RESEARCH ARTICLE PHARMACOLOGIA

DOI: 10.5567/pharmacologia.2015.79.87

Phytochemical Screening and *in vitro* Antitrypanosomal Activity of Aqueous and Methanol Leaf Extract of *Clutia abyssinica* (Euphorbiaceae) Against *Trypanosoma congolense*

[1]Ermias Mergia, [2]Getachew Terefe, [3]Tilahun Teklehaymanot and [4]Workineh Shibeshi

[1]Department of Pharmacy, Faculty of Health Sciences, Rift Valley University College, Ethiopia

[2]Department of Parasitology and Pathology, College of Veterinary Medicine and Agriculture, Addis Ababa University, Ethiopia

[3]Aklilu Lemma Institute of Pathobiology, Addis Ababa University, Ethiopia

[4]Department of Pharmacology and Clinical Pharmacy, College of Health Sciences, Addis Ababa University, Ethiopia

ABSTRACT

Background and Objectives: There is urgent need of search for alternative compounds for chemotherapy of African trypanosomiasis, a disease of major economic and public health importance. The current study investigated the phytochemical composition and *in vitro* antitrypanosomal activity of aqueous and methanolic leaf extracts of *Clutia abyssinica* (*C. abyssinica*) against *Trypanosoma congolense* (*T. congolense*) isolated from natural infection of cattle. **Materials and Methods:** The aqueous and methanol extracts were screened for various secondary metabolites using standard methods. The *in vitro* antitrypanosomal assay was carried out by monitoring the extract concentrations of 4, 2, 1, 0.4 and 0.2 mg mL^{-1} for cessation or reduction in motility of trypanosomes followed by inoculation of incubation mixtures to healthy mice and monitoring development of infection for 21 days. **Results:** Phytochemical screening revealed presence of alkaloids, anthraquinones, flavonoids, glycosides, phenolic compounds, saponins, steroids, terpenes and tannins. An appreciable *in vitro* activity was attained by the methanol extract of *C. abyssinica* at 4 mg mL^{-1} concentration which ceased motility of trypanosomes within 30 min and caused loss of infectivity of trypanosomes to mice which remained aparasitaemic for 21 days after the inoculation of the *in vitro* mixtures. **Conclusion:** The results obtained suggest ethno-pharmacological usefulness of the plant and necessitate further *in vivo* studies to be carried on isolated active substances from these plants.

Key words: Traditional medicine, crude extract, *in vitro* activity

Pharmacologia 6 (3): 79-87, 2015

INTRODUCTION

African trypanosomiasis is a serious parasitic disease caused by trypanosomes, found in the blood and other tissues of vertebrates including livestock, wildlife and people[1]. Trypanosomes are able to infect a wide variety of domestic animals and more than 30 species in the wild. The pattern of the disease is mainly affected by differences in the distribution of the pathogenic trypanosomes. Trypanosoma congolense (*T. congolens*), Trypanosoma *vivax* (*T. vivax*) and Trypanosoma *brucei* (*T. brucei*) are always found within tsetse infested areas. *T. congolense* is considered the most important cause of African animal trypanosomiasis in East Africa and *T. vivax* in West Africa. In Ethiopia, trypanosomiasis is one of the most significant and costly disease hindering the effort made for food sufficiency[1]. In Ethiopia, about 220,000 km^2 in the South West and North West part of the country following the greater river basins of Abay, Omo, Ghibe and Baro, having a high potential for agricultural development are infested with tsetse flies[2,3]. About 10-15% of the land believed to be suitable for livestock production is affected by one or two species of the tsetse flies[4].

Only the salts of the three compounds, diminazene, homidium and isometamidium are currently in use to

Corresponding Author: Workineh Shibeshi, Department of Pharmacology and Clinical Pharmacy, College of Health Sciences, Addis Ababa University, Ethiopia Tel: +251 927361143 Fax: +25111513099

which resistance has been developed a decade after their introduction to the market[5]. The therapeutic and prophylatic use of trypanocides is beset by numerous limitations, including toxicity and the development of resistance by the parasites[6]. The emergence of drug resistant trypanosome strains is considered a very serious problem in control of trypanosomiasis particularly for the resource-poor at risk populations and farmers in Africa. Recent surveys in Eastern and Southern Africa and in West Africa have shown that the prevalence of trypanocidal drug resistance might even be higher than hitherto expected[7,8].

