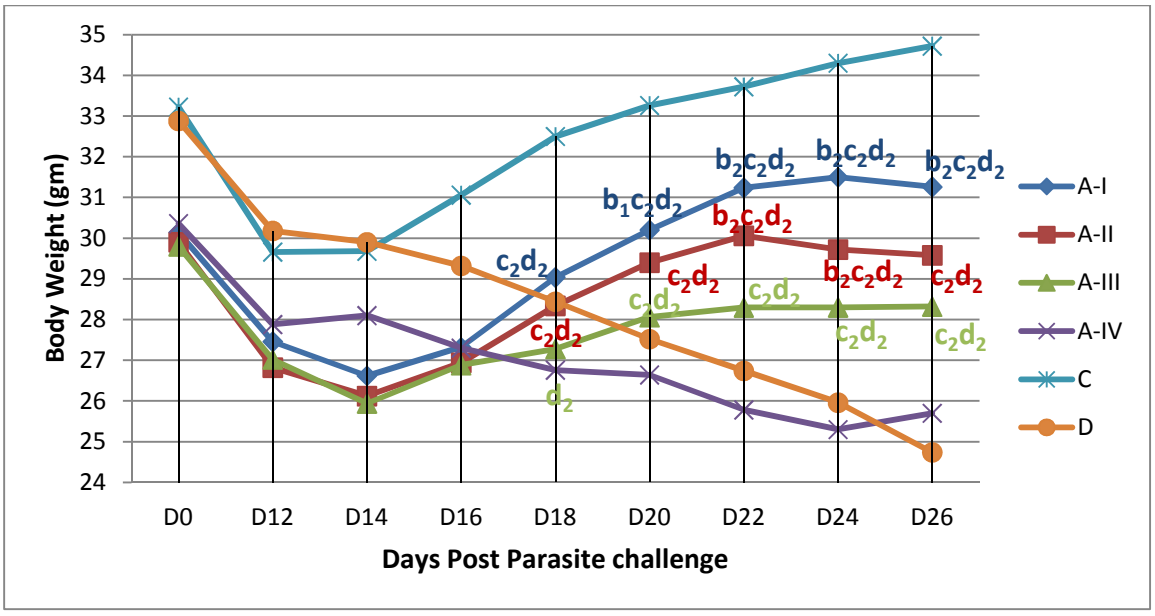
Comparative analysis (Fig 5a & 5b) indicated that DCM250 and DCM200 were superior to respective MOH250 and MOH200 doses in ameliorating rectal temperature elevation, though the difference failed to reach statistical significance ($p>0.05$). In spite of this none, except DCM250 on day 8 PI, of these doses was comparable ($p<0.01$) to 28 mg/kg diminazene aceturate in preventing rectal temperature rise associated with the infection.

4.6.4 Effect of Extracts on Body Weight

The mean body weight (MBW) values, in gram, of all groups of mice from day of parasite challenge to the last monitoring day are depicted in Figures 6a and 6b. MBW of *T. congolense* infected mice during parasite challenge and treatment initiation are 25.34±0.80 to 33.22±0.70 and 23.44±0.79 to 30.18±1.2, respectively; clearly indicating a statistically significant ($p<0.01$) weight loss associated with parasitemia.

