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Original Research Article

Antitrypanosomal Evaluation of Methanol Fractions of Stem Bark Extract of *Acacia nilotica* (Linn) against *Trypanosoma congolense* Infection in Albino Mice

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ABSTRACT

Trypanosomiasis is considered a plague in sub-Saharan Africa and chemotherapy of the disease is unsatisfactory. This study was conducted to explore an alternative source of an antitrypanosomal agent from the stem bark of *Acacia nilotica.* The methanol extract of *A. nilotica* stem bark was subjected to a qualitative phytochemical screening and fractionation by column chromatography using gradient elution with hexane, ethyl acetate, and methanol solvent mixtures. The acute toxicity of the crude extract was evaluated. The antitrypanosomal assay was performed on the column fractions (coded 1-9). Fifty-eight albino mice were used with diminazene aceturate as a standard drug for the *in vivo* assay. The mice were divided into eleven groups and the effect of each fraction on parasitaemia, packed cell volume (PCV), white blood count (WBC), red blood cell (RBC), body weight, and percentage survival of the animals were monitored over a period of 21 weeks. Treatment with fractions 4 and 5 of the ethyl acetate: methanol (90:10 and 70:30, respectively) resulted in prolonged infection, extension of life, and recovery 14 weeks beyond the negative control. Meanwhile, treatment with other fractions showed lower potency. The animals treated with fractions 4 and 5 also showed higher levels of PCV, RBC, and WBC. It was also observed that 20 and 40 % of mice treated with fractions 4 and 5 appeared to have recovered. The findings of this study revealed that fractions 4 and 5 can control anaemia, boost immunity, and prolong the life of infected mice beyond the standard drug, indicating that they offer promising prospect for lead compounds in the chemotherapy of trypanosomiasis.

Keywords: *Acacia nilotica*, Antitrypanosomal activity, *Trypanosoma congolense*, Trypanosomiasis, Alternative therapy.

Introduction

Trypanosomiasis is a group of important human and animal diseases caused by parasitic protozoa of the genus *Trypanosoma*.[1] The condition is endemic in the 36 sub-Saharan African and 21 Latin American countries. Human African Trypanosomiasis (HAT) has two forms. The first is *Trypanosoma brucei gambiense*, a chronic form of the disease that accounts for 95% of reported cases and is prevalent in Western and Central Africa. The second form is *Trypanosoma brucei rhodesiense* which is the acute form of the disease and occurs in Eastern and Southern African countries.[2] African Animal Trypanosomiasis (AAT) is caused by different species of trypanosomes. It is a major constraint to the health and productivity of cattle and other domestic animals in sub-Saharan Africa.[3] Lethargy, weight loss, oedema, anaemia, paralysis, decreased milk production, decreased fertility, and increased death are all indications of AAT.[4] The disease has serious socioeconomic effects since it lowers the quality of food and milk output. The agricultural gross domestic product loss is expected to be USD 4.7 billion per year.[5,6]
The use of naturally derived trypanotolerant breed,[7] as well as drugs are some of the approaches used to control the disease.

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Trypanotolerance is the capacity of certain animals such as N'Dama and Muturu cattles,[8] West African Dwarf goats, and sheep to remain productive despite AAT infection. However, many farmers prefer the more productive trypanosusceptible breeds such as Zebu cattle.[8] Drugs used for the treatment of HAT include pentamidine, suramin, melarsoprol, eflornithine, nifurtimox, and fexinidazole, while the AAT drugs are isometamidium chloride, diminazene aceturate, and homidium salts. However, most of the drugs are outdated, have low efficacy, are difficult to obtain, and have unacceptable toxicity, and the parasites are resistant to them.[9] Presently, despite the free donations of HAT drugs by manufacturers, coordinated by WHO, they hardly get to beneficiaries, necessitating the search for alternative sources of medicines.
Plants have been a rich source of phytochemicals stored in different plant parts such as the root, stem bark, juice, flowers, fruits, and seeds. Our ancestors had "cures" made from medicines,[10] many of which were formulated from plants and other materials. These remedies are mostly made by boiling plant materials or simply soaking them in water, alcohol, palm wine, palm oil, or kernel oil. Pastes, pomades, and ointments in oiled media could also be used to present the preparations. The plant, *Acacia nilotica* (Linn) belongs to the family Fabaceae,[11] and consists of six sub-species.[12] The plant has been used to treat cancers and other tumors,[13] and the poultice made from the leaves has been used to treat ulcers. At the same time, the root is claimed to be used for the treatment of tuberculosis.[14] The antidiabetic and hypolipidemic effects of the aqueous-methanol extract of the pods have also been reported.
The current interest in *A. nilotica* in the search for new trypanocides stems from different claims for its usage in alternative medicine, as well as previous findings on its *in vitro* antitrypanosomal activity of crude methanol extracts.[15]

