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ARTICLE

Anti-trypanosomal Activity of Bufonidae (Toad) Venom Crude Extract on *Trypanosoma brucei brucei* in Swiss Mice

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ABSTRACT

Trypanosomiasis afflicts about 6 ~ 7 million people globally and to a large extent impedes livestock production in Africa. Naturally, trypanosomal parasites undergo genetic mutation and have developed resistance over a wide range of therapies. The utilization of animals and plants products has presented therapeutic potential for identifying novel anti-trypanosomal drugs. This study evaluated toad venom for anti-trypanosomal potency in-vivo in Swiss mice. Toads were collected from July to August 2019. The acute oral toxicity and biochemical characterization of the toad venom were determined. The experimental mice were administered various doses (130 mg/kg, 173 mg/kg and 217 mg/kg) of the toad venom crude extract and 0.75 mg/mL of Diamizan Plus standard drug for the treatment of trypanosomiasis, once daily for 3 days. The in-vivo anti-trypanosomal activity was evaluated by a curative test, after infecting the mice with *Trypanosoma brucei brucei*. The pre-patent period was 72 hours before treatment commenced. The overall results showed that trypanosomal load was highest in the control group while the group treated with Diamizan drug had the least trypanosomal load. As such, the mean trypanosomal load in relation to treatments showed a very high significant difference ($P<0.05$). Also, the mean trypanosomal load in Swiss mice in relation to the highest dosage of toad venom versus Diamizan drug showed a very high significant difference ($P<0.05$). The mean change in relation to the haematological parameters across treatments groups varied significantly ($P<0.05$) with the exception of Hb which showed no significant difference ($P>0.05$) across treatment groups. The over 50% reduction in the trypanosomal load in the 130 mg/kg group in comparison with the control group brings to bare the anti-trypanosomal potency of the toad venom. The anti-trypanosomal activity demonstrated by the toad venom has provided basis for development of new therapeutic agents from different toad species. The study recommends further studies (both in-vivo and in-vitro) followed by the characterization of the active compounds present in the toad venom responsible for the anti-tyrpanosomal activity observed alongside the management and conservation of these species.

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1. Introduction

Trypanosomiasis is a disease caused by microscopic parasites belonging to the species *Trypanosoma brucei* which invades the blood plasma and various body tissues, lymph and cerebrospinal fluid. In sub-Saharan Africa, tsetse fly serves as the insect vector responsible for the transmission of this parasite. They appear to be harmful to only mammals including man though they also infect many animals [1]. Human African trypanosomiasis (HAT), or sleeping sickness, remains a life-threatening disease that mostly affects poor rural populations. Two subspecies of *Trypanosoma brucei* cause disease: *T. b. gambiense* in West and Central Africa, and *T. b. rhodesiense* in East Africa. HAT transmission requires the interaction of humans, tsetse flies and parasite reservoirs (humans, and domestic and wild animals) [2].

African animal trypanosomiasis (AAT) is a parasitic disease that causes very significant economic losses in livestock, from anemia, loss of condition and effects on reproduction whereas cattle production, accounts for a greater percentage of these losses [3]. Trypanosomes infect a large number of wild fauna (antelope species, warthogs, elephants, hippopotamus, lions, hyenas, jackals, caracals, and wild ruminants etc.) [1,4,5] and domestic ungulate species (cattle, sheep, goats, horses, pigs, camels and dogs) [6]. Infections in wildlife are influenced by species and habitat [7]. Trypanosome species commonly found in wildlife species include *T. vivax, T. brucei brucei, T. congolense* and *T. evansi* [5].

The human and animal trypanosomiasis continue to present significant global health burden in human and animal (domesticated and wildlife communities) thus far, chemotherapy and chemoprophylaxis represent the mainstay for its control [8,9]. Worryingly, the inherent potential of trypanosomal parasite to undergo genetic mutation has led to its ability to successfully develop resistance over a wide range of therapies.

Uptake of natural products from animals and plants presents explorable therapeutic potentials and earlier studies of toad venoms from different species and their chemical basis have demonstrated new perspectives for their pharmaceutical use including the development of new therapeutic agents [10]. Over the years yet, a novel licensed compound is unlikely to be available [1]; hence the rationale for this study.

2. Materials and Methods

2.1 Toad Collection

The toads used for this study were collected from the month of July to August 2019 in a well-ventilated container between 07:00 a.m. and 10:00 a.m. hours daily in the rice fields at Gandu, Lafia LGA, Nasarawa State and conveyed to the Laboratory unit of the Department of Zoology, Faculty of Science, Federal University of Lafia, Nasarawa State for extraction.

2.2 Ethical Permit

Ethical permit with the Project Identification Code (PIC) – FUL/FS/ZLY/2019/002 was obtained for the research from the Ethical Committee of the Department of Zoology, Faculty of Science, Federal University of Lafia, Nasarawa State.

2.3 Extraction of Crude Venom Extract from Toad

The extraction process of bufonidae was achieved by massaging and pressing the parotoids macro-glandules and the secretion was collected using a petri dish [11]. The collected secretion was lyophilized and stored in a freezer (–20 °C) at the Federal University of Lafia, Lafia, Nasarawa State.

2.4 Characterization of Bioactive Compounds

Determination of the quantitative and qualitative chemical components of the venom was achieved by Mass Spectrometry using GC-MS techniques at the Spectral Laboratory and Services, Tudun, Wada Kaduna South, Kaduna, Nigeria. The Shimadzu Fourier transform Infrared Spectrophotometer- FTIR 8400 S was used for the determination of the functional units present in the venom. Gas Chromatography Analysis of toad venom was done using gas chromatography (Perkin-Elmer 8500). The Scanning Electron Microscope energy dispersive X-ray spectroscopy (SEM-EDS) Phenom Prox, manufactured by phenom World Eindhoven (Netherlands) was used to carry out the morphology analysis (that is; analysis of the chemical elements present, in the toad venom).

2.5 Experimental Animals

Thirty Laboratory Swiss mice of the same age, weighing between 12 g to 45 g were purchased from National Veterinary Research Institute (NVRI) Vom, Jos, Plateau State. All animals were fed with formulated feeds and water was administered *ad libitum*. The caring and experimental use of the mice was in accordance with the National Institutes of Health Guidelines for Care of Laboratory Animals. The animals were acclimatized for 7 days prior to their randomization into the various experimental groups.

2.6 Coding and Weighing of Animals

Different codes were given to every animal that was

employed in this study using a permanent marker to create marks of identification on a particular part of the body that is head (HD), Bark (BK), Tail (TL), Right side (RS), Left Side (LS), Right ear (RE), Left Ear (LE) etcetera. The weight of each animal was taken using top animal precision balance.

2.7 Toxicity Study

The median oral lethal dose of the toad venom was determined in mice using Lorke's method 1983 [12]. This method has two phases.

Phase 1 requires 9 animals. The nine animals were divided into three groups of three animals each. Each animal was administered with different doses (10, 100 and 1000 mg/kg) of the toad venom and animals, placed under observation for 24 hours to monitor their behavior as well as mortality.

Phase 2 involves the use of 3 animals, distributed into three groups of one animal each. The mice were intoxicated with different doses (250 mg/kg, 500 mg/kg and 750 mg/kg) of the toad venom and observed for 24 hours for behavior and mortality as well.

Then the LD_{50} is calculated by 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.

2.8 Parasite Species and Standard Inoculation

Parasite was obtained from National Veterinary Research Institute (NVRI) Vom, Plateau State, Nigeria. Parasites were maintained through serial blood passage in mice wherein the mice previously infected with Nigerian strain of *Trypanosoma brucei brucei* and with high parasitemia level served as the donor. Donor mouse was anaesthetized with chloroform and blood (1 mL) was extracted through cardiac puncture using 1ml needle and syringe and made up to 20 mL with normal saline. Blood samples were taken such that 0.2 mL injected subcutaneously into the experimental animals [13].

