Antitrypanosomal Activity of Selected Medicinal Plants against *Trypanosoma congolense* Field Isolate

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This is to certify that the thesis prepared by Beza Dereje, entitled: "**Antitrypanosomal activity of selected medicinal plants against *Trypanosoma congolense* field isolate**." submitted in partial fulfillment of the requirements for the Degree of Master of Science in Pharmacology complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abstract

Antitrypanosomal activity of selected medicinal plants against *Trypanosoma congolense* field isolate.

Beza Dereje

Addis Ababa University, 2023

Trypanosomiasis is among the most common neglected tropical diseases (NTDs) of humans and animals. It mainly affects countries with poor health infrastructures and the actual disease burden is unknown. It is estimated that 10 to 14 million heads of cattle, goats and a million equines are at risk of contracting the disease in Ethiopia. Trypanocidal drugs are currently facing a number of problems like toxicity, resistance and availability issues. These limitations have prompted the search for new, safe and effective drugs. In Ethiopia, the seed of *Brucea antidysentrica,* the leaf of *Clematis hirsuta* and the root of *Rumex nepalensis* are used to treat animal trypanosome infection by traditional healers. The study aimed to investigate the *in vitro* activity of selected medicinal plants against *Trypanosoma congolense* and *in vivo* antitrypanosomal activity of the most active plant. The plants were extracted by 80% methanol maceration and tested for their *in vitro* activity using motility test (at concentration of 4, 2, 0.4 and 0.1 mg/ml) for cessation or reduction in motility of trypanosomes followed by monitoring for loss of infectivity of mice. After 12 days of *T. congolense* field isolate inoculation of mice and peak parasitaemia level (~10^8 trypanosomes/ml) was reached, 80% methanol extract of roots of *Rumex nepalensis* was administered at doses of 100, 200 and 400mg/kg orally once daily for 7 days.

The packed cell volume, body weight, parasitaemia level and rectal temperature were used as parameters for monitoring *in vivo* activity by comparing with the positive control: 28 mg/kg dose of diminazene aceturate and negative control: 1% Dimethyl Sulfoxide (DMSO) treated groups. The statistical significance was determined by one-way ANOVA followed by Tukey post hoc test. The motility of *T. congolense* was ceased by *R. nepalensis, B. antidysentrica,* and *C. hirsuta* at concentration of 4mg/ml within 10, 25 and 35min, respectively. Mice treated with 4mg/ml of *R. nepalensis* and Diminazene aceturate caused loss of infectivity of trypanosomes in mice for 21 days after the inoculation of the *in vitro* mixtures. The 80% methanol extract of roots of *Rumex nepalensis* at dose of 2000 mg/kg did not show acute toxicity signs and symptoms. Highly significant ($p<0.001$) reduction in pre-treatment parasitaemia from (7.30±0.06) to (2.70±1.21) trypanosomes/ml on day 8 of treatment and increased PCV from (45.83±0.31) to (48.00±0.26) and body weight increased from (22.63±0.55) to (26.60±1.14) gram at day 14 was recorded in mice treated with 80% methanol extract of roots of *R. nepalensis* at the dose of 400 mg/kg. The results revealed that the selected medicinal plants showed antitrypanosomal activity that supports their traditional claim and prompted further studies on isolated active substances from these plants.

Key words: Antitrypanosomal activity, *Trypanosoma congolense*, *Rumex nepalensis, Brucea antidysentrica, Clematis hirsuta*.

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LIST OF ABBREVIATIONS AND ACRONYMS

AAU	Addis Ababa University
ANOVA	Analysis of Variance
BBB	Blood Brain Barrier
CNS	Central Nervous System
DA	Diminazene aceturate
DMSO	Dimethyl Sulfoxide
HAT	Human African Trypanosomiasis
ILRI	International Livestock Research Institute
OECD	Organization of Economic Cooperation and Development
PCV	Packed Cell Volume
ROS	Reactive Oxygen Species
WHO	World Health Organization

1. Introduction

1.1 Overview of Trypanosomiasis

Trypanosomiasis is disease of both humans and animals caused by the genus *Trypanosoma*, unicellular flagellated protozoa of the phylum Sarcomastigophora, order Kinetoplastida, and family Trypanosomatidae. It is the most significant livestock diseases in Africa and South America, transmitted by the bite of infected tsetse fly. African trypanosomiasis is endemic in 36 sub-Saharan African countries. The disease is fatal if left untreated (WHO, 2022).

Human African Trypanosomiasis also known as sleeping sickness is a vector-borne parasitic disease which takes two forms depending on the characteristic feature of the subspecies *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*.

T. b. gambiense (West African sleeping sickness) is predominantly common in 24 countries in western and central Africa. Humans are the most important reservoir of *T. b. gambiense*, though it also infects domestic animals (e.g.- cattle, sheep, camels, goats, horses, pigs, dogs) (CDC, 2022; WHO, 2022). The prolonged asymptomatic first stage of *T. b. gambiense* infection prompted the search for an exhaustive active screening of the population at risk which are not possible in rural areas with poor health infrastructure. Therefore, infected people could pass away before being diagnosed and treated (WHO, 2022).

T. b. rhodesiense (East African sleeping sickness) is restricted to 13 Eastern and Southern African countries, and it causes an acute infection which leads to death within 6 months (CDC, 2022; Büscher *et al.*, 2017). Domestic and wild animals are the main reservoir of infection. Cattle have been implicated in the spread of the disease. A wild animal reservoir is responsible for sporadic transmission to hunters and visitors (WHO, 2022).

T. vivax is also found in areas where tsetse flies are not existed, which could be transmitted by mechanical vectors as biting flies (Abebe, 2005). Other species and sub-species of Trypanosoma genus are pathogenic to animals that it infects cattle, sheep, camels, goats, horses, and many other domestic and wild mammals. Nagana (Bovine Trypanosomiasis) is the most serious disease of cattle in Ethiopia caused by *Trypanosoma congolense*, *Trypanosoma vivax,* and *Trypanosoma brucei*. They are distributed mainly in the tsetse belt of western, southwestern, and southern part of the country, where the primary vector exists (Gelaye and Fesseha, 2020).

The prevalence varies depending on climatic conditions, seasons and activities which are required to control the disease. It causes great socioeconomic crisis such as morbidity and mortality including abortion and stillbirth of the livestock population, decreased dairy and meat production, and prevention and treatment costs of the disease condition, which is a major obstacle to the economic development of the country (Abebe, 2005).

2. Literature Review

2.1 Epidemiology of the disease

Human African Trypanosomiasis (HAT) resulted in mortality of millions of people in the late 1990s (Büscher *et al.*, 2017). Trypanosomiasis was listed among the top twenty cattle diseases of developing countries by the International Livestock Research Institute (ILRI) (Perry *et al.,* 2002). Many cases are reported from more than 30 countries residing in Africa, where it causes substantial mortality, morbidity, limits livestock productivity and agricultural development, annual losses that run into billions of dollars. The disease cause hunger and poverty since it causes a serious impediment to livestock production (Franco *et al.*, 2014; Erick et *al*., 2012). In Ethiopia, Nagana is prevalent in the most arable and fertile land following the greater river basins of Abay, Omo, Ghibe and Baro (Gelaye and Fesseha, 2020). It is estimated that 20,000 heads of cattle die every year and 10 to 14 million heads of cattle in Ethiopia are at risk of contracting the disease (Fassel, 2016; Leta *et al.*, 2016).

The vector of trypanosomiasis, tsetse flies cover areas over ten million square kilometers in Africa, representing 38 countries of the continent. Approximately 377,000 species out of the total 147,000,000 species of animals in different countries, have been susceptible to tsetse flies (Hundessa *et al.,* 2021). The fly has infested an estimated 130,000-200,000 square kilometers of fertile land. The most important trypanosomes in Ethiopia are *T. vivax* and *T. congolense* (Fassel, 2016).

2.2 Pathogenesis

"African trypanosomes" or "Old World trypanosomes" are hemoflagellates of the genus Trypanosoma. Trypanosomes live in the blood and other body fluids of vertebrate hosts. They use flagellum to swim, possess a kinetoplast and undergo development in an arthropod vector. Trypanosomes mostly are 20–30 µm long and 2–5 µm wide spindle shaped cells characterized by wriggling movement in the blood and tissues of mammals. A dense coat of glycoprotein dimers covered its cell membrane and shields against innate immunological attacks. These highly immunogenic glycoproteins induce an antibody response which triggers destruction of antibody-opsonized trypanosomes. For the sake of survival, they developed variation of an antigen, where the glycoprotein coat is replaced by a different coat (Büscher *et al.*, 2017).

The most important species affecting livestock in Ethiopia are: *T. congolense*, *T. vivax* and *T. brucei* in cattle, sheep and goats, respectively. *T. evansi* and *T. equiperdum* affecting horses and camels (Gelaye and Fesseha, 2020; Brun *et al.*, 2010; Awoke, 2000). *T. brucei* has three subspecies: *Trypanosoma brucei brucei* causes African Animal Trypanosomiasis. *T. b. gambiense* and *T. b. rhodesiense* causes chronic and acute African trypanosomiasis, respectively. *T. b. brucei* primarily infects cattle and occasionally other animals, does not infect humans (WHO, 2022).

