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ORIGINAL ARTICLE

In Vitro Anti-Trypanosomal Activity of *Bitis arietans* Crude Venom Against *Trypanosoma brucei brucei*

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SUMMARY
African Animal trypanosomosis (AAT) otherwise known as Nagana is a resurgent disease in Africa. It is caused by *Trypanosoma brucei brucei, Trypanosoma congolense, Trypanosoma vivax, Trypanosoma evansi* among others. It is transmitted cyclically by tsetse flies (Diptera: Glossinidae) or mechanichally by other biting flies. In the absence of efficient vaccines, chemotherapy, together with vector control, remains the most important measure to control the disease. The parasites have developed resistance against the disease due to improper exposure to chemotherapeutic agents. These present challenges necessitate the development of alternative therapeutic agents against the disease. Therefore, the aim of this study was to test for the *in vitro* anti-trypanosomal activity of *Bitis arietans* (Puff adder) venom against *Trypanosoma brucei brucei* species. Protein profiles of the crude snake venoms were determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS- PAGE), and the results revealed the venoms contain 10 different proteins. The IC_{50} of the venom was also determined by 3(4,5-dimethylthiozol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay and was found to be 0.3085µg/ml. The *in vitro* anti-trypanosomal activity of the graded dose of the venom was evaluated over a period of 5 hours in comparison with two standard drugs; Diminaveto and Babezene (12mg/15ml) as positive control and phosphate buffered saline glucose (PBSG) pH 7.4 as negative controls. The venom lysed the parasites across all the different concentration within 30 minutes of incubation. It was also found to have mild or no lytic effects on the erythrocytes for the period of the incubation. The results show that *Bitis arietans* has anti-trypanosomal activity and can be a potential drug target against the disease.

Keywords: *Bitis arietans* venom, anti-trypanosomal activity, *in vitro, Trypanosoma brucei brucei*,

INTRODUCTION
African trypanosomosis is one of the 20 neglected tropical disease that is still of great public relevance, and a severe impediment to livestock and agricultural development in endemic areas (Ebiloma *et al.,* 2018). Trypanosomosis is a disease caused by single cell protozoan parasites of the genus *Trypanosoma*. Tsetse flies which are the primary vectors of African trypanosomes are responsible for the cyclical transmission of this parasitic protozoan between numerous vertebrate hosts (Baral, 2010). This disease is generally known as sleeping sickness or Human African Trypanosomosis (HAT) in humans and Nagana in cattle and other ruminants (Mwandiringana *et al.*, 2012; Rosa *et al.,* 2012; Baldissera *et al.,* 2016; Cai *et al.,* 2016). AAT is a resurgent disease of livestock that directly hinders livestock production and therefore impedes the socio-economic development of sub-Saharan Africa (Isaac *et al.*, 2017). Animals

suffering Nagana develop fever, weight loss and progressively become weak and unproductive. Breeding animals may abort or become infertile, if left untreated. Most animals die of anaemia, heart failure or concomitant bacterial infections as a result of the animal's weakened immune status. (Chechet, 2015). The direct losses to livestock production caused by AAT amounts to more than $20 million spent per annum on trypanocidal drugs and indirect losses related to the opportunity cost of land and other resources currently not used for livestock production due to the presence of tsetse flies (Eze *et al.*, 2015).

Effective treatment of AAT has been hampered by the development of drug resistance by most *Trypanosoma* species (Meyer *et al*., 2016). The use of alternative drugs has been recommended as a measure to avoid the development of resistant pathogenic organisms including trypanosomes. The resistance mechanisms employed by trypanosomes are major factors that contribute to the difficulties encountered in the management and treatment of the disease. More so, the side effects caused by trypanocidal drugs and high cost of the drugs calls for an urgent search for a novel chemotherapy for the treatment and management of AAT.

Snake venoms and poisons are one of the most concentrated peptides, protein, enzymes and several bioactive compounds with potentials to yield novel drugs or drug candidates for pharmacotherapeutics (Leon, 2011; Fox, 2007).

