

## Evaluation of *In vivo* Antitrypanosomal Activity of Aqueous and Methanol Leaf Extracts of *Clutia abyssinica* (Euphorbiaceae) against *Trypanosoma congolense* Field Isolate

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### Abstract

Aqueous and methanol leaf extracts of *C. abyssinica* were investigated for their *in vivo* activity against *Trypanosoma congolense*, the main causative agent of African animal trypanosomiasis in Sub-Saharan Africa and Ethiopia. The *in vivo* antitrypanosomal efficacy of the aqueous and methanol extracts was evaluated in Swiss albino mice infected with *T. congolense* field isolate. The leaf extracts were administered 12 days post-infection at peak parasitaemia level of  $\sim 10^8$  trypanosomes/ml at doses of 100, 200 and 400 mg/kg by intraperitoneal injection once daily for 7 days. Parasitaemia, packed cell volume (PCV), mean survival time and change in body weight were used as indices for monitoring the efficacy of the extracts by comparing with the positive control: 28 mg/kg dose of diminazene aceturate and negative control: 2% tween 80 treated groups. The extracts had a lethal dose greater than 2000 mg/kg and there were no evidences of acute toxicity at the doses tested. Highly significant ( $p < 0.001$ ) reduction in pre-treatment parasitaemia by 3.91% ( $7.38 \pm 0.18$ ), increase in PCV by 1.12% ( $48.66 \pm 0.20$ ), body weight improvement by 1.36% ( $22.34 \pm 0.27$ ) and mean survival time of  $39.20 \pm 0.37$  days was observed in the group treated by 400 mg/kg methanol leaf extract of *C. abyssinica*. In general, the results obtained suggest ethno-pharmacological usefulness of the plant and necessitate further studies to be carried on isolated active substances from the plant.

**Keywords:** Trypanosomiasis; *Trypanosoma congolense*; *Clutia abyssinica*; Parasitaemia; Packed cell volume; Body weight; Mean survival time

### Introduction

African trypanosomiasis is a protozoan disease of man and livestock. It is caused by trypanosomes and transmitted by tse tse fly. While *Trypanosoma rhodiense* and *Trypanosoma gambiense* cause African Human Trypanosomiasis (AHT, sleeping sickness), *Trypanosoma brucei brucei*, *Trypanosoma vivax*, *Trypanosoma congolense*, *Trypanosoma evansi* and *Trypanosoma equiperdum* are the agents of African Animal Trypanosomiasis (AAT) [1]. The economic impacts of trypanosomiasis 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]. AAT is associated with fever, depression, weakness, anemia, salivation, lacrimation, nasal discharges, and subcutaneous edema of the mandible and prominent jugular pulse. The appetite is decreased and there is a rapid weight loss which progresses to an extreme emaciation. Often, death is related to severe anemia and circulatory collapse [3].

Chemotherapy, the main means of controlling the disease is under threat due to parasite resistance [4] and toxicity of the trypanocidal drugs [5]. 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 [6,7]. Although recent reports indicate antitrypanosomal activity exists in some medicinal plants [8-10], the potentials of many other plants used in folkloric medicine in Ethiopia are yet to be investigated. This study was designed to determine the *in vivo* antitrypanosomal activity of *Clutia abyssinica* against *T. congolense* infected mice.

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

Euphorbiaceae is a large and fascinating family of about 300 genera and 8,000-10,000 species, mostly found in the tropics of both hemispheres. *Clutia* is a genus having about 60 species. *Clutia abyssinica* called by the Amharic name 'fyele fe' is herb 1-2 m high [11]. Traditionally it is used in treatment of venereal and skin diseases, chest problems, cancer [12]; Skin fungal infections [13,14]; Yellow fever and malaria [15]; management of ear, nose and throat diseases [16]; diarrhoea [17]; gonorrhoea, 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 [14]. Moreover, maceration of the crushed leaves of *C. abyssinica* given orally has traditionally been used for the treatment of animal trypanosomiasis [18].