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Due to the importance of both human and animal trypanosomiasis in public health, the present study was conducted to evaluate the potency of the fractions obtained from chromatographic purification of the stem bark extract of *A. nilotica* against *Trypanosoma congolense*-infected albino mice.

Materials and Methods

Plant materials

The stem bark of *A. nilotica* was obtained in March 2019 from Hwol Buji in Bassa Local Government area of Plateau State located at Latitude 10.01° N and longitude 008.89° E. The plant was authenticated by Mr. Azila J. J. of the Department of Horticulture, Federal College of Forestry, Jos, Nigeria. A Specimen Voucher (FHJ 270) was deposited in the departmental Herbarium. The Plant materials were air-dried under shade, pulverized to a fine powder, and stored in plastic containers for subsequent use.

Source of animals and ethical clearance

Albino mice used in this study were obtained and raised in the Animal House Unit of the Nigerian Institute for Trypanosomiasis Research (NITR), Vom, Plateau State. The animals were kept in cages and given access to food and water *ad libitum.* They were kept under the protocol of the University of Jos Institutional Animal Care and Use, with reference number F17-00379, and internationally accepted principles for laboratory animal use and care.

Preparation of the methanol extract of Acacia nilotica

One kilogram of powdered material was exhaustively macerated by cold extraction in 2 L of 98% methanol. The maceration was carried out for 48 hours. Then, the extract was decanted and filtered using a muslin cloth and subsequently with Whatman No. 1 filter paper. Using a fresh solvent, the extraction process was repeated 3 times. The combined extracts were concentrated at 35 ^{o}C and the percentage yield was calculated using the formula below:

$$\% \text{ yield of extract} = \frac{Weight\ of\ total\ extract}{Weight\ of\ powdered\ material} \times 100$$

Phytochemical screening of methanol extract of Acacia nilotica

The methanol crude extract of *A. nilotica* was subjected to qualitative tests to identify the plant's major secondary constituents using standard methods.[16]

Column chromatographic analysis of methanol extract of Acacia nilotica

The extract (10g) was dissolved in 20 mL of methanol and then adsorbed on 20 g of silica gel. Hexane (500 mL) was mixed with 260 g of the adsorbent silica gel (70-200 mesh) to make a slurry which was gently poured into a chromatographic column, ensuring that no air bubbles were trapped. The sample was applied on top of the column and eluted accordingly with solvents in increasing order of polarity; n-hexane:ethyl acetate in a ratio (100:0, 95:5, 90:10, 85:15, 80:20, 70:30, 50:50, 30:70 and 10: 90) to obtain fractions F1-F2, F3-F6, F7-F11, F12-F16, F17-F20, F21-F25, F26-F30, F31-F35, and F36-F40, respectively. This was followed by ethyl acetate: methanol in a ratio (90:10, 70:30, 50:50, 30:70, and 0:100) to obtain fractions F41-F45, F6-F50, F51-F55, F56-F60, and F61-F67, respectively. The eluates were divided into 100 mL portions. TLC analysis was used to closely monitor the elution, and similar fractions were combined to create nine fractions based on their TLC profiles. F1, F2, F3, F4, F5, F6, F7, F8, and F9 were used to code the fractions.