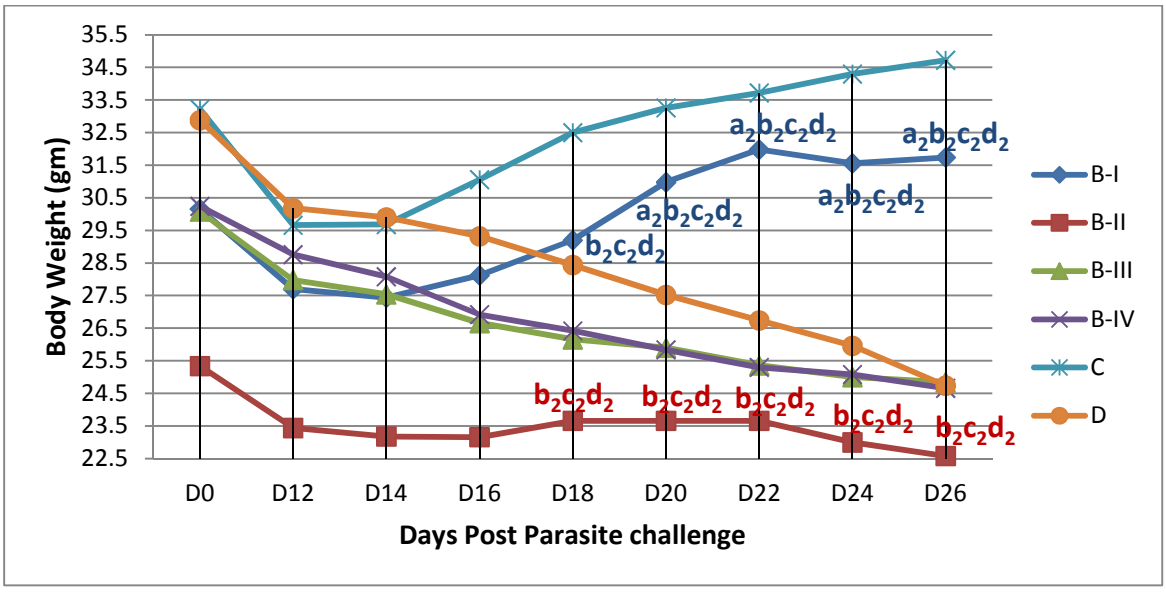
Compared to the negative control, groups treated with all doses of dichloromethane extract, except 100 mg/kg, significantly ($p<0.05$) improved body weight of infected mice throughout the monitoring period starting from 6^{th} day post-treatment initiation. Within this follow up period MBW improvement in the groups treated with DCM250 and DCM200 was not significantly different ($p>0.05$), though better increment was produced by DCM 250 mg/kg (Fig 6a).

On the other hand, only MOH250 and MOH200 were able to significantly improve MBW compared to the control group ($p<0.05$), though MOH250 was statistically superior ($p<0.01$) to MOH200 in improving body weight of mice. By contrast, there was consistent body weight lose in mice treated with MOH150 and MOH100 (Fig 6b).



Values are Mean±SEM; N=5; [1] p<0.05; [2] p<0.01; [b] against 150 mg/kg; [c] against 100 mg/kg; [d] against DMSO D0= day of parasite inoculation; 12=day of treatment initiation; A= dichloromethane extract; I=250 mg/kg; II=200 mg/kg; III=150 mg/kg; IV=100 mg/kg; C= diminazene aceturate; D= dimethylsulfoxide.

Figure 6a: Effect of crude dichloromethane leaf extract of *D. abyssinica* on body weight of mice infected with *T. congolense*.