The limited availability and affordability of pharmaceutical medicines and parasite resistance emphasizes the need for research into a more comprehensive, formidable and cheaper sources of trypanocide. Plants have provided the basis for traditional treatment for different types of diseases and still offer an enormous potential source of new cheaper chemotherapeutic agents. Euphorbiaceae is a large and fascinating family of about 300 genera and 8,000-10,000 species, mostly found in the tropics of both hemispheres. *Clutia* is a genus (Euphorbiaceae family) having about 60 species. *Clutia abyssinica* called by the Amharic name 'fyele fej' is herb 1-2 m high[9]. Traditionally it is used in treatment of venereal and skin diseases, chest problems, cancer[10], skin fungal infections[11,12], yellow fever and malaria[13]; management of ear, nose and throat diseases[14]; diarrhoea[15]; gonorrhea, burns, pneumonia, enlarged spleen and kidney, shock, elephantiasis, diarrhoea and tachycardia[12]. The maceration of the crushed leaves of *C. abyssinica* given orally has traditionally been used for the treatment of animal trypanosomosis[16]. The aim of the present study was to investigate the phytochemical constituents and *in vitro* antitrypanosomal activity of aqueous and methanolic extract of Clutia abyssinica.

MATERIAL AND METHODS

Reference drug: Diminazene aceturate (*veriben*® containing 1.05 g diminazene aceturate + 2.36 g antipyrine, (Ceva Santé Animale, France; batch number-719A1) a commercial trypanocidal drug was used as reference drug.

Test organism: The test organism *T. congolense* was isolated from infected cattle in Sebategna kebele, Ilu-Aba-Bora-Zone, Bedele town, Dabo Hana woreda, 480 km south west of Addis Ababa. The presence of *T. congolense* in the screened cattle was detected from blood samples collected from the ear vein of the animals. The slide was examined for *T. congolense* based on their type of motility in the microscopic field of 40 X objective and confirmations of *T. congolense* species by morphological characteristics was done by staining the blood smear with Giemsa stain and examination under a microscope using oil immersion 100 X objective[17,18]. Then the infected blood was collected from the jugular vein of the animal using Ethylene diaminetetraacetic acid (EDTA) coated tubes and heavily inoculated to laboratory mice and transported to laboratory.

Experimental animals: Healthy Swiss albino mice (weighing 20-30 g and age of 8-12 weeks) were obtained from Ethiopian Health and Nutrition Research Institute (EHNRI) and School of Pharmacy, Addis Ababa University. Animals were housed in polypropylene cages (6-10 animals per cage), maintained under 12 h light and 12 h dark cycle and allowed free access to pellet diet and clean water *ad libitum*. All procedures complied with the guide for the care and use of laboratory animals[19]. The experimental protocols and ethics of handling of animals were approved by research and ethics committee of Department of Pharmacology and clinical pharmacy.

Extraction of plant materials: The leaves of *C. abyssinica* were collected from Debre Libanos Monastery in Amhara regional state, Ethiopia. The fresh leaves were wrapped by plastic sheets during transportation. Taxonomic identification was done and a voucher specimen was deposited (Voucher specimen number EM/001) at the National Herbarium, College of Natural sciences, Addis Ababa University. The leaves of the plant materials were washed with distilled water and were dried under shade. The dried leaves were pulverized using mortar and pestle at laboratory of Aklilu Lemma Institute of Pathobiology. For preparation of extracts, 200 g of dried leaf powder of *C. abyssinica* was separately macerated with 1000 mL of distilled water and absolute methanol for 48 h with frequent agitation in orbital shaker and the resulting liquid was filtered using Whatman No. 3 filter paper (Whatman Ltd., England). Extraction was repeated three times and the filtrates of all portions were pooled in one vessel. The aqueous extract was placed in a petridish and lyophilized for one week to yield a solid residue while the methanol extract was concentrated using Rota vapor (BÜCHI Rota-vapor, Switzerland) at 40°C in order to obtain dry extract. The resulting dried mass was then powdered, weighed and stored in a desiccators until use. The percentage yield of 12.92 and 17.21% was obtained for aqueous and methanol extracts, respectively.

Phytochemical screening: Aqueous and methanol extracts of *C. abyssinica* were screened for the presence of active principles such as alkaloids, anthraquinones, flavonoids, glycosides, phenolic compounds, saponins, steroids, tannins and terpenes according to Briggs[20], Dermarderosian and Liberti[21], Evans[22], Rafauf[23], Sofowora[24] and Tyler *et al*.[25].