2.9 Determination of Parasites

Blood samples were collected by bleeding the tail vein of the infected mice. Thin blood smears were made on clean glass microscope slides. The films were dried in air and then fixed in methanol and stained with 10% Giemsa solution [14]. The stained film was then observed under the binocular compound microscope and viewed for parasitemia. The percentage parasitemia were determined by counting the number of parasites on four or five fields.

2.10 Preparation of Treatment Solution

The treatment doses of the venom were calculated based on the lethal dose (LD50), which is 30% of the LD_{50} [15]. Therefore, the study calculated 15%, 20% and 25% of the lethal dose as the curative doses, which gave 130 mg/kg, 173 mg/kg and 217 mg/kg respectively.

2.11 Curative Study

The animals were acclimatized 7 days prior to their randomization into various groups infected with *T. brucei-brucei* and then divided into five groups of five mice per group. The presence of parasites was confirmed in the mice 72 hours after inoculation and was taken as day 0, thereafter, treatment commenced once daily for three days. Group 1, 2, and 3 were orally treated with 130 mg/kg, 173 mg/kg, and 217 mg/kg dose of crude toad venom extract respectively while group 4 received 0.75 mg/mL of Diamizan Plus (treatment drug for trypanosomiasis) intradermally, whereas group 5 (infected and untreated) was regarded as the control. The parasitemia of experimental mice were established before treatment was administered [16].

2.12 Hematological Parameters

Using the methods described by Cheesbrough [17], the Packed Cell Volume (PCV), haemoglobin (Hb) and erythrocyte (RBC) counts were determined. These parameters were determined for each mouse before infection and after treatment. Blood samples were collected from the tail of each mouse with a heparinized capillary tube with one end sealed with plasticine.

2.13 Statistical Analysis

Data obtained were analyzed using R Console software (Version 3.2.2). Shapiro-Wilk normality test was carried out to determine normality in the distribution of the data. Thereafter, Kruskal-Wallis rank sum test was used to compare the mean of pooled trypanosomal load in Swiss mice in relation to toad venom treatments and standard drug. One-way analysis of variance was used to compare daily changes in the mean load of trypanosomes in Swiss mice in relation to toad venom treatments and diamizan plus standard drug. Mean change in body weight as well as in haematological parameters was compared using Kruskal-Wallis rank sum test. Level of significance was set at $P < 0.05$. Wilcoxon rank sum test with Bonferroni correction was used as post-hoc test for multiple pairwise comparisons of means where there was a significant difference between the treatments in pooled trypanosomal

load, change in weight and as well as haematological parameters. While Turkey's Honest Significant Difference (Turkey HSD) post-hoc test was used for multiple comparisons of means in daily trypanosomal load changes between the treatments.

3. Results

3.1 Determination of the Bioactive Compounds in the Toad Venom Crude Extract

The biocharacterization analysis of the toad venom crude extract involving GC-MS, revealed that the most dominant chemical compound present was 9,12-Octadecadienoic acid (Z,Z) at peak 3 as depicted in Figure 1 which had a rate of 38.615, at a proportion of 49.76% followed by n-Hexadecanoic acid (29.04%) then Octadecanoic acid (7.03%), Squalene (5.48%), 1-Hexadecyne (3.87%), Butyl 9,12-octadecadienoate (2.95%) while Hexadecanedioic acid was the least compound (1.87%). These findings are properly presented in Table 1.

Furthermore, as depicted in Figure 2, the characterization procedures using FTIR showed that the toad venom crude extract contains 18 active functional groups, in which Nitrogen-Hydrogen Oxygen stretch of 93.18% was the most active functional group. However, the toad

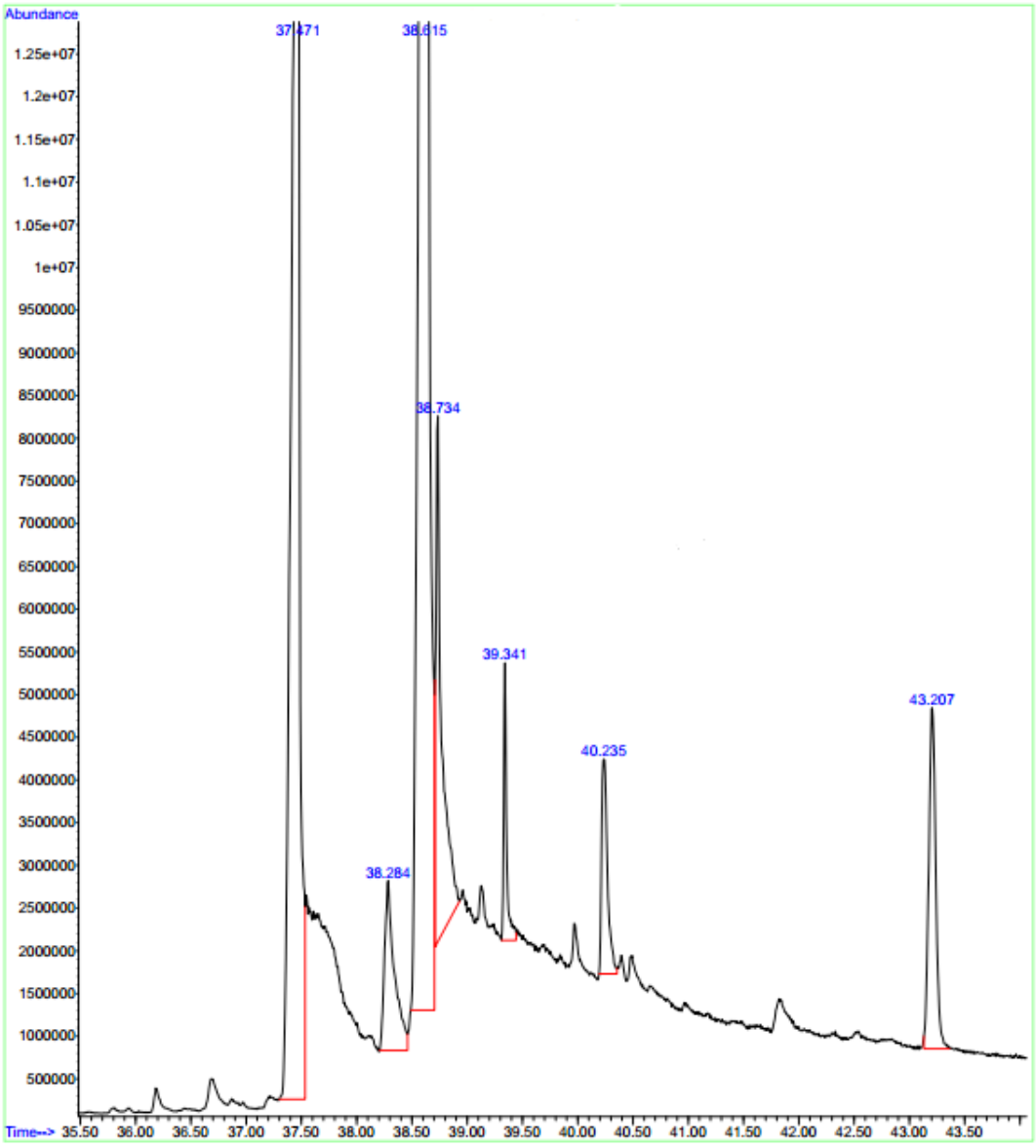

Figure 1. Mass spectroscopy of toad venom crude extract showing the abundance of the chemical compounds at different peak

venom crude extract was predominantly characterized by the presence of the amino group made up of 8 functional groups, followed by the nitro-group having 6 functional groups while the non- protein group was made up of only 4 functional group as shown in Table 2.