T. brucei infection mostly induces polyclonal B-cell activation, which results in extremely elevated (up to 14 times normal) Immunoglobulin M (IgM) concentrations and various antibodies including autoantibodies. Infection of hosts begins after the bite of tsetse fly, where metacyclic trypanosomes injected with tsetse fly saliva into the skin. Trypanosomes spread via the blood and lymph to various organs and tissues invade the brain parenchyma, where they will trigger inflammation and neurological damage (Büscher *et al.*, 2017).

T. b. gambiense is anthroponotic (human to human transmission) and provide the main reservoir. The infection can be transmitted by blood transfusion, sexual contact, organ transplantation, accidental exposure and from pregnant woman to her baby (CDC, 2022; Brun *et al.*, 2010).

The healthy human plasma contains trypanosome lytic factor that destroys trypanosomes pathogenic for animals, although *T. b. gambiense* and *T. b. rhodesiense* are resistant. The high degree of antigenic variation makes development of vaccine, unlikely to be feasible (Brun *et al.*, 2010).

Bovine trypanosomiasis is transmitted cyclically or mechanically. Trypanosomiasis transmitted by blood sucking flies other than tsetse flies is called noncyclical transmission. Mechanical vectors transmit pathogens on the bite site through contamination with infected blood. The primary vector for *T. congolense*, *T. vivax*, and *T. b. brucei* is the tsetse fly, where the trypanosomes replicate and are transmitted through saliva while feeding. *T. vivax* can be transmitted by "syringe passage" of infective blood experimentally (Gelaye and Fesseha, 2020).

Most wild and some domestic animals remain clinically normal carriers. Some breeds of indigenous cattle in Africa can tolerate challenge of tsetse flies by limiting the replication of trypanosomes and by warding off the infection especially *T. vivax.* The trypanotolerance level depends on genetic and environmental origin. Cattles susceptibility to trypanosomiasis depends on age, previous exposure, behavior, health status and type of breed. The indigenous zebus and West African Bos taurus breeds are trypanosusceptible and trypanotolerant, respectively. Exotic imported ruminants are more severely affected than local genotype (Gelaye and Fesseha, 2020).

Compared with other species, *T. vivax* produces a higher parasitaemia level in cattle, which facilitate mechanical transmission. Since, the life cycle of *T. vivax* in tsetse fly is shorter; it is transmitted more readily (Gelaye and Fesseha, 2020).

The transmission of animal trypanosomiasis is mainly determined by Tsetse flies (genus Glossina) which is found only in tropical Africa from about latitude 15° N to 29° S. In Ethiopia, tsetse flies are confined to the regions between longitude 33° and 38°E and latitude 5° and 12°N covering about 220,000 km² area. Different regions of Ethiopia are infested with more than one species of tsetse flies (Gelaye and Fesseha, 2020; Oyda and Hailu, 2018). The distribution of tsetse flies depends on climate, altitude, vegetation, and presence of suitable host animals (Gelaye and Fesseha, 2020). The density of tsetse fly and the level of their contact with the host determines the level of infection. Agricultural and industrial developments lower tsetse density by destroying its habitat (Gelaye and Fesseha, 2020).

Currently areas of about 220,000 km^2 are infested with five species of Tsetse flies. The new areas are being invaded and settled communities are continually evicted by the advancing tsetse (Gelaye and Fesseha, 2020). Tsetse flies are narrow, yellow to dark brown flies and 6 to 15mm in length (Gelaye and Fesseha, 2020). Both sexes of tsetse flies are hematophagous, viviparous and can transmit trypanosomes. The tsetse species are classified according to differences in morphology, habitat, and abilities to transmit *T. b. gambiense* or *T. b. rhodesiense* sleeping sickness. Only 0·01% of tsetse flies carry and transmit *T. brucei* infection but feeding every 3 days with 2–3month lifespan, it can infect many people (Büscher *et al.*, 2017; Brun *et al.*, 2010).

Once ingested, trypanosomes undergo a complex journey, then reach the salivary glands and develop into free-swimming and short stumpy metacyclic forms. Eliminating tsetse flies or reducing their contact with humans or animals serve as one way to interrupt its transmission (Büscher *et al.*, 2017).

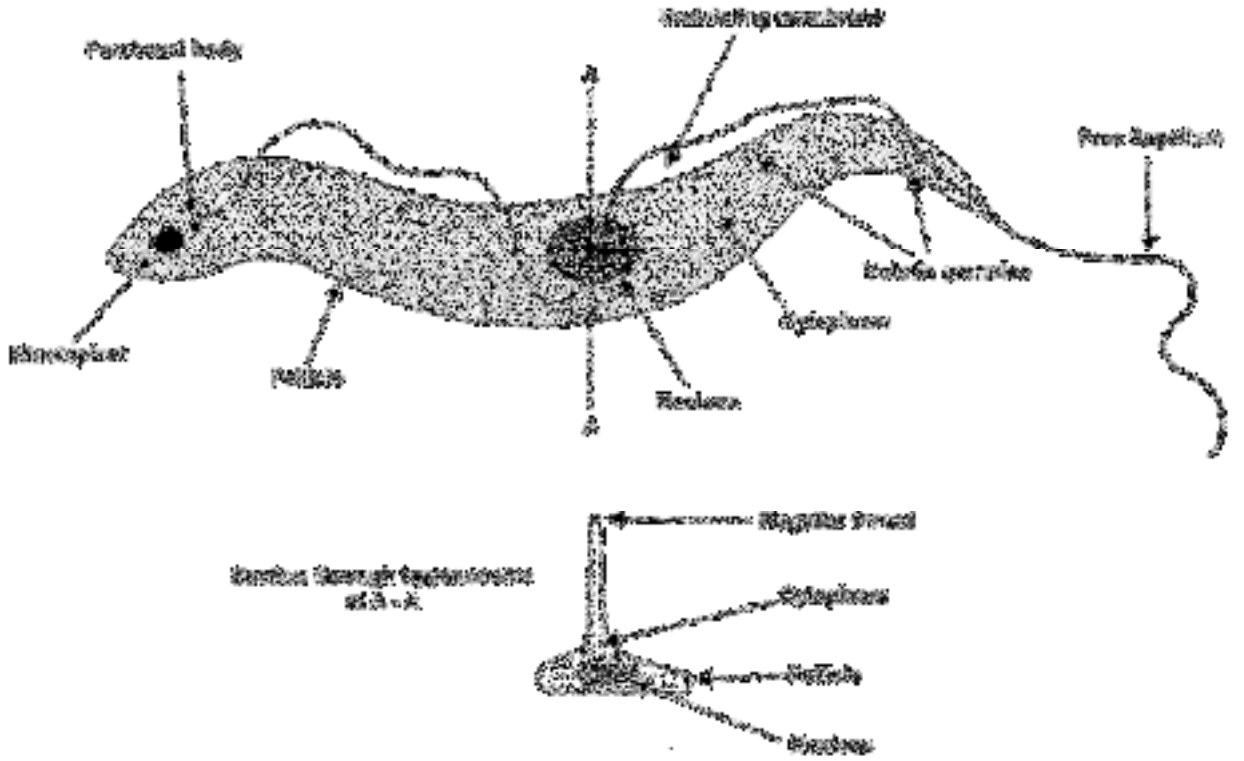

Figure 1. A diagram illustrating the different parts of a trypanosome (Uilenberg G, 1998)

2.3 Clinical features of trypanosomiasis

The basic clinical signs and symptoms occurs following an incubation period of 8-20 days. Infected animals are dull, lack appetite, emaciated, and have watery ocular discharge. Swollen lymph nodes, pale mucous membrane, diarrhea and throat edema. The animal becomes very emaciated, cachectic and dies within 2-4 months or longer. Thin, rough-coated, anemic, lethargic cattle with generalized lymph node enlargement are said to have 'Fly struck' appearance (Gelaye and Fesseha, 2020).

The Human African Trypanosomiasis goes through two stages: The first (hemolymphatic) stage, trypanosomes replicate in blood, lymph and subcutaneous tissues, which entails chronic and intermittent fever, headaches, pruritus, lymphadenopathy, hepatosplenomegaly, joint pains and itching (Brun *et al.*, 2010). The febrile illness results from type 1 inflammatory reaction, activation of macrophage-1 cells and high interferon γ, tumor necrosis factor (TNF), Reactive Oxygen Species (ROS), and nitric oxide concentrations (Brun *et al.*, 2010).

In the second stage (neurological or meningoencephalic stage) the parasites cross the blood-brain barrier (BBB) and infect the central nervous system (CNS). Signs and symptoms include: behavioral change, confusion, poor coordination, sleep disturbance (disrupted sleep/wake cycle, daytime somnolence, sudden sleep urges, and nocturnal insomnia). Neurological signs include: tremor, choreiform, fasciculation, weakness, ataxia, akinesia, speech disorders, emotional lability, lack of attention, indifference apathy, aggressive and stereotypic behavior, manic episodes, melancholia, confusion, and dementia (WHO, 2022; Büscher *et al.*, 2017). In severe cases secretion of prolactin, renin, growth hormone, and cortisol disappear (Brun *et al.*, 2010).