***In vitro* antitrypanosomal activity:** The *in vitro* assay was performed in triplicates in 96 well micro-titter plates (Flow laboratories Inc.). Infected blood obtained by cardiac puncture of mice at peak parasitaemia ($\sim 10^8$ trypanosomes mL^{-1})[26] was put into EDTA coated tube. Stock solutions of the aqueous and methanol leaf extracts of *C. abyssinica* were first prepared in 2% tween 80 in Phosphate Buffered Saline Glucose (PBSG) as used elsewhere[27,28]. Aliquot of 50 μL of crude extracts solution of 20.0, 10.0, 5, 2.0 and 1 mg mL^{-1} were mixed with 200 μL of blood containing about 20-25 trypanosomes/field ($\sim 10^8$ trypanosomes mL^{-1}) in micro-titter plates to produce effective test concentrations of 4, 2, 1, 0.4 and 0.2 mg mL^{-1}, respectively. The negative and positive controls included 200 μL of infected blood suspended in 50 μL of 2% tween 80 in PBSG and similar effective test concentrations of diminazene aceturate, respectively[29,30,31]. After 5 min incubation in covered micro-titter plates maintained at 37°C, a drop of the test mixture was placed on separate microscope slide covered with cover slip and the motility of the trypanosomes was observed under the microscope (400 X) at 10 min interval for 2 h. The procedure was carried out separately for the aqueous and methanol extracts of the plant in triplicates. Cessation or drop in motility of the trypanosomes in extract-treated blood compared to that of parasite-loaded control blood without extract was taken as a measure of antitrypanosomal activity. Time (minute) after which motility ceased or reduced drastically was recorded for comparison. The movement of the parasite were grouped as; actively motile (motile parasite in ≤5 microscopic fields), drastically reduced motility (motile parasite in the range of 10-20 microscopic fields), ceased (no motile parasite in 10-20 microscopic fields). The shorter the time of cessation of motility of the parasite, the more active the extract was considered to be. Under this *in vitro* system, parasites survived for about 4 h when no extract was present[28, 32].

Blood incubation infectivity assay: For the validation of the *in vitro* antitrypanosomal activity, concentrations of extracts that ceased or drastically reduced motility of trypanosomes in the *in vitro* study were assessed for blood incubation infectivity test. Parasite suspension was incubated in the presence of the aqueous/methanol leaf extract of *C. abyssinica* as described in the *in vitro* study, then the remaining contents of the *in vitro* mixtures in the micro-titter plates were inoculated intraperitoneally into healthy mice (n = 5) and the level of parasitaemia was assessed every other day by collecting blood from tail of each mouse and checked for the presence of trypanosomes using the wet blood film by microhaematocrit buffy coat technique (Woo, 1970). The loss of infectivity of the trypanosome to mice was concluded if no trypanosome was detectable within 21 days[32,33,34]. The effect of the extracts in prolongation of establishment of infection was monitored by comparing with the negative control.

Data analysis: Descriptive statistics was used for summarizing the data. The data obtained are expressed as Mean±Standard error. The p value of less than 0.05 was considered statistically significant.

RESULTS

Phytochemical constituents: Phytochemical screening of the aqueous and methanol leaf extracts of *C. abyssinica* had revealed the presence of different secondary metabolites (Table 1).

***In vitro* antitrypanosomal activity:** As shown in Table 2, the methanol leaf extract of C. *abyssinica* had ceased motility of the parasites within 30 and 40 min at 4 and 2 mg mL^{-1} test concentrations, respectively. Drastic reduction in motility of trypanosomes was observed after 50 min at 4 mg mL^{-1} aqueous extract of *C. abyssinica* . However, the standard drug (diminazine aceturate) immobilized trypanosomes within 20, 30 and 60 min at 4, 2 and 1 mg mL^{-1}, respectively. Whereas the negative control 2% tween 80 and lower test

Table 1: Phytochemical constituents for the aqueous and methanol leaf extracts of *Clutia abyssinica*

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

+: Present, -Absent

Table 2: *In vitro* antitrypanosomal effect of the aqueous and methanol leaf extracts of *Clutia abyssinica* on motility of *Trypanosoma congolense*

		Time of cessation or drastic reduction in motility (min)				
		Test concentrations (mg mL^{-1})				
Treatments	Extract	4	2	1	0.4	0.2
C. abyssinica	Aqueous	50**	NE	NE	NE	NE
	Methanol	30*	40*	NE	NE	NE
Positive control	DA	20*	30*	60*	NE	NE
Negative control	2% Tween 80	NE				

*Ceased motility, **Drastically reduced motility, NE: No noticeable effect on motility, DA: Diminazene aceturate

Table 3: Effect of aqueous and methanol leaf extract of *Clutia abyssinia* on blood incubation infectivity test