Venom-based drug is not a new idea, and it has been used in human history for more than hundreds of years which have been effectively used against arthritis, cancer, neurological disorders as well as heartattacks (Ma *et al*., 2017; Peichoto *et al.*, 2011). Snake venoms have shown and proven to be interesting sources of potential novel agents against neglected diseases, including nagana disease (Adade *et al*., 2012). Different snakes have different types of venoms, which depend upon the species, geographical location, habitat, climate, age etc. *Bitis arietans* commonly known as the puff adder is a medically important snake with a much wider distribution than, but overlapping with that of *E. ocellatus* (Fiona and Simpson, 2017). Studies have revealed that the crude venom of South American Bothrops snakes inhibit the growth of *Leishmania* major promastigotes and *T. cruzi* epimastigotes and induce programmed cell death in *T. cruzi* (Adade *et al.*, 2011). Although, *in-vitro* and *in-vivo* trypanocidal activities of different snake venoms have been documented, there are less or no sufficient published data on the activity of these crude snake venoms against the virulent *T. b. brucei.* Looking at previous data obtained for other snake venoms against protozoa parasites such as *T. cruzi,* we attempted to investigate the anti-trypanosomal activity of *Bitis arietans* venom against *T. b. brucei.*

MATERIALS AND METHODS

Trypanosoma b. brucei

Trypanosoma brucei brucei (Federe strain) originating from a naturally infected cow. was obtained from the Nigerian Institute for Trypanosomosis and Onchosachiasis Research (NITOR), Kaduna State, Nigeria. The parasite was maintained by serial passage in rats (0.3ml of aliquot 5×10^6 parasites/ml of trypanosomes by intraperitoneal injection) at the Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria.

Albino rats (*Rattus albus*) were obtained from the Department of Pharmacology Ahmadu Bello University, Zaria, Nigeria. The rats had no history or symptoms of trypanosomosis, and were infected intraperitoneally with *T. brucei bruce*i at the concentration of 2×10^6/ml. The parasitaemia was monitored for the period of

five days until massive parasitaemia was obtained.

Determination of Parasitaemia

The number of parasites was determined microscopically at x40 magnification using the "Rapid Matching" method of Herbert and Lumsden, (1976). Briefly, the method involves microscopic counting of parasites per field in whole blood or blood appropriately diluted with phosphate buffer saline (PBS, pH 7.4). Logarithmatric values of these counts were obtained by matching with the table of Herbert and Lumsden (1976) and converted to antilog to provide absolute number of trypanosomes per ml of blood (Atawodi *et al.*, 2003)

Collection of blood

When the parasitemia level was massive as determined by the "Rapid Matching" method, the rats were sacrificed using a sharp surgical lancet after the animals were anesthetized and their blood collected in sample bottle containing phosphate buffer saline mixed with glucose (pH 7.4) at ratio 1:1. The buffer prevented blood coagulation and glucose was source of energy for the parasites.

Venoms and standard drugs preparation

Lyophilized *Bitis arietans* venom was graciously provided by Dr. Peter Yusuf of the Department of Toxicology and Pharmacology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. The crude venom was diluted fourfold to obtain concentrations of 5.00, 1.25, 0.31, 0.05 and 0.02µg/ml from the stock solution.

Standard trypanocidal drugs

Standard drugs, Diminaveto (Diminazene diaceturate + Antipyrine) and Babezene (Diminazene + Phenazone) were prepared at a standard concentration of 161µg/ml as specified by the manufacturer and used for this experiment as positive controls. 5µg/ml and the concentration obtained by 3(4,5-dimethylthiozol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used for the *in vitro* study.

Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in order to determine the protein/enzymes profiles present in the venom. This was carried out according to Laemmli, 1970 in a Mini Protean II Cell (Bio-Rad, Munich, Germany) in the presence of a reducing agent (dithiothreitol). The polyacrylamide gels consisted of 15% resolving and 4% stacking gel. A molecular weight marker, pre-stained SDS-PAGE standards from Fermentas (for immunoblotting) or SDS-PAGE Marker High Range from Sigma was used for staining.

Determination IC_{50} of the venom Using MTT Assay

Principle of the assay

This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase to generate NADH or NADPH. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored (dark purple) formazan product. The cells are then solubilized with an organic solvent (e.g. isopropanol) and then released, solubilized

formazan reagent is measured spectrophotometrically at 570 nm. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.

