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Isolation and Purification of *Bitis arietans* (Puff Adder) Venom Proteins and Its Effect on Blood Chemistry of Envenomated Wistar Rats

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Abstract

Puff adders (*Bitis arietans*) are one of the most medically important African snake species, because they are likely responsible for a large portion of the 32,000 snake bite deaths that occur in sub-Saharan Africa annually. These snakes have highly modified saliva known as snake venom which is an extremely hemotoxic venom that prevents formation of platelets in the bloodstream and also leads to extreme pain, swelling, tissue necrosis, and spontaneous bleeding. This study isolated and purified the proteins from the snake venom of the Puff adder (*Bitis arietans*) and to ascertain the effect of blood chemistry on the envenomized Wistar rats. The protein concentration was determined using Bradford Assay, and purified using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Twenty Wistar rats were envenomated and their blood chemistry examined. The concentration of proteins in the venom of the Puff Adder (*Bitis arietans*) was 121.66 ± 7.94 µg/ml. Five protein bands were obtained in regions between 45 kDa and 66.2 kDa, 25 kDa, 18.4 kDa using Sephadex gel chromatography, between 14.4 kDa and 18.4 kDa and 14.4 kDa molecular weights. The five different proteins were identified in the snake venom on distinct bands on the SDS-PAGE Electrophoregram. There were significant differences in some of the hematological parameters of the Wistar rats envenomated with the snake venom (study group) when compared to that of the control group. Differences between the groups were considered significant at $P<0.05$. The results showed significantly higher lymphocytes, White Blood Cells (WBCs) and neutrophils blood level in the envenomated group which serves as indications of an infection in the body system. There was also a significant reduction in Red Blood Cells (RBCs) of the study group, which indicates possible hemorrhage. The serum AST, ALP, ALT activities and creatinine levels of the envenomated rats were also found to be significantly lower than the control group. Results of this study suggest that the snake venom proteins which were isolated and purified could serve as protein markers for anti-venom therapy and could be used for the development of new therapeutic and diagnostic tools in the pharmaceutical industry relevant for human medicine.

Keywords: *Bitis arietans*; Venom proteins; Envenomated proteins; Hematological; Biochemical parameters; Rats

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Introduction

Snake venoms are secretions of venomous snakes produced by a pair of specialized exocrine glands [1-3]. They are poisonous, typically yellow fluids stored in the modified salivary glands of venomous snakes. There are hundreds of venomous snake species that rely on the venom they produce to debilitate and immobilize their prey [4-9]. The potent harmful effects of snake venoms have intrigued mankind for centuries, inspiring in many cultures both fear and fascination [2]. These venoms are complex mixtures of proteins and peptides that play vital roles in the survival of venomous snakes. As with their diverse pharmacological activities, snake venoms can be highly variable, hence the importance of understanding the compositional details of different snakes [3,10-13]. Venoms have evolved over millions of years to facilitate prey capture and/or defense from predators and rivals [14,15]. Snake venoms, in particular, have evolved a wide diversity of peptides and proteins that induce harmful inflammatory and neurotoxic effects, including severe pain and paralysis, hemotoxic effects, such as hemorrhage and coagulopathy, and cytotoxic/myotoxic effects, such as inflammation and necrosis [16-20].

The puff adder (*Bitis arietans*) is a venomous snake of the Viperidae species found in savannah and grasslands from Morocco and western Arabia throughout Africa except for the Sahara and

rainforest regions [21-25]. This specie is responsible for more snakebite fatalities than any other African snake, due to a combination of factors, including its wide distribution, common occurrence, large size, potent venom that is produced in large amounts, long fangs, and their habit of basking by footpaths and sitting quietly when approached [14,23,26].

In humans, bites from this specie can produce severe local and systemic symptoms. Serious bites cause limbs to become immovably flexed as a result of significant hemorrhage or coagulation in the affected muscles [27-31]. Other bite symptoms that may occur in humans include edema, which may become extensive, shock, watery blood oozing from the puncture wounds, nausea and vomiting, subcutaneous bruising, blood blisters that may form rapidly, and painful swelling of the regional lymph nodes. Swelling usually decreases after a few days, except for the area immediately around the bite site. Hypotension, together with weakness, dizziness, and periods of semi- or unconsciousness is also reported [14,32,33]. When left untreated, much envenomation result in death or severe morbidity in humans, and despite advances in management, snakebite remains a major public health problem, particularly in developing countries.