Plant	Extract	Test concentration (mg mL^{-1})	No. of mice which developed infection	Infection interval in days (Mean±SEM)
C. abyssinica	Methanol	4.0	0/5	Ni
		2.0	3/5	16.66±0.66
		1.0	4/5	14.50±0.50
		0.4	5/5	14.00±0.00
		0.2	5/5	13.20±0.48
	Aqueous	4.0	5/5	14.80±0.48
		2.0	5/5	14.40±0.40
		1.0	5/5	13.60±0.40
		0.4	5/5	12.80±0.48
		0.2	5/5	12.40±0.40
Positive control	DA	4.0	0/5	Ni
		2.0	3/5	18.66±0.66
		1.0	4/5	16.50±0.50
		0.4	5/5	15.60±0.40
		0.2	5/5	14.80±0.48
Negative control	2% Tween 80		5/5	11.80±0.37

Values are Mean±SEM, N: 5, Ni: No infection, DA: Diminazene aceturate

concentrations (1, 0.4 and 0.2 mg mL^{-1}) of both extracts neither immobilized nor reduced motility of trypanosomes.

Blood incubation infectivity test: The mice which received the test concentrations containing 4 mg mL^{-1} of methanol extract of *C. abyssinica* and diminazine aceturate were found to be aparasitaemic after 21 days monitoring period. In addition, test concentrations containing 2 mg mL^{-1} methanol extract of *C. abyssinica* and 1 and 2 mg mL^{-1} diminazine aceturate lost infectivity to some of the animals and had prolonged establishment of infection as compared to the negative control (Table 3).

DISCUSSION

For several decades, trypanosomiasis has continued to contribute adversely to the economic and social well-being of sub-Saharan Africans[35]. Despite the enormity of the health and economic implication of African trypanosomiasis, current chemotherapeutic options are very limited and far from ideal for both human and livestock[36]. Therefore, the need to source for safer, cheaper and readily available sources of medications cannot be over-emphasized. Literature surveys and field studies have shown that plants are used in traditional medicine in Africa to treat trypanosomes in humans and animals[37]. With this regard, the objective of this study was to evaluate phytochemical constituents and the *in vitro* antitrypanosomal activity of aqueous and methanol leaf extracts of *C. abyssinica,* a plant reported to have several medicinal uses in treatment of infectious diseases including trypanosomosis[38].

The aqueous extracts was prepared by macerating the dried leaves in distilled water in order to simulate the way the plant is traditionally used[38] and with the assumption that some of the active ingredients responsible for the claimed antitrypanosomal activity might not be soluble in water adequately. Methanol leaf extract of the plants was also included in the study. The yield obtained from the methanol extract was found to be higher as compared to the aqueous extract which could be an indication of the extracting power of the solvent with respect to semipolar and nonpolar components which was also noticed in the phytochemical screening. The high yield if the extracts are found to be active and promising for further development can add advantage to the commercial

production of the plant. According to the results of the phytochemical screening study, various secondary metabolites were found in the aqueous and methanol extract of *C. abyssinica*. The methanol extract of *C. abyssinica* showed positive test for the presence of alkaloids, flavonoids, glycosides, steroids, tannins and terpenes while the aqueous extract showed a positive result for anthraquinones, saponins and phenolic compounds.

Numerous *in vitro* studies conducted on the antitrypanosomal activities of the class of compounds listed above reported the potential of each class of compounds in killing or inhibiting the growth of wide ranges of trypanosomes. Phytochemical screening on both extracts had shown the presence of phenolic compounds. Phenolics and polyphenols have been reported in the literature to have antitrypanosomal potential. For instance, ascofuranone, phenol antibiotic isolated from a phytopathogenic fungus, *Ascochyta visiae*, was found to be effective against *T.b.brucei*. Inhibition of the Trypanosome Alternative Oxidase (TAO) enzyme was thought to be responsible for antitrypanosomal activity of phenolic compounds[39].

Phytochemical screening of *C. abyssinica* had shown presence of anthraquinones in the crude aqueous and methanol leaf extracts (Table 1). Similarly Jeruto *et al*.[10] reported that anthraquinones are the main constituents in *C. abyssinica* leaves. Cenas *et al*.[40] reported that quinones, especially 1,4-naphthoquinones can induce oxidative stress in trypanosomes (*T. congolense* and *Trypanosoma cruzi*). This may be explained by their reduction to semiquinone radicals by enzymes such as those present in the mitochondrial electron transport chain and the trypanothione reductase[41]. As a result, there is a great potential for quinones to serve as antitrypanosomal agents[29,42]. As shown in Table 1, methanol leaf extracts of *C.abyssinica* had a positive test for flavonoids which is in agreement with the report of Abdel[43], who reported that the family *Euphorbiaceae* is rich in flavonoids, particularly flavones and flavonols. Even though anti-trypanosomal activity of the isolated flavonoids from *C. abyssinica* are not yet reported. Flavonoids and flavonoid-derived plant natural products have long been known to function as free radical scavengers and metal chelators[44]. The findings of the phytochemical tests had shown that only the methanol extract of *C. abyssinica* contain terpenes.