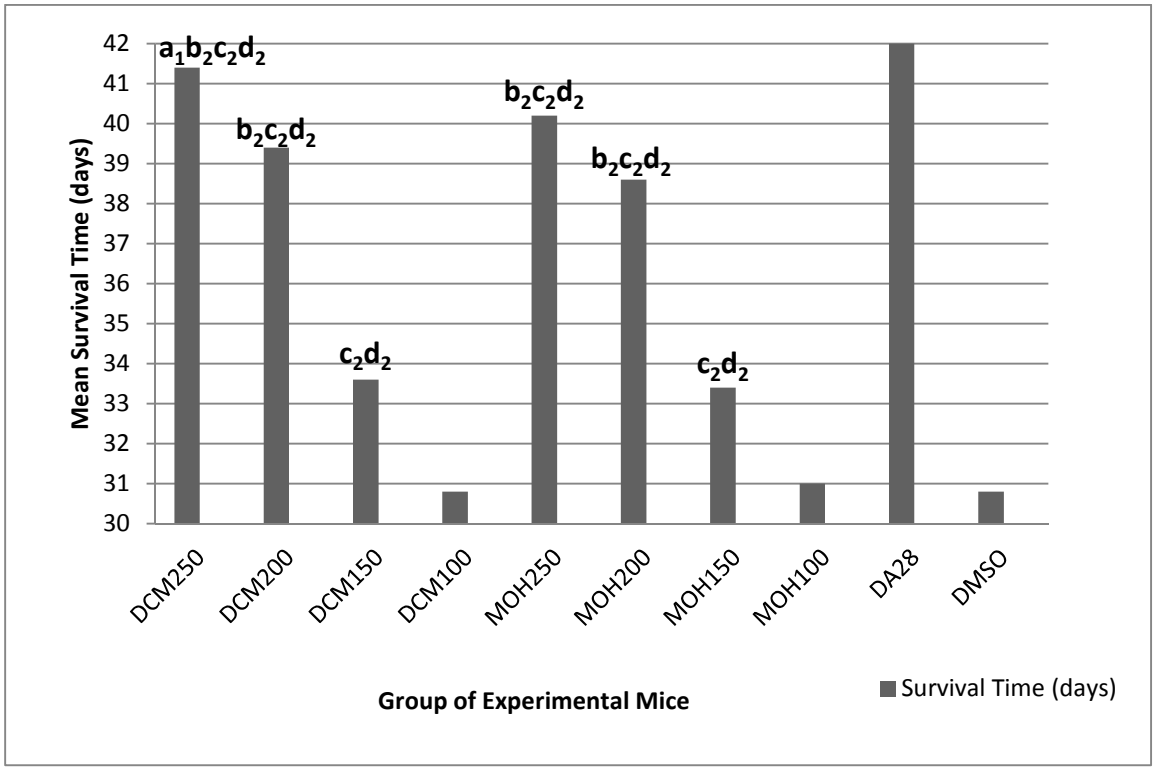
Values are Mean±SEM; N=5; [2] p<0.01; B= methanol extract; I=250 mg/kg; II=200 mg/kg; III=150 mg/kg; IV=100 mg/kg; [a] against 200 mg/kg; [b] against 150 mg/kg; [c] against 100 mg/kg; [d] against DMSO; C= diminazene aceturate; D= dimethylsulfoxide; D0= day of parasite inoculation.

Figure 6b: Effect of crude methanol leaf extract of *D. abyssinica* on body weight of mice infected with *T. congolense*.

Comparative analysis revealed that mice treated with DCM250, DCM200, MOH250, and DA 28 mg/kg significantly ($p<0.05$) improved body weight compared to those treated with MOH200 on days 10, 12 and 14 post-treatment initiation. However, the difference in body weight improvement produced by these doses were statistically insignificant ($p>0.05$). Even though diminazene aceturate produced the highest body weight gain, body weight improvement in mice treated with diminazene aceturate failed to reach statistical significance ($p>0.05$) compared to DCM250, DCM200, and MOH250, except at the last monitoring day which was significantly superior ($p<0.05$) to that produced by treatment with DCM200 (Figures 6a and 6b).

4.6.5 Effect of Extracts on Mean Survival Time

Higher doses of dichloromethane extracts (250 and 200 mg/kg) significantly ($p<0.05$) prolonged mean survival time of infected mice compared to that conferred by treatments with DCM150, DCM100, and DMSO which was below 34 days. Similarly, mice treated with MOH250 and MOH200 survived longer time than those treated with lower MOH doses (150 and 100 mg/kg) and DMSO. Significantly longer ($p<0.05$) survival time (41.40±0.24 days) was recorded in mice treated with DCM250 compared to those treated with MOH250 (40.20±0.49), DCM 200 mg/kg (39.40±0.24) and MOH200 (38.60±0.51) which were statistically indifferent ($p>0.05$) amongst themselves. Even, mice treated with 28 mg/kg diminazene aceturate survived not statistically longer (42±0.00 days) than those treated with DCM250 ($p>0.05$). Out of 40 mice treated with both extracts, only three (2 DCM250 and 1 MOH250-treated) mice survived up to the end of the monitoring period of 42 days (Fig 7).



Values are Mean±SEM; N=5; [1] $p<0.05$; [2] $p<0.01$; [a] compared to DCM200 and MOH200; [b] compared to DCM150 and MOH150; [c] compared to DCM100 and MOH 100; [d] compared to DMSO; DA= diminazene aceturate; DCM=dichloromethane extract; MOH= methanol extract; DMSO= dimethylsulfoxide.