Procedure

Fifty microliters of Trypanosomes suspension ($5x10^6$ parasites ml^{-1}/well) was incubated for 2 h at 37^0C with 50µl different concentrations (4-fold dilutions) of each of the crude venom (5.00, 1.25, 0.31, 0.05 and 0.02µg/ml) in a 96-well flat bottom plate. 50µl of PBSG (pH=7.4) without the parasites was used as a control. After that, 10µl of the MTT solution (5mg/ml) was added and the preparation was incubated for 4 hours before it was reaction was stopped by the addition of 100µl acidic isopropanol. The optical density (OD) was measured at 570 nm.

Cell viability was calculated using the following formula according to FCG (19XX):

Cell viability %= [(test - control) /control)] x100

Data (Dose-response inhibition) were transformed by logarithm and the half-inhibitory concentration IC_{50} was determined by non-linear regression. Tests were carried out in duplicate. All data were expressed as Means±SD of the duplicate measurements using Graph Pad Prism 7 software Version 7.0a.

Assessment of *in vitro* anti-trypanosomal activity of the crude *Bitis arietans* venoms

Assessment of *in vitro* anti-trypanosomal activity was performed in duplicate in 96 well micro titre plates. 50µl of blood containing approximately $5x10^6$ parasites ml^{-1}/well was incubated with an equal volume of the crude venom solution (containing 5.0 and 0.3085mg/ml high and low concentrations respectively). Also, equal volume of the parasitized blood was incubated with equal volume of standard drugs and PBSG, which served as positive and negative controls respectively. Immediately after the above step, aliquots of test mixtures were placed on separate microscope slides and covered with cover slips and the parasites observed at intervals of 10 minutes starting from zero minutes for a total duration of 5 hours. It should be noted that under this *in vitro* system adopted, parasites survived for up to 6 hours when no venom was present. Cessation or drop in motility of the parasites in venom incubate blood compared to that of parasite-loaded control blood without crude venom was taken as a measure of anti-trypanosomal activity (Umar *et al.*, 2010). All analyses were performed in duplicates.

Cytotoxicity of the crude snake venoms

Fifty microliters of the parasitized blood and 50µl of diluted *Bitis arietans* (5 and 0.3085µg/ml) was incubated at room temperature in a 96 well microtitre plate to test for the cytotoxicity of the venom. 50µl of PBS-G, and two standard drug solutions were also incubated at room temperature to serve as positive and negative control respectively. The numbers of parasites per microscopic field were monitored for 0, 10, 30 minutes followed by hourly checks for about 5-6 hours.

RESULTS

SDS-PAGE Analysis

The 18% SDS-PAGE analysis of crude *B. arietans* snake venom under reducing conditions resolved the venom components into 10 different protein bands of approximate molecular masses ranging from 10 to 180 kDa (Plate 1).

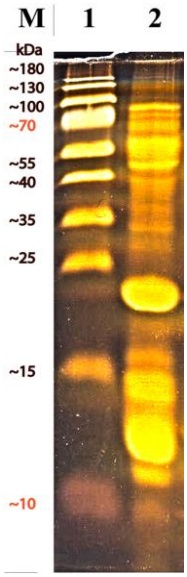

Plate 1: SDS-PAGE (18%) protein profile containing the crude ***Bitis arietans*** venom under reducing conditions. The gel was stained with Coomassie blue. Lane 1 molecular weight markers (PageRuler Prestined protein ladder, Thermofischer scientific).

IC_{50} determination

Figure 1 presents the IC_{50} values of *Bitis arietans* crude venom, which was found to be 0.3085µg/ml. Data (Dose-response Inhibition) were transformed by logarithm and the half-Inhibitory concentration IC_{50} was determined by Non-linear regression (Figure 1).

***In vitro* activity**

After 10 minutes, a reduction of 33.3% and 16.7% of live trypomastigotes was observed at

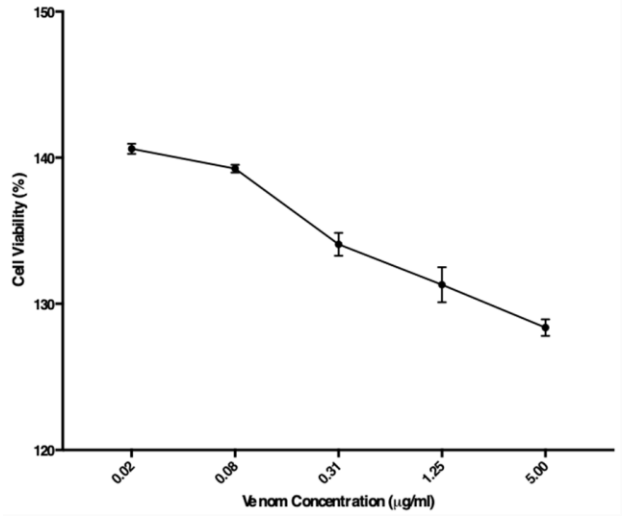

Figure 1: The cytotoxic effect of crude *Bitis arietans* venom evaluated by MTT assay.