### Material and Methods

#### Reference Drug

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

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## Test organism

The test organism, *T. congolense* was isolated from infected cattles at Sebategna kebele of Ilu-Aba-Bora-Zone, 480 km from Addis Ababa in South west direction. Blood samples were collected from the peripheral ear vein of each animal using heparinized microhaematocrit capillary tubes that filled 3/4 of the height and sealed with cristaseal. The presence of *T. congolense* was detected by Microhaematocrit Buffy Coat Technique (MHBCT) [19] where the sealed microhaematocrit capillary tubes were centrifuged immediately in microhaematocrit centrifuge for 5 min at 12000 rpm. After centrifugation, the capillary tube was cut by diamond pencil 1 mm below the buffy coat to include the top layer of red cells. The content of the capillary tube was expressed onto a clean microscope slide, mixed and covered with a 22 × 22 mm cover slip.

Then the slide was examined for *T. congolense* based on their type of motility in the microscopic field 400X objective. Confirmations of *T. congolense* species by morphological characteristics was done after a thin blood smear was prepared from the buffy-coat examination and stained with Giemsa stain and examined under a microscope using oil immersion 100X objective [20,21]. Then an animal with peak parasitaemia of ( $\sim 10^8$  trypanosomes/ml) [22] was selected and blood was collected to the ethylene diamine tetra acetic acid (EDTA) coated tube from the jugular vein of the animal and diluted with PBS.

Then 0.2 ml of the blood containing  $\sim 10^4$  trypanosomes/ml was injected intraperitoneally to 6 laboratory mice and transported to the laboratory at Akililu Lemma Institute of Pathobiology (ALIPB), Addis Ababa University for serial passage to other mice for the experimental part of the study. The development of trypanosomes in the infected mice was determined by wet film examination of blood obtained from the tail of the mice by buffy coat method [19]. Following syringe challenge all the donor mice had shown parasitaemia 7 days post-infection. After establishment of infection heavily ( $\sim 10^8$  trypanosomes/ml) (21 days post- inoculation), the donor mice were then subjected to cardiac puncture and blood was collected with an EDTA coated tube and immediately diluted with phosphate buffered saline. Then about 0.2 ml of blood collected from the donor mice containing ( $\sim 10^4$  trypanosomes/ml) [22] was injected intraperitoneally in to mice that were acclimatized to laboratory conditions and were used for *in vivo* evaluation of the crude leaf extracts of *C. abyssinica*.

## Experimental animals

Healthy Swiss albino mice (weighing 20-30 gm and age of 8-12 weeks) were obtained from the animal house of the Ethiopian Health and Nutrition Research Institute (EHNRI) and School of Pharmacy, Addis Ababa University. Animals were housed in polypropylene cages (6-10 animals per cage), maintained 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 [23].

## Collection of plant specimens

The leaves of *C. abyssinica* were collected in the month of April 2013 from Debre Libanos 'gedam', a Monastery in Amhara regional state, Ethiopia. The fresh leaves were wrapped by plastic sheets during transportation. Taxonomic identification was done and a voucher specimen was deposited (Collection EM/001) at the National Herbarium, College of Natural sciences, Addis Ababa University. The leaves were thoroughly washed with distilled water to remove dirt, soil and any other foreign materials and left to drain off. Then they were then spread on laboratory bench and dried under shade. The dried

leaves were pulverized using mortar and pestle at medicinal plants laboratory of ALIPB.

## Preparation and storage of extracts

200g of dried leaf powder of *C. abyssinica* was separately macerated with 1000 ml of distilled water and 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 not more than 40°C in order to obtain dry extract. Then the resulting dried mass was then powdered, weighed and packed into a glass vial and stored in a desiccator over silica gel until use.

## 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 using standard methods [24-29].

## Acute toxicity study

The acute toxicity study was conducted in accordance with the Lorke's method [30]. The study was conducted for each extract in two phases using female swiss albino mice after 7 days of adaptation. In the first phase, nine mice were divided into 3 groups of 3 mice each. Each group was given 10, 100, and 1000 mg/kg body weight of the test substance respectively. In the second phase, further specific doses (1600, 2900, and 5000 mg/kg) of each extract were administered to nine mice (three mice per dose) to further determine the correct lethal dose ( $LD_{50}$ ) value. In addition, a fourth group of six mice was set up as control group and received the reconstituting solvent 2% tween 80 in sterile water.