The SEM-EDS method uncovered the dynamics of the 11 chemical elements contained in the toad venom crude extract. Carbon was top of the list with atomic concentration and weight of 61.76 mol/dm^3 and 41.04 g/mol respectively, followed by oxygen (25.04 mol/dm^3 and 22.29 g/mol) while iodine and aluminium had the least atomic concentration and weight of 0.24 mol/dm^3 and 0.90 g/mol respectively. The details of these results were properly captured in Table 3.

Table 1. The chemical components in the crude extract of the toad venom using the GC-MS

Peak	Rate	Library/ID of Compounds	Percentage of Total Compounds (%)
1	37.471	n-Hexadecanoic acid n-Hexadecanoic acid n-Hexadecanoic acid	29.04
2	38.284	1-Hexadecyne 5-Hexadecyne 5-Eicosyne	3.87
3	38.615	9,12-Octadecadienoic acid (Z,Z)- 9,12-Octadecadienoic acid (Z,Z)-9,17-Octadecadienal, (Z)	49.76
4	38.734	Octadecanoic acid Octadecanoic acid Pentadecanoic acid	7.03
5	39.341	Hexadecanedioic acid Dodecanoyl chloride 4-Cyclopropylme thylbenzonitrile	1.87
6	40.235	Butyl 9,12-octadecadienoate 6-Dodecane 7,10-Hexadecadienoic acid, methyl ester	2.95
7	43.207	Squalene Squalene Supraene	5.8

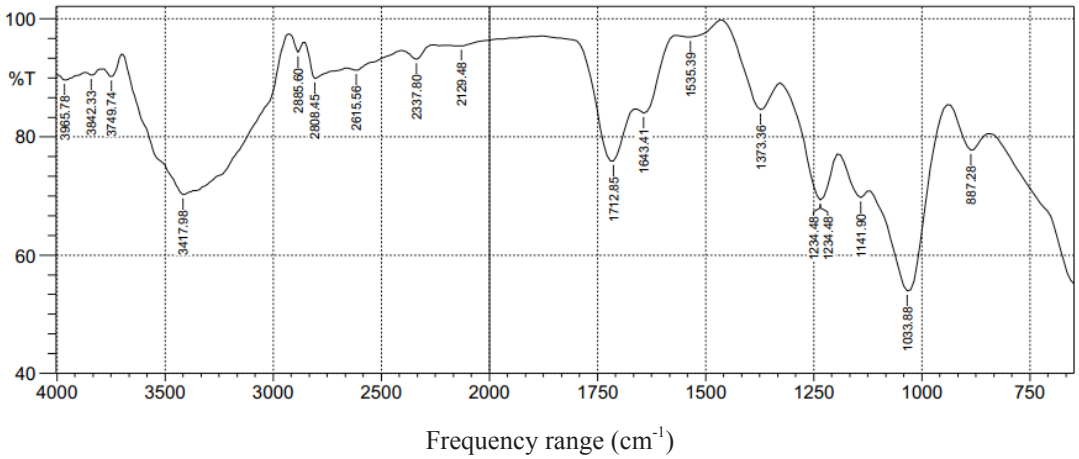

Figure 2. The functional groups present in the toad venom crude extract at different peaks

Table 2. The different protein functional groups present in the toad venom crude extract at different peaks using FTIR

Amino-group	Nitro-group	Non-protein group
Triethylamine	Nitro group with broad stretching of NO_2	Aromatic group (Peak 4)
Diethylamide	M-nitrotoluene	Aromatic group (Peak 14)
Aniline with a concentration of 84.653%	Nitrile group	Carbon Hydrogen stretch with strong intensity of 89.60%
Secondary Amines	Nitrogen Hydrogen Oxygen stretch of 93.18%	Olefins with weak band of 53.975%
n-butylamine & Benzamide with intensity (concentration) of 90.201%	Nitro Methane with concentration of 91.31%	
Nitrogen Hydrogen strong bond (amide) with concentration of 90.462	Nitrile group with Concentration of 89.86 %	
N-H bending		

Table 3. Chemical elements present in the toad venom crude extract using the SEM-EDS

Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
6	C	Carbon	61.76	41.26
8	O	Oxygen	25.04	22.29
25	Mn	Manganese	4.12	12.59
82	Pb	Lead	0.94	10.84
24	Cr	Chromium	1.43	4.13
7	N	Nitrogen	2.67	2.08
53	I	Iodine	0.24	1.68
11	Na	Sodium	1.12	1.44
16	S	Sulfur	0.80	1.44
9	F	Fluorine	1.28	1.36
13	Al	Aluminium	0.60	0.90

3.2 Oral Toxicity (LD_{50}) of the Toad Venom Crude Extract on the Laboratory Swiss Mice

The oral toxicity study resulted in mortality of lab animals after one hour of administration of 1000 mg/kg dose of toad venom crude extract. Prior to their mortality, symptoms like hyperactivity, convulsion and constant stooling were observed before finally resulting in death. However, on the administration of 750 mg/kg, hyperactivity, diarrhea and sedation were observed within one hour, but there was no mortality recorded. Following the Lorke's method computation, the oral toxicity of the toad crude venom extract was established as 866 mg/kg.

3.3 Trypanosomal Load at Day Zero

Pre-patency was observed after 72 hours that was noted as day zero prior to the commencement of first treatment. Prior to treatment, the mean of trypanosomal load in Swiss mice at day zero in relation to treatments with toad venom and Diamizan plus drug showed no significant difference (F_{20} = 0.9545, Adjusted R^2 = –0.007644, P = 0.4537, Figure 3). Though trypanosomal load was found to be highest in mice group treated with 173 mg/kg of the toad venom (group 2) followed by those designated in group 1 (130 mg/kg), then individuals for group 4 treatment (0.75 mg/mL of Diamizan plus), whereas parasitemia was very low in those set aside for group 3 (217 mg/kg) trial.

3.4 Trypanosomal Load in Swiss Mice in Relation to Treatments with Toad Venom and Diamizan Plus Drug

A very high significant variation (Kruskal-Wallis χ^2 = 42.189, df = 4, P < 0.0001, Figure 4) was observed in the mean trypanosomal load in Swiss mice in relation to treatments with toad venom alongside Diamizan plus drug. Thus, the overall trypanosomal load was highest in group 2 treated with 173 mg/kg of toad venom crude extract followed by group 3 treated with 217 mg/kg then group 1 treated with 130 mg/kg while it was least in group 4 treated with Diamizan plus. Table 4 shows multiple comparisons between means of trypanosomal load in Swiss mice in which the highest dosage of toad venom versus

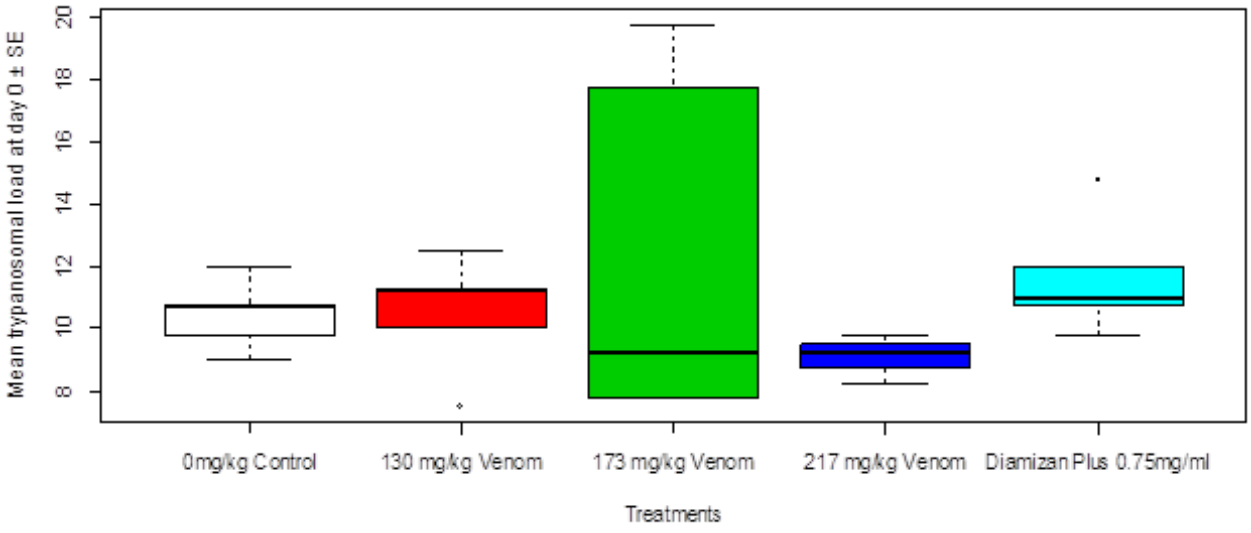

Figure 3. Mean trypanosomal load in Swiss mice in relation to treatments with toad venom and Diamizan plus drug at day zero