Figure 7: *In vivo* anti-trypanosomal activity of dichloromethane and methanol crude leaf extracts of *D. abyssinica* on mean survival time of mice infected with *T. congolense.*

4.7 Suppressive *In Vivo* Anti-trypanosomal Effect

Mice treated with 250 and 200 of each extracts (DCM and MOH) for 12 days 24 hours post-infection were shown to posses significantly reduced density of *T. congolense* at the end of the treatment period compared to DMSO-treated ones ($p<0.05$). But none of these extracts doses were able to completely abrogate the progression of infection ($p>0.05$) as there was a consistent rise in parasitemia after discontinuation of treatment. Mice treated with diminazene aceturate had no noticeable parasitemia in their blood until the end of monitoring period ($p<0.05$) in contrast to all groups (Table 7).

Table 7: *In vivo* anti-trypanososmal activity of dichloromethane and methanol crude leaf extracts of *D. abyssinica* to suppress the parasitemia level of mice infected with *T. congolense*.

Treatment	Dose(mg/kg)	Parasitemia level (log number/ml)							
		D0	D2	D4	D6	D8	D10	D12	D14
DCM	250	4.32±1.08[a]	4.56±1.14	5.82±.12	6.06±.11	6.18±.07	6.48±.07	6.78±.07	7.14±.11
	200	5.52±.073[a]	5.82±.073	6.12±.073	6.42±.073	6.72±.073	6.90±.09	7.20±.09	7.50±.09
MOH	250	5.40±.00[a]	5.64±.06	5.94±.06	6.06±.11	6.06±.06	6.36±.06	6.66±.06	7.20±.09
	200	5.76±.06[b]	6.06±.06	6.30±.09	6.42±.15	6.54±.17	6.84±.17	7.36±.17	7.74±.11
DA	28	.00±.00	.00±.00	.00±.00	.00±.00	.00±.00	.00±.00	.00±.00	.00±.00
DMSO	-	7.32±.07	7.62±.07	7.86±.06	8.10±.09	8.28±.07	8.40±.09	8.52±.07	8.34±.11

Values are mean±SEM; N= 5; $p<0.05$; [a] compared to MOH 200 mg/kg and DMSO; [b] compared to DMSO; DCM=dichloromethane extract; MOH; methanol extract; DA=diminazene aceturate; DMSO=dimethylsulfoxide; D=day; D0=day of treatment termination.

5 DISCUSSION

In this study the activity of crude dichloromethane and methanol leaf extracts of *D. abyssinica* were studied in terms of *in vitro* motility and *in vivo* parameters, including prasitemia, packed cell volume, rectal temperature, body weight and mean survival time of infected mice. In addition, acute toxicity and phytochemical constituents were tested.

The result of Freiburghaus et al. (1996) has clearly indicated that different solvent extracts of the same plant may exhibit different trypanocidal activity just as extracts of different parts of the same plants. Therefore, the statement that a plant is trypanocidal or not should be taken within the context of the solvent used and the parts investigated (Atawodi et al., 2003). Hence, the leaves of *D. abyssinica* were extracted using dichloromethane and methanol solvents; the selection of which was performed based on the promising results of previous work (Nibret and Wink 2011).

Acute toxicity was studied per oral (OECD, 2001) and intraperitoneal (Lork, 1983) routes in female mice. Oral 300 mg/kg administration of dichloromethane and methanol extracts was safe as there were no visible toxicity signs. In contrary, mice could not tolerate oral 2000 mg/kg. Though all mice also survived administration of IP 10, 100, and 1000 mg/kg, IP 1600 mg/kg dose level caused the death of a mouse. As a result the median lethal dose was 1,265 mg/kg and the plant can be regarded as being of low toxicity or relatively safe (Nuglde et al., 2013). For pharmacological evaluation, therefore, 250, 200, 150 and 100 mg/kg dose levels were selected.