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

## Parasite inoculation and extract administration

40 healthy Swiss albino mice that are infected intraperitoneally with 0.2 ml of *T. congolense* infected blood ( $\sim 10^4$  trypanosomes/ml) collected by cardiac puncture from donor mice was divided into eight groups: *C. abyssinica* aqueous extract (CAAE 100, CAAE 200, CAAE 400), *C. abyssinica* methanol extract (CAME 100, CAME 200, CAME 400), Diminazine aceturate (DA28), and 2% tween 80 (TW80) each comprising of 5 mice.

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

toxicity study. The middle dose was one tenth of the lethal dose (~2000 mg/kg) which was 200 mg/kg. Higher dose was calculated as twice the middle dose, which was 400 mg/kg. The lower dose level was calculated by taking half of the middle dose, which was 100 mg/kg. For the positive control, diminazine aceturate (Veriben®) (DA28), dissolved in sterile water as recommended by the manufacturer (Ceva Santé Animale, France) was administered at the dose of 28 mg/kg intraperitoneally based on previous reports, while for the negative control, 2% tween 80 in sterile water (TW80), was administered intraperitoneally. Volume administered was determined based on the organization of Economic Co-operation and Development (OECD) guideline that states 2 ml/100 gm of body weight of the animal.

### Determination of parasitaemia

Parasitaemia was monitored every other day by microscopic examination of blood obtained from the tail of each mouse that was pre-sterilized with methylated spirit. The tail tip was cut to extrude blood and drop of blood was placed on microscope slide and covering with a cover-slide (22 × 22 mm) (OIE, 2013) [31]. The blood was examined microscopically at 400X total magnification. The degree of parasitaemia was determined using the “Rapid Matching” method of [22]. 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 [22].

### Determination of packed cell volume (PCV)

PCV was measured using Wintrobe’s method [32] to predict the effectiveness of the test extracts in preventing hemolysis resulting from increasing parasitaemia associated with trypanosomosis. It was monitored before infection and three times till the 14<sup>th</sup> day (on day 0, 7 14). Briefly, blood was collected from tail of each mouse in heparinized microhaematocrit capillary tubes filled up to 3/4<sup>th</sup> of their length. The tubes were then sealed immediately by cristalseal and centrifuged in a microhaematocrit centrifuge (Hettich Haematokrit, Germany) for 5 min at 12,000 rpm. After centrifugation, the height of the red blood cell column were measured by use of haematocrit reader and compared to the total height of the column of the whole blood (Wernery et al., 2001) [33]. 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

### Yield for plant extraction

The yield of aqueous and methanol leaf extracts of *Clutia abyssinica* (Table 1)

### Phytochemical screening

The results of phytochemical screening results for the aqueous and methanol leaf extracts of *Clutia abyssinica* is shown in (Table 2)

### Acute toxicity test

The acute toxicity bioassay had shown that the lethal dosage (LD<sub>50</sub>) of the aqueous and methanol leaf extracts of *C. abyssinica* was above 2000 mg/kg and there were no evidences of acute toxicity at the doses tested indicating good safety margin.

### Effect 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 3), while animals treated with the methanol extract at 200 and 400 mg/kg dose had statistically significant low parasitaemia on day 6, 8, 10 and 14 (p<0.001) of treatment as compared to the negative control group (Table 4).

### Effect on packed cell volume (PCV)

The mean PCV% value of animals treated with 400 mg/kg of the aqueous extract of *C. abyssinica*, with 200 and 400 mg/kg dose of the methanol extract had a statistically significant (p<0.001) higher PCV value as compared to the negative control groups on day 14 of treatment (Table 5). Analysis of change in percentage of PCV from day 7 to day 14 of treatment also showed that the methanol extracts at 200 and 400 mg/kg doses had significantly increased PCV value of treated animals by 1.29 and 1.12%, respectively as compared to the negative control groups which had a drop in PCV by 9.38 % from day 7 to 14 of treatment (Table 6). This finding was consistent with the effect shown by the extracts on parasitaemia level of *T. congolense* infected mice (Figures 1 and 2).

| Plant species        | Part extracted | Solvent         | Percentage yield (%w/w) |
|----------------------|----------------|-----------------|-------------------------|
| <i>C. abyssinica</i> | Leaf           | Distilled water | 12.92%                  |
|                      |                | Methanol        | 17.21%                  |