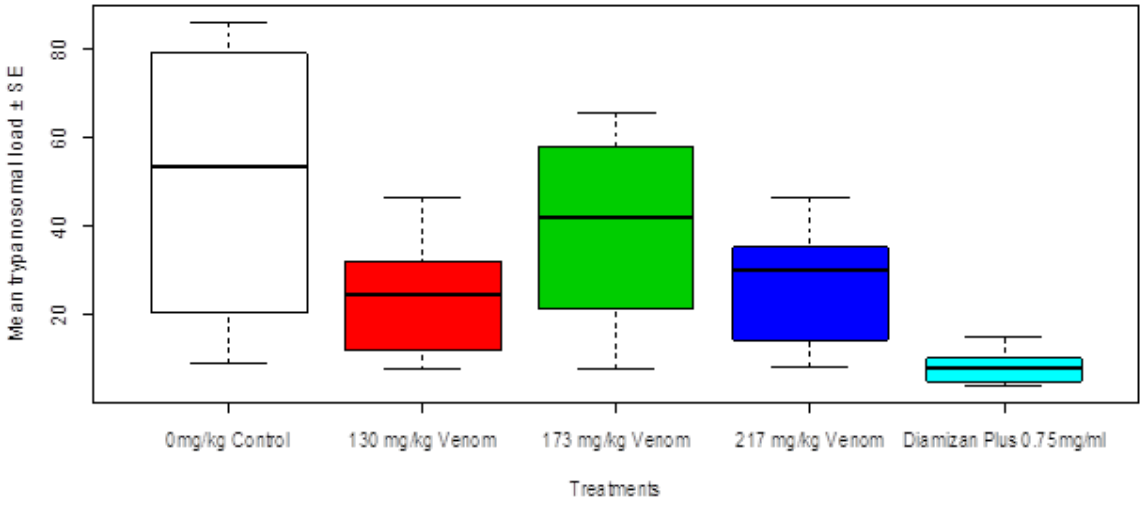

Figure 4. Mean Trypanosomal Load in Swiss mice in Relation to Treatment with Toad Venom and Diamizan Plus Drug

Table 4. Pairwise Comparisons between Means of Pooled Trypanosomal Load in Swiss mice in Relation to Treatments with Toad Venom and Diamizan Plus Drug using Wilcoxon Rank Sum Test

	Trypanosomal Load and Treatments			
	0 mg/kg	**130 mg/kg**	**173 mg/kg**	**217 mg/kg**
130 mg/kg	0.038	0	0	0
173 mg/kg	0.156	0.038	0	0
217 mg/kg	0.038	0.626	0.093	0
Diamizan Plus 0.75 mg/mL	1.0e-05	1.0e-05	1.0e-05	2.7e-05

P Value Adjustment Method: BH

Diamizan plus drug showed a very high significant difference ($P < 0.0001$). Also, trypanosomal load in Swiss mice between control group and the highest dosage of toad venom treatment was significant ($P = 0.038$).

3.5 Trypanosomal Load at Day One

At the 24th hour, the mean trypanosomal load in Swiss mice showed a very high significant difference ($F_{20} = 19.43$, Adjusted $R^2 = 0.7544$, $P = 0.000001157$, Figure 5) in relation to treatments with toad venom and Diamizan plus. Therefore, the trypanosomal load at day one (after 24 hours of treatment) was highest in group 2, followed by group 1, then group 3, whereas it was least in group 4. Furthermore, the comparison of trypanosomal load in Swiss mice in relation to the highest dosage of toad venom versus Diamizan plus treatments in day 1 showed a very high significant difference ($P < 0.0001$) as shown Table 5.

3.6 Trypanosomal Load at Day Two

At 48 hours of treatment, the trypanosomal load was highest in group 2 followed by group 3, then group 1, whereas it was least in group 4. Thus, there was a very significant difference ($F_{20} = 107$, Adjusted $R^2 = 0.9464$, $P < 0.00001$, Figure 6) in the mean of trypanosomal load in Swiss mice at 48 hours of treatment with varying concentrations of toad venom and Diamizan plus drug. Trypanosomal load in Swiss mice in relation to multiple comparison of means between treatments at day 2 showed a significant difference ($P < 0.0001$) as shown in the Table 6.

3.7 Trypanosomal Load at Day Three

Group 2 at 72 hours of treatment had the highest trypanosomal load followed by group 1, then group 3 whereas it was least in group 4. Hence, mean trypanosomal load in Swiss mice in relation to treatments with toad venom and Diamizan plus drug at day three of treatment had a very significant difference ($F_{20} = 145.2$, Adjusted $R^2 = 0.9601$, $P < 0.00001$, Figure 7). Trypanosomal load in Swiss mice in relation to multiple comparison of means between treatments at day 3 showed a high significant difference ($P < 0.0001$) with the exception of between 130 mg/kg and 217 mg/kg as shown in the Table 7.

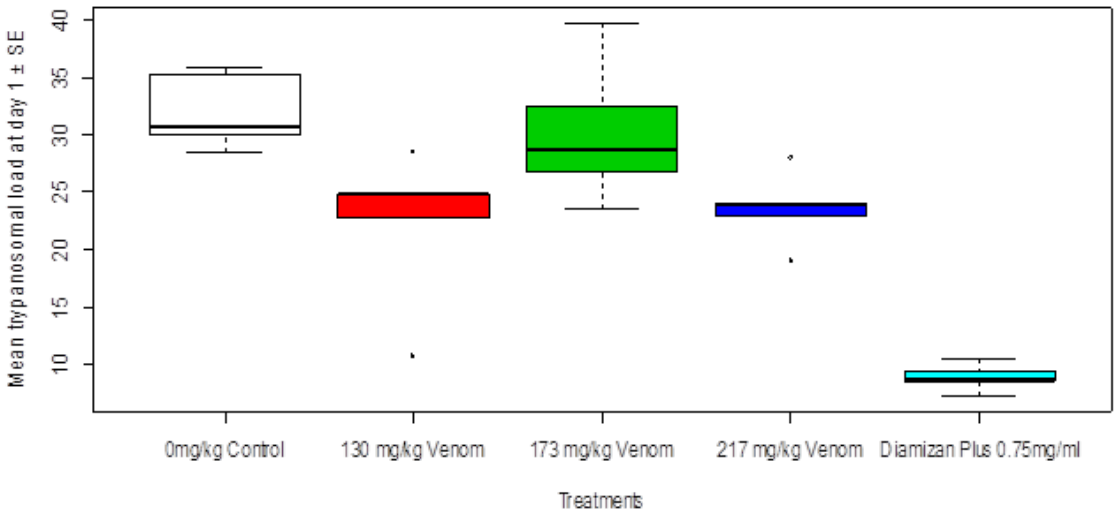

Figure 5. Mean trypanosomal load in Swiss mice in relation to treatments with toad venom and Diamizan plus treatments at day one

Table 5. Turkey Multiple Comparisons of Means of Trypanosomal Load at 95% Family-Wise Confidence Level –for Day One

