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Research Article

Evaluation of *in vivo* Antitrypanosomal activity of aqueous and methanol leaf extracts of *Clutia abyssinica* (Euphorbiaceae) against *Trypanosoma congolense*

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Abstract

Introduction: African trypanosomiasis is a major disease of economic and public health importance affecting agricultural and human development. The search for alternative compounds against African trypanosomiasis is essential as existing chemotherapeutic agents have several drawbacks. The objective of the study reported was to evaluate aqueous and methanol leaf extracts of *Clutia abyssinica* for *in vivo* activity in *Trypanosoma congolense* infected mice.

Materials and Methods: The *in vivo* antitrypanosomal efficacy of the aqueous and methanol extracts was evaluated in Swiss albino mice infected with *T. congolense* isolated from naturally infected cattle. The leaf extracts were administered 12 days post-infection at doses of 100, 200 and 400 mg/kg by intraperitoneal injection once daily for 7 days. Parasitaemia, packed cell volume (PCV), mean survival time and change in body weight were used as indices for monitoring the efficacy of the extracts by comparing with the positive control (28 mg/kg dose of diminazene aceturate) and negative control (2% tween 80) treated groups.

Results: Highly significant ($p<0.001$) reduction in pre-treatment parasitaemia by 3.91% (7.38 ± 0.18), increase in PCV by 1.12% (48.66 ± 0.20), body weight improvement by 1.36% (22.34 ± 0.27) and mean survival time of 39.20 ± 0.37 days was observed in the group treated with 400 mg/kg methanol leaf extract of *C. abyssinica* on day 14 of treatment while the mice treated with aqueous extracts of *C.abyssinica* at 400 mg/kg dose had low parasitaemia on day 6 ($p<0.01$), day 8, 10 and 14 ($p<0.001$) of treatment as compared to the negative control.

Conclusion: The results obtained confirm ethno-pharmacological usefulness of the plant in treatment of trypanosomiasis and possible indications in development of alternative drugs.

Keywords: *Clutia abyssinica; In vivo* antitrypanosomal activity; Mice; *Trypanosoma congolense*

Abbreviations

T. congolense: Trypanosoma Congolense; *C. abyssinica:* Clutia Abyssinica; CAAE: *C. Abyssinica* Aqueous Extract; CAME= *C. Abyssinica* Methanol Extract; DA: Diminazine Aceturate

Introduction

African trypanosomosis is a protozoan disease of humans and livestock caused by trypanosomes and transmitted by tsetse fly vector. While *Trypanosoma rhodiense* and *Trypanosoma gambiense* cause African Human Trypanosomosis, *Trypanosoma brucei brucei, Trypanosoma vivax, Trypanosoma congolense, Trypanosoma evansi* and *Trypanosoma equiperdum* are the agents of African Animal Trypanosomosis (AAT) [1].

The economic impacts of trypanosomosis in Africa are diverse and complex, with direct effects on animal production and human health, as well as indirect effects on settlement patterns, land use, animal husbandry and farming [2]. Chemotherapy, the main means of controlling the disease is under threat due to parasite resistance [3] and toxicity of the trypanocidal drugs [4]. The poor prospect for a vaccine due to antigenic variation of the parasite is further compounded by unwillingness of the pharmaceutical industry to develop new compounds because of uncertain and unprofitable market or perhaps the localized nature of the disease.

The few commercial trypanocides (diminazene aceturate, isometamidium and homidium) have been in use for well over 40 years. Thus, the search for medicinal plants with trypanocidal activities continues to generate a lot of research interest [5,6]. Although recent reports indicate antitrypanosomal activity exists in some medicinal plants [7-11], the potentials of many other plants used in folkloric medicine in Ethiopia are yet to be investigated. 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 within a family Euphorbiaceae, having about 60 species. *Clutia abyssinica* called by the Amharic name 'fyele fej' is herb 1-2 m high [12]. Traditionally it is used in treatment of venereal and skin diseases, chest problems, cancer [13]; Skin fungal

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infections [14,15]; yellow fever and malaria; management of ear, nose and throat diseases [16]; diarrhoea [17]; gonorrhea, cough and fever, headache, toothache, menstrual pain, burns, pneumonia, enlarged spleen and kidney, shock, abdominal problems- as a laxative and to expel intestinal worms, elephantiasis, diarrhoea and tachycardia [15]. The maceration of the crushed leaves of *C. abyssinica* given orally has been traditionally used for the treatment of animal trypanosomosis [18]. Therefore, this study was aimed to evaluate the *in vivo* antitrypanosomal activity of *Clutia abyssinica* in mice infected with *T. congolense* isolated from natural infection of cattle.