Although, the antitrypanosomal activity of some herbs is attributed to a specific chemical compound, labeling the activity, especially those commonly used in traditional therapy, to a single compound is a difficult undertaking and it is very unlikely that the activity is due to a single compound only. The possible explanation one can propose for their effectiveness in treating various infectious diseases is that each class of compounds might act synergistically contributing their own share to the total activity of the herbal drug[45,46]. Therefore, the observed antitrypanosomal activity of *C. abyssinica* might be attributed to either the individual class of compounds present in the herb or to the synergistic effect that each class of compounds exert to give the observed biological activity. Hence, further in-depth investigations should be carried out to isolate the active compounds.

Parasites motility constitutes a relatively reliable indicator of viability of most trypanosomes[47] and a complete elimination or reduction in motility of trypanosomes when compared to the control could be taken as index of trypanocidal activity[48]. *C. abyssinica* crude leaf extracts had shown appreciable *in vitro* antitrypanosomal activity with the methanol extract exhibiting the highest activity. The *in vitro* antitrypanosomal activity of the methanol extract could be attributed either to the solubility of the active ingredient(s) responsible for the observed *in vitro* activity or the variations in the types of phytochemicals among the two solvent extracts as revealed by the results for the phytochemical screening. The *in vitro* activity of methanol extracts of *C. abyssinica* which immobilized motility of the trypanosomes within 30 min at 4 mg mL^{-1} concentration is comparable with diminazine aceturate which had shown similar effect within 20 min of incubation. In addition, the activity of methanol extract of *C. abyssinica* at 2 and 4 mg mL^{-1} concentration which ceased motility of the trypanosomes within 40 and 30 min, respectively (Table 2) is higher as compared with the *in vitro* antitrypanosomal activity of methanol extract of *Ximenia americana*[49] which at effective concentration of 9 mg mL^{-1} inhibited motility of *T. congolense* within 45 min of incubation. It is not known why 4 mg mL^{-1} aqueous extracts of *C. abyssinica* drastically reduced trypanosome motility within 50 min but could not completely eliminate motility. However, it appears reasonable to speculate that these extracts may belong to the group that acts by static action affecting growth and multiplication of trypanosomes rather than eliminating them altogether[50].

The mechanism by which the extracts immobilized or reduced motility of the trypanosome is not known at this stage of the work. However, accumulated evidences suggested that many natural products exhibited their antitrypanosomal activity by virtue of their interference with the redox balance of the parasites acting either on

the respiratory chain or on the cellular defenses against oxidative stress[34,51]. Respiration of trypanosomes is obligatory for their motility as well as for managing the energy reserve required for the synthesis of the variable surface glycoproteins. The inhibition of cellular and mitochondrial respiration by any chemotherapeutic agent will obviously compromise all the energy dependent processes. This was confirmed by the microscopy of the trypanosomes which showed a cessation or reduction in motility after incubation with different concentrations of extracts[41]. The positive control diminazine aceturate immobilized trypanosome within 20, 30 and 60 min[49], in which even lower concentration (0.1 mg mL^{-1}) of diminazine aceturate ceased motility of the trypanosomes within 20 min. The difference might be due to the *T. congolense* isolate that could have developed resistance to the drug as reported by Chaka and Abebe[52]. Comparison analysis revealed that the standard drug exhibited superior *in vitro* antitrypanosomal activity even at lower concentration (2 mg mL^{-1}) when compared to the extracts. This is consistent with several reports made on other medicinal plant extracts[31,34,53,54]. The observation that incubation of trypanosome with the 4 mg mL^{-1} methanol extract of *C. abyssinica* inhibited healthy mice from developing infection in the observation period agrees with earlier reports which showed that plant extracts can cause trypanosomes to lose their infectivity to rodents[55].

However the *in vitro* antitrypanosomal activities of other concentrations were not confirmed by blood incubation infectivity. Yusuf *et al.*[55] suggested that complete immobility of the parasites *in vitro* may not necessarily indicate that the parasites were dead but rather the parasites may have lost their infectivity. This may be due to the respective concentration might have only min at test concentrations of 4, 2, 1 mg mL^{-1}, respectively. This finding is not in agreement with immobilized but not killed the parasite by causing unfavorable conditions. The parasites might have recovered and become infective at time in contact with suitable physiological conditions. Prolongation of the prepatent period of animals inoculated with the *in vitro* mixtures containing lower test concentrations of methanol extract of *C. abyssinica* (2 mg mL^{-1}) is in agreement with the findings of Feyera *et al.*[28] and Yusuf *et al.*[55]. It may appear to contemplate that the highest (4 mg mL^{-1}) concentration level either killed the parasites or caused them to lose their infectivity coupled to cease in motility of the trypanosome *in vitro*. Loss of infectivity may be by abrogating some vital metabolic processes in the parasites or could be due to some morphological changes on the parasites induced by the extract at this concentration that render them more susceptible to the mice immune defense systems.