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

| Constituents       | <i>C. abyssinica</i> |                  |
|--------------------|----------------------|------------------|
|                    | Aqueous extract      | Methanol extract |
| Alkaloids          | -                    | +                |
| Antraquinones      | +                    | +                |
| Flavonoids         | -                    | +                |
| Glycoside          | -                    | +                |
| Saponins           | +                    | -                |
| Steroids           | -                    | +                |
| Phenolic compounds | +                    | +                |
| Tannins            | -                    | +                |
| Terpenes           | -                    | +                |

+ =present, - =absent

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

| Days                                | Parasitaemia (log number/ml) |                          |                           |                           |                                 |
|-------------------------------------|------------------------------|--------------------------|---------------------------|---------------------------|---------------------------------|
|                                     | DA28                         | TW80                     | CAAE100                   | CAAE200                   | CAAE400                         |
| Day 0                               | 7.68 ± 0.18                  | 7.44 ± 0.17              | 7.38 ± 0.07               | 7.68 ± 0.18               | 7.33 ± 0.14                     |
| Day 2                               | 0.00 ± 0.00 <sup>3</sup>     | 7.74 ± 0.17              | 7.62 ± 0.07               | 7.92 ± 0.15               | 7.56 ± 0.11                     |
| Day 4                               | 0.00 ± 0.00 <sup>3</sup>     | 7.86 ± 0.11              | 7.86 ± 0.11               | 7.98 ± 0.07               | 7.68 ± 0.18                     |
| Day 6                               | 0.00 ± 0.00 <sup>3</sup>     | 8.16 ± 0.11              | 7.68 ± 0.12               | 7.80 ± 0.13               | 7.44 ± 0.19 <sup>b2</sup>       |
| Day 8                               | 0.00 ± 0.00 <sup>3</sup>     | 8.28 ± 0.07 <sup>3</sup> | 7.44 ± 0.11 <sup>b3</sup> | 7.62 ± 0.07 <sup>b3</sup> | 6.96 ± 0.17 <sup>b3,c1,d2</sup> |
| Day 10                              | 0.00 ± 0.00 <sup>3</sup>     | 8.52 ± 0.12 <sup>3</sup> | 7.80 ± 0.13 <sup>b3</sup> | 7.86 ± 0.06 <sup>b3</sup> | 7.26 ± 0.14 <sup>b3,c1,d2</sup> |
| Day 12                              | 2.16 ± 1.32 <sup>3</sup>     | 8.64 ± 0.06              | 8.22 ± 0.07               | 8.04 ± 0.06               | 7.74 ± 0.11                     |
| Day 14                              | 5.52 ± 0.07 <sup>3</sup>     | 8.82 ± 0.12              | 8.52 ± 0.07               | 8.28 ± 0.07               | 8.04 ± 0.06 <sup>b3,c2</sup>    |
| % Change in Parasitaemia (Day 0-14) | -27.3                        | 18.55                    | 15.45                     | 7.81                      | 9.68                            |

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

**Table 3:** The effect of aqueous leaf extract of *Clutia abyssinica* on parasitaemia level of *Trypanosoma congolense* infected mice.

| Days                                | Parasitaemia (log number/ml) |             |             |                              |                                 |
|-------------------------------------|------------------------------|-------------|-------------|------------------------------|---------------------------------|
|                                     | DA28                         | TW80        | CAAE100     | CAAE200                      | CAAE400                         |
| Day0                                | 7.68 ± 0.18                  | 7.44 ± 0.17 | 7.56 ± 0.15 | 7.32 ± 0.12                  | 7.68 ± 0.18                     |
| Day2                                | 0.00 ± 0.00 <sup>3</sup>     | 7.74 ± 0.17 | 7.68 ± 0.18 | 7.50 ± 0.13                  | 7.62 ± 0.20                     |
| Day4                                | 0.00 ± 0.00 <sup>3</sup>     | 7.86 ± 0.11 | 7.92 ± 0.24 | 7.38 ± 0.07                  | 7.32 ± 0.22                     |
| Day6                                | 0.00 ± 0.00 <sup>3</sup>     | 8.16 ± 0.11 | 7.68 ± 0.18 | 7.26 ± 0.22 <sup>b3</sup>    | 6.54 ± 0.11 <sup>b3,c1,d1</sup> |
| Day8                                | 0.00 ± 0.00 <sup>3</sup>     | 8.28 ± 0.07 | 7.92 ± 0.18 | 6.78 ± 0.07 <sup>b3,c3</sup> | 5.94 ± 0.24 <sup>3</sup>        |
| Day10                               | 0.00 ± 0.00 <sup>3</sup>     | 8.52 ± 0.12 | 8.16 ± 0.17 | 7.32 ± 0.15 <sup>b3,c2</sup> | 6.24 ± 0.22 <sup>3</sup>        |
| Day12                               | 2.16 ± 1.32 <sup>3</sup>     | 8.64 ± 0.06 | 8.28 ± 0.15 | 7.74 ± 0.17                  | 7.26 ± 0.11                     |
| Day14                               | 5.52 ± 0.07 <sup>3</sup>     | 8.82 ± 0.12 | 8.52 ± 0.15 | 7.92 ± 0.15 <sup>b3,c1</sup> | 7.38 ± 0.18 <sup>b3</sup>       |
| % Change in Parasitaemia (Day 0-14) | -27.3                        | 18.55       | 12.69       | 8.19                         | -3.91                           |