T. b. rhodesiense disease is typically an acute disease, progresses rapidly to second stage and death within weeks and months (Büscher *et al.*, 2017). Enlarged lymph nodes, edema, trypanosomal chancre, thyroid dysfunction, adrenal insufficiency, hypogonadism, hepatomegaly and jaundice sometimes with ascites are frequent. The more severe and fatal myocarditis, perimyocarditis and congestive heart failure can occur (Büscher *et al.*, 2017; Brun *et al.*, 2010). High Tumor necrosis factor α and transforming growth factor β concentrations were seen (Brun *et al.*, 2010). *T. b. gambiense* follows a chronic progressive condition with an estimated duration of 3 years. The headache, pruritus, lymphadenopathy, and an intermittent fever lasting 1 day to 1 week (Büscher *et al.*, 2017; Brun *et al.*, 2010).

In travelers from non-endemic countries the incubation period is less than 3 weeks and less than 1 month for *T. b. rhodesiense* and *T. b. gambiense,* respectively. Chancre, non-itching rash, irregular erythematous macules, headache, lymphadenopathy, hematological disorders, kidney impairment, electrolyte imbalance, an elevated C-reactive protein and liver enzymes, hepatomegaly, and splenomegaly occur. In *T. b. rhodesiense* disease, gastrointestinal symptoms are more frequent, with jaundice reported in 28% of cases. Multiorgan failure, disseminated intravascular coagulopathy, and coma are less frequent complications (Büscher *et al.*, 2017; Brun *et al.*, 2010). The seeds of *B. antidysenterica* J.F. Mill*,* roots of *R. nepalensis* Spreng and leaves of *C. hirsuta* Perr. & Guill. were previously reported for their traditional use against trypanosomiasis (Iwaka *et al*., 2022; Kunwar, 2021; Weldegerima *et al*., 2008).

Drug resistance: Polymorphisms associated with trypanocide resistance in the TbAT1 gene were detected (Kulohoma *et al.*, 2020). Mutations in the *T. b. gambiense* genome confer resistance to Melarsoprol and Pentamidine have been reported. Melarsoprol resistance generated serious concern when its failure rates rose in several HAT foci (Büscher *et al.*, 2017). A *T. brucei* adenosine transporter is well-known for its role in the uptake of Melarsoprol and Pentamidine. The loss of aquaglyceroporin 2 (AQP2) (channel that facilitate drug accumulation) function was linked to Melarsoprol-Pentamidine cross-resistance (Baker *et al.*, 2013). The possibility that parasites resistant to melarsoprol and eflornithine indicates that genes capable of conferring drug resistance to both drugs are in circulation (Barrett *et al.*, 2011). Insecticide resistance against tsetse has not been reported (Büscher *et al.*, 2017).

2.3.1 Prevention and Control measures

There is no vaccine against trypanosome infection and chemoprophylaxis is not recommended because of toxicity and low risk of infection. The only preventive measure is reduction of tsetse fly bites. The control measure involves control of tsetse fly population, prophylactic treatment, husbandry of animals at risk and use of trypanotolerant animals. Each of these approaches has important limitations as expense, environmental pollution and drug resistance. Earliest methods to control tsetse flies as bush clearing and elimination of game animals on which tsetse feed and recent methods involve using insecticide-impregnated nets and screens, fly traps, spraying insecticides and use of sterile male flies (Gelaye, 2020). Controlling wild animal reservoir is far more challenging. Integration of several methods is recommended (Büscher *et al.*, 2017).

The Pan-African Tsetse and Trypanosomiasis Eradication Campaign (PATTEC) was founded as a taskforce uniting African countries to eliminate the disease with main strategy of elimination of the tsetse fly. In 2002, the WHO HAT control and surveillance program established worldwide alliance to eliminate sleeping sickness. (Brun *et al.*, 2010; Büscher *et al.*, 2017).

The prerequisite for elimination is reliable methods for diagnosis and staging, and safe and effective drugs. To guarantee sustainability, additional partners are needed to maintain the effort (Brun *et al.*, 2010). The elimination process faces challenges as sustaining commitment of stakeholders; limitations of diagnostic and treatment tools; integrating disease control in peripheral health facilities and reaching populations in civil unrest (Büscher *et al.*, 2017).

2.4 Statement of the Problem

Human African Trypanosomiasis (sleeping sickness), protozoan disease of humans as well as animals, is fatal if left untreated. The disease is endemic in sub-Saharan Africa mostly in central Africa, where it is a huge burden on rural communities (Büscher *et al.*, 2017). Large areas of Africa, approximately 4 million km^2, have been rendered unsuitable for livestock production by trypanosomes (Ikenna, 2008). It caused devastating epidemics and the estimated population at risk was 55 million people for the period 2016–2020; with 3 million people at moderate or higher risk (WHO, 2022). It causes the death of 3 million cattle with an estimated cost of $ 6-12 billion US dollars, annually (Maikai *et al.*, 2010). In 2015, fewer than 3000 cases of human trypanosomiasis were reported as it was difficult to assess the exact situation because of instability of social circumstances and inaccessibility for surveillance and diagnosis. WHO targeted for the elimination of the disease (WHO, 2022; Büscher *et al.*, 2017).

African Animal Trypanosomiasis affects health, livestock and agricultural production, national economy, and the environment (Adly *et al.*, 2013). In Ethiopia, trypanosomiasis known as Gendi (Giday and Ameni, 2003) is caused by *T. congolense*, *T. vivax* and *T. brucei*, in cattle, sheep and goats, *T. evansi* in camels and *T. equiperdium* in horses (Getachew, 2005). It is one of the main constraints to livestock and agricultural production affecting the development of the nation (Abebe, 2005).

Trypanosomiasis resulted in the annual losses to the national economy estimated to exceed US$200 million. The disease and its vector are excluding 180,000-220,000 km^2 land of Ethiopia suitable for agriculture, 10 to 14 million heads of cattle, an equivalent number of goats and nearly of a million equines are at risk (Gelaye and Fesseha, 2020).

Although substantial amount of resource spent for trypanosomiasis control (Tikubet, 2000), modern medicines have serious adverse effects, lack efficacy, availability and affordability (Ketema *et al*., 2013; Legros, 2002), ongoing discoveries and trials are required for safe, effective and affordable treatment (Büscher *et al.*, 2017). Ethiopian farmers and pastoralists highly rely on traditional knowledge, practices and available medicinal plants to control livestock diseases. The medicinal plants illicit very effective medicinal value provides a vital contribution to health care needs of the country for ailments of human and animals (Temeche and Asnakew, 2020).

2.5 Medicinal Plants Used for Management of Trypanosomiasis

Traditional medicinal plants are still the most important sources of therapeutics for most of the population in developing countries (Awas and Demissew, 2009). Reports revealed that about 80% of the population depend on traditional medicinal plants to fulfill their primary healthcare need, largely due to its cultural acceptability, affordability and efficacy (WHO, 2001). In Ethiopia, about 90% of livestock depends on traditional medicine for treatment of various ailments (Birhan *et al*., 2011).

Table 1. Medicinal plants traditionally used for trypanosomiasis treatment.

Medicinal plants traditionally used for treatment of trypanosomiasis	**Reference**
Dalbergia lacteal, Coffea arabica, Terminalia brownii, Phaseolus vulgaris and *Albizia gummifera*	(Hansha *et al.,* 2020)
Lepidium sativum L, Allium sativum, Aloe vera, Aeschnomene elaphroxylon Guill, *Loblia giberroa Hemsl, Myrica salicifolia Hochst ex A. Rich, Withania somnifera (L.),* *Vernonia amygdalina Del*. *Nicotiana tabacum, Trifolium burchellianum, Thuja orientalis, Piper.L, Citrus aurantifolia., Echinops kebericho*	(Shilema *et al.,* 2013), (Kidane *et al.,* 2014)
Solanum dasyphyllum Schumach.	(Bekalo *et al.,* 2009)
Khaya senegalensis, Afzelia africana, *Vitellaria paradoxa*, *Crossopteryx febrifuga*, *Cassia sieberiana*, *Vernonia amygdalina*, and *Crossopteryx febrifuga*	(Shilema *et al.,* 2013); (Dassou *et al*., 2014); (Dassou *et al*., 2015); (Ogni *et al*., 2016); (Azokou *et al*.,2016); (Noudèkè *et al.,* 2017)
Allium sativum, *Annona senegalensis*, *Ximenia Americana*, *Detarium microcarpum*, *V. amygdalina*, *Guieria senegalensis*, *B. africana*, *P. erinanceus*, *B. costatum*, *Cissus quandrangularis*, *C. myxa*, *Securidaca longipedunculata*, and *Ocimum lamiifolium*	(Abiodun *et al*., 2012)
Ocimum gratissimum Linn. *Trema orientalis* (L.), *Pericopsis laxiflora* (Benth.), *Jatropha curcas* Linn.,	(Abedo *et al.,* 2013) and (Ahamidé *et al*., 2017)