CONCLUSION

This study evaluated phytochemistry and *in vitro* antitrypanosomal activity of crude leaf extracts of *C. abyssinica* against *T. congolence*. The higher concentration (4 mg mL^{-1}) of the methanol extract of *C. abyssinica* showed superior *in vitro* activity than aqueous extract by immobilizing trypanosomes within 30 min and abrogated infectivity of trypanosome to mice which remained aparasitaemic for 21 days after the inoculation of the *in vitro* mixtures. Generally, the current study established that leaves of *C. abyssinica* could have potential antitrypanosomal activity which can be considered as a potential source for new drugs in chemotherapy of African animal trypanosomiasis.

ACKNOWLEDGMENT

We would like to thank staffs of the National Tsetse and trypanosomiasis Investigation and Control Center for technical assistance during isolation of the test organism; Vice President for research and Technology transfer of Addis Ababa university for partly funding the research under the framework of the thematic research project, Animal Health Improvement and staffs of Aklilu Lemma Institute of Pathobiology for their cooperation and unreserved help during the laboratory study.

REFERENCES

1. Abebe, G., 2005. Trypanosomosis in Ethiopia. Ethiopia J. Biol. Sci., 4: 75-121.
2. Kebede, N. and A. Animut, 2009. Trypanosomosis of cattle in selected districts of Awi zone, Northwestern Ethiopia. Trop. Anim. Health Prod., 41: 1353-1356.
3. Rundassa, M.D., S. Menkir and A. Kebede, 2013. Prevalence and seasonal incidence of bovine trypanosomosis in Birbir valley, Baro Akobo River system, Western Ethiopia. J. Vet. Med. Anim. Health Acad. J., 5: 138-143.
4. NTTICC., 2004. National tsetse and trypanosomiasis investigation and control centre. Final Report, June 7th, 2003-July 6th, 2004, Bedelle, pp: 1-3.