Materials and Methods

Chemicals and drugs

The chemical reagents used were diminazine aceturate (Ceva Santé Animale, France; batch number- 719A1), Giemsa stain, tween-80 (BDH Ltd, England), 40% glucose (Pharmacure, Ethiopia), methanol (Carlo ebra reagents, Italy).

Test organism

The test organism T. congolense was isolated from infected cattle in Sebategna kebele, 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 40x objective and confirmations of T. congolense species by morphological characteristics was done by staining with Giemsa stain, and examination under a microscope using oil immersion 100x objectives [19,20]. Then the infected blood was collected from the jugular vein of the animal using EDTA coated tubes and heavily inoculated to laboratory mice and transported to laboratory at Akililu Lemma Institute of Pathobiology, Addis Ababa University for subsequent serial passage to other mice.

Experimental animals

Healthy Swiss albino mice (weighing 20–30 gm and age of 8–12 weeks) were obtained from the animal house of the Ethiopian Health and Nutrition Research Institute and School of Pharmacy, Addis Ababa University. Animals were housed in polypropylene cages (6–10 animals per cage), maintained less than 12 hr light and 12 hr dark cycle and allowed free access to pellet diet and clean water ad libitum. All procedures complied with the guide for the care and use of laboratory animals [21]. The experimental protocols and use and handling of animals were approved by research and ethics committee of Department of Pharmacology and clinical pharmacy.

Preparation 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 (Collection EM/001) at the National Herbarium, College of Natural sciences, and Addis Ababa University. The leaves were washed with distilled water and were dried under shade. The dried leaves were pulverized using mortar and pestle. For preparation of extracts, 200g of dried leaf powder of *C. abyssinica* was separately macerated with 1000 ml of distilled water and absolute methanol for 48 hours with frequent agitation in orbital shaker and the resulting liquid was filtered using Whatman No. 3 filter paper (Whatman Ltd., England). Extraction was repeated three times and the filtrates of all portions were pooled in one vessel. The 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 no more than 40°C in order to obtain dry extract. Then the resulting dried mass was then powdered and weighed resulting in yield of 12.92% and 17.21% for aqueous and methanol extracts respectively.

Acute toxicity study

The acute toxicity study was conducted in accordance with the Lorke's [22] method. The study was conducted for each extract in two phases using female Swiss albino mice after 7 days of adaptation. In the first phase, nine mice were divided into 3 groups (n=3). Each group was given 10, 100, and 1000 mg/kg body weight of the test substance respectively. In the second phase, further specific doses (1600, 2900, and 5000 mg/kg) of each extract were administered to nine mice to estimate the lethal dose (LD_{50}) value. The control group received the reconstituting solvent 2% tween 80 in sterile water. The extract was dissolved in 2% tween 80 in sterile water and given through intraperitoneal route. All animals were kept under strict observation for behavioral, neurological, autonomic or physical changes such as alertness, motor activity, restlessness, convulsions, coma, diarrhea and lacrimation for 24 h. These observations continued for further 14 days for any signs of overt toxicity. Then the lowest dose which killed one mouse (minimum toxic dose) and the highest dose which had not killed any mouse (maximum tolerated dose) were noted, and the geometric mean of these two doses gave LD_{50}. The detailed procedures for determining the doses for testing acute toxicity are shown in annex 1.

