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Proteomic analysis of three medically important Nigerian Naja (*Naja haje, Naja katiensis* and *Naja nigricollis*) snake venoms

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ABSTRACT

Proteomics technologies enable a comprehensive study of complex proteins and their functions. The venom proteomes of three medically important Nigerian *Elapidae* snakes *Naja haje, Naja katiensis* and *Naja nigricollis* was studied using HILIC coupled with LC-MS/MS analysis. Results revealed a total of 57, 55, and 46 proteins in the venoms of *N. haje*, *N. katiensis,* and *N. nigricollis*, respectively, with molecular mass ranging between 5 and 185 kDa. These snakes have 38 common proteins in addition to 3 uncommon proteins: actiflagelin, cathelicidin, and cystatin identified in their venoms. The identified proteins belonged to 14 protein families in *N. haje* and *N. katiensis*, and 12 protein families in *N. nigricollis*. Of the total venom proteins, 3FTx was the most abundant protein family, constituting 52% in *N. haje* and *N. katiensis*, and 41% in *N. nigricollis*, followed by PLA_2, constituting 37% in *N. nigricollis*, 26% in *N. haje*, and 24% in *N. katiensis*. Other protein families, including LAAO, CRISPs, VEGF, PLB, CVF, SVMP, SVH, AMP, PI, Globin, Actin, and C-type lectins, were also detected, although, at very low abundances. Quantification of the relative abundance of each protein revealed that alpha and beta fibrinogenase and PLA_2, which constituted 18–26% of the total proteome, were the most abundant. The 3 uncommon proteins have no known function in snake venom. However, actiflagelin activates sperm motility; cystatin inhibits angiogenesis, while cathelicidin exerts antimicrobial effects. The three Nigerian *Naja* genus proteomes displayed 70% similarity in composition, which suggests the possibility of formulating antivenom that may cross-neutralise the venoms of cobra species found in Nigeria. These data provide insights into clinically relevant peptides/proteins present in the venoms of these snakes. Data are available via ProteomeXchange with identifier PXD024627.

1. Introduction

Due to the high number of deaths from venomous snakebites, the World Health Organization has reconsidered snakebite envenomation as a neglected tropical disease (Chippaux, 2017). Envenomation is predominant in some tropical and subtropical areas (Gutiérrez et al., 2010). Envenomation by *Elapidae* snakes is one of the leading causes of morbidity and mortality in Sub-Saharan Africa (Gutierrez et al., 2006). Approximately 5.4 million people are bitten by snakes annually, with an estimated 2.7 million cases of envenomation (World Health Organization, 2019). About 138,000 fatalities due to snake envenomation are reported annually, with a 3-fold increase in the cases of amputation and permanent physical disability. In Nigeria, *Elapidae* is the second most venomous snake family. The incidence of bites by *Elapidae* snakes, especially *N. nigricollis*, constitutes the estimated 497 cases of envenomation for 100,000 inhabitants annually (Habib et al., 2001; Habib, 2013). This scourge raises public health concerns and constitutes occupational hazards for most youths of productive age, outdoor workers, and agriculturists (Habib et al., 2001; Habib, 2013; Kunalan et al., 2018).

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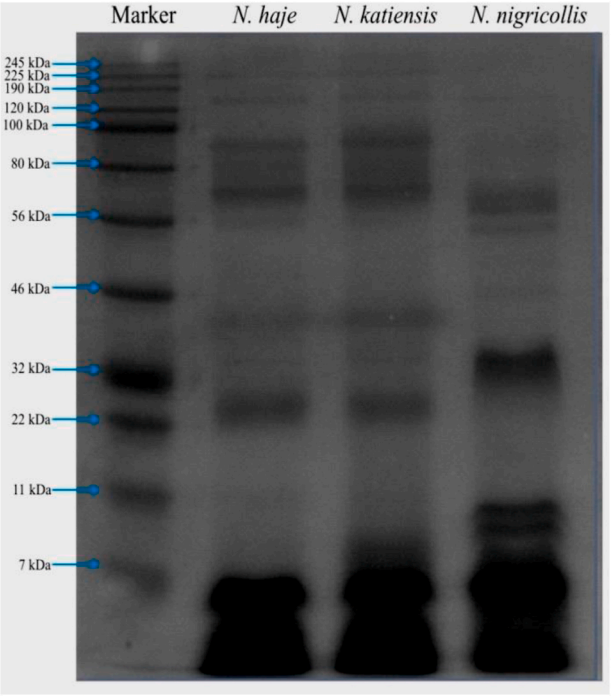

Fig. 1. 1D SDS-PAGE profile of Nigerian *N. haje, N. katiensis,* and *N. nigricollis* venoms.