Treatment	Diff	Lower	Upper	P – Adjusted
130 mg/kg – 0 mg/kg	–9.75	–18.5138779	–0.9861221	0.0246790
173 mg/kg – 0 mg/kg	–1.80	–10.5638779	6.9638779	0.9710613
217 mg/kg – 0 mg/kg	–8.45	–17.2138779	0.3138779	0.0621310
Diamizan Plus 0.75 mg/mL – 0 mg/kg	–23.15	–31.9138779	–14.3861221	0.0000013
173 mg/kg – 130 mg/kg	7.95	–0.8138779	16.7138779	0.870134
217 mg/kg – 130 mg/kg	1.30	–7.4638779	10.0638779	0.9913229
Diamizane Plus 0.75 mg/mL – 130 mg/kg	–13.40	–22.1638779	–4.6361221	0.0015377
217 mg/kg – 173 mg/kg	–6.65	–15.4138779	2.1138779	0.1954923
Diamizan Plus 0.75 mg/mL – 173 mg/kg	–21.35	–30.1138779	–12.5861221	0.0000043
Diamizan Plus 0.75 mg – 217 mg/kg	–14.70	–23.4638779	–5.9361221	0.0005646

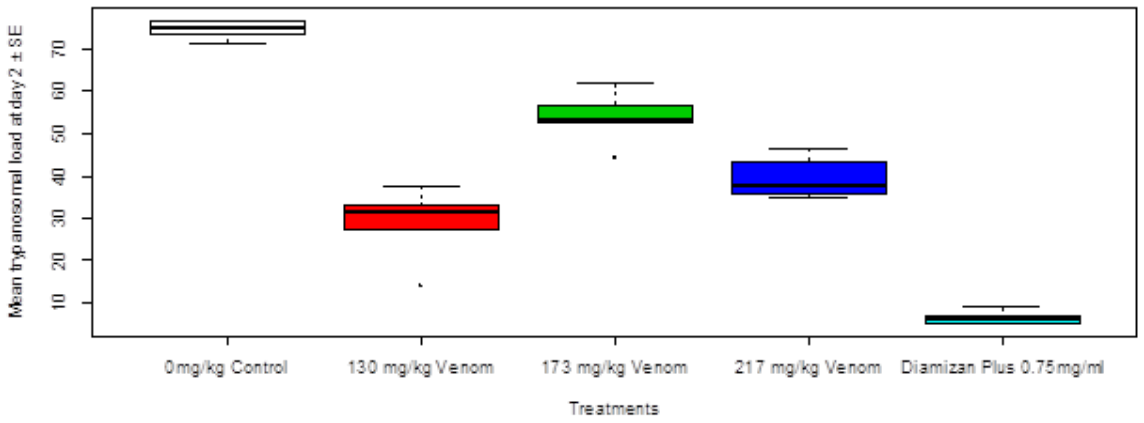

Figure 6. Mean trypanosomal load in Swiss mice in relation to treatments with toad venom and Diamizan plus treatments at day 2

Table 6. Turkey Multiple Comparisons of Means of Trypanosomal Load at 95% Family-Wise Confidence Level for Day Two

Treatment	Diff	Lower	Upper	P – Adjusted
130 mg/kg – 0 mg/kg	–46.02	–56.5505483	–35.489452	0.0000000
173 mg/kg – 0 mg/kg	–20.97	–31.5005483	–10.439452	0.0000705
217 mg/kg – 0 mg/kg	–35.07	–45.6005483	–24.539452	0.0000000
Diamizan Plus 0.75 mg/mL – 0 mg/kg	–68.32	–78.8505483	–57.789452	0.0000000
173 mg/kg – 130 mg/kg	25.05	14.5194517	35.580548	0.0000061
217 mg/kg – 130 mg/kg	10.95	0.4194517	21.480548	0.290893
Diamizane Plus 0.75 mg/mL – 130 mg/kg	–22.30	–32.8305483	–11.769452	0.0000313
217 mg/kg – 173 mg/kg	–14.10	–24.6305483	–3.569452	0.0055509
Diamizan Plus 0.75 mg/mL – 173 mg/kg	–47.35	–57.8805483	–36.819452	0.0000000
Diamizan Plus 0.7 5mg – 217 mg/kg	–33.25	–43.7805483	–22.719452	0.0000001

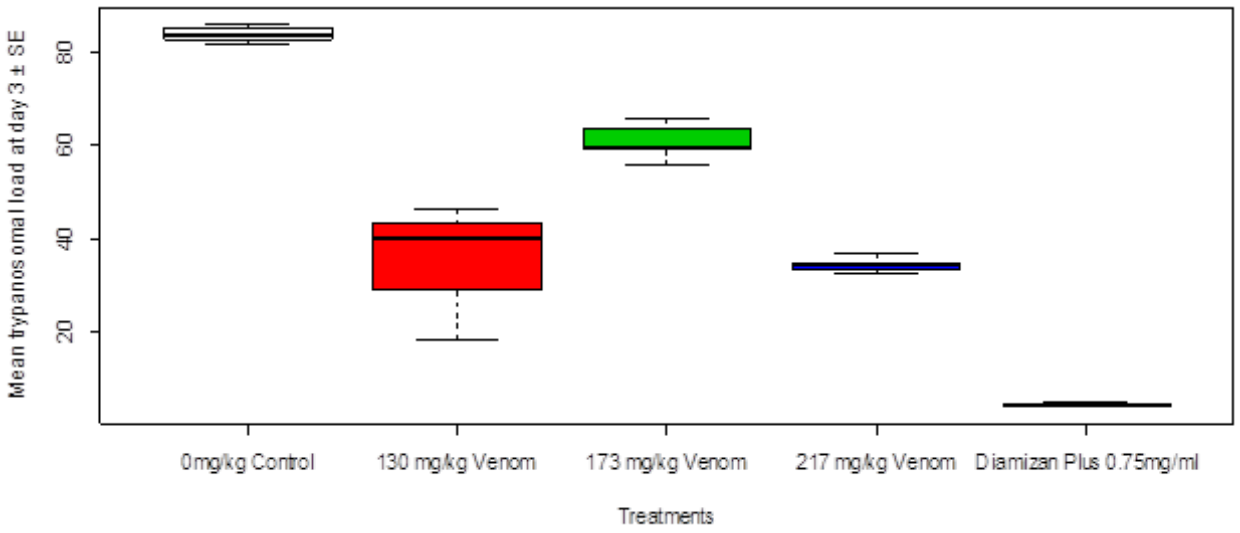

Figure 7. Mean trypanosomal load in Swiss mice in relation to treatments with toad venom and Diamizan plus treatments at day 3

Table 7. Turkey Multiple Comparisons of Means of Trypanosomal Load at Day 3

Treatment	Diff	Lower	Upper	P – Adjusted
130 mg/kg – 0 mg/kg	–48.45	–59.01312	–37.886875	0.0000000
173 mg/kg – 0 mg/kg	–23.00	–33.56312	–12.436875	0.0000214
217 mg/kg – 0 mg/kg	–49.50	–60.06312	–38,936875	0.0000000
Diamizan Plus 0.75 mg/mL – 0 mg/kg	–79.60	–90.16312	–69.036875	0.0000000
173 mg/kg – 130 mg/kg	25.45	14.88688	36.013125	0.0000051
217 mg/kg – 130 mg/kg	–1.05	–11.61312	9.513125	0.9981429
Diamizan Plus 0.75 mg/mL – 130 mg/kg	–31.15	–41.71312	–20.586875	0.0000002
217 mg/kg – 173 mg/kg	26.50	–37.06312	–15.936875	0.0000028
Diamizan Plus 0.75 mg/mL – 173 mg/kg	–56.60	–67.16312	–46.036875	0.0000000
Diamizan Plus 0.75 mg – 217 mg/kg	–30.10	–40.66312	–19.536875	0.0000004

3.8 Change in Body Weight of Swiss Mice after Treatment with Toad Venom and Diamizan Plus

After treatment with the toad venom crude extract of different dosages and Diamizan plus drug, change in body weight was highest in Group 4 treated with 0.75 mg/mL of Diamizan plus, followed by Group 3 treated with 217 mg/kg of toad venom, then Group 1 treated with 130 mg/kg of toad venom whereas it was least in Group 2 treated with 173 mg/kg of the toad venom. Thus, the mean change in body weight of Swiss mice after treatment in relation to toad venom as well as Diamizan plus drug respectively showed a high significant difference (Kruskal-Wallis χ^2= 15.779, df = 4, P = 0.00333, Figure 8). The multiple comparisons of means of change in body weight between 173 mg/kg treatment and Diamizan plus drug showed a significant difference (P< 0.05, Table 8).