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

**Table 4:** The effect of methanol leaf extract of *Clutia abyssinica* on parasitaemia of *Trypanosoma congolense* infected mice.

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

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

**Table 5:** The effect of the aqueous leaf extract of *Clutia abyssinica* on packed cell volume of *Trypanosoma congolense* infected mice.

| Days                 | PCV% Values               |                           |                            |                            |                                  |
|----------------------|---------------------------|---------------------------|----------------------------|----------------------------|----------------------------------|
|                      | DA28                      | TW80                      | CAAE100                    | CAAE200                    | CAAE400                          |
| Pre-infection        | 51.18 ± 0.37              | 50.88 ± 0.14              | 51.10 ± 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 <sup>3</sup> | 44.78 ± 0.37              | 46.06 ± 0.22 <sup>b1</sup> | 46.22 ± 0.32 <sup>b2</sup> | 48.12 ± 0.15 <sup>b3,c3</sup>    |
| % change PCV day 7-0 | -1.05                     | -8.69                     | -6.30                      | -5.82                      | -1.87                            |
| Day 14               | 50.08 ± 0.15 <sup>3</sup> | 40.58 ± 0.27 <sup>3</sup> | 45.66 ± 0.22 <sup>b3</sup> | 46.82 ± 0.34 <sup>b3</sup> | 48.66 ± 0.20 <sup>b3,c1,d2</sup> |
| %change PCV day 7-14 | 2.45                      | -9.38                     | -0.86                      | 1.29                       | 1.12                             |

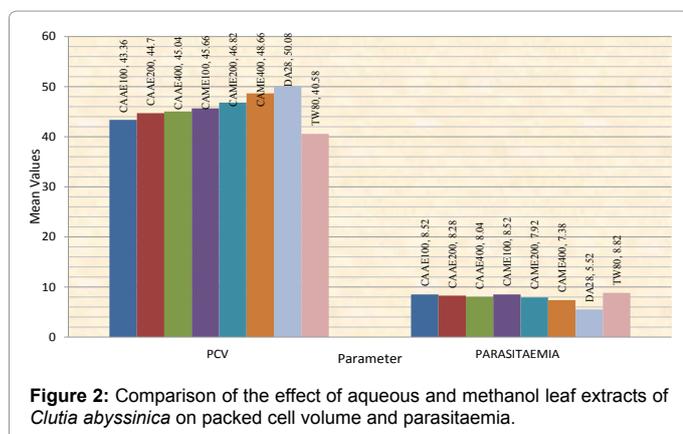
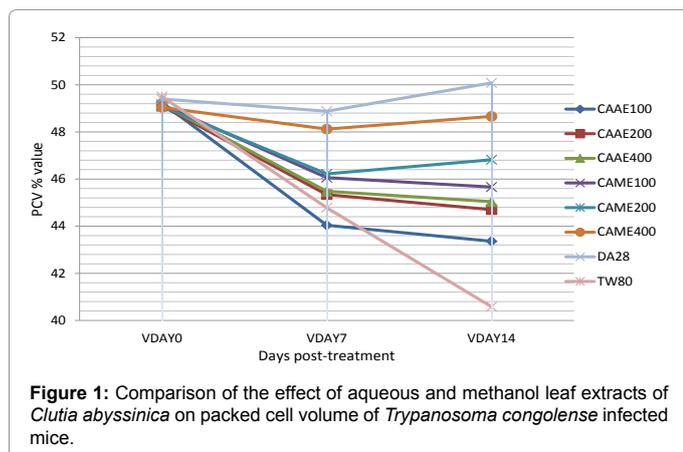
Values are expressed in Mean ± S.E.M (n=5) performed with ANOVA followed by Tukey's Post hoc multiple comparison test; DA28=diminazine acetate 28 mg/kg-the positive control, CAME400=C. *abyssinica* methanol extract 400 mg/kg; <sup>b</sup>compared to TW80=2% tween 80-the negative control, <sup>c</sup>compared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