Terminalia catappa Linn. and *Vitex doniana* Sweet	
Prosopis africana, *Acacia polyacantha*, *Combretum collinum*, *Crossopteryx febrifuga*, *K. africana*, *Zea mays, A. africana*, *C. sieberiana*, *D. microcarpum*, *K. senegalensis*, *Pseudocedrela kotschyi* and *X. americana*	(Ogni *et al.,* 2014)
Annona senegalensis	(Okoye *et al*., 2012)
X. americana	(Siddaiah *et al.,* 2011; Monte *et al.,* 2012)
Allium cepa, *Phytolacca dodecandra*, *Clutea abyssinica*, *Clausena anisata*, *Rumex nepalensis*, *Verbascum sinaticum*, and *Salvadora persica*	Fullas (2010)
root bark of *Morinda morindiodes,* leaves of *Tithonia diversifolia and Acalypha wilkesiana*	(Olukunle et al., 2010)
Verbascum sinaiticum Benth and *Phytolacca dodecandra* L.	(Weldegerima *et al.,* 2008; Yigezu *et al.,* 2014)
Enantia chlorantha	(Odoh *et al.,* 2010)
Annickia kummeriae *Pseudospondias macrocarpa* *Artemisia annua* *Drypetes natalensis* *Maytenus senegalensis* *Neurautanenia mitis*	(Malebo *et al*., 2009)
Azadirachta indica	(Mbaya *et al.,* 2010)

2.6 Overview of the Experimental Plants

2.6.1 *Rumex nepalensis* Spreng.

The name Rumex originated from the Latin word for dart, alluding to the habit of Romans sucking the leaves to allay thirst (Jain and Parkhe, 2018). Rumex is a genus comprised of about 200 species (Vasas *et al*., 2015). *R. nepalensis* Spreng. known as Nepal Dock belong to the family Polygonaceae, is widely distributed throughout Himalayas in 35 districts of Nepal ranging between 900 and 4000 m in moist and shady places. Flowering April–May, fruiting June–July (Kunwar, 2021). It is locally known as "Yeberamilase" (Alemayehu *et al*., 2015), "Tult" (Bizualem *et al.,* 2023; Alemayehu, 2017) and "Yewushamilas" (Alemneh, 2021) in Amharic and "Shuultii" in Oromiffa (Kefalew *et al.,* 2015). A perennial, ascending herb that has a wide range of activity.

Table 2. Traditional use of *Rumex nepalensis* Spreng.

***Rumex nepalensis* Spreng traditional use**	**Reference**
Febrile illness, Retained placenta	(Birhan *et al.,* 2017)
Wound	(Bitew *et al.,* 2019*;* Shaikh *et al.,* 2018*;* Giday*,* 2007*;* Handa *et al.,* 2006*)*
Abdominal cramp, diarrhea, purgative, constipation, stomachache	(Bekalo *et al.,* 2009*;* Giday*,* 2009), (Chekole, 2015; Shen*,* 2010*;* Yineger *et al.,* 2007*;* Wirtu *et al.,* 1999*),* (Gautam *et al.,* 2011*;* Ghosh *et al.,* 2002) (Gairola *et al.,* 2014) (Amsalu *et al.,* 2018*;* Chekole*,* 2015*;* Abera*,* 2014*;* Megersa *et al.,* 2013*;* Etana*,* 2010*;* Teklehaymanot*,* 2009)
Leishmaniasis, Trypanosomiasis	(Teklehaymanot*,* 2009), *(*Iwaka *et al.,* 2022*;* Nibreta and Wink*,* 2011)
Eye infection, toothache	(Giday, 2009) (Etana, 2010; Handa *et al*., 2006)
Liver disease, evil eye, spider poison, blackleg	(Giday, 2009) (Megersa *et al*., 2013; Yineger *et al*., 2007; Wirtu *et al*., 1999)
Tonsillitis	(Kunwar, 2021; Eshetu *et al*., 2020; Chekole, 2015; Mekonnen *et al*., 2015; Teklehaymanot and Giday, 2009)

Abortion, blood clot, treat blood pressure	(Eshetu *et al.,* 2020*;* Etana*,* 2010*;* Abera*,* 2003). (Amsalu *et al.,* 2018)
Antioxidant, antifungal, Antiviral, antibacterial, antiprostaglandin, anti-inflammatory, antialgal, insecticidal, analgesic	(Esubalew *et al.,* 2017)*,* (Ghosh *et al.,* 2002)
CNS depressant, diabetic nephropathy, muscle relaxant, cytotoxic and phytotoxic activities	(Shaikh*,* 2018*;* Esubalew *et al.,* 2017)
Antidote	(Getahun, 1976)
Parasites	(Shen, 2010)
Swelling	(Yineger *et al.,* 2007*;* Wirtu *et al.,* 1999)
Skin irritation, Dislocated joints, bone, antiarthritic activity, increases immunity, and regulates genitourinary system	(Kunwar, 2021*;* Ur-Rahman *et al.,* 2018*;* Gairola *et al.,* 2014)

The roots of *R. nepalensis* are used as hepatoprotective, antipyretic, anthelmintic, for mental tension and disturbance, as hypotensive agent (Ghosh *et al*., 2002), for treatment of dysmenorrhea, abdominal cramps and ear infection (Giday *et al*., 2010). Fresh roots and leaves for leishmaniasis, and while giving birth (Kunwar, 2021; Teklehaymanot and Giday, 2007), ascariasis, uterine bleeding (Eshetu *et al*., 2020), Cytotoxic (Tesfaye *et al*., 2020) and laxative (Getahun, 1976). In Ethiopia, fresh roots of *R. nepalensis* Spreng. were pulverized and given to calves orally or through nasal route for protection and treatment of trypanosomiasis (Kunwar, 2021; Elto, 2019; Weldegerima *et al.,* 2008).

Figure 2. Photograph of *Rumex nepalensis* Spreng. (by Tasisa Ketema, 2020)

2.6.2 ***Clematis hirsuta*** **Perr. & Guill.**

Clematis hirsuta Perr. & Guill. belongs to the family Ranunculaceae, locally known as Nech Azo Hareg (Yimam *et al*., 2022; Moges and Moges, 2019; Tuasha *et al*., 2018) is used for treatment of bone and tissue cancer (Ashagre *et al*., 2016), tumor on the neck (Abate *et al*., 2022; Moges and Moges, 2019) and wound (Romha *et al*., 2015). In topical leishmaniasis, the leaf is pounded and applied on the affected area with salt (Yimam *et al*., 2022; Teklehaymanot *et al.,* 2006).

It is used in Asia as anti-inflammatory, analgesics and anti-rheumatics (Habtamu and Mekonnen, 2017). For the treatment of anthrax (Birhan *et al*., 2017), fungus, leprosy, yaws (Cos *et al*., 2002), trachoma, elephantiasis, hemorrhoids, wound (Abesh, 2021) and ascariasis (Giday *et al*., 2007). The root extract had antioxidant and antibacterial activities (Abdisa and Kenea, 2020). It is used to treat headache, sweating, diarrhea, and breast cancer (Tuasha *et al*., 2018; Woldeab *et al*., 2018).

Figure 3. Picture of *Clematis hirsuta* Perr. & Guill. (by Betelhem Sirak, 2021)

2.6.3 ***Brucea antidysentrica***

Brucea antidysenterica J.F. Mill (Simaroubaceae) named after a Scottish scholar and traveler James Bruce (1730-1794) (Jansen, 1981). It is commonly known as "Waginos" (in Geez) or "Aballo" (in Amharic), is used for treatment of hemorrhoids, weight loss, fever, itching, diarrhea (Amuamuta *et al., 2014)* and wound (Taye *et al*., 2011). It is used to treat evil eye, cutaneous leishmaniasis, leprosy (Wubetu *et al*., 2017), antineoplastic activity (Abebe, 2016) and antimalarial activity (Teklehaymanot *et al*., 2016; Fassel *et al.,* 2016; Karunamoorthi and Tsehaye, 2012). The crude extracts and compound isolates obtained from other members of the genus Brucea revealed activities against *T. cruzi and T. b. brucei* (Ketema *et al*., 2023; Ehata *et al.,* 2016).

Figure 4. Photograph of Seeds of *Brucea antidysentrica* J.F. Mill (by Tasisa Ketema, 2020).

3 Objectives

3.1 General objective

To investigate antitrypanosomal activity of selected medicinal plants against *Trypanosoma congolense* field isolate.

3.2 Specific objectives

- To examine the *in vitro* antitrypanosomal activity of the selected medicinal plants
- To determine the acute oral toxicity profile of the extract with best *in vitro* activity
- To investigate *in vivo* antitrypanosomal activity of the extract with best *in vitro* activity

4 Materials and Methods

4.1 Plant material

The *Brucea antidysentrica* seeds were collected from Dega Damot district of Amhara region, located 399 km away from Addis Ababa. The roots of *Rumex nepalensis* was collected from Dek Island, located in Bahir Dar Zuria woreda, 600 km far from Addis Ababa. Leaves of *Clematis hirsuta* Perr and Guill. were collected from the highland of Ochollo village, Chencha woreda in Gamo Zone located 520 km South from the capital, Addis Ababa.