There is paucity of information on the snake venom proteins from *Bitis arietans* in Nigeria, and the effect on blood chemistry of envenomized rats. The findings from this study will provide valuable lead on the pharmaceutical potential of anti-snake venom as drug targets and development.

Materials and Methods

Venom collection

A fresh venom sample was obtained from the Puff adder (*Bitis arietans*) from a herpetarium or snake farm in its habitat at Badagry, Lagos State, Nigeria. The snakes were reared under standard environmental and ethical conditions in conformity of National Research Council Guide for care and use of Experimental animals [34]. The Puff adder was authenticated by a herpetologist at the Department of Zoology, Faculty of Science, University of Lagos, Nigeria, with Voucher Specimen No: S021/002. The person responsible for the venom collection was an expert in venom extraction. After sampling, the venom was air-dried and stored at -20°C until the chemical analysis was performed.

Bradford assay

The total protein concentration in the snake venom was determined using the dye-binding technique of Bradford with Bovine Serum Albumin (BSA) at 1.0 mg/mL. A six-point calibration curve in the range 20 µg/mL to 100 µg/mL was performed using a BSA solution. The venom was diluted 100 times using phosphate buffer saline for analysis, 1 mL of Bradford reagent was added to the sample, after 20 min, absorbance was measured at 595 nm.

Purification of Snake Venom Proteins

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

400 µl of snake venom was resuspended in 400 µl of Tris-Buffered Phenol (Saturated Phenol), at pH 8.0. 400 µl of SDS buffer was added, then 4 µl of Protease Inhibitor. It was vortexed rigorously for 10 min. Centrifugation at 12,000 rpm for 5 min at 4°C was done. The top phenol layer (300 µl) was transferred into a new tube and 3x the volume of the supernatant with precooled 0.1 M ammonium acetate was added in methanol. It was then vortexed and incubated at -20°C overnight.

The proteins were made to pellet by centrifugation (12,000 rpm for 5 min at 4°C). Supernatant was discarded. The pellet was washed once by adding 1000 µl of pre-cooled 0.1M ammonium acetate in methanol, then vortexed. Centrifugation was done at 12,000 rpm for 5 min. Supernatant was discarded. The pellet was washed by adding 1000 µl of precooled 80% v/v acetone, vortex. Centrifugation was done at 12,000 rpm for 5 min. Pellets were then air dried, 25 µl of PBS was added. Vortexing was done till it homogenized and 25 µl of 2x-SDS sample buffer was added, the solution was then boiled at 100°C for 10 min. Immediately, it was placed on ice and leave on ice and left for 30 min to overnight and 8 µl of the sample was loaded for SDS-PAGE [35,36] (Table 1, 2).

Experimental care

Adult male Wistar rats weighing between 93 g and 136 g were obtained from the Laboratory Animal Centre, at the College of Medicine, University of Lagos, Idiaraba, Lagos and were placed in polypropylene cages, with laboratory grade pine shavings as bedding. The rats were allowed to acclimatize to the experimental room condition (temperature 25°C and 12 h light/dark cycle) for 1 week prior to commencement of experiments. The rats were fed with rat chow and tap water throughout the period of experiment. The animals were randomly allocated into two groups (n=10 per group) on the basis of administration of distilled water *via* the intravenous route (control group); group1, and administration of the snake venom solution *via* the intravenous route; Group 2. Two (2) ml of solution were administered in each case.

Blood collection and chemistry

After 10 h post administration, the blood of the Wistar rats were collected through retro-orbital sinus bleeding using a capillary tube into plain and EDTA bottles. The experimental animals died upon blood collection. The following biochemical indices were later determined on the blood samples: Aspartate Amino Transferase (AST), Alanine Amino Transferase (ALT), Alkaline Phosphatase (ALP), urea, creatinine, Sodium (Na), Potassium (K), Chlorine (Cl), Bicarbonate (HCO3), cholesterol, triglycerides, High Density Lipoproteins (HDL), Low Density Lipoproteins (LDL) and direct bilirubin contents. They were determined using commercially available kits (Randox laboratories, UK). The full blood count of the blood samples; Packed Cell Volume (PCV), White Blood Cells (WBC), neutrophils, lymphocytes, Red Blood Cells (RBC) were

Table 1: The 10% separating gel was mixed in the following order.

H_2O	41 mL
Acrylamide/bis (30%)	33 mL
Tris-HCl (1.5 m, pH 8.8)	25 mL
SDS, 10%	1000 µL
N,N,N′,N′-tetramethylethylenediamine (TEMED)	100 µL
Ammonium Persulfate (APS), 10%	320 µL

Table 2: The stacking gel was then prepared in the following order.