Acute toxicity determination of methanol extract of Acacia nilotica

The assessment of the lethal dose (LD_{50}) of the methanol stem bark crude extract of *A. nilotica* was determined using the Lorke's method.[17] In the first phase of the study, 9 albino mice were divided into 3 groups of 3 animals each. The extract was given to each group of mice in doses of 10, 100, and 1000 mg/kg, and then, the animals were placed under observation for 24 hours to monitor for mortality and behavioural changes. Afterward, the experiment moved on to the second phase, which involved employing fresh animals. Three (3) animals were placed into 3 groups, each with one animal. The animals were administered higher doses of 1600, 2900, and 5000 mg/kg of the extract and observed for 24 hours for behavioural changes and mortality. One animal served as a control in both phases of the experiment.

The LD_{50} was calculated with the formula:

$$LD_{50} = \sqrt{(D_0 \times D_{100})}$$

D_0: Highest dose that gave no mortality; D_{100}: Lowest dose that produced mortality

In vivo analysis

Whole blood containing *Trypanosoma congolense* was collected from an albino mice. The number of parasites was determined microscopically at x40 magnification using the "Rapid Matching" method.[18] A suspension of trypanosome was prepared in normal saline. The concentration was adjusted to 1 x 10^6 organisms per 0.1 mL. Fifty-eight (58) albino mice bred in the Animal House of the NITR, Vom, Plateau State were used for the *in vivo* assay**.** Fifty-five (55) albino mice were divided into 11 groups of 5 animals each. These animals were inoculated intraperitoneally with 0.1 mL of the inoculum containing the trypanosome cells. Groups 1-9 were treated orally with different fractions of the extract at 150 mg/kg. Treatment was repeated for 7 consecutive days after the establishment of parasitaemia. Group 10 was treated with the standard drug (diminazene aceturate), group 11 served as the negative control (infected, untreated group), while group 12 containing three (3) albino mice were not inoculated, serving as the positive control (uninfected, untreated).

Haematological parameters

Blood samples for haematological evaluation were placed into ethylenediaminetetraacetic acid (EDTA) sample bottles to prevent coagulation. Thereafter, the levels of white blood cell (WBC), red blood cell (RBC), and packed cell volume were monitored using the automatic haematology BC-2800-Vet analyzer.

Parasitaemia evaluation

Parasitaemia was monitored daily using the wet film method, and the number of parasites was determined microscopically at x40 magnification. For daily parasitaemia load estimation, the logarithm equivalent value (LEV) of parasitaemia was utilized.

Effect of extract fractions on survival of albino mice

The animals were monitored for mortality daily throughout the experiment, and the percentage of survival for each group was recorded.

Statistical analysis

Statistical Package for Social Sciences (SPSS) version 24 was used in analyzing the data obtained from the experiments. Data were analyzed using one-way Analysis of Variance (ANOVA). Means were separated using Duncan's post hoc test at $P \leq 0.05$.

Results and Discussion

Percentage yield and phytochemical screening of extracts of the stem bark of Acacia nilotica

The powdered stem bark of *A. nilotica* (1 kg) extracted with 98% ethanol gave a percentage yield of 21.21% of the extract. The percentage yield of column fractions produced from 30 g of the crude extract is presented in Table 1. In *A. nilotica*, phytochemical screening revealed the presence of anthraquinones, tannins, glycosides, cardiac glycosides, terpenes, alkaloids, and saponins, among other phytochemical constituents (Table 2). Earlier reports have identified the presence of cardiac glycosides, tannins, and saponins in plants with trypanocidal activities,[19,20] and these phytochemicals may be responsible for the observed antitrypanosomal activity in this study.

Acute toxicity of methanol extract of Acacia nilotica

Oral administration of the methanol stem bark extract of *A. nilotica* at the highest dose of 5000 mg/kg did not induce any mortality in the experimental animals as indicated in Table 3.