Parasite inoculation and extract administration

Healthy Swiss albino mice that are infected intraperitoneally with 0.2 ml of T. congolense infected blood (~10^4 trypanosomes/ml) collected by cardiac puncture from donor mice was divided into eight groups (n =5): C. abyssinica aqueous extract (CAAE 100, CAAE 200, CAAE 400), C. abyssinica methanol extract (CAME 100, CAME 200, CAME 400), diminazene aceturate (DA28), and 2% tween 80 (TW80). Treatment with the extracts began on the 12th day post-infection (day 0 of treatment), when the infected mice showed peak parasitaemia of (~10^8 trypanosomes/ml). On each day of drug administration, the aqueous and methanol extracts of C. abyssinica were freshly prepared by dissolving in 2% Tween-80 in sterile water for injection and administered intraperitoneally daily at 9 am for seven days at the doses of (100, 200 and 400 mg/kg). The doses were selected based on the acute toxicity study. For the positive control, diminazine aceturate (DA28), dissolved in sterile water as recommended by the manufacturer was administered at the dose of 28 mg/kg intraperitoneally based on previous reports of Moti et al. [23], Feyera et al. [11], while for the negative control, 2% tween 80 in sterile water (TW80), was administered intraperitoneally. Volume administered was 2 ml/100 gm of body weight of the animal (OECD, 2001).

Determination of parasitaemia

Parasitaemia was monitored every other day by microscopic

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

LD_{50}=*3807.886* mg/kg

examination of blood obtained from the tail of each mouse that was pre-sterilized with methylated spirit. The tail tip was cut to extrude blood and drop of blood was placed on microscope slide and covered with a cover-slide. The blood was examined microscopically at 400x total magnification. The degree of parasitaemia was determined using the "Rapid Matching" method of Herbert and Lumsden [24]. Wet smear were prepared in triplicates from each animal and the mean value of slide counts were taken per sample examined microscopically. Logarithm values of these counts were obtained by matching with the table given by Herbert and Lumsden [24].

Determination of packed cell volume (PCV)

PCV was measured [25] to predict the effectiveness of the test extracts in preventing hemolysis resulting from increasing parasitaemia associated with trypanosomosis. It was monitored before infection and three times till the 14th day (on day 0, 7 14). Briefly, blood was collected from tail of each mouse in heparinized microhaematocrit capillary tubes filled up to 3/4th of their length. The tubes were then sealed immediately by cristal seal and centrifuged in a microhaemtocrit centrifuge (Hettich Haematokrit, Germany) for 5 min at 12,000 rpm. After centrifugation, the height of the red blood cell column were measured by use of haematocrit reader and compared to the total height of the column of the whole blood [26]. The effect of extracts in improving PCV of treated animals was compared with the controls.

Determination of body weight

The body weight of each mouse in all groups was measured before infection, on the day treatment commenced (day 0) and every other day up to day 14.

Determination of mean survival time

Mortality was monitored daily and the number of days from the time of inoculation of the parasite up to death was recorded for each mouse in the treatment and control groups throughout the follow up period for six weeks.

Statistical analysis

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

Results

Acute toxicity test

The acute toxicity bioassay had shown that the lethal dosage (LD_{50}) of the aqueous and methanol leaf extracts of *C. abyssinica* was above 2000 mg/kg and there were no evidence of acute toxicity at the doses tested indicating good safety margin. As shown on annex 1, the lethal dosage (LD_{50}) for aqueous and methanol extract of C.abyssinica were 2154.065 mg/kg and *3807.886* mg/kg body weight respectively.

Effect of extracts on parasitaemia

Mice treated with aqueous extract of *C. abyssinica* at 400 mg/kg dose had low parasitaemia on day 6 ($p<0.01$), day 8, 10 and 14 ($p<0.001$) (Table 1), while mice treated with the methanol extract at 200 and 400 mg/kg dose had statistically significant low parasitaemia

Table 1: The effect of aqueous leaf extract of *Clutia abyssinica* on parasitaemia of *Trypanosoma congolense* infected mice.

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

Values are expressed in Mean ± S.E.M (n=5); DA28= diminazine aceturate 28 mg/kg, [b]compared to TW80= 2% tween 80; [c]compared to CAAE100= *C. abyssinica* aqueous extract 100 mg/kg, [d]compared to CAAE200= *C.abyssinica* aqueous extract 200 mg/kg, *compared with all groups; [1]p < 0.05, [2]p < 0.01 and [3]p < 0.001

Table 2: The effect of methanol leaf extract of *Clutia abyssinica* on parasitaemia of *Trypanosoma congolense* infected mice.