H_2O	61 mL
Acrylamide/bis (30%, 37.5:1)	13 mL
Tris-HCl (0.5 m, pH 6.8)	25 mL
SDS, 10%	1000 µL
TEMED	100 µL
Ammonium Persulfate (APS), 10%	1000 µL

also determined. The manual hematocrit centrifuge was used for PCV determination, Wright's stain was used for RBC count and lymphocytes and neutrophils determination. The Türk's solution was used for the WBC counts which were examined under a light microscope [37].

Results

Concentration of protein in snake venom of Puff adder (*Bitis arietans*)

The Bradford protein assay was used to measure the concentration of total proteins in the venom sample. The protein, Bovine Serum Albumin (BSA) was used as a standard for the venom sample protein quantification. Concentrations of 20 µg/ml to 100 µg/ml of BSA were created, and a spectrophotometer was used to calculate the absorbance of BSA at 595 nm. The snake venom protein concentration was computed from the calibration curve.

The precise protein concentration of the venom sample was determined by the extrapolation from the standard curve made by measuring the concentration of the sample protein from the standard curve whose absorbance was already determined. The snake venom protein was computed as 121.66 ± 7.94 µg/ml.

Purification of venom proteins of puff adder (*Bitis arietans*) using SDS-PAGE

The Figure 1 is an electrophoregram showing the purification of venom proteins from the Puff adder (*Bitis arietans*) through the process of Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Blood chemistry analysis of Wistar rats administered with the venom of puff adder (*Bitis arietans*) snake and distilled water

The hematological profile and biochemical parameters determined from the blood of the Wistar rats administered the venom of the Puff adder (*Bitis arietans*) and distilled water are presented in Tables 3-5.

Discussion

This study assessed the proteins present in the snake venom of the Puff adder (*Bitis arietans*) and its effect on the blood chemistry of Wistar rats upon administration. The results revealed the presence of various proteins; it also showed significant changes in some hematological parameters of the Wistar rat's envenomated with snake venom when compared to that of the control group which was administered with distilled water. The concentration of the snake venom proteins was 121.66 ± 7.94 µg/ml. This confirms the presence of proteins in the snake venom of Puff adder. These proteins are of great value, due to their diversified and distinct pharmacological activity, high affinity and selectivity towards their receptors [13,28,37]. Five specific proteins were identified from the snake venom of *Bitis arietans*, with molecular weights of 45, 66.2, 25, 18.4 and 14.4 kDa. The Puff adder (*Bitis arietans*) venom has shown to contain mostly lower molecular weight proteins, with one significantly higher molecular weight protein which lies between 45 kDa and 66.2 kDa. The composition of *Bitis arietans* venom studied at the proteomic level by [37], contains the following families: disintegrin, kunitz-type inhibitor (venom Kunitz-type), PLA2 (phospholipase A2), cystatin, serine proteinase (peptidase S1), C-type lectin-like (snaclec) and venom metalloproteinase. Isolated proteins from *Bitis arietans* venom have been shown to interact particularly with platelets [15,24,33].

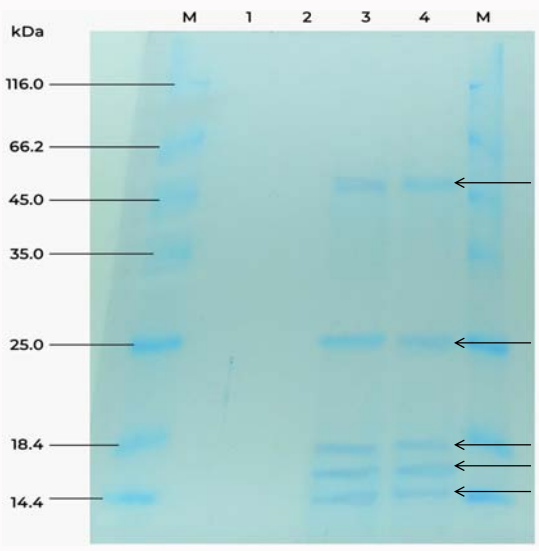

Figure 1: Sodium dodecyl sulphate-poly-acrylamide gel Electropherogram of the puff adder (*Bitis arietans*) venom proteins.
*→= Separated proteins

Data of the study show statistically significant quantitative differences in some hematological parameters in the blood of the envenomated rats compared to the control rats. The results showed significantly higher lymphocytes, White Blood Cells (WBCs) and neutrophils blood level in the group administered with the snake venom than the control group (Lymphocytes: study group =26%, control group =31%, P=0.01; WBC: Control group =5000 MM^3, study group =25000 MM^3, P=0.01; Neutrophils: study group =71%, control group =69%, P=0.03). A high WBC count, and lymphocyte count, also called lymphocytosis, is usually due to an infection or other inflammatory conditions [7,8,38].