The different parts of the medicinal plants were identified & authenticated by Mr. Melaku Wendafrash taxonomist at National Herbarium, Department of Biology, College of Natural and Computational Sciences, Addis Ababa University (AAU). The voucher numbers TK-002, TK-004 and BS-006 were given to *R. nepalensis* Spreng, *B. antidysenterica* J.F. Mill and *C. hirsuta* Perr. and Guill. specimens, respectively and kept in National Herbarium of AAU for future reference.

4.2 Chemicals and Instruments

Chemicals: Distilled water, Normal saline, immersion oil, Diminazene aceturate BP Vet [1.05g (4,4-diazoamino, dibenzamidine diaceturate) + 1.31g phenazone BP (DIMINASHISH) aceturate], Dimethyl sulfoxide, Giemsa stain, and methanol (Sisco Research Laboratories, India).

Apparatuses: Syringes, Scissor, Aluminum foils, oral gavage, balance, mortar and pestle, gloves, microscope, slides, cover slides, 96 well plate, Whatman No.1 filter paper, beakers, desiccator, incubator, water bath, heparinized capillary tube, centrifuge, rotavapor (Heidolph Instruments GmbH and Co., Germany), oven, micropipettes, refrigerator, polypropylene cages, spatula and flasks were used.

4.3 Test organism

Trypanosoma congolense field isolate obtained from sick cattle in Abulo Kebele, Gamo Gofa zone, Arba Minch city, Southern Ethiopia. *Trypanosoma congolense* infected blood was taken from jugular vein of cattle in heparinized microhematocrit capillary tubes to $3/4^{th}$ of the height and then sealed with sealing clay. For 5 minutes, the blood was centrifuged at 12,000 revolutions per minute. The contents of the capillary tube's buffy coat zone were placed on a microscope slide and covered with a coverslip (22 x 22 mm) (Abdeta *et al*., 2020).

The motility of *Trypanosoma* was examined under a 400x objective microscope on the slide. (Abdeta *et al*., 2020). An oil immersion 100x objective microscope was used to examine (Uilenberg, 1998). An infected blood (0.2 ml) was injected intraperitoneally into six healthy mice. The blood was collected on an EDTA coated tube after the donor mice undergo cardiac puncture. The parasites were maintained using serial blood passage.

4.4 Experimental Animals

Swiss albino mice (25–35 g) of both sexes aged 8–10 weeks were obtained from Arba Minch University. Mice were housed in polypropylene cages and allowed free access to water and pellet and kept at room temperature at Biochemistry laboratory of Arba Minch University. All mice were acclimatized to the working environment for a week. All procedures and techniques were conducted in accordance with the internationally accepted guidelines for the Care and Use of Laboratory Animals.

4.5 Plant Extraction

After collection, the plants were washed with tap water, dried in the shade and mechanically grounded with mortar and pestle. Prior to extraction, the material was sieved and weighed. The course powder of *B. antidysentrica* 400gm obtained using mortar and pestle was extracted by maceration using 1.2 liter of 80% methanol (1:3) in volumetric flask. After 72 hrs of maceration with regular shaking it was filtered with Whatman No 1 filter paper.

The 400 g of seeds of *B. antidysenterica* and roots of *R. nepalensis* were macerated in separate volumetric flasks with 1.2 liters of 80% methanol (1:3) and shaken occasionally and was filtered first with sterile gauze. After that, Whatman No. 1 filter paper was used. By adding fresh solvents, the mark was re-macerated twice to maximize yield, and then filtered in the same manner. At 40ºC, the combined filtrates were concentrated using a rotary evaporator. The concentrated extracts were frozen overnight in a refrigerator at -20ºC and then dried using a lyophilizer. The prepared solid extracts were transferred to a beaker and stored in a refrigerator at 4°C until the experimental procedures conducted.

Fresh leaves (600g) of *Clematis hirsuta* were rinsed with water to remove dirt. It was cut into small pieces and macerated in a sufficient amount of 80% methanol at room temperature for 24 hours with continuous agitation before being filtered with sterile gauze and Whatman no. 1 filter paper. This was done twice, and the combined filtrate was concentrated in a rotavapor at 40 °C. The dried extract was labeled as CH-M and stored in an amber-colored bottle in a refrigerator at 4 °C until use.

Lastly, a solid extract of golden colored *B. antidysenterica*, a brown colored solid extract of *R. nepalensis* with a sticky nature and the clingy *C. hirsuta* were obtained and their percentage yields were computed using the formula:

$$Yield\ \% = \frac{Weight\ of\ extract\ obtained}{Weight\ of\ extracted\ plant\ sample}$$

4.5.1 **Parasite inoculation and extract administration**

Blood collected from infected mice with high parasitaemia level through cardiac puncture on EDTA coated tubes was mixed with normal saline. Two hundred microliter of blood with approximately 16-32 organisms per field was obtained and mixed with 5 µl of each of the test substances at 4.0, 2.0, 0.4, and 0.1 mg/ml concentration determined from previous *in vitro* studies (Tewabe *et al.,* 2014; Yusuf *et al*., 2012).

Thirty healthy Swiss albino mice infected intraperitoneally with 0.2 ml of *T. congolense* infected blood (~10^4 trypanosomes/ml) collected by cardiac puncture from donor mice were divided into *R. nepalensis* 80% methanol extract 100, 200 and 400 mg/kg (RN 100, RN 200, RN 400), the positive control Diminazene aceturate, and the negative control 1% DMSO. Treatment with the extracts began on the 12^{th} day post-infection (Day 0 of treatment), when the infected mice show peak parasitaemia (~10^8 trypanosomes/ml). On each day of drug administration, the 80% methanol extracts of *B. antidysenterica* seeds*, R. nepalensis* roots and *C. hirsuta* leaves were freshly prepared by solubilizing in 1 % DMSO and doses of (100, 200, and 400 mg/kg) were given orally every day for seven days. An acute toxicity tests on the substance determined by Organization of Economic Cooperation and Development (OECD) guideline helped in the selection of an appropriate starting dose (OECD, 2001).

4.6 Acute oral Toxicity Test

Acute oral toxicity test was conducted according to the OECD 2001 guideline with 2 g/kg dose. A group of five female Swiss albino mice 30-35 g (age 10-12 weeks) fasted for 4 hours before and 2 hours after oral administration of the extract. The mice were monitored for 1 hour after the extract was administered, then intermittently for 4 hours, and for a period of 24 hours for death and behavioral changes, physical appearance, feeding activities, erection of hair, lacrimation, decreased motor activity, and other signs of acute toxicity and mortality were observed and documented then follow up was continued for 14 days. The 80% methanol extract of *R. nepalensis* roots was not toxic at the dose administered, which indicates that its 50% lethal dose (LD_{50}) is above 2000 mg/kg (OECD, 2008).

4.7 Inoculum preparation

The donor mice were infected with *Trypanosoma congolense* field isolates obtained directly from trypanosome-infected cattle. The donor mice reached peak parasitaemia level after 12 days of infection. Blood was drawn from donor mice via cardiac puncture into an EDTA-coated tube. To increase the volume of the inoculum, collected blood was diluted with normal saline. A 0.2 ml injection containing 10^7parasites/ml was given to healthy mice intraperitoneally (Feyera *et al.*, 2014).

The experimental mice were tested twelve days after infection and only positive mice were allowed to take part in the experiment. After infection was established (~10^8 trypanosomes/ml), blood was collected from the donor mice using cardiac puncture (Atawodi and Ogunbusola, 2009). Blood (0.2 ml) containing ~10^4 trypanosomes/ml (Herbert and Lumsden, 1976) was injected intraperitoneally to acclimatized mice and used for *in vitro* and *in vivo* antitrypanosomal activity test.

After 2 weeks the mice were randomly divided into five groups of six mice each, with the average parasitaemia reaching 7.20 (Log number). Treatment began on the 14th day post infection (day 0 of treatment). Mice in Group 1 received a vehicle injection (1% DMSO, 10 ml/kg/day), while mice in Group 2 injected Diminazene aceturate (28 mg/kg) as a positive control (Mergia *et al.,*2016; Maikai *et al.*, 2010). The other three groups were used as treatment groups. Animals in Groups 3–5 received RN-M injections of 100, 200, and 400 mg/kg/day, respectively.

Each test substances were dissolved in 1% DMSO and given orally every morning for seven consecutive days, with parasitaemia examined every other day until the experiment ended on the 14th day (Mergia *et al*., 2016). The level of parasitaemia (expressed as Log number of parasites per ml of blood) in those treated groups were compared to those in the control group to assess the antitrypanosomal effect of the test substance (Mustapha *et al.,* 2013).