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

Values are expressed as Mean ± S.E.M (n=5); DA28= diminazine aceturate 28 mg/kg, [b]compared to TW80= 2% tween 80; [c]compared to CAME100= C. abyssinica methanol extract 100 mg/kg, [d]compared to CAME200= C. abyssinica methanol extract 200 mg/kg, *compared with all groups; [1]p < 0.05, [2]p < 0.01 and [3]p < 0.001

on day 6, 8, 10 and 14 (p<0.001) of treatment as compared to the negative control group (Table 2). Comparison of the percentage change in parasitaemia on day 14-0 showed that only 400mg/kg dose of the methanol extract significantly (p<0.05) reduced parasitaemia by 3.91%. The lowest mean parasitaemia value (5.94+0.24) was observed in the same group on day 8 of treatment which was highly significant (p<0.001) when compared to the 100 and 200mg/kg dose. Animals treated with diminazene have cleared the parasites from circulation though parasitemia relapsed as of day 12 of treatment.

Effect of extracts on packed cell volume

The animals treated with 400 mg/kg of the aqueous extract of *C. abyssinica,* and with 200 and 400 mg/kg dose of the methanol extract had significantly (p<0.001) higher PCV value as compared to the negative control groups on day 14 of treatment (Table 3). Analysis of change in percentage of PCV from day 7 to day 14 of treatment also showed that the methanol extract at 200 and 400 mg/kg doses had significantly increased PCV value of treated animals by 1.29 and 1.12%, respectively as compared to the negative control groups which had a drop in PCV by 9.38 % from day 7 to 14 of treatment (Table 4). This finding was consistent with the effect shown by the extracts on parasitaemia of *T. congolense* infected mice (Figure 2). Animals treated with diminazene showed significantly improved PCV compared to all groups at days 7 and 14 of treatment (Figure 1).

Effect of extracts on body weight

Animals treated with 400 mg/kg dose of the aqueous extract of *C.abyssinica* had shown significant improvement on their body weight on day 6 (p<0.01), days 8-14 (p<0.001) of treatment, while animals treated with the methanol extract at 400 mg/kg dose had improved body weight (p<0.001) as compared to the negative control group and lower dose (100 and 200 mg/kg) treated groups at p<0.05. In addition animals treated with 400 mg/kg dose of the methanol extract had shown an improvement in their body weight by 1.36% (Figure 3).

Effect of extracts on mean survival time

Animals treated with 400 mg/kg of the methanol extract of *C. abyssinica* had highest mean survival time of 39.20±0.37 days as compared to the negative control group (25.40 ± 0.43) (Table 5), while animals that received the standard drug (diminazine aceturate) had mean survival time of 44.00 ± 0.63 days (Figure 4).

Discussion

Since the few trypanocides developed over 40 years a go are expensive and toxic [4], it has become necessary to search for new compounds that are safe and efficacious, especially those of plant origin. The plant screened in the present study has folkloric medicinal

Table 3: The effect of the aqueous leaf extract of *Clutia abyssinica* on packed cell volume of *Trypanosoma congolense* infected mice.

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

Values are expressed in Mean ± S.E.M (n=5); DA28= diminazine aceturate 28 mg/kg; CAAE200=*C. abyssinica* aqueous extract 200 mg/kg, CAAE400=*C. abyssinica* aqueous extract 400 mg/kg; [b]compared to TW80= 2% tween 80, [c]compared to CAAE100=*C. abyssinica* aqueous extract 100 mg/kg, *compared with all groups; [1]p < 0.05, [2]p < 0.01 and [3]p < 0.001

Table 4: The effect of the *methanol* leaf extract of *Clutia abyssinica* on packed cell volume of *Trypanosoma congolense* infected mice.

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

Values are expressed in Mean ± S.E.M (n=5); DA28= diminazine aceturate 28 mg/kg, CAME400=C. abyssinica smethanol extract 400 mg/kg; [b]compared to TW80= 2% tween 80, [c]compared to CAME100=C. abyssinica methanol extract 100 mg/kg, [d]compared to CAME200=C. abyssinica methanol extract 200 mg/kg; *compared with all groups; [1]p < 0.05, [2]p< 0.01 and [3]p < 0.001