During an infection or illness, the body often produces extra lymphocytes to help fight it. Like other types of white blood cells, lymphocytes play a key role in helping the body's immune system fight cancers or foreign viruses, bacteria and parasites. Having a high percentage of neutrophils in the blood is called neutrophilia. This is usually a sign that the body has an infection. Neutrophilia can point to a number of underlying conditions and factors, including infection, most likely bacterial [39,40].

The hematological profile of the envenomated rats shows a significant reduction in the Red Blood Cell (RBC) count of the study group (study group =3.2 × 10^{12}/L, control group =7.5 × 10^{12}/L, P=0.01). The common causes are hemolysis or hemorrhage, which results in a sudden reduction in RBCs. Hemorrhage caused by snake venom is often complicated and aggravated in patients presenting with blood clotting disturbances [39,41].

The serum concentrations of urea, creatinine, Alkaline Phosphatase (ALP), Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), activities, HCO_3, Na, Cl and total bilirubin levels of the envenomated rats were significantly lower than in the control rats. Low activity of AST is normally found in the blood when body tissue or an organ such as the heart or liver is diseased or damaged, additional AST is released into the bloodstream [5,18,20]. The amount of AST in the blood is directly related to the extent of the tissue damage. ALT is also an indicator of liver damage and is the enzyme found in the bloodstream, it helps break down proteins in the body and exists in different forms, depending on where it originates.

Table 3: Hematological profile of envenomated male Wistar albino rats.

Group	PCV (%)	WBC (MM^3)	Lymphocytes (%)	Neutrophils (%)	RBC ($\times 10^{12}$/L)
Distilled water (control)	34 ± 2.11	5000 ± 1.03	31 ± 3.15	69 ± 4.99	7.5 ± 0.28
Snake venom (2 ml/kg body weight)	35 ± 4.70	25000 ± 2.82	26 ± 2.35	74 ± 1.87	3.2 ± 3.0

Table 4: Serum Lipid profile of envenomated male Wistar albino rats.

Group	Cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
Distilled water (control)	87 ± 0.06	40 ± 1.50	34 ± 0.30	45 ± 1.30
Snake venom (2 ml/kg body weight)	89 ± 0.04	38 ± 1.70	30 ± 0.70	50 ± 1.72

Table 5: Serum biochemical parameters of envenomated male Wistar albino rats.

Group	Urea (mg/dl)	Creatinine (mg/dl)	ALP (U/L)	ALT (U/L)	AST (U/L)	HCO_3 (Meq/l)	Na (Meq/l)	Cl (Meq/l)	Total Bilirubin (md/dl)
Distilled water (control)	41 ± 1.48	0.3 ± 0.17	610 ± 13.1	133 ± 2.14	375 ± 87.16	33 ± 1.14	152 ± 3.80	129 ± 11.92	0.8 ± 0.03
Snake venom (2ml/kg body weight)	30 ± 1.61	0.6 ± 1.07	453 ± 21.2	115 ± 2.30	185 ± 86.13	30 ± 0.55	158 ± 3.40	117 ± 14.67	0.4 ± 0.02

Abnormal levels of ALP in the blood most often indicate a problem with the liver, gallbladder, or bones [13,41].

Conclusion

Data of the present study indicate that the snake venom proteins isolated and purified from *Bitis arientan* (Puff adder) in Nigeria contains 5 specific proteins with low molecular weights between 14.4 kDa and 66.2 kDa. In addition, the envenomized rats with this snake venom resulted in and may serve as potential anticoagulants, whose mechanism of action could be explored in the development of therapeutic agents as Antivenom, in the management of snake bites [4].

Understanding the biological effects of snake venom and the complete proteome analysis of particular snake venom is beneficial, because it helps for easier identification of novel venom components by database search. It also aids in prediction of the consequences of snakebites and their effective neutralization [38,42,43].

This has led to a better understanding of the pathological effects of a snake bite and important analysis of the physiological processes in which the venom components are involved [39-41]. Venom components can serve as potential anticoagulants and their mechanism of action can be used for design of new therapeutic agents for blood coagulation and other hematological disorders.

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