3.9 Change in Haematological Parameters of Swiss Mice after Three Days of Parasitemia Treatment

Hemoglobin (Hb)

After treatment with the toad venom crude extract of different dosages and Diamizan plus drug, the result showed that there was no change in the blood concentration of hemoglobin of the Swiss mice in the different groups. However, the mean change in Hb level in Swiss mice after three days of trypanosomal treatment between dosages of toad venom and Diamizan plus drug showed no significant difference (Kruskal-Wallis χ^2 = 8.141, df = 4, P = 0.08655, Figure 9).

RBC

After treatment with the toad venom crude extract of different dosages and Diamizan plus drug, the result showed a change in the RBC level of the Swiss mice in the different treatment groups where group 4 had the highest level followed by group 3 then group 2 while group 1 had the least. Thus, the mean change in RBC level in Swiss mice after three days of trypanosomal treatment between dosages of toad venom and Diamizan plus drug showed a high significant difference (Kruskal-wallis χ^2 = 14.326, df = 4, P = 0.006324, Figure 10). Table 9 shows that multiple comparisons of means of change in RBC level between treatments between dosages of toad venom and Diamizan plus drug showed a high significant difference (P= 0.006324).

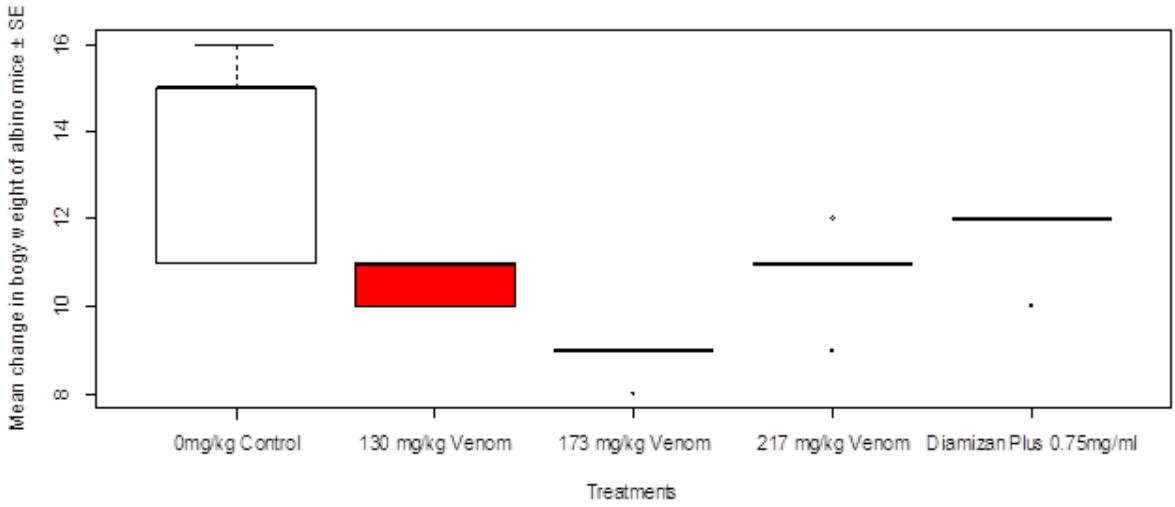

Figure 8. Mean change in body weight of Swiss mice after treatment with toad venom and Diamizan plus drug

Table 8. Turkey Multiple Comparisons of Means of Change in Body Weight Between Treatments

	Treatments			
	0 mg/kg	**130 mg/kg**	**173 mg/kg**	**217 mg/kg**
130 mg/kg	0.087	0	0	0
173 mg/kg	0.031	0.031	0	0
217 mg/kg	0.168	0.555	0.060	-
Diamizan Plus 0.75 mg/mL	0.428	0.128	0.031	0.219

P Value Adjustment Method: BH

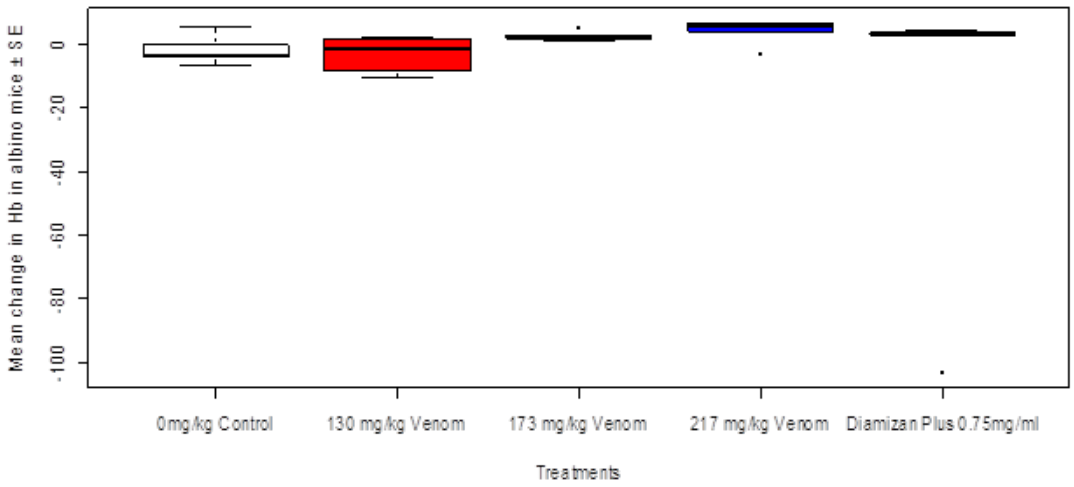

Figure 9. Mean change in hemoglobin level in Swiss mice in relation to treatments with toad venom and Diamizan plus drug after three days of trypanosomal inoculation

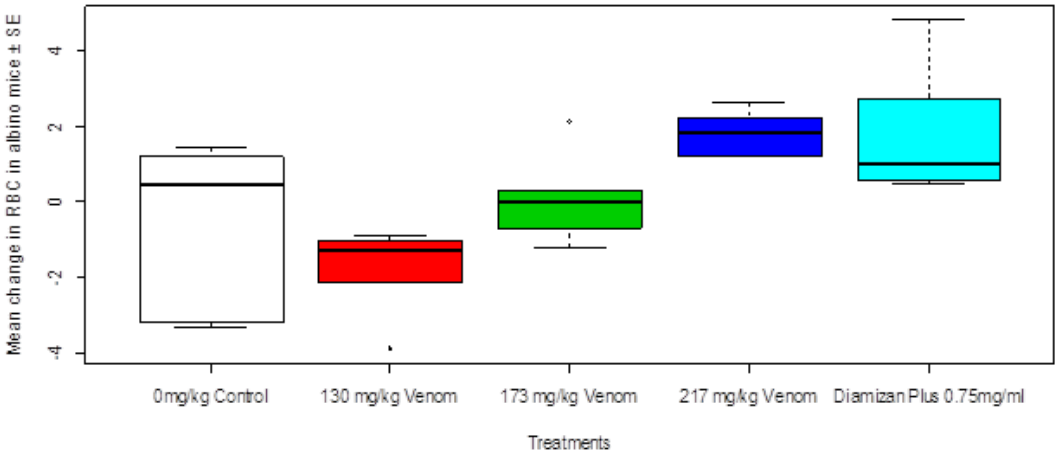

Figure 10. Mean change in RBC level in Swiss mice in relation to treatments with toad venom and Diamizan plus drug after three days of trypanosomal inoculation