5. Delespaux, V., D. Geysen, P. Van den Bossche and S. Geerts, 2008. Molecular tools for the rapid detection of drug resistance in animal trypanosomes. Trends Parasitol., 24: 236-242.
6. Afewerk, Y., P.H. Clausen, G. Abebe, G. Tilahun and D. Mehlitz, 2000. Multiple-drug resistant Trypanosoma congolense populations in village cattle of Metekel district, North-West Ethiopia. Acta Trop., 76: 231-238.
7. Chitanga, S., T. Marcotty, B. Namangala, P. van den Bossche, J. van den Abbeele and V. Delespaux, 2011. High prevalence of drug resistance in animal trypanosomes without a history of drug exposure. PLoS Negl. Trop. Dis., Vol. 5., 10.1371/journal.pntd.0001454
8. Moti, Y., R. Fikru, J. van den Abbeele, P. Buscher, P. van den Bossche, L. Duchateau and V. Delespaux, 2012. Ghibe river basin in Ethiopia: Present situation of trypanocidal drug resistance in Trypanosoma congolense using tests in mice and PCR-RFLP. Vet. Parasitol., 189: 197-203.
9. Edwards, S, M. Tadesse and I. Hedberg, 1995. Flora of Ethiopia and erteria canellacea to euphorbiacea. Nat. Herbarium, Addis Ababa Uppsala, 2: 286-289.
10. Jeruto, P., C. Mutai, C. Lukhoba and G. Ouma, 2011. Phytochemical constituents of some medicinal plants used by the Nandis of South Nandi district, Kenya. J. Anim. Plant Sci., 9: 1201-1210.
11. Runyoro, D.K.B., O.D. Ngassapa, M.I.N. Matee, C.C. Joseph and M.J. Moshi, 2006. Medicinal plants used by Tanzanian traditional healers in the management of Candida infections. J. Ethnopharmacol., 106: 158-165.
12. Matu, E.N., 2008. Clutia Abyssinica Jaub and Spach. In: Medicinal Plants/Plantes Medicinales, Schmelzer, G.H. and A. Gurib-Fakim (Eds.). PROTA, Wageningen, Netherlands.
13. Fowler, D.G., 2006. Traditional fever remedies: A list of Zambian plants. pp: 1-61. http://www.giftsofhealth.org/ritam/news/Traditional Fever_remedie1.pdf.
14. Njoroge, G.N. and R.W. Bussmann, 2006. Traditional management of Ear, Nose and Throat (ENT) diseases in central kenya. J. Ethnobiol. Ethnomed., Vol. 2. 10.1186/1746-4269-2-54
15. Yineger, H., E. Kelbessa, T. Bekele and E. Lukekal, 2007. Ethnoveterinary medicinal plants at Bale Mountains National Park, Ethiopia. J. Ethnopharmacol., 112: 55-70.
16. Fullas, F., 2010. Ethiopian medicinal plants in veterinary healthcare a mini-review. Ethiopian E-J. Res. Innov. Foresight, 2: 48-58.
17. Murray, M., P.K. Murray and W.I. McIntyre, 1977. An improved parasitological technique for the diagnosis of African trypanosomiasis. Trans. R. Soc. Trop. Med. Hyg., 71: 325-326.
18. Uilenberg, G., 1998. Field Guide for Diagnosis, Treatment and Prevention of African Animal Trypansomosis. Food Agriculture Organization, United Nations Publications, Rome, Itlay.
19. ILAR., 2000. Humane endpoints for animals used in biomedical research and testing. ILAR J., 41: 59-123.
20. Briggs, M.H., 1970. Advances in Steroid Biochemistry and Pharmacology. Academic Press, New York, USA., Pages: 24.
21. Dermarderosian, A. and L.E. Liberti, 1988. Natural Product Medicine: A Scientific Guide to Foods, Drugs, Cosmetics. 1st Edn., Geo. F. Stickley Co., Philadelphia, USA., pp: 46-66.
22. Evans, W., 1996. Trease and Evans' Pharmacognosy. 14th Edn., W.B. Saunders Ltd., USA., pp: 105-766.
23. Rafauf, R., 1970. Handbook of Alkaloids and Alkaloid Containing Plants. 2nd Edn., Academic Press, New York, USA.
24. Sofowora, A., 1982. Medicinal Plants and Traditional Medicine in Africa. 1st Edn., John Wiley and Sons, Chichester, New York, ISBN-10: 0471103675, Pages: 256.
25. Tyler, V.E., L.R. Brady and J.E. Robbers, 1988. Pharmacognosy. 9th Edn., Lea and Febiger, Philadelphia.
26. Herbert, W.J. and W.H.R. Lumsden, 1976. Trypanosoma brucei: A rapid matching method for estimating the host's parasitemia. Exp. Parasitol., 40: 427-431.
27. Johnson, T.O., K.O. Ijeoma, E.E. Ekanem, E. Nelson and B. Mohammed, 2011. *In vitro* studies on the Trypanocidal activities of various Phytochemical fractions obtained from Garcinia kola seed. J. Med. Trop., 13: 124-128.
28. Feyera, T., G. Terefe and W. Shibeshi, 2011. Phytochemical screening and *in vitro* antitrypanosomal activity of the aerial parts of Artemisia abyssinica against Trypanosoma congolense field isolate. Ethiopian Pharm. J., 29: 137-142.