**Table 6:** The effect of the methanol leaf extract of *Clutia abyssinica* on packed cell volume of *Trypanosoma congolense* infected mice.

## Effect 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), 8-14 (p<0.001) of treatment, while animals treated with



the methanol extract at 400 mg/kg dose had improved body weight of treated animals ( $P < 0.001$ ) 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 on mean survival time

Animals treated with 400 mg/kg of the methanol extract of *C. abyssinica* had highest mean survival time of  $39.20 \pm 0.37$  days as compared to the negative control group ( $25.40 \pm 0.43$ ) (Table 7), while animals that received the positive control diminazine aceturate had mean survival time of  $44.00 \pm 0.63$  days (Figure 4).

### Discussion

Since the few trypanocides developed over 40 years ago are expensive and toxic [5], 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 uses as fever remedies and treatment of infectious diseases including trypanosomiasis [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.

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 negative result for the same test.

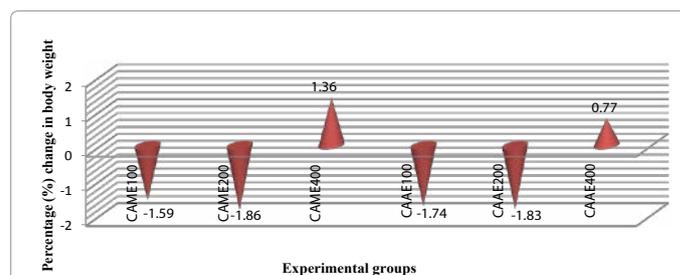
Numerous *in vivo* studies conducted on the antitrypanosomal activities of the class of compounds listed above reported the potential of each class of compounds in killing or inhibiting the growth of wide ranges of trypanosomes.

Inhibition of the trypanosome alternative oxidase (TAO) enzyme was thought to be responsible for antitrypanosomal activity of phenolic compounds [34]. Quininos can induce oxidative stress in trypanosomes (*T. congolense* and *T. cruzi*). This may be explained by their reduction to semiquinone radicals by enzymes such as those present in the mitochondrial electron transport chain and the trypanothione reductase [35]. Flavonoids and flavonoid-derived plant natural products have long been known to function as free radical scavengers and metal chelators: they inhibit lipid peroxidation and exhibit various physiological activities, including antihypertensive and anti-arthritis activities [6,36]. 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 [37]. The proposed mechanism for antitrypanosomal activity of terpenes include: by formation of aldehyde-thiol adducts with sulphur containing components thereby decreasing the buffering agents which can create oxidative stress in cells [38,39]; by oxidation of glutathione, pyruvic and alpha-ketoglutaric acids and the oxidative decarboxylation of pyruvic acid by hydroperoxy group which makes them toxic [40].

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 exert to give the observed biological activity. Hence, further in-depth investigations should be carried out to resolve this issue [41,42].

The results obtained during the monitoring period had shown that the higher dose (400mg/kg) of the methanol extracts of *C. abyssinica* exhibited appreciable 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).

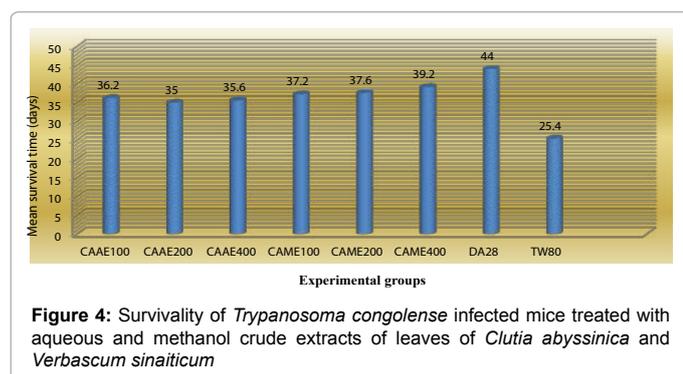
This trypanostatic effect might correlate with the type of antiviral activity reported by Colegate and Molyneux [43,44]. In that report,