the concentrations of 0.3085 and 5.0 µg/ml of *Bitis arietans* crude venom, respectively, when compared with control group. Whereas, there was 100% reduction of motility in the positive and negative control wells. After 30 minutes, a reduction of 25.89% and 40.47% of live trypomastigotes occurred at concentrations of 0.3085 and 5µg/ml of *Bitis arietans* crude venom, respectively, when compared with control groups. After 60 minutes, a significant decrease ($P<0.05$) of live parasites was observed at both concentrations of crude venom with control group (Figure 2). After 180 minutes in maintenance medium, there was 100% reduction of motile parasites at either concentration tested while the PBSG group showed high numbers of live trypomastigotes.

Lytic effect of crude venom on RBCs and parasite motility

Table I shows the effect of *Bitis arietans* crude venom on the motility of *Trypanosoma brucei brucei* and lysis of RBCs. After 10

TABLE I: Effect of crude *Bitis arietans* crude venom on the motility of *Trypanosoma brucei brucei* and its lytic effect on RBCs and parasites

Time (min)	Concentration	Motility	ANMP (mean ± SD)	ANIMP (mean ± SD)	Lysis	
					RBC	Parasite
0	5 µg/ml	+++	50.00±0.00	0	*	*
	0.3085µg/m	+++	50.00±0.00	0	*	*
	PBSG	+++	50.00±0.00	0	*	*
	Diminaveto	+++	50.00±0.00	0	*	*
	Babezene	+++	50.00±0.00	0	*	*
10	5 µg/ml	++	9.33±1.08	8.13±0.54	√	√
	0.3085µg/m	++	16.56±1.04	26.38+0.35	*	*
	PBSG	+++	50.00±0.00	0	*	*
	Diminaveto	-	0	49.13±0.22	√	*
	Babezene	-	0	46.25±0.53	√	*
30	5µg/ml	++	0.81±0.52	8.13±0.54	√	√
	0.3085µg/m	++	1.19±1.08	0.63±0.72	*	√
	PBSG	+++	50.00±0.00	0	*	*
	Diminaveto	-	0	49.13±0.22	√	*
	Babezene	-	0	46.25±0.53	√	*
60	5 µg/ml	+	<0.0625	2.33±0.58	√	√
	0.3085µg/ml	+	0.0625	0.13±0.58	*	√
	PBSG	+++	48.9±0.56	<0.0625	*	*
	Diminaveto	-	0	49.13±0.22	√	*
	Babezene	-	0	46.25±0.53	√	*
120	5 µg/ml	-	0.00	0.33±0.58	√	√
	0.3085µg/ml	+	<0.0625	0.07±1.00	*	√
	PBSG	+++	48.06±1.21	1.063±0.54	*	*
	Diminaveto	-	0	49.13±0.22	√	*
	Babezene	-	0	46.25±0.53	√	*
180	5 µg/ml	-	0.00	0.06±0.042	√	√
	0.3085µg/ml	-	0.00	<0.07	*	√
	PBSG	+++	47.88±0.08	2.13±0.21	*	*
	Diminaveto	-	0	47.98±0.14	√	*
	Babezene	-	0	46.02±0.11	√	*
240	5 µg/ml	-	0.00	<0.06	√	√
	0.3085µg/ml	-	0.00	0.00	*	√

	PBSG	+++	47.60±0.24	2.38±0.08	*	*
	Diminaveto	-	0	47.98±0.14	√	*
	Babezene	-	0	46.02±0.11	√	*
300	5 µg/ml	-	0	0.00	√	√
	0.3085µg/ml	-	0	0.00	*	√
	PBSG	+++	47.01±1.04	2.01±0.14	*	*
	Diminaveto	-	0	47.18±0.12	√	*
	Babezene	-	0	45.92±0.58	√	*