In the present study incubation of infected blood with high concentrations (20, 10, and 2 mg/ml) of both extracts had ceased motility of *T. congolense* within 1 hour, suggesting that *D. abyssinca* has promising *in vitro* anti-trypanosomal activity since motility of parasites constitutes a relatively reliable indicator of viability of most zooflagellate parasites (Peter et al., 1976; Kaminsky, et al., 1996). This result is consistent with previous reports (Atawodi et al., 2011; Maikai, 2011; Feyera et al., 2013). Previous works (Freiburghaus et al., 1997) have shown that the mean MIC value of common trypanocidal drugs is 10.7 mg/ml and that agent with MIC value between 5–20 mg/ml could be regarded as very active. In this study, *D. abyssinica* can then be considered active comparable to the standard drug (diminazene aceturate). This effect perhaps may originate from inhibition, by *D. abyssinca,* of cellular and mitochondrial respiration which obviously compromise all the energy dependent processes (Nok, 2002). Respiration of trypanosome parasite is obligatory for rhythmic flagella movement as well as for managing the energy reserve required for the synthesis of the variable surface glycoproteins (Atawodi et al., 2003). This was confirmed by the microscopy of the cells, which showed an immediate cessation of flagella movement after incubation with the dichloromethane and methanol extracts.

Furthermore, to complement the *in vitro* effect on motility, subsequent administration of mice with the remaining *in vitro* mixtures revealed that the mice received concentrations which abrogated motility *in vitro* did not result in infection for the monitoring period of 21 days. This result is in agreement with Eze et al. (2012). Interestingly, even the lowest diminazene aceturate concentration (0.015 mg/ml), which did not cease parasite motility, turned out to eliminate infectivity. The effect at this concentration may be attributed to

abrogation of some vital metabolic processes or some morphological changes in the parasites that render them more susceptible to the mice immune defense systems. The result indicated that the extracts eliminated parasite motility *in vitro* which corroborates previous report by Nibret and Wink (2011).

Even though the lower two concentrations of both extracts drastically reduced motility of the parasite, mice administered with these mixtures contracted the infection. It, thus, appears reasonable to speculate that these concentrations may belong to the group that acts by static action affecting growth and multiplication of trypanosomes rather than eliminating them completely (Atawodi et al., 2003).

Higher doses (250 and 200 mg/kg) of both extracts substantially reduced parasitemia level in mice, despite the failure to completely clear bloodstream form of the parasite. The result is consistent with other reports (Maikai, 2011; Feyera et al., 2012) that medicinal plants reduce parasitemia. The effect might be attributed to the presence in them of one or more of secondary metabolites which may exert their effects through the additive or synergistic action of several chemical compounds acting at a single or multiple target sites associated with a physiological process (Kaufmann et al., 1999; Tyler, 1999). Even though detailed characterization and isolation of different compounds that could be responsible for the observed activity was not carried out, preliminary phytochemical screening indicated the presence of alkaloids, phenolic compounds, flavonoids, sapponins, and steroids. Several possible mechanisms, therefore, working separately or in concert may account for the observed effect (Paul et al., 2012).

D. abyssinica contains spermidine alkaloids (Rasmussen et al., 2006) that may in part account for the observed anitrypanosomal activity of this plant (Nibret and Wink, 2011). The aryl moiety in the spermidine molecule interacts with the hydrophobic region of trypanothione reductase so that the spermidine would adopt a non-extended bound conformation (Omar and Khan, 2007), could be responsible for the observed effect of the alkaloid. Besides, the extracts may increase oxygen consumption and stimulation of hydrogen peroxide production in the protozoan cell (Atawodi et al., 2003). Hence, the parasite might have been exposed to oxidative stress that couldn't be dealt with the inhibited trypanothione pathway. Interestingly, spermidine in part resembles the chemical structure of pentamidine (Nibret and Wink, 2011). Binding to nucleic acids in DNA and RNA, or promotion of cleavage of the parasite's circular DNA in a manner similar to that of topoisomerase II inhibitors might have also been responsible (Wang, 1995).

But this may not rule out the actions of the other secondary metabolites. Flavonoids targeting the replicating forms of trypanosomes which are totally dependent on glycolysis for energy production (Opperdoes, 1987) might have been responsible mechanisms. It may also be proposed that phenolic compounds, though only found in dichloromethane extract, might have killed the parasite through specific inhibition of the glycerol-3-phosphate-dependent mitochondrial oxygen consumption or formation of reactive oxygen species acting as a pro-oxidant (Hoet et al., 2004) . Moreover, steroids should not be neglected in this regard since sterols, such as vernoguinosterol and vernoguinoside, have been reported to have anti-trypanosomal activities (Tchinda et al., 2002). On the other hand, although the anti-trypanosomal activity of saponins is controversial, saponins with

detergent properties can dissolve in biomembranes and disturb their fluidity and the function of membrane proteins of parasites (Wink, 2012).