29. Maikai, V.A., J.A. Nok, A.O. Adaudi and C.B.I. Alawa, 2008. *In vitro* antitrypanosomal activity of aqueous and methanolic crude extracts of stem bark of Ximenia Americana on Trypanosoma congolense. J. Med. Plants Res., 2: 55-58.
30. Atawodi, S.E. and F. Ogunbusola, 2009. Evaluation of anti-trypanosomal properties of four extracts of leaves, stem and root barks of Prosopis africana in laboratory animals. Biokemistri, 21: 101-108.
31. Ene, A.C., S.E. Atawodi, D.A. Ameh, C.N. Nnamani and Y.E. Apeh, 2009. Antitrypanosomal effects of petroleum ether, chloroform and methanol extracts of Artemisia maciverae Linn. Indian J. Exp. Biol., 47: 981-986.
32. Wurochekke, A.U. and A.J. Nok, 2004. *In vitro* and *in vivo* antitrypanosomal activity of the leaf of Lawsonia inermis against Trypanosoma brucei brucei infection in mice. J. Med. Sci., 4: 236-239.
33. Maikai, V.A., 2011. Antitrypanosomal activity of flavonoid extracted from Ximenia Americana stem bark. Int. J. Biol., 1: 115-121.
34. Atawodi, S.E., T. Bulus, S. Ibrahim, D.A. Ameh, A.J. Nok, M. Mamman and M. Galadima, 2003. *In vitro* trypanocidal effect of methanolic extract of some Nigerian savannah plants. Afr. J. Biotechol., 2: 317-321.
35. WHO., 2006. African trypanosomiasis (sleeping sickness). Fact Sheet 259, World Health Organization, Geneva, Switzerland.
36. Legros, D., G. Ollivier, M. Gastellu-Etchegorry, C. Paquet, C. Burri, J. Jannin and P. Buscher, 2002. Treatment of human African trypanosomiasis-present situation and needs for research and development. Lancet Infect. Dis., 2: 437-440.
37. Mbaya, A.W. and U.I. Ibrahim, 2011. In vivo and *in vitro* activities of medicinal plants on haemic and humoral trypanosomes: A review. Int. J. Pharmacol., 7: 1-11.
38. Teklehaymanot, T., 2009. Ethnobotanical study of knowledge and medicinal plants use by the people in Dek Island in Ethiopia. J. Ethnopharm., 124: 69-78.
39. Yabu, Y., A. Yoshida, T. Suzuki, C.I. Nihei and K. Kawai et al., 2003. The efficacy of ascofuranone in a consecutive treatment on Trypanosoma brucei brucei in mice. Parasitol. Int., 52: 155-164.
40. Cenas, N.K., D. Arscott, C.H. Williams and J.S. Blanchard, 1994. Mechanism of reduction of quinones by Trypanosoma congolense trypanothione reductase. Biochemistry, 33: 2509-2515.
41. Nok, A.J., 2002. Azaan thraqinone inhibits respiration and *in vitro* growth of long slender blood stream forms of T. congolense. Cell Biochem. Funct., 20: 205-212.
42. Eze, J.I., G.N. Anosa and C.A. Ozota, 2012. *In vitro* and *in vivo* trypanocidal activity of Combretum racemosum leaves. Afr. J. Biotechnol., 11: 10611-10616.
43. Abdel, F.M.R., 1987. The Chemical Constituents and Economic Plants of the Euphorbiaceae. In: The Euphorbiales: Chemistry, Taxonomy and Economic Botany, Jury, S.L. (Ed.). Academic press, USA., pp: 293-296.
44. Harborne, J.B. and C.A. Williams, 2000. Advances in flavonoid research since 1992. Phytochemistry, 55: 481-504.
45. Pelttari, E., J. Matikainen and H. Elo, 2002. Antimicrobial activity of the marine alkaloids haminol, puluo' upone and related Compounds. Z. Naturforsch., 57: 548-552.
46. Abd-El-Hady, F.K. and A.G. Hegazi, 2002. Egyptian propolis: 2. Chemical composition, antiviral and antimicrobial activities of East Nile Delta propolis. Z. Naturforsch C, 57: 386-394.
47. Freiburghausa, F., R. Kaminsky, M.H.H. Nkunya and R. Brun, 1996. Evaluation of African medicinal plants for their *in vitro* trypanocidal activity. J. Ethnopharmacol., 55: 1-11.
48. Atawodi, S.E., J. Joseph-Idrisu, U.S. Ndidi and L. Yusufu, 2011. Phytochemical and antitrypanosomal studies of different solvents extracts of Boswellia dalzielii. Int. J. Biol., 3: 179-184.
49. Maikai, V.A., 2010. *In vitro* and *in vivo* evaluation of anti-trypanosomal activity of stem bark of Ximenia Americana. Int. J. Biol., 2: 50-54.
50. Atawodi, S.E. and H. Shehu, 2010. Antitrypanosomal property of some extracts of different parts of Moringa oleifera lam. Electr. J. Biol., 6: 19-23.
51. Antia, R.E., J.O. Olayemi, O.O. Aina and E.O. Ajaiyeoba, 2009. *In vitro* and *in vivo* animal model antitrypanosomal evaluation of ten medicinal plant extracts from south west Nigeria. Afr. J. Biotechnol., 8: 1437-1440.

52. Chaka, H. and G. Abebe, 2003. Drug resistant trypanosomes: A threat to cattle production in the southwest of Ethiopia. Revue Elevage Med. Vet. Trop., 56: 33-36.
53. Hoet, S., Opperdoes, B. Brun and J. Quetin-Leclercq, 2004. Natural products active against African trypanosomes: A step towards new drugs. Nat. Prod. Rep., 21: 353-364.
54. Maikai, V.A. and P.I. Kobo, 2008. Preliminary studies on the *In vitro* antitrypanosomal activity of aqueous and methanolic crude extracts of stem bark of Nauclea latifolia on Trypanosoma congolense. J. Med. Plants Res., 2: 115-118.
55. Yusuf, A.B., I.A. Umar, U.B. Musa and A.J. Nok, 2012. Screening of Vernonia amygdalina and Hymenocardia acida extracts and 1, 3-diaminopropane for their antitrypanosomal activities: *In vitro* model. J. Med. Plants Res., 6: 3573-3578.