As a result of the findings, oral administration of *A. nilotica* crude extract at a dose of 5000 mg/kg may be considered safe in mice. However, restlessness and loss of appetite were observed immediately after administration of the extracts, which lasted for 2 hours. The results of this study agree with an earlier report,[21] which recorded no deaths or adverse effects after administering daily oral dose (3000 mg/kg) of the crude methanol extract of *A. nilotica* to rats for 14 days. However, another study,[22] reported 50% mortality after intraperitoneal administration of 2000 mg/kg of the extract to the experimental animals for 14 days. Toxicity from *A. nilotica* appears to be associated with long-term exposure and a greater dose, as well as the method of administration.

Effects of Acacia nilotica extract fractions on parasitaemia

The results (Figures 1 and 3) of the *in vivo* studies revealed that parasitaemia was established in all the infected groups after the first-week post-inoculation. There was a significant difference ($p \leq 0.05$) in the logarithm equivalent value (LEV) of parasitaemia in the group of animals treated with fractions F2 and F9 compared to fractions F1, F3, F4, F5, F6, F7, and F8. The animals treated with fractions F2 and F9 suffered an acute crisis and were the least active fractions, with parasitaemia lasting only a few weeks.[23] Meanwhile, the animals treated with fractions F1, F3, F4, F5, F6, F7, and F8 showed a chronic pattern of the disease profile lasting into several weeks, characterized by low frequency and intensity of parasitaemia.[23] The parasitaemic profiles of animals treated with fractions F4 and F5 (Figure 2) were the most active fractions, lasting for 21 weeks post-inoculation and persisting throughout the experiment. The observations suggest a potential trypanocidal activity of fractions F4 and F5 used in the treatment of the animals in these groups.

Effects of Acacia nilotica extract fractions on white blood cells

Generally, the WBC count increased across the groups' post-inoculation as demonstrated in Figure 4. This may be due to the animals' defence mechanism against the infection. However, with the disease progression, there was an observed low WBC count. This is in agreement with the observations made by other authors.[24,25] Leucopoenia (low WBC count) is associated with trypanosomiasis infection which arises from bone marrow granulocyte hypoplasia as a result of significant depression of precursor cells.[23] Other reasons for this observation have been attributed to massive peripheral utilization, phagocytosis in the bone marrow and other organs such as the liver and spleen.[24,25] However, there was a significant increase in WBC count (leucocytosis) in the animals treated with fractions F1, F3, F4, F5, and F7 in the 9th-week post-inoculation (Figure 4). This may have resulted from the effect of the phytochemicals in these fractions in mobilizing leucocytes from the bone marrow of the animals in these groups. The animals treated with fractions F4 and F5 survived throughout the period of the study (Figure 4). There was no significant increase in WBC count in the uninfected, untreated (positive) control group.

Effect of Acacia nilotica extract fractions on PCV and RBC

The results (Figures 5 and 6) of the PCV and RBC indicated a reduction across the groups as the infection was established. These observations suggest anaemia,[26,27] which is a well-recognized and inevitable consequence of an infection with pathogenic trypanosomes, including *Trypanosoma congolense*.[28,29] Anaemia in trypanosomiasis usually sets in during the first wave of parasitaemia.[23,30] The low PCV observed may be as a result of acute haemolysis due to growing infection. Previous studies have shown that infection with trypanosomes resulted in increased susceptibility of the RBC membrane to oxidative damage probably as a result of depletion of reduced glutathione on the surface of the red blood cell.[31-33] This study reveals that the baseline values were within an acceptable range (Figures 5 and 6). However, upon the development of parasitaemia as evident in their profiles (Figure 5-6), there was a gradual decline in PCV and RBC estimations. Groups treated with fractions (F1, F2, F3, F4, F5, F7, F8, F9) and the standard drug, had an increase in the PCV post-treatment compared to the untreated group (Figure 6). This may be attributed to the ability of the phytochemicals in these fractions to suppress anaemia in the experimental animals. The group treated with fraction F6 did not show any increase in PCV post-treatment.