Table 9. Turkey Multiple Comparisons of Means of Change in RBC Level between Treatments

	Treatments			
	0 mg/kg	**130 mg/kg**	**173 mg/kg**	**217 mg/kg**
130 mg/kg	0.526	0	0	0
173 mg/kg	1.000	0.079	0	0
217 mg/kg	0.099	0.060	0.099	0
Diamizan Plus 0.75 mg/mL	0.136	0.060	0.079	0.750

P Value Adjustment Method: BH

PCV

After treatment with the toad venom crude extract of different dosages and Diamizan plus drug, the result showed a change in the PCV level of Swiss mice in the various treatment groups in which group 3 had the highest PCV level, followed by group 4 then group 2 whereas group 1 had the lowest PCV level. Thus, the mean change in PCV level in Swiss mice after three days of trypanosomal treatment between dosages of toad venom and Diamizan plus drug showed a very high significant difference (Kruskal-Wallis $\chi^2 = 18.513$, df = 4, P = 0.0009793, Figure 11). Table 10 shows multiple comparisons of means of change in PCV level between treatments which the highest dosage of toad venom versus Diamizan plus drug showed no significant difference (P = 0.317).

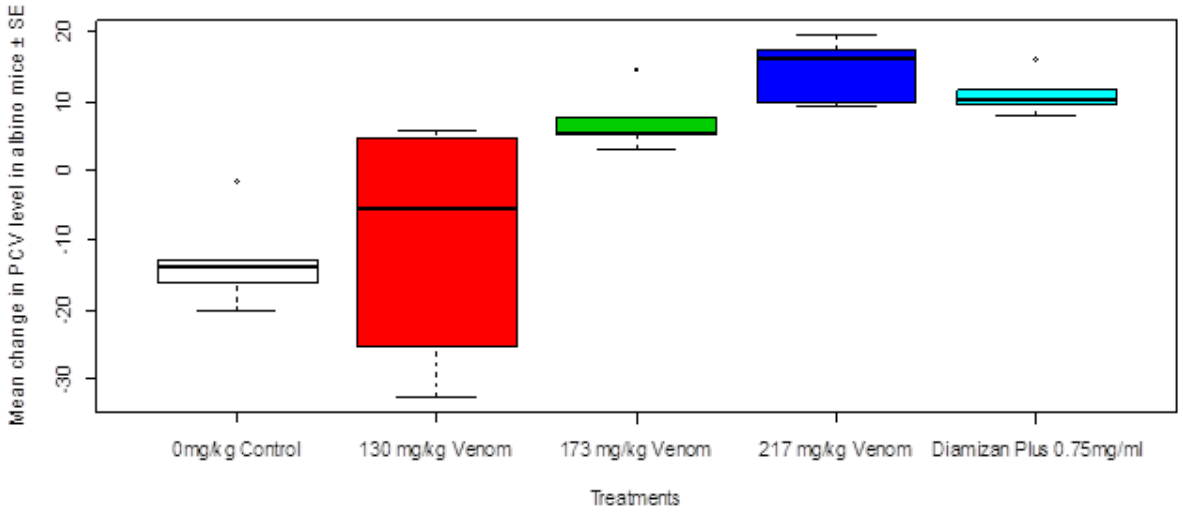

Figure 11. Mean change in PCV level in Swiss mice in relation to treatments with toad venom and Diamizan plus drug after three days of trypanosomal inoculation

Table 10. Turkey Multiple Comparisons of Means of Change in PCV Level Between Treatments

	Trypanosoma Load and Treatments			
	0 mg/kg	**130 mg/kg**	**173 mg/kg**	**217 mg/kg**
130 mg/kg	0.841	0	0	0
173 mg/kg	0.026	0.136	0	0
217 mg/kg	0.026	0.026	0.053	0
Diamizan Plus 0.75 mg/mL	0.032	0.032	0.139	0.317

P Value Adjustment Method: BH

4. Discussion

4.1 Bioactive Compounds Present in Bufonidae Toad Venom Crude Extract

The toad's parotoid secretions have been reported to be of a high chemical complexity. They have diverse types of biomolecules as recorded in this study, such as proteins, chemical components and elements, peptides, biogenic amines, and alkaloids and other unidentified classes. Previous studies had established that, bufadienolides have several activities against bacteria, fungi, protozoa, virus, HIV, cancer cells and some of its components identified as modulators of blood coagulation, neurotransmission, analgesic, and anti-inflammatory [18-21].

4.2 Biochemical Compounds Present in the Toad Venom Crude Extract

Using GC-MS techniques, compounds identified in this study like n-Hexadecanoic acid; 9,12-Octadecadieonic acid (Z,Z); 9,17-Octadecadienal (Z); Hexadecanoic acid 2-hydroxy-1-(hydroxymethyl) ethyl ester, are in harmony with the findings of Ajanaku *et al*. [22] who reported that the methanolic extract of the leaf of *C. adansonii* having the same compounds, has cancer preventive, anti-microbial, anti-fungal and other therapeutic properties which could suppose that toad venom crude extract equally possesses anti-trypanosomal therapeutic potentials as observed by Aparna *et al*. [23] and Sanni and Omotoyinbo [24].

4.3 The Protein and Non-Protein Functional Groups Present in the Toad Venom Crude Extract

According to Sakate and Oliveira [25], the various components that make up the toad venom can be divided into basic compounds (biogenic amines) and steroid derivatives. Consequently, the biochemical analysis using FTIR procedures employed in this study revealed the presence of amines like aniline and triethylamine which is in agreement with the earlier discoveries of Andrade *et al.* [26] who reported that amino acids and polyamines are established membrane transporters in *T. cruzi*. These findings apparently suggest that the amino-group (aniline

and triethylamine) were easily absorbed by *T. brucei brucei* and could be potential therapeutic targets due to the anti-trypanosomal activity of the toad venom crude extract [27]. Tempone *et al*. [28] reported antiparasitic activity of the steroids isolated from toad venom crude extract and only hellebrigenin from the biogenic amines was active against trypomastigote of *T. cruzi*, which could be attributed to the activity against *T. brucei brucei* as seen in this study.

4.4 The Chemical Elements Present in the Toad Venom Crude Extract

Except for lead (Pb) and aluminium (Al), every other element identified in this study using SEM-EDS are essential for the human body, and this agrees with the findings of Lingamaneni *et al*. [29] who stated that these elements function in the stabilization of cellular structures (immune systems) at normal levels but in deficiency, may stimulate alternate pathways and cause diseases. Thus, these elements could be considered to be chemotherapeutic and chemo-preventive agents which stimulate the immune systems in the treatment of other diseases and may be, trypanosomal infection as demonstrated in this study. However, variation in the toad's diet can influence the molecules uptake by feeding, thereby modifying the secretion composition as well as activity. Thus, this requires further and sustained research in diverse locations, looking for constituents of toad venoms from various species [30].

4.5 Oral Toxicity (LD_{50}) of the Toad Venom Crude Extract on the Laboratory Swiss Mice

Prior to the mortality of the laboratory animals, symptoms such as hyperactivity, convulsion and constant stooling were observed which suggests that the toad venom extract was lethal and even up to 1000 mg/kg after an hour, however on the administration of 750 mg/kg, hyperactivity, diarrhea and sedation were observed within one hour, but there was no mortality recorded. Following the Lorke's method computation, the oral toxicity of the toad crude venom extract was established as 866 mg/kg. This finding agrees with Tubaro *et al.* [31] who investigated the acute oral toxicity of a new palytoxin congener in mice which induced scratching, jumping, respiratory distress, cyanosis, paralysis and death in mice within 24 hours but not in accordance with Al-Afifi *et al*. [32] who reported that *Dracaena cinnabari* resin methanol extracts in rats, showed no treatment related mortality.