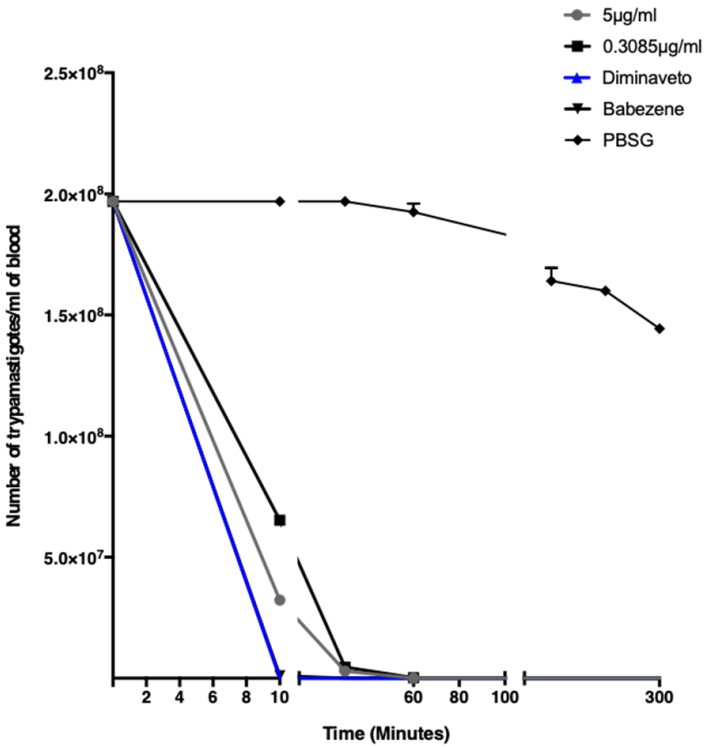

Figure 2: A dose-dependent trypanocidal effect of *Bitis arietans* crude venom on *Trypanosoma brucei brucei* trypomastigotes.

Mins of incubation, there was significant ($P<0.05$) reduction of the average number of motile parasites (ANMP) in the test concentrations (5.0 and 0.3085µg/ml). The average number of immotile parasites (ANIMP) increased (16.56±1.04 to 26.38+0.35) in the lower crude venom concentration whereas no significant change was observed in the higher venom concentration. RBCs and parasites lysis was observed in the higher crude venom concentration medium. In the standard drugs medium, the parasites remained unlysed while RBC lysis was observed. After 30 minutes of incubation, parasite lysis was observed in both low and high crude venom concentrations. RBC lysis was also observed in the standard drugs and higher crude venom mediums. After 60 minutes in the maintenance medium, parasite lysis was observable only in both concentrations the crude venom. RBC lysis however, was only observed in the medium containing the standard drugs and higher concentration of the crude venom.

DISCUSSION

The protein profile of *B. arietans* venom obtained in this study corresponds to 10 different protein fractions ranging from 10-180 kDa contrary to the 9 protein fractions earlier reported under similar experimental condition

(Guidlolin *et al*., 2010). One or more of these fractions may be working singly or synergistically and could be responsible for the anti-trypanosomal effect of the crude venom observed. A similar finding was reported for *Bothrops mattogrossensis* crude venom. The authors stated that Phospholipase A_2 isolated from the crude venom might be responsible for the *in vitro* trypanocidal activity on *T. cruzi* (Tohamy *et al.,* 2014). In another study,. Steven *et al.* (2014), showed that the crude venom of *B. arietans* contained some toxic substance, namely; Bitinarin, a post synaptic neurotoxin with PLA_2, Bitiscetin, a platelet aggregation inducer and Ba100, a toxin with fibrogenase activity. Furthermore, Adade *et al.* (2014) reported that Crovirin, a cysteine-rich secretory protein (CRISP) contained in snake venom showed promising activity against *Trypanosoma* and *Leishmania* spp. Additionally, some snakes venom enzyme/proteins has been reported to contain complex mixture of peptides, enzymes and toxins, including metalloproteases (41%–44%), phospholipases A_2 (PLA_2) (29%–45%), serine proteases (4%–18%), L-amino acid oxidases (5%–59%), disintegrins (1%–2%) C-type lectin-like proteins (0.5%) and cysteine-rich secretory proteins (CRISP) (0.1%) (Alape-Giron *et al.,* 2008). Some of these enzymes/proteins for instance, PLA_2 has been reported to show anti-plasmodium activity and therefore suggested to be a potential drug candidate (Castillo *et al.,* 2012). Our results corroborate the observations of Adade *et al.,* 2011; Vulfius *et al*., 2011) who reported that a crovirin and Bitanarin toxin which have molecular weights of 24 and 14 kDa respectively under reduced conditions are present in the crude venom of ***Bitis arietans.*** Although the proteins obtained in this study were not fractionated or sequenced, it could be speculated that crovirin and Bitanarin, a novel post-synaptic neurotoxin with PLA_2 activity and other related proteins with similar activities may be responsible in the clearing of the parasites. This study suggests that anti-trypanosomal chemotherapy could possibly and probably be developed from the screening and investigation of natural products such as animal venoms (Adade *et al.,* 2011). Animal venoms and toxins, including snake venoms, can provide compounds directly useful as drugs, or with potential as drug candidates for the synthesis of novel therapeutic agents (Tempone *et al.,* 2007). The anti-trypanosomal activity of a certain snake venom of the species *C. viridis viridis*, has been reported to inhibit the developmental stages of different forms of *T. cruzi*, the etiologic agent of Chagas disease (Adade *et al.,* 2011).