Table 1: Yield of column fractions of *Acacia nilotica* extract

Fraction	Yield (%)
F1	7.00
F2	0.70
F3	1.07
F4	21.67
F5	2.60
F6	23.67
F7	6.33
F8	8.07
F9	4.40
Total	**75.43**

Table 2: Phytochemical constituents of methanolic stem bark extract of *Acacia nilotica*

Phytochemicals	Observation
Saponins	+
Tannins	+
Cardiac glycosides	+
Anthraquinones	+
Alkaloids	+
Glycosides	+
Triterpenoids	-
Steroids	-
Terpenes	+
Flavonoids	-

+: Present; -: Absent

Table 3: Acute toxicity effect of methanolic stem bark extract of *Acacia nilotica*

Stage	Dosage (mg/kg)	No. of Animal	Observation
1st stage	10	3	No mortality
	100	3	No mortality
	1000	3	Restlessness, No mortality
2nd stage	1600	3	Restlessness, No mortality
	2900	3	Restlessness, No mortality
	5000	3	Restlessness, No mortality
Control	No extract administered	1	Normal, no mortality

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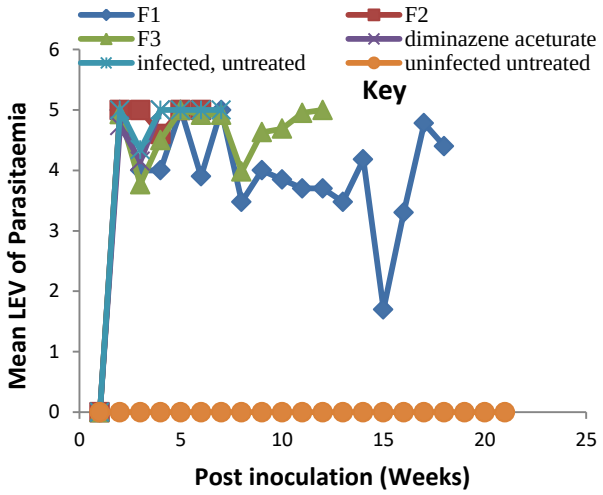

Figure 1: Parasitaemia profile of *Trypanosoma congolense* in mice after treated with Fractions 1, 2, and 3 of methanolic stem bark extract of *Acacia nilotica.*

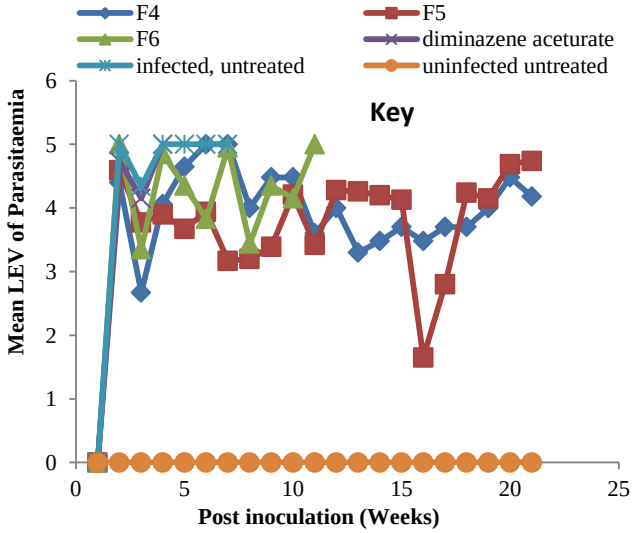

Figure 2: Parasitaemia profile of *Trypanosoma congolense* in mice after treatment with Fractions 4, 5, and 6 of methanolic stem bark extract of *Acacia nilotica.*

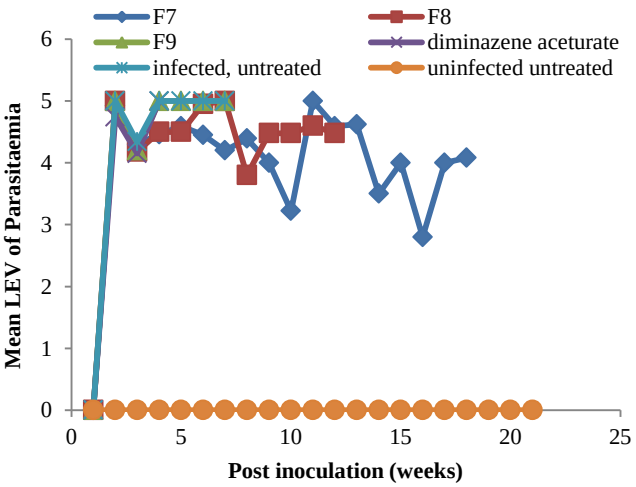

Figure 3: Parasitaemia profile of *Trypanosoma congolense* in mice after treatment with Fractions 7, 8, and 9 of methanolic stem bark extract of *Acacia nilotica.*