A wet smear on microscope slides from a drop of blood obtained from a mouse's tail was made and parasitaemia level monitored every other day at 400x magnification. Herbert and Lumsden's "Rapid Matching" technique was used to determine the level of parasitaemia. The wet blood smear from mice was prepared in triplicate, and the slide counts mean value was calculated for each sample (Herbert and Lumsden, 1976).

4.8 *In vitro* Antitrypanosomal activity

In vitro test was performed on 96 well plate to detect motility of trypanosomes. The 200 µl blood obtained from cardiac puncture containing approximately 16-32 trypanosomes per field were mixed with 50µl of the test substances in an amount of 0.5, 2, 10 and 20mg/ml to produce 0.1, 0.4, 2 and 4.0mg/ml respectively and similar doses of standard drug (Diminazene aceturate) as positive control and 1% DMSO as negative control were used. The diluted blood's parasite load was estimated to be 1 x 10^5 parasites/ml (Abdeta *et al*., 2020).

The incubator was used to incubate the mixture of blood and extracts in the 96-well plates at 37^0C for 5 minutes, then 2 µl of test mixtures from the plate was placed on separate microscope slides and covered with a coverslip. The parasites were examined at X400 magnification every 5 minutes for a total of 1 hour (Maikai *et al*., 2008; Nok, 2002; Freiburghaus *et al*., 1996). The motility test was performed three times and two times for each concentration (Abdeta *et al.,* 2020; Adeiza *et al*., 2009).

The parasites movement are classified as reduced motility (parasite moves in 10-20 fields) and ceased motility (no parasite moves in 10-20 fields). The extract was thought to be more active the sooner the cessation of the parasite (Feyera *et al*., 2011; Wurochekke and Nok, 2004). For comparison, the time at which motility stopped reduced was recorded and grouped as; no effect, reduced motility and ceased motility.

The various amounts of extracts that stopped or reduced trypanosome motility were evaluated for blood incubation infectivity test. From the parasite and extract mixture in the micro-titer plates, 0.2 ml of each preparation was injected intraperitoneally into six healthy mice for each group. The presence of trypanosomes was checked using a wet blood film by taking blood from each mouse's tail every other day starting from the 10^{th} day of inoculation till day 21. Parasitemia, PCV, body weight and rectal temperature were measured to predict the effectiveness of the test extracts (Woo, 1970). When compared to the negative control, the loss of trypanosome infectivity was labeled if no trypanosome was detectable after 21 days (Maikai, 2011).

4.9. *In vivo* Antitrypanosomal activity of *R. nepalensis*

Body weight, rectal temperature and PCV were measured pre-infection, before starting treatment (day 0), as the treatment subsided (day 7), and as the experiment finished (day 14). Six uninfected and untreated mice were used for comparison (Abdeta *et al*., 2020; Mergia *et al*., 2016).

4.9.1 Determination of parasitaemia level

The level of parasitaemia in the treated group was compared to that of the control group to assess the extracts' anti-trypanosomal effect (Maikai, 2011). Starting on the first day of treatment, parasitaemia level was monitored every other day (Day 0, 2, 4, 6, 8, 10, 12 and Day 14) by microscopic examination of blood from the tail of mice. Wet smear was prepared in triplicates from each mouse and the mean value of slide counts was taken per sample examined microscopically. The parasites of infected blood were counted at 400x magnification per field. By utilizing the "Rapid Matching" technique, the logarithm values of the trypanosome number obtained (Herbert and Lumsden, 1976).

4.9.2 Determination of Body Weight

Each mouse's body weight (in grams) was recorded using digital balance pre-infection, before starting treatment (day 0), as the treatment subsided (day 7) and as the experiment finished (day 14).

4.9.3 Assessment of rectal temperature

Rectal temperature was measured using digital rectal thermometer during pre-infection, before starting treatment (day 0), as the treatment subsided (day 7) and on day 14.

4.9.4 Evaluation of Packed Cell Volume

The packed cell volume (PCV) was measured to determine the effectiveness of the test extracts in preventing hemolysis resulting from increased parasitaemia level. It was monitored before infection and Day 0 (parasite inoculation day), day 7 and 14 of post-treatment initiation. Blood collected from tail of each mouse in heparinized microhematocrit capillary tubes filled up to 3/4th of their length. The tubes were sealed with crystal seal and placed in a slot in the centrifuge head with sealed end outward and centrifuged for 5 minutes at 12,000 rpm and the PCV was measured using hematocrit reader. The extract's effect on improving PCV in treated mice was compared to control groups.

4.10 Statistical analysis

SPSS (Statistical Package for Social Sciences) Statistics for Windows, Version 25.0, was used to analyze the data. The results were reported as mean ± standard error of mean (M ± SEM). To compare different parameters between the treatment and control groups, statistical significance was determined using one-way ANOVA followed by the Tukey post hoc test. $P < 0.05$ was considered significant.

5. Results

5.1 Percent yield of plant

The seeds of *B. antidysenterica,* roots of *R. nepalensis* and fresh leaves of *C. hirsuta* were weighed both before and after extraction. The yields of 80% methanol extracts of seeds of *B. antidysenterica,* roots of *R. nepalensis* and leaves of *C. hirsuta* were computed.

Table 3. Percentage yields of seeds of *B. antidysenterica* J.F. Mill*,* roots of *R. nepalensis* Spreng. and leaves of *C. hirsuta* Perr. & Guill.

Plant name	Original weight (gm)	Weight of the extract (gm)	Yield of the extract
B. antidysenterica J.F. Mill	400	32.68	8.17%
R. nepalensis Spreng.	400	69.76	17.44%
C. hirsuta Perr. & Guill.	600	4.87	0.81%

5.2 Antitrypanosomal activity

5.2.1 *In vitro* activity of the extracts

As shown in the Table below, roots of *Rumex nepalensis* Spreng. showed reduced motility at 5, 10, 20 and 30 minutes and the motility ceased at 10, 35, 40 and 45 min for concentrations of 4, 2, 0.4 and 0.1 mg/ml, respectively. The seeds of *B. antidysentrica* showed reduced motility at 10, 15, 10, 15 minutes and motility ceased within 25, 35, 40, 45 min at 4, 2, 0.4 and 0.1 mg/ml concentrations, respectively (Table 4).

The 80% methanol extract of leaves of *C. hirsuta* showed no effect at 5min for all concentrations and reduced at 10, 20, 40 and 45 min and ceased at 35, 45, 50 and 60 min for 4, 2, 0.4 and 0.1 mg/ml concentrations, respectively. Diminazene aceturate showed reduced motility at 5 min and ceased motility within 10, 20, 25, and 30 min at 4 mg/ml, 2 mg/ml, 0.4 mg/ml and 0.1 mg/ml while viewed under microscope. The negative control group (1% DMSO), neither ceased nor reduced motility of *T. congolense* within an hour.

Table 4. *In vitro* antitrypanosomal effect of *Rumex nepalensis* Spreng*., Brucea antidysentrica* J.F. Mill *and Clematis hirsuta* Perr. & Guill. against *Trypanosoma congolense* motility.

Treatment	Time (min) of cessation or drastic reduction in motility Test concentrations			
	4 mg/ml	2 mg/ml	0.4 mg/ml	0.1 mg/ml
RN-M	10	35	40	45
BA-M	25	35	40	45
CH-M	35	45	50	60
DA	10	20	25	30
1% DMSO	NE	NE	NE	NE

RN-M: 80% methanol extract of *R. nepalensis*, BA-M: 80% methanol extract of *B. antidysentrica,* CH-M: 80% methanol extract of *C. hirsuta*, DA: diminazene aceturate; 1% DMSO (dimethyl sulfoxide): NE: no effect on motility.

5.2.2 Blood incubation infectivity test

The mice treated with 4 mg/ml of *R. nepalensis* and Diminazene aceturate were discovered to be free of trypanosome when compared with mice treated with 4 mg/ml, 2 mg/ml, 0.4 mg/ml, and 0.1 mg/ml of *B. antidysentrica, C. hirsuta* and 1 % DMSO.

Table 5. The effect of extract of seeds of *B. antidysentrica*, roots of *R. nepalensis* and leaves of *C. hirsuta* on blood incubation infectivity test.

Plant	Number of mice, which developed infection on different Test Concentration			
	4 mg/ml	2 mg/ml	0.4 mg/ml	0.1 mg/ml
B. antidysentrica	1/6	2/6	2/6	4/6
R. nepalensis	0/6	1/6	2/6	2/6
C. hirsuta	2/6	4/6	4/6	5/6
Diminazene aceturate	0/6	2/6	3/6	3/6
1 % DMSO	6/6	6/6	6/6	6/6

1% DMSO (dimethyl sulfoxide): DA: diminazene aceturate

5.2.3 *In vivo* activity of *Rumex nepalensis* Spreng.

Since 80% methanol extract of roots of *R. nepalensis* showed best *in vitro* antitrypanosomal activity compared with seeds of *B. antidysentrica* and leaves of *C. hirsuta*, it was further investigated for *in vivo* antitrypanosomal activity.

5.2.4 Acute oral toxicity test

The result of acute oral toxicity test showed that all mice taking the extract of roots of *R. nepalensis* Spreng. at a dose of 2000 mg/kg did not show any observable signs of toxicity.