At similar *in vitro* concentrations and *in vivo* doses dichloromethane extract was superior to its methanol counterpart, which is consistent with previous report (Nibret and Wink, 2011). This may be credited to the presence of phenolic compounds in the dichloromethane, but not in the methanol, extract as these compounds were shown to exhibit anti-trypanosomal activity (Otoguro et al., 2011). Other report has shown similar observation that superior activity was obtained from the dichloromethane plant extracts (Feyera et al., 2012).

The observation that parasitemia was relatively elevated and that trypanosomes were persistently present in blood of extract treated animals after withdrawal of treatment (day 12, 14) may suggest recovery of the parasites from the suppressive effect of the extracts. This resurgence in mean parasitemia may be due to the waning effect of the treatment (Feyera et al., 2012) or the release of trypanosomes from the tissues which can occur when treatment is delayed or the dose rate is inadequate (Eze et al., 2012). Despite the temporary clearance of blood stream forms of trypanosomes, relapse occurred in all mice treated with diminazene aceturate at day 12 post-treatment initiation. This observation is not surprising as resistance to diminazene aceturate has been widespread in South West Ethiopia (Chaka and Abebe, 2003).

Consistent with reduction in parasitemia, it is apparent from results of the present study that administration of both extracts at higher doses (250 and 200 mg/kg) led to remarkable improvement of anemia, evident from the significant difference in the levels

of the PCV of the extract treated animals and those treated with DMSO. The result is consistent with previous works (Inabo and Fathuddin, 2011; Feyera et al. 2012). The increase in PCV after treatment may, therefore, be due to mechanisms associated with reduction in trypanolytic crisis which enhances red blood cells damage and destruction leading to anemia (Anosa, 1988). This, in turn, may be mediated by extracts ability to eliminate parasites from the blood, probably by reaching the site of action or rapid metabolization (Wurochekke et al., 2005), or neutralization of the toxic metabolites produced by trypanosomes (Abubakar et al., 2005). Certain components of the plant might have helped stabilize the membrane of erythrocytes; specifically, the anti-oxidant or free radical scavenging properties of phenolic compounds and flavonoids (Saeed et al., 2012), may play vital roles in this regard.

Animals infected with trypanosomes characteristically exhibit fever shown in the present study by the occurrence of febrile peaks which reflected the response to successive waves of parasitemia that the high body temperature itself is detrimental to the trypanosomes (Zwart et al., 1990). However, following treatment with high doses (250 and 200 mg/kg) of both extracts pyrexia was reduced with attendant rise in packed cell values (Figs 4, 5a, 5b). The body temperature set point in the hypothalamus, which was changed under the influence of pyrogenic stimuli released during infection (Pathak, 2009), might have, then, been kept in check.

Further deduction of the anti-trypanosomal activity of the plant can be made from the weight gain observed in mice treated with the higher (250, 200 mg/kg) doses of extracts. In the present study, infected mice manifested weight loss and similar findings have been reported in *T. congolense* infected West African dwarf goats (Faye et al., 2005) and in

mice infected with *T. congolense* (Feyera et al., 2012). The weight gain might be linked to the treatment-induced reduction of parasitemia, which otherwise would lead to depressed appetite and food intake during fever peaks. Moreover, fever is associated with increase heat production and increase metabolizable energy so that proportion of protein that is used for growth is reduced, as it is metabolized for animals to provide the extra energy required (Pathack, 2009). Fever reduction might, therefore, have decreased synthesis of protein that would occur at the expense of muscle protein catabolism and loss in body weight (Dargie, 1980). The increased supply of oxygen and nutrients because of the improved PCV level may also be an important factor in the weight gain.