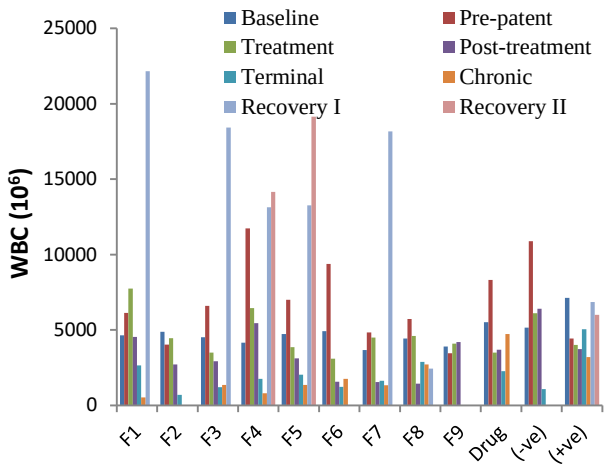

Figure 4: Changes in Total WBC in mice infected with *T. congolense* after Treatment with Different Fractions of *Acacia nilotica*

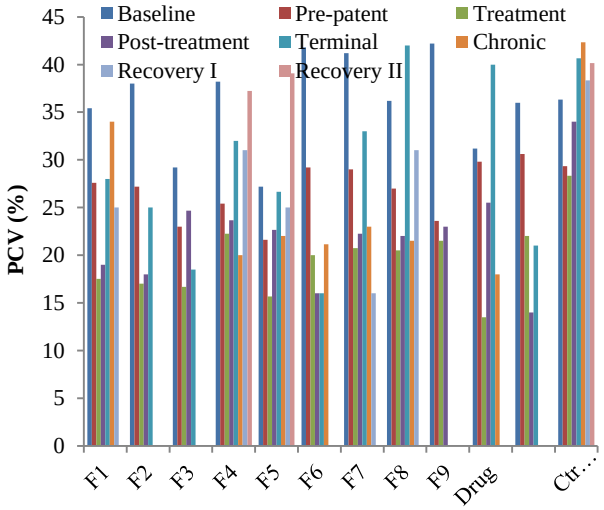

Figure 5: Changes in Packed Cell Volume in Mice Infected with *T. congolense* after Treatment with Different Fractions of *Acacia nilotica*

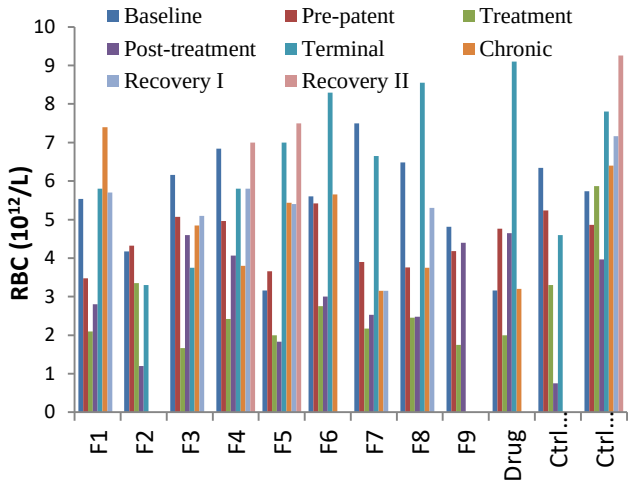

Figure 6: Changes in Red Blood Cell in Mice Infected with *T.congolense* After Treatment With different Fractions of *Acacia nilotica*

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Effects of Acacia nilotica extract fractions on the weight of experimental animals

The animals treated with fractions F1, F2, F4, F7, F8, and the infected untreated group (negative control) suffered weight loss after the establishment of the disease (Figure 7). Reductions in voluntary feed intake were common in parasitized animals.[34] The remarkable increase in body weight of the animals in Groups 4 and 5, perhaps suggests that phytochemicals in the fractions boosted the appetite of the animals and contributed to their recovery.