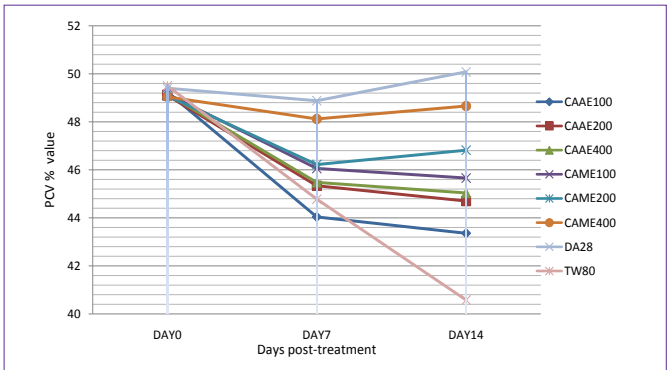

Figure 1: Comparison of the effect of aqueous and methanol leaf extracts of *Clutia abyssinica* on packed cell volume of *Trypanosoma congolense* infected mice.

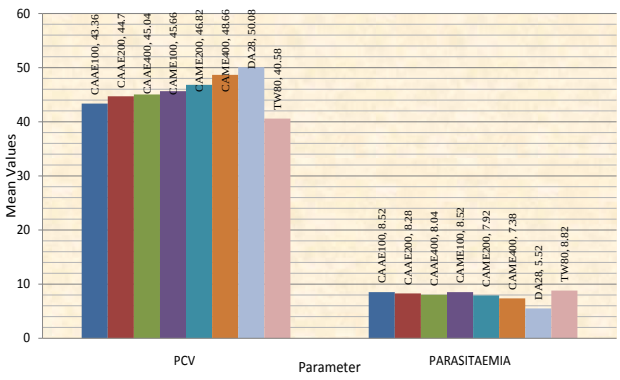

Figure 2: Comparison of the effect of aqueous and methanol leaf extracts of *Clutia abyssinica* on packed cell volume and parasitaemia.

uses as fever remedies and treatment of infectious diseases including trypanosomosis [17,18]. Based on this, the aqueous extract was prepared by macerating the dried leaves in distilled water in order to simulate the way it is traditionally used [17,18]. With the assumption that some of the active ingredients responsible for the claimed antitrypanosomal activity might not be soluble in water adequately; the methanol leaf extract of the plant was also included in the study. Based on the results of the acute toxicity study, the plant extracts had shown LD_{50} greater than 2000 mg/kg. Thus, since *C. abyssinica* is believed to have several traditional medicinal uses by different traditional healers, the experimental determination of this good safety margin would justify that the plant is safe at the dose levels (100, 200 and 400 mg/kg) used in the study which is an additional proof for the medicinal value of the plant in folk medicine, as toxic plants will not be used for generations.

According to the results of the phytochemical screening study, 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 only for anthraquinones, phenolic compounds and polyphenols (unpublished report).

Numerous *in vivo* studies conducted on the antitrypanosomal activities of the class of compounds listed above reported the potential of each class of compounds in killing or inhibiting the growth of wide ranges of trypanosomes. Inhibition of the trypanosome alternative oxidase (TAO) enzyme was thought to be responsible for antitrypanosomal activity of phenolic compounds [27]. Quininos can

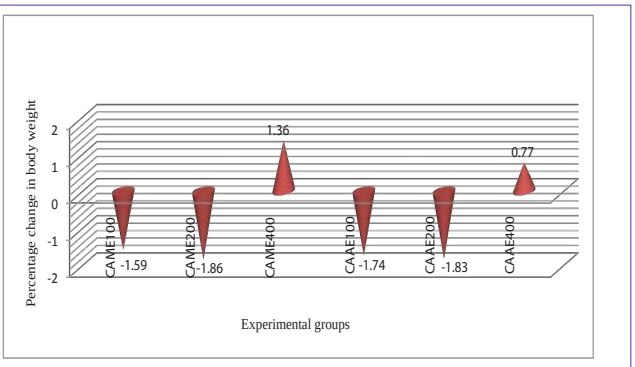

Figure 3: Comparison of the effect of aqueous and methanol leaf extracts of *Clutia abyssinica* on percentage change in body weight of *Trypanosoma congolense* infected mice.

induce oxidative stress in trypanosomes (*T. congolense* and *T. cruzi*). This may be explained by their reduction to semiquinone radicals by enzymes such as those present in the mitochondrial electron transport chain and the trypanothione reductase [28]. Flavonoids and flavonoid-derived plant natural products have long been known to function as free radical scavengers and metal chelators which inhibit lipid peroxidation [29].