At the calculated IC_{50}, which was 0.3085µg/ml the crude *Bitis arietans* venom showed little or no lytic effect on the RBCs but it was able to induce mortality of at least 50% of the parasites. A similar observation had been reported where 10 µg/mL of *B. arietans* venom caused a significant inhibition in cell viability of L6 cells (Steven *et al*., 2014). That was what informed our inclusion of the 5 µg/mL concentration in the *in vitro* tests besides the IC_{50} concentration, which we determined.

The *in vitro* anti-trypanosomal activity of the venom with two standard drugs alongside a negative control (PBSG pH 7.4) is presented in figure 3. The crude venom used in this study showed a significant ($P<0.05$) decrease in the number of motile parasites compared with the PBSG control. At the concentration of 5µg/ml, a significant ($P<0.05$) decrease in number of parasites were observed after 60 minutes post incubation and complete ceassation of parasites after 2 hours post incubation. Similar effect was also observed at the concentration of

0.3085µg/ml. The two standard drugs; Diminaveto (purple) and Babezene selectively lysed the erythrocytes within 10 minutes incubation. The slight change in the number of motile parasite seen with time may be as a result of the complete utilization of the glucose (contained in PBSG) without replenishing or unequal distribution of parasite in wells of the microtitre plate. For the negative control that is parasites + PBSG pH 7.4, the number of the parasites remained relatively constant throughout the period of incubation. Cessation or drop in motility of the parasite in the blood incubated with crude venom compared to the negative control was taken as a measure of anti-trypanosomal activity of the crude venom(Umar *et al*., 2010). This decrease in the number of parasites over time suggested that the crude venom has an anti-trypanosomal activity.

Interestingly, only the trypomastigotes were lysed after 30 minutes of incubation with the lower concentration of the venom while the RBCs remained intact. This could suggest that the crude venom at low concentration could act as a better trypanocide than it would at higher concentrations without affecting body cells. On the other hand, the standard drugs completely lysed the RBCs while the trypomastigotes remained intact but immotile. This could be the reason why current trypanocides are toxic and harmful to the animal receiving treatment and the unlysed parasites may be immotile but not necessarily dead hence this possibly could be reason why trypanosomes develop resistance against current trypanocides.

Strydom *et al.*(2016) observed that RBCs showed eryptosis during puff adder (*Bitis arietans*) envenomation which impacts on the cellular structure and triggers eryptotic pathways. Perhaps we didn't observe eryptosis in our experiment because of the low concentration of crude venom used. Adade *et al.* (2014) reported the anti-trypansomal effects of crovirin on both the procyclic and the bloodstream form of *T. brucei rhodesiense* and suggested that crovirin might be useful in the development of new anti-HAT chemotherapeutics. Based on this study, there is no doubt therefore that this venom could serve as potential anti-AAT agent.

CONCLUSION

The findings in this study agree with earlier reports on the ability of *B. arietans* crude venom at low concentrations to selectively degrade parasites without necessarily affecting the RBCs. We recommend that further studies are required to explore the potentials of the crude venom of *B. arietans* as potent trypanocidal candidate.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

ACKNOWLEDGEMENTS

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