5.3. Effect of the *Rumex nepalensis* Spreng. extract on body weight

The result of the current study revealed that *R. nepalensis* regained the lost body weight of mice due to its antitrypanosomal activity, animals might have not lost much weight compared to untreated group at all dose levels on 14^{th} day ($p < 0.001$) compared with 1% DMSO. All mice in untreated control group showed gradual decrease in body weight as shown below in Figure 5. The extract of roots of *R. nepalensis* 400 mg/kg had significantly ($p < 0.001$) increased body weight of animals compared with 1% DMSO (negative control) and lower dose (100 and 200 mg/kg) administered groups on day 14 of treatment. There was a statistically significant ($p < 0.001$) improvement in body weight of groups treated with Diminazene aceturate (28 mg/kg) (29.86±1.10) followed by 80% methanol extract of *R. nepalensis* 400 mg/kg (26.60±1.14) on day 14 of treatment.

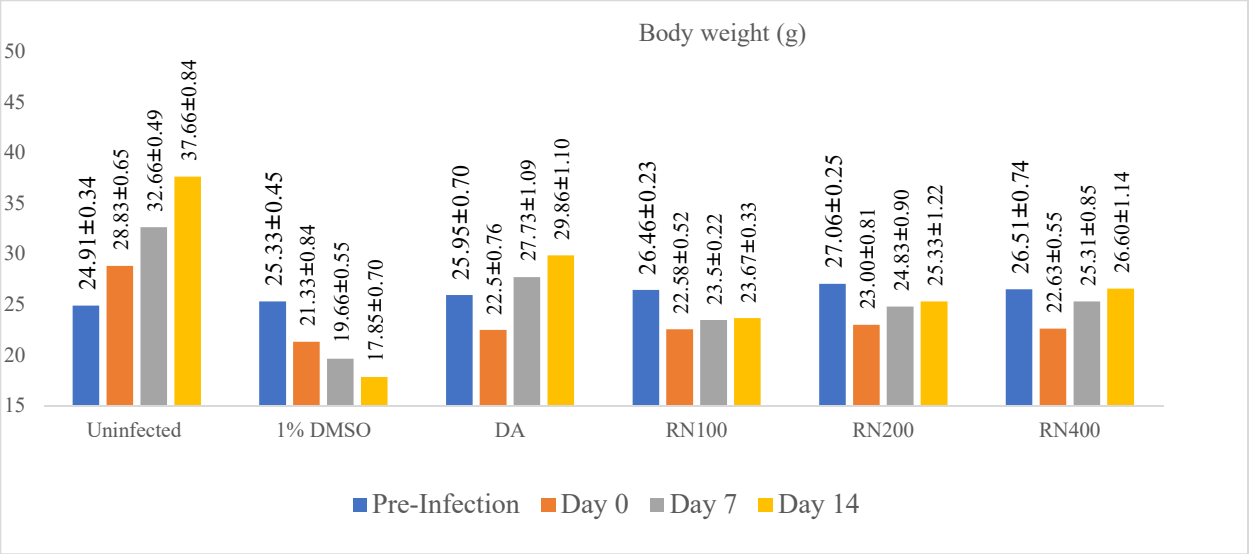

Figure 5. Body weight (g) of healthy and *T. congolense* infected mice treated with *R. nepalensis* Spreng.

Values are expressed in Mean ± SEM (n=6) performed with ANOVA followed by Tukey's Post hoc test. 1% DMSO (dimethyl sulfoxide): DA: diminazene aceturate; RN: 80% methanol extract of *R. nepalensis*

5.4 Effect of the *Rumex nepalensis* Spreng. extract on rectal temperature

The rectal temperature showed relatively constant values in untreated uninfected mice. Animals in negative control (DMSO) group showed increased rectal temperature throughout the study period. The extract at all doses and the positive control showed decreased rectal temperature at day 7 (Figure 6).

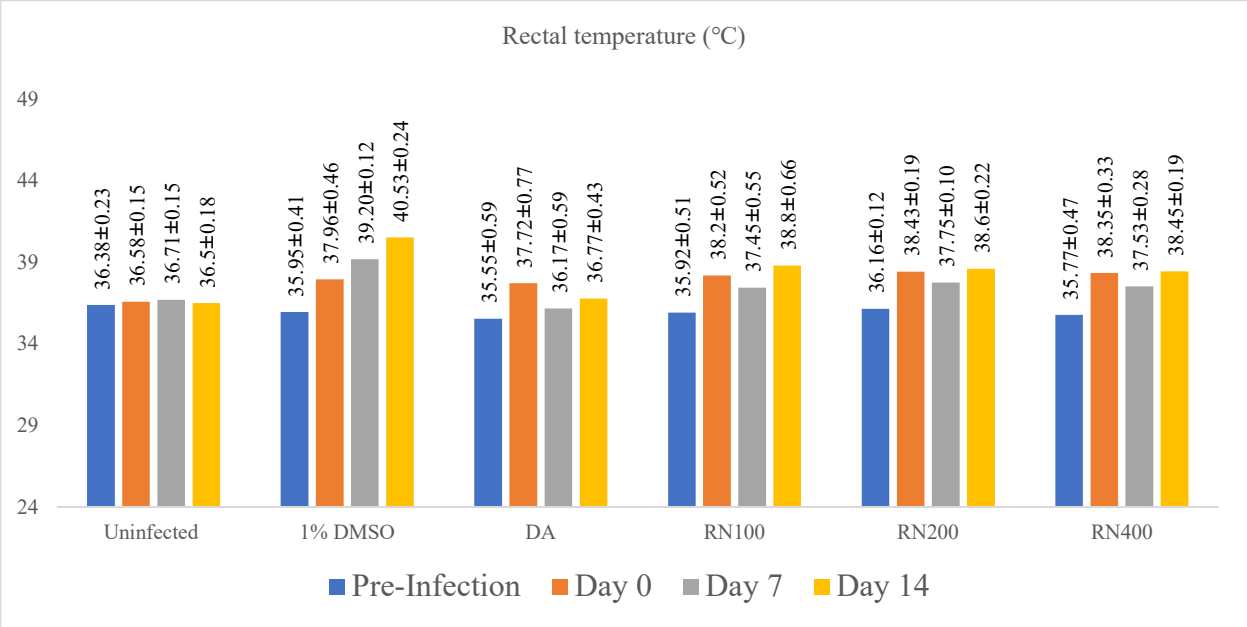

Figure 6. Rectal temperature (°C) of healthy and *T. congolense* infected mice treated with *R. nepalensis* Spreng.

5.5 Effect of the extract on parasitaemia level

The result of the present study revealed that mice treated with different concentrations of *R. nepalensis* extract reduced parasitaemia level. The mean parasite counts of all pre-treatment groups was 7.30±0.06 trypanosomes/ml. Treatment with the extracts reduced parasitaemia levels between days 2-8 when compared to the negative control in which parasitaemia levels were increased. Beginning on day 2, animals treated with 400 mg/kg and 200mg/kg reduced parasitaemia level (6.8±0.10) ($p<0.001$) compared with compared to DA which reduced parasitaemia level to (2.80±1.25), and lowest mean parasitaemia value of (2.70±1.21) was observed on day 8 of treatment ($P< 0.001$) compared with 1% DMSO.

On day 14 of treatment, mean parasitaemia had significantly ($p< 0.001$) lowered (6.35±0.09) as compared to the negative control group (8.91± 0.05) significantly ($p< 0.05$). When compared to the negative control group, treated animals had lower parasitaemia level on days 8, 10, and 12. Treatment of mice with 200 mg/kg and 100 mg/kg of *R. nepalensis* resulted in statistically significant reduction in parasite load on the 8th day compared with the negative and positive control. Relapse in standard drug group was recorded at day 12 of treatment initiation (Table 6).