**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 .

| Plant                | Extract              | Dose (mg/kg) | Mean Parasitaemia | Mean PCV value | Mean Body weight | Mean survival time |
|----------------------|----------------------|--------------|-------------------|----------------|------------------|--------------------|
| <i>C. abyssinica</i> | 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          | 2 ml/100 gm  | 8.82 ± 0.12       | 40.58 ± 0.27   | 19.09 ± 0.34     | 25.40 ± 0.43       |

**Table 7:** Comparison of the effect of aqueous and methanol extract of *Clutia abyssinica* on parasitaemia, packed cell volume, body weight, and survival time of *Trypanosoma congolense* infected mice at the end of the experimental study.



**Figure 4:** Survivability of *Trypanosoma congolense* infected mice treated with aqueous and methanol crude extracts of leaves of *Clutia abyssinica* and *Verbascum sinaiticum*

it was shown that the ethanolic leaf, stem and root extracts of *C. abyssinica* showed moderate antiviral activity against polio virus and Coxsackie virus [43], while the leaf extracts exhibited anti-HIV-1 activity [44]. Although we do not yet know the mechanism by which the extract exerts this remarkable trypanostatic effect, our speculation is that the extract may be interfering with cell cycle progression in the parasite, possibly causing cell cycle arrest and thereby halting cell proliferation, which is a similar mechanism for currently available antitrypanosomal agents. It is also possible that the extract might be exerting its trypanostatic effect through the modulation of the animal's immune system, which in turn enables the animal to withstand the ravaging parasites for a long time. It may well be the interplay of both effects that resulted in the observed tremendous trypanostatic effect.

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 [8,9,45] and concluded that high parasite load could mask the efficacy of crude extract [46]. Also, the reduced efficacy of the crude extracts in clearing trypanosomes from blood circulation could be due to enzymatic inactivation of active compounds and impaired absorption from the site of administration [47]. 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 [48] 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 [9,49-52]. The relapse of parasitaemia might be due to trypanocidal drug resistance by trypanosomes or the ability of *T. congolense* to sequester in small vessels and capillaries of the heart, skeletal and other tissues [53-55]. 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 (MPS) of the host resulting in anemia [56]. Thus, scavenging the trypanosome associated free radicals may ameliorate anemia.

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 [46,59-63].

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 [59,60].

The trypano suppressive 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 and 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 by the extracts physical status of the treated mice improved. They were therefore more able to resist weight loss that is usually associated with trypanosomosis. Similar observations have been made by other researchers [46,59,60-63].

Based on our findings, animals in the negative control group lost 11.03% of their body weight which might be due to the significant drop in PCV associated with high level of parasitaemia in this group. The majority of trials provide evidence of the negative effect of trypanosomosis on body weight. During the high levels of parasitaemia the appetite is decreased and the animal losses condition as a result there is wasting. There is consumption of the fat reserves but there are also severe degenerative changes of the muscle cells and other tissue cells, and there is an increased breakdown of protein in muscles and elsewhere, leading to atrophic degeneration. The decreased supply of oxygen because of the anemia is also an important factor [64-66].

## Conclusion

This study gave indications of *in vivo* antitrypanosomal activity of methanol crude leaf extracts of *C. abyssinica* against *T. congolense* field isolate. The work demonstrated good safety margin of the crude extracts in mice suggesting their ethnopharmacological usefulness. The aqueous extract exhibited lower *in vivo* antitrypanosomal activity, while the methanol extracts have shown higher *in vivo* activity at higher doses. However, both extracts were not effective enough to eradicate the parasites completely. The study revealed that methanol leaf extract of *C. abyssinica* has promising effect by reducing pre-treatment parasitemia by 3.91% ( $7.38 \pm 0.18$ ), increasing PCV by 1.12% ( $48.66 \pm 0.20$ ), increasing pretreatment body weight by 1.36% ( $22.34 \pm 0.27$ ) and prolonging survival time by  $39.20 \pm 0.37$  days 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 African animal trypanosomiasis.

## Acknowledgment

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