4.6 Pre-patency Period of *Trypanosoma brucei brucei*

The lack of variation observed at day zero prior to treatment in the mean of trypanosomal load in Swiss mice in relation to treatments with toad venom and diamizan plus drug respectively could be because the Swiss mice were yet to receive any form of treatment at this stage. The results possibly suggests that the pre-patent period of *Trypanosoma brucei brucei* was 3 days, which is in line with the findings by Turay *et al*. [33] and Udensi and Fagbenro-Beyioku [34].

4.7 Trypanosomal Load in Swiss Mice in Relation to Treatments with Toad Venom and Diamizan Plus Drug

The observed variation in the three (3) days pooled trypanosomal load in Swiss mice in relation to treatments possibly suggests that the standard drug (diamizan plus) is effective. This is in consonance with the finding of Ezeh *et al*. [35] who reported that treatment with Diminazene aceturate (Berenil) resulted in the reduction of parasitemia load in infected mice after two days of treatment. Although the toad venom did not actively reduce the *Trypanosoma* parasitaemia as much as diamizan plus drug did when both of their mean load was compared, however, the highest dosage of toad venom treatment still yielded over 50% reduction in trypanosomal load in Swiss mice in comparison with the control group. This suggests that the toad venom has the potential to relatively reduce trypanosomal load in the Swiss mice which corroborates with Tempone *et al*. [28] who reported antiparasitic activity of steroids (telocinobufagin and hellebrigenin) from toad venom crude extract and also leishmanicidal activity against *L. infantum* promastigotes with IC50 of 126.2 μg/mL and 61.2 μg/mL. This report possibly suggests that the activity of the steroids may involve mitochondrial degradation and perturbation of the parasite membrane, resulting in cell death. These results indicate the range of biochemical molecules and possible applications.

The observed variation after 24 hours (day one) of treatment in the mean trypanosomal load in Swiss mice in relation to treatments with toad venom and diamizan plus perhaps suggests that the diamizan plus drug was more effective in reducing trypanosomal load in the infected Swiss mice. The toad venom did not successfully reduce the trypanosomal load in the mice as evident in Figure 5. With respect to the highest dosage of toad venom and diamizan plus treatments at day one, the high significant difference recorded suggests that although the toad venom was able to reduce the trypanosomal load in the mice, diamizan plus drug actively and considerably reduced the trypanosomal parasitaemia in the Swiss mice examined. Freiburghaus *et al.* [36] have shown that the mean minimum inhibitory concentration value of common trypanocidal drugs is 10.7 mg/mL and that agent with minimum

inhibitory concentration value between 5 mg/mL ~ 20 mg/mL could be regarded as very active.

The high difference detected after 48 hours (day two) of treatment in the mean of trypanosomal load in Swiss mice in relation to treatments with toad venom and diamizan plus drug possibly connotes that the toad venom did not successfully reduce the trypanosomal parasitaemia in the mice models as much as diamizan plus drug did. This is possibly because of poor inactivation of the active components contained in toad venom. Thoughvariation was observed between the mean trypanosomal load of toad venom treatment and control group, which possibly suggests that the toad venom treatment could be used to reduce *Trypanosoma* parasitaemia. The present finding is not in agreement with Habila *et al*. [37] who examined the anti-trypanosomal potentials of *A. indica* seeds methanolic extract against *T. evansi* at 25 mg/mL, 50 mg/mL and 100 mg/mL and immobilized the parasites within 14 mins, 8 mins and 3 mins, respectively.

The 72 hours (day 3) decline in the trypanosomal load in Swiss mice in relation to treatments suggests that *Trypanosoma* become relatively susceptible to bufonid product over a longer period.

4.8 Impact of Trypanosomes on Body Weight of Swiss Mice after Three Days of Treatment

Body weight of Swiss mice after a three (3) day treatment in relation to toad venom as well as diamizan plus drug respectively was much more a positive increase in body weight for group treated with diamizan plus drugs than the toad venom groups. But, the change in the mean body weight in control (untreated) group went the negative direction. Kifleyohannes *et al*. [38] reported that the weight in the untreated infected mice group started to decrease after 12 days post infection till all the mice died by day 18 where as those standard drug and extract of *A. absinthium* and *M. stenopetala* treated mice generally showed a gradual increase in mean weight until the end of the experimental period. The finding is also in agreement with finding of Tadesse *et al*. [39] who found out that the treatment with the crude extracts of *A. absinthium* and *D. abyssinica* prevented loss in body weights, particularly at higher doses. The aqueous and methanolic extracts of *V. sinaiticum* were capable of improving body weight of treated animals on day 8–14 as compared to the untreated control group [40]. On the other hand, reports by Ngure *et al*. [41] showed that extract of *A. indica* and suramin-treated animal groups had a significant decline in body weight.

4.9 Impact of Trypanosomes on Haematological Parameters of Swiss Mice after Three Days of Parasitemia Treatment

Haemoglobin (Hb)

The lack of variation observed in the mean change in Hb level in Swiss mice after three days of trypanosomal treatment between dosages of toad venom and diamizan plus drug respectively suggests that the treatments has some haematopoetic property, and also trypanosomal infection in the Swiss mice has no effect on haemoglobin. This result is not in discordance with Alli *et al*. [42] who recorded high Hb concentration when he compared group treated with diminazene and *M. lucida* leaf extract increased haemoglobin.

Red Blood Cells (RBC)

The observed high variation in the mean change in RBC level in Swiss mice after three days of trypanosomal treatment between dosages of toad venom and diamizan plus drug possibly suggests that the presence of trypanosomes in the Swiss mice had a major effect on the RBC of the mice. It equally suggests that if left untreated, the impact of the infection on the mice would possibly be deleterious. This result is in consonance with the finding of Alli *et al.* [42] who recorded lowest RBC count in the group infected but not treated and this is in keeping with trypanosomal infection.

Packed Cell Volume (PCV)

The negative in the mean change in PCV level of the control (untreated) wiss mice group after three days of possibly suggests that the trypanosomes had a negative impact on the immunity of the mice with resultant effect on PCV. The trypanosomes affect the immune-response of animals as observed in this study leading to anaemia which is the most outstanding clinical and laboratory feature of African trypanosomiasis and the primary cause of death [43].

The PCV of the mice treated with the toad venom crude extracts and diamizan plus drug stayed within constant range. This result illustrates that toad venom crude extract has the capacity to improve the PCV even if it declines after relapse of parasites. The finding is in agreement with finding of Abubakar *et al*. [44] who reported *M. balsamina* and *S. longipenduculata* possess the highest anti-trypanosomal potential since they are able to control anemia by resisting sudden drop in PCV level.

The mean PCV between diamizan plus drug and different dosages of toad venom crude extract were relatively comparable which is in agreement with Kifleyohannes *et al*. [38] and Tadesse *et al*. [39] who studied on *A. absinthium* and *D. abyssinica* respectively in which PCV level was significantly improved with comparable potential to diminazene aceturate since they are able to control anemia as well as minimize the decline in PCV level.

5. Conclusions

Results of the in-vivo anti-trypanosomal activities of the toad venom crude extract on laboratory mice showed that the overall parasitemia was highest in the control group and the least parasitemia was observed in the group treated with Diamizan Plus drug. Toad venom has the potential to relatively reduce the *Trypanosoma* infection in Swiss mice as observed in one of the toad venom treatment group (130 mg/kg) where trypanosome parasitemia was about thrice less than the control group. The 3-day treatment shows that trypanosomal load in Swiss mice have a negative impact on the RBC and PCV levels. There is a need for further studies (both in-vivo and in-vitro) based on the output on trypanosomal potential of bufonidae product recorded in this study. Also, research should be conducted on the bio-active compounds of toad venom crude extract and their pharmacological activity.

Conflict of Interest

There is no conflict of interest.

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