Table 6. *In vivo* antitrypanosomal activity of roots of *Rumex nepalensis* extracts on parasitaemia on *T. congolense* infected mice

Test substance (mg/kg/day)	*Log number trypanosomes*/ml							
	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
1% DMSO	7.30±0.06	7.80±0.13	8.30±0.06	8.60±0.06	8.65±0.05	8.70±0.00	8.77±0.04	8.91±0.05
DA 28	7.30±0.06	2.80±1.25^{a3}	0.00±0.00^{a3}	0.00±0.00^{a3}	0.00±0.00^{a3}	0.00±0.00^{a3}	0.90±0.90^{a3}	2.70±1.21^{a3}
RN 100	7.30±0.06	6.95±0.09^{b3}	6.65±0.05^{a3,b3,e3}	6.20±0.17^{a3,b3,e1}	6.10±0.20^{b3,e1}	6.15±0.17^{a1,b3,e2}	6.30±0.19^{b3}	6.90±0.08^{b3}
RN 200	7.30±0.06	6.80±0.10^{b3}	6.50±0.06^{a3,b3,e2}	6.00±0.20^{a3,b3}	4.75±0.96^{a2,b3}	6.10±0.17^{a1,b3,e2}	6.3±0.08^{b3,e1}	6.70±0.06^{b3}
RN 400	7.30±0.06	6.80±0.10^{b3}	6.20±0.06^{a3,b3}	5.60±0.13^{a3,b3}	2.70±1.21^{a3}	2.70±1.21^{a3,b1}	5.45±0.05^{a3}	6.35±0.09^{a1,b3}

Data are expressed as mean ± SEM; n = 6; a: compared to 1% DMSO, b: compared to DA, c: compared to RN 100, d: compared to RN 200, e: compared to RN 400; 1: $p < 0.05$, 2: $p < 0.01$, 3: $p < 0.001$; 1% DMSO (dimethyl sulfoxide): DA: Diminazene aceturate, RN: 80% methanol extract of *Rumex nepalensis*

5.6 Effect of the extract on packed cell volume

The mice in the control group had lower mean PCV than the mice treated with extract inoculums and standard drug. When compared to the negative control group (36.50±0.53), the PCV of mice treated with *R. nepalensis* (400 mg/kg) extract was higher (48.00±0.26) on day 14 of treatment. Groups of mice treated with 100 mg/kg, 200 mg/kg, 400 mg/kg of *R. nepalensis* and the positive control DA 28 mg/kg showed significant increase in mean PCV values compared with 1% DMSO.

The results obtained after 100mg/kg treatment show decreased PCV until the mice died. The change in percentage of PCV from day 7 to day 14 of treatment revealed that extracts at 200 and 400 mg/kg doses significantly increased the PCV value of treated animals when compared to the negative control groups.

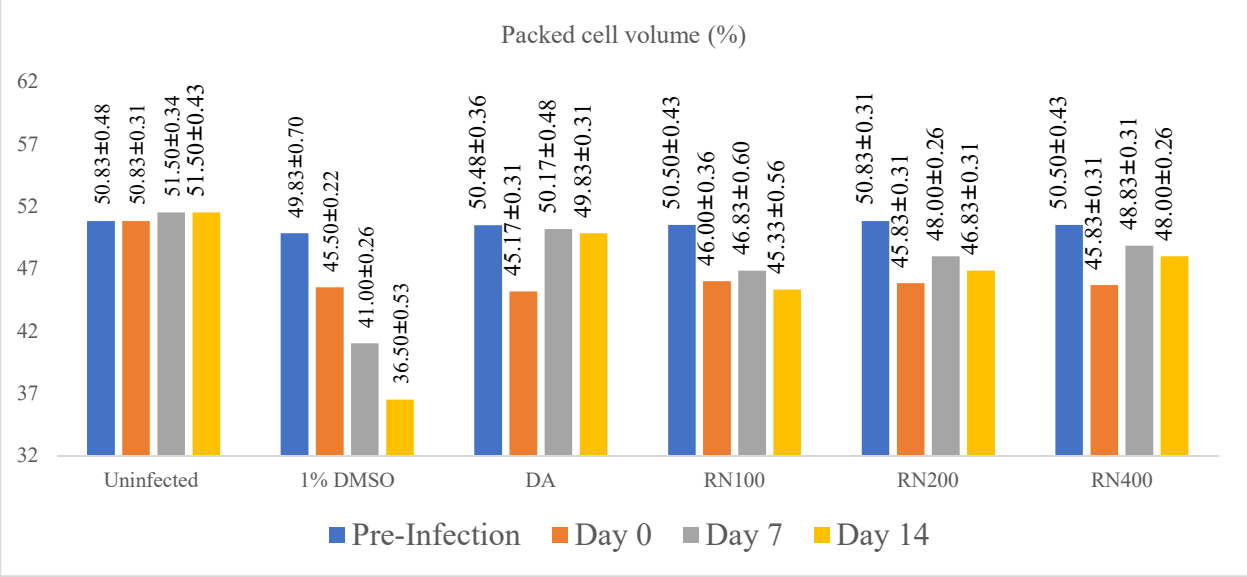

Figure 7. Packed cell volume (%) of uninfected and *Trypanosoma congolense*-infected mice treated with *Rumex nepalensis* Spreng.

6 Discussion

In the present study 80% methanol extracts of *R. nepalensis* were tested for acute oral toxicity on Swiss albino mice. At 2000 mg/kg dose oral administration, no visible signs of toxicity and death of the mice were observed. The middle dose of 200 mg/kg was selected in the present study, by considering that 10 % of the test dose 2000 mg/kg in the acute toxicity study was tolerated by experimental animals. Respiration of trypanosome is needed for the movement of flagella (Atawodi *et al.,* 2003). Trypanosomes elimination or reduction in motility is a relatively reliable indicator of trypanocidal activity (Atawodi *et al*., 2009; Wurochekke and Nok, 2004; Freiburghaus *et al*, 1996). *R. nepalensis* reduced trypanosome motility at 5, 10, 20 and 30 min, at 4, 2, 0.4 and 0.1 mg/ml concentrations whereas the motility ceased at 10, 35, 40 and 45 min for the respective concentration. *T. congolense* infected mice lost weight, as had previously been reported by Feyera *et al.*, 2011. The weight gained in mice treated with the *R. nepalensis* extract was statistically significant between days 7 and 14 and it increased body weight of mice at all dose levels on 14th day compared with the negative control. The weight gain could be linked to a decrease in parasitaemia, which would result in a decrease in appetite (Pathak, 2009) and an increased supply of oxygen and nutrients due to an improved PCV level (Dargie, 1980). Relapse in group which treated with standard drug and different doses of the extract was recorded in all mice at day 12 of treatment as shown by elevated rectal temperature and parasitaemia level. Relapse occurred approximately on days 12-14 of treatment could be due to ability of *T. congolense* to sequester in small vessels and capillaries of the heart, skeletal muscles, and other tissues due to drug resistance or inhibition of cellular and mitochondrial respiration, which compromise all energy dependent processes (Mbaya *et al*., 2007; Nok, 2002; Afewerk *et al*., 2000).

In the present study, infected mice showed elevated rectal temperature as stated by Zwart *et al.,* (1990). After treatment with *R. nepalensis* rectal temperature decreased at day 7. The hypothalamic temperature set point may be altered as a result of pyrogenic stimuli released during infection (Pathak, 2009). The Packed cell volume value of mice treated with 400 mg/kg of roots of *R. nepalensis* was higher compared to the negative control groups on day 14 of treatment. Groups of mice treated with 100 mg/kg, 200 mg/kg and 400 mg/kg of *R. nepalensis* and the positive control DA 28 mg/kg showed an increase in mean PCV values compared with 1% DMSO (the negative control). Infection resulted in significant drop in PCV (42-52%). in the negative control groups by 14^{th} day of treatment. The low PCV value in the infected groups could be attributed to acute hemolysis caused by the infection. Trypanosomes increases susceptibility of red blood cell membranes to attack by reactive oxygen species resulting in and oxidative damage (Karori *et al*., 2008). By reducing parasite load and neutralizing toxic metabolites, scavenging trypanosome associated free radicals may help to alleviate anemia (Ogoti *et al.,* 2009; Ekanem *et al*., 2008; Mpiana *et al*., 2007) by phenolic compounds and flavonoids (Saeed *et al.,* 2012). The results of the present study showed that *R. nepalensis* (400 mg/kg) maintained PCV and ameliorated anemia. The increase in PCV after treatment could be attributed to a decrease in trypanolytic crisis, which increases red blood cells destruction or due to antioxidant activity of the flavonoids and glycosides as observed from *Verbascum sinaiticum* (Akdemir *et al*., 2004; Anosa, 1988). The infected mice were given diminazene aceturate stayed within constant range of PCV due to its ability to eliminate parasites from the blood on days 2-10 (Inabo and Fathuddin, 2011; Umar *et al*., 2010). Despite the temporary clearance of trypanosomes, relapse occurred at day 12 post-treatment initiation. This could be due to the treatment's diminishing effect (Feyera *et al.,* 2011) or the expulsion of trypanosomes from tissues (Eze *et al.,* 2012).

7. Conclusion

The 80% methanol extracts of roots of *R. nepalensis* at higher concentrations showed best *in vitro* antitrypanosomal activity against *T. congolense* field isolate than *Brucea antidysenterica* and *Clematis hirsuta*. Administration of roots of *R. nepalensis* showed *in vivo* activity as demonstrated by lost infectivity, reduced parasitaemia level, decreased rectal temperature and enhanced packed cell volume and body weight.

The observed *in vitro* and *in vivo* trypanocidal activity of roots of *R. nepalensis* showed supports that plant extracts possess activity against trypanosomes providing scientific evidence for the traditional use of these plants as an antitrypanosomal agents and it can be a possible source of new drugs for the treatment of trypanosomiasis.

8. Recommendation

Future areas investigation should be for phytochemical analysis of the plants to isolate novel antitrypanosomal drugs. Comparative studies on various *T. congolense* and *Trypanosoma* strains need to be carried out to determine the effect of variation on anti-trypanosomal activity on different species of trypanosomes. Antitrypanosomal activity of the solvent fractions of roots of *R. nepalensis* needs to be investigated.

9. References

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