One of the molecular actions of tannins is by complexing proteins through the so-called nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation [30]. The proposed mechanism for antitrypanosomal activity of terpenes include formation of aldehyde-thiol adducts with sulphur containing components thereby decreasing the buffering agents which can create oxidative stress in cells [31]; oxidation of glutathione, pyruvic and alpha-ketoglutaric acids and the oxidative decarboxylation of pyruvic acid by hydroperoxy group which makes them toxic [32]. Therefore, the observed antitrypanosomal activity of *C. abyssinica* might be attributed to either the individual class of compounds, or to the synergistic effect that each class of compounds exerts to give the observed biological activity. Hence, further in-depth investigations should be carried out to resolve this issue [33,34].

The results obtained during the monitoring period had shown that the higher dose (400mg/kg) of the methanol extracts of C. *abyssinica* exhibited significant antitrypanosomal activity by reducing the level of parasitaemia by 3.91% as compared to the 18.55% increment in the negative control group and prolonging the lifespan of the test animals beyond that of the negative control by more than 10 days (Table 4). This trypanostatic effect might correlate with the type of antiviral activity reported by Colegate and Molyneux [35], and Cos et al. [36]. In that report, it was shown that the ethanolic leaf, stem and root extracts of *C. abyssinica* showed moderate antiviral activity against polio virus and Coxsackie virus [35], while the leaf extracts exhibited anti-HIV-1 activity [36].

Although we do not yet know the mechanism by which the extract exerts this remarkable trypanostatic effect, our speculation, based on current literature on mechanisms of anti-trypanosomal compounds, is that the extract may be interfering with cell cycle progression in the parasite, possibly causing cell cycle arrest and thereby halting cell proliferation, which is a similar mechanism for currently available antitrypanosomal agents. It is also possible that the extract might be exerting its trypanostatic effect through the modulation of the animal's immune system, which in turn enables the animal to withstand the ravaging parasites for a long time. It may well be the interplay of both effects that resulted in the observed tremendous trypanostatic effect.

In addition, the findings of this study had shown that the plant extracts did not completely eliminate parasites from the blood stream of infected mice, but only reduced the level of parasitaemia. Several researchers made similar observations on reduction in parasitaemia [7,8,37]; and concluded that high parasite load could mask the efficacy of crude extract [38]. The reduced efficacy of the crude extracts in clearing trypanosomes from blood circulation could be due to enzymatic inactivation of active compounds and impaired absorption from the site of administration [38]. In addition, failing to reach target organs in sufficient concentration and duration to effect a cure; short half life of the constituents making them unable to stay long enough to exert pronounced effect on the parasites [39] could also be attributed to the failure of the extracts to clear the blood of infected mice from trypanosomes.

In the group of mice treated with diminazine aceturate, there was no parasite development from day 2 to 10 although relapse occurred in all mice approximately on days 12-14 of treatment. Similar observations were made by Afewerk et al. [40], Assefa and Abebe [41], Delespaux et al. [42], Ibrahim et al. [8], Miruk et al. [43]. The relapse of parasitaemia might be due to drug resistance by trypanosomes or the ability of *T. congolense* to sequester in small vessels and capillaries of the heart, skeletal and other tissues [44,45,46].

The study on packed cell volume (PCV) gave results that were fairly consistent with the observations made on parasitaemia (Figure 2). Infection caused significant drop in PCV in the negative control groups approximately by day 25 post-infection (14th day of treatment) with mean value being below the reference values (42-52%). The low PCV value in the infected groups may be due to acute hemolysis and is a result of the growing infection. In addition infection with trypanosomes results in increased susceptibility of red blood cell membrane to oxidative damage. Reactive oxygen species generated by trypanosomes can also attack red blood cells' membranes, induce oxidation and subsequently hemolysis. This phenomenon subjects RBC to massive erythrophagocytosis by an expanded and active mononuclear phagocytic system of the host resulting in anemia [47].

The effect of extracts in ameliorating anemia is possibly by reducing the parasite load, neutralizing the toxic metabolites produced by trypanosomes or scavenging the trypanosome associated free radicals which could be attributed to the secondary metabolites present in the extracts [48-50]. The infected mice treated with the diminazine aceturate showed significant improvement in PCV. This is because the drug was able to eliminate parasites from the blood to levels detectable by microscopy on days 2-10. This is in harmony with previous reports [51,52].