Compared to DMSO treatment, administration of higher doses (250 and 200 mg/kg) of both extracts conferred prolongation of lives of mice. The result agrees with other reports (Ngure et al., 2009; Feyera et al., 2012) that medicinal plants prolong survival of trypanosome infected mice. This could be related to effects of the active compound found in *D. abyssinica* on red blood cells and/or antioxidant activity. The amelioration of anemia, which is primarily responsible for the death of infected animals (Ngure et al., 2009), shown by the ability of these extracts to improve PCV may have then rescued the lives of mice. Additionally, the improved survival of trypanosome infected mice could be due to the effects of *D. abyssinica* leaf extracts on pathogenesis of the disease. Indeed polyphenols in tea have been shown to attenuate cytokine induced (Courtiou et al., 2006) and also nitric oxide inflammation (Mabbott and Sternberg, 1995), both of which initiate the pathological effects of trypanosomosis (Ngure et al., 2009)

Though lower parasite load was observed at the end of suppressive treatment (250 and 200 mg/kg), after termination of treatment parasitemia indifferently rose steadily in all

groups. Moreover, parasitemia reduction was higher in mice treated on the established infection (curative) than those treated 24 hours PI (suppressive) (Tables 6a, 6b, and 7). This may suggests that the extract might strengthen the host defense, which might had already been activated due to the presence of parasites in circulation in the mice with established infection, by eliciting antibody production or enhanced phygocytosis (Abubakar et al., 2012). Unlike curative doses, suppressive diminazene aceturate cleared the parasites from the blood till the end of the monitoring day. This may be attributed to the variation in sensitivity of *T. congolense to* diminazene aceturate during early and established infection (Mamman et al., 1993a) or greater maximum plasma drug concentration during the former than the later (Mamman et al., 1993b).

6 CONCLUSION

In the present study, crude dichloromethane and methanol leaf extracts of *D. abyssinica* at higher concentrations (20, 10, 2 mg/ml) caused marked *in vitro* motility cessation and eliminated subsequent infectivity in mice. Furthermore, administration of higher curative doses (250 and 200 mg/kg) of both extracts considerably displayed *in vivo* activity as evidenced by reduction in parasitemia level, and rectal temperature and enhanced packed cell volume, body weight, and survival time. However, suppressive doses did not prevent parasitemia development. Therefore, it could be concluded that crude dichloromethane and methanol leaf extracts of *D. abyssinica* displayed anti-trypanosomal activity that may cause abrogation of *T. congolense* motility *in vitro* and reduce parasite density *in vivo.*

7 RECOMMENDATIONS

- It will be appropriate to experiment further toxicological studies to establish safety.
- Isolation of *D. abyssinica* components and hence their respective effects of the dichloromethane extract will provide ample knowledge on the anti-trypanosomal activity of *D. abyssinica.*
- Hematological studies and bioassays on different *in vivo* parameters other than those studied in this study have to be conducted in order to further ascertain the anti-trypanosomal effect of *D. abyssinica*.
- Strain identification and comparative studies on the various strains of *T. congolense* has to be performed in order to rule out the impact of strain variation on anti-trypanosomal activity of *D. abyssinica*.

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Annex 1

Materials

The following supplies were used; Digital balance (Mettler Toledo, Switzerland), Filter paper (Wattman International, England), Syringe (Shandock zibo Shanchuan medical instrument, Germany), Evacuated EDTA tube (Taurus Bio medical Aids, Bhosori Pone), Cover slide and slide (Vardhman Electron Devices, India), Glove (Sara health Care, India), Refrigerator (Acira, China), Hematocrite centrifuge (Nuve Sanayi Malze Mel Eri Imaltve, Turkey), microhematocrit reader (Hawksley, England), Microscope (Olympus optical, Taiwan), 96 well micro titer plate (flat bottomed), oven, umber glass vials, crucible dishes, clamps, metal stands and spatula, Digital rectal thermometer.

Chemicals

Analytical grade solvents and reagents used in this work were; Dichloromethane (BDH laboratory supplies, England), Methanol (Sigma Aldrich, Germany), Diethyl ether (Blulux Laboratories, India), Dimethylsulphoxide (DMSO), Diminazene aceturate (BerenilÒ, Hoechst, Germany).

Study area

The study was conducted in Aklilu Lemma Institute of Pathobiology (AL-IPB), Addis Ababa University, Addis Ababa.