Effects of Acacia nilotica extract fractions on survival of experimental animals

As shown in Figure 8, treatment with fractions F4 and F5 resulted in the extension of the life of the animals by 20 and 40 %, respectively by 100 days compared to the negative control (infected, untreated group). The animals in Groups 1, 3, 6, 7, and 8 survived 80, 40, 27, 79, and 36 days, respectively compared to the negative control group. In terms of survival time, Groups 2 and 9 had no advantage over the negative control. A longer survival period than the negative control is one of the indices of the trypanocidal activity or other beneficial effects of an agent.

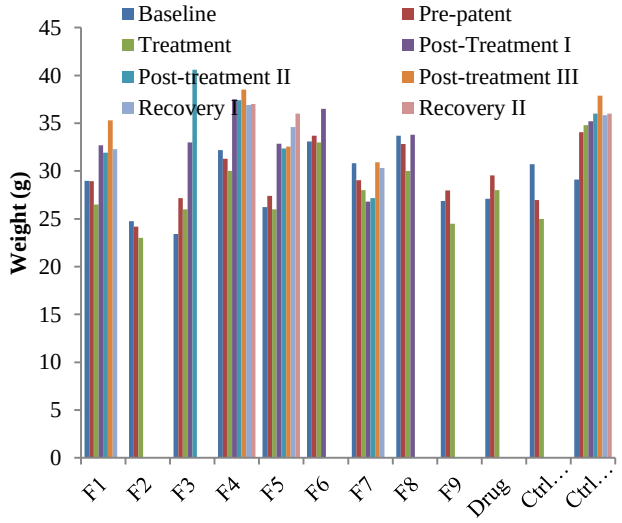

Figure 7: Changes in Body Weight of Mice Infected with Different Fractions of *Acacia nilotica*

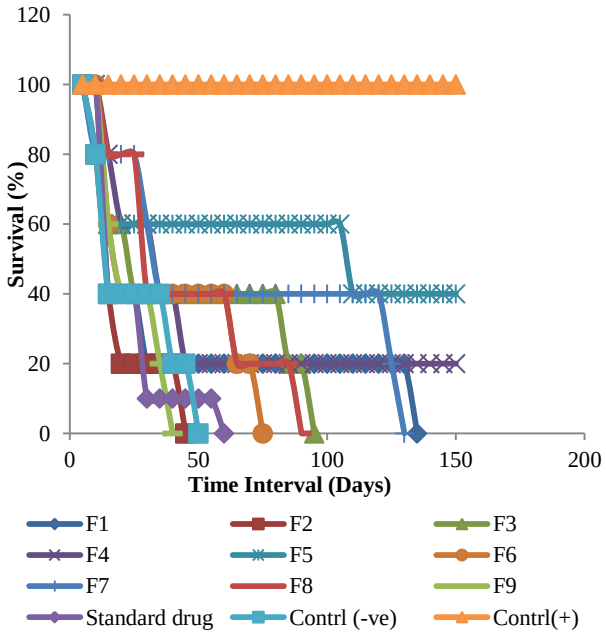

Figure 8: Percentage survival of albino mice infected with *Trypanosoma congolense* after treatment with Fractions F1- F9 of *Acacia nilotica* extract.

Conclusion

The present study has established that two fractions (F4 and F5) from the methanol stem bark extract of *A. nilotica* possess the ability to control anaemia, boost immunity, and prolong the life of infected mice beyond the standard drug, diminazene aceturate. These fractions offer prospects for lead compounds in the therapy of trypanosomiasis. This will contribute to the promotion of food security, rural development, improvement of human and animal health, as well as the facilitation of sustainable agricultural practices in Nigeria and on the continent.

Conflict of Interest

The authors declare no conflict of interest.

Author's Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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