The trypanosuppresive effect of the extracts against trypanosome infection can further be inferred from the weight status of the treated animals. At day 14 post-treatment, animals that received 400 mg/kg dose of the aqueous and methanol extracts of *C. abyssinica* gained weight by 0.77% and 1.36%, respectively (Tables 3 & 4) at ($p<0.001$) as compared to the negative control groups. This shows that as a result of reduction in parasitaemia and prevention of drop in PCV

Table 5: Comparison of the effect of aqueous and methanol extract of *Clutia abyssinica* on all indices of *Trypanosoma congolense* infected mice at the end of the experimental study.

Plant	Extract	Dose (mg/kg)	Mean Parasitaemia	Mean PCV value	Mean Body weight	Mean survival time (days)
C. abyssinica	Aqueous	100	8.52 ± 0.07	43.36 ± 0.24	21.25 ± 0.20	36.20 ± 0.66
		200	8.28 ± 0.07	44.70 ± 0.28	21.25 ± 0.18	35.00 ± 0.31
		400	8.04 ± 0.06	45.04 ± 0.31	22.18 ± 0.22	35.60 ± 0.40
	Methanol	100	8.52 + 0.15	45.66 ± 0.22	21.28 ± 0.21	37.20 ± 0.66
		200	7.92 + 0.15	46.82 ± 0.34	21.31 ± 0.14	37.60 ± 0.50
		400	7.38 + 0.18	48.66 ± 0.20	22.34 ± 0.27	39.20 ± 0.37
Positive control	Diminazine aceturate	28	5.52 + 0.07	50.64 ± 0.15	22.61± 0.17	44.00 ± 0.63
Negative control	2% Tween 80	2ml/100gm	8.82 + 0.12	40.58 ± 0.27	19.09 ± 0.34	25.40 ± 0.43

Values are expressed in Mean ± S.E.M (n=5).

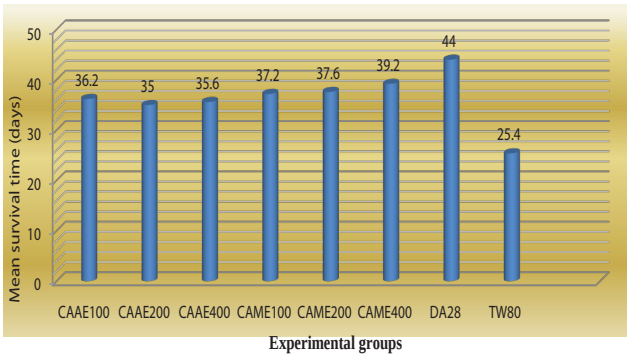

Figure 4: Survivality of *Trypanosoma congolense* infected mice treated with aqueous and methanol crude extracts of leaves of *Clutia abyssinica.*

by the extracts and physical status of the treated mice improved. They were therefore more able to resist weight loss that is usually associated with trypanosomosis. Similar observations have been made by other researchers [51-55]. Based on our findings, animals in the negative control group lost 11.03% of their body weight which might be due to the significant drop in PCV associated with high level of parasitaemia.

The majority of trials provide evidence of the negative effect of trypanosomosis on body weight. During the high levels of parasitaemia the appetite is decreased and the animal losses condition resulting in wasting. There is consumption of the fat reserves but there are also severe degenerative changes of the muscle cells and other tissue cells, and there is an increased breakdown of protein in muscles and elsewhere leading to atrophic degeneration. The decreased supply of oxygen because of the anemia is also an important factor [56,57].

Conclusion

This study evaluated the *in vivo* antitrypanosomal activity of aqueous and methanol crude leaf extracts of *C. abyssinica* against *T. congolence.* The aqueous extract exhibited lower *in vivo* antitrypanosomal activity, while the methanol extracts have shown better activity at higher doses. The methanol leaf extract of *C. abyssinica* has promising effect by reducing pre-treatment parasitemia, increasing PCV, increasing pretreatment body weight and prolonging survival time in *T. congolense* infected mice. Generally, the current study established that leaves of *C. abyssinica* could have potential antitrypanosomal activity which can be considered as a potential source for the search of new drugs against Africal animal trypanosomosis.

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