Snake venoms, which are complex cocktails secreted by a pair of specialised glands, have a peculiar intricacy compared to spider, scorpion, and cone snail venoms (Casewell et al., 2013; Zelanis and Tashima, 2014; Utkin, 2015). The venoms of this group of animals exert pharmacological effects on the victims mainly by the disulfide-bridged peptides, while snake venoms are composed of large and numerous biologically active proteins and peptides (Casewell et al., 2013; Utkin, 2015; Kalia et al., 2015). Venom and venom glands have been extensively explored using well-defined proteomic and transcriptomic approaches (Oldrati et al., 2016; Pla, 2017) to unravel the toxin and non-toxin components and identify the major toxic components. These approaches have facilitated the understanding of disease mechanisms, thus enhancing therapeutic targets in envenomed individuals. Snake venom from over 100 snake species have been analysed using proteomic approaches (Fry et al., 2003; Fox and Serrano, 2008; Tasoulis and Isbister, 2017), but data on the venom composition of Nigerian *Elapidae* snakes *N. haje, N. katiensis*, and *N. nigricollis* (Petras et al., 2011) are scarce and not yet fully elucidated.

It is believed that factors such as the snake's diet (Barlow et al., 2009), age (Dias et al., 2013), geographical location (Durban et al., 2017; Goncalves-Machado et al., 2016), type of natural habitat, (Menezes et al., 2006; Zelanis et al., 2016), and the season in which the snakes are most active (Alape-Giro et al., 2008; Nunez et al., 2009) may determine the venom composition. Mutations in the venom-related genes may influence the venom composition. These factors may also cause variations in snake venom at the levels of species, genera (Fry et al., 2008; Tasoulis and Isbister, 2017) and families (Alape-Giro'n et al., 2008; De Lima et al., 2005). In this study, we used HILIC coupled with LC-MS/MS to analyse the venoms of the Nigerian *Naja haje* (Broadley, 1998), *Naja* (Afronaja) *katiensis* (Wallach et al., 2009), and *Naja nigricollis nigricollis* (Spawls et al., 2018). To the best of our knowledge, this is the first study to investigate the venom profile of Nigerian *Elapidae* snakes using proteomic analysis.

2. Materials and methods

2.1. Venom samples

Adult snakes from each species (*N. haje*, *N. katiensis*, and *N. nigricollis)* were captured from different regions of Nigeria. Venoms were extracted from individual snakes of the same species, pooled together, lyophilised, and stored at −20 °C until further use.

2.2. Chemicals and reagents

Hydrophilic interaction liquid chromatography (HILIC) magnetic bead particles were purchased from Sigma Aldrich USA. Standard marker proteins and analytical-grade trypsin were obtained from Agilent Technologies (Santa Clara, CA, USA). All chemicals and reagents used in this study were of analytical grade.

2.3. Protein extraction from the crude venom and pellet solubilisation

Two milligrams (dry-weight) of the lyophilised crude venom (from each species) were solubilised in 50 μL 1X PBS (pH 7.4), vortexed for 10 min, and centrifuged at 15,700×*g* for 5 min at 4 °C. Acetone precipitation was performed on the supernatant by the addition of 600 μL cold acetone, and the samples were incubated at −20 °C for 15 min. Precipitated samples were centrifuged at 15,700×*g* for 15 min at 4 °C. Protein pellets were air-dried and resuspended in 100 μL 1X PBS buffer (pH 7.4). Protein concentration of each sample was quantified using the RC DC Protein Assay Kit 11 (Bio-Rad Laboratories) and confirmed with the microvolume protein concentration determination method previously described (Desjardins et al., 2009). Data was acquired on a Nanodrop Spectrophotometer 2000c (Thermo Fisher Scientific, USA).

2.4. 1D SDS-PAGE analysis

A fraction of each sample (15 μg) was denatured and fractionated on a 12% SDS-PAGE gel for 90 min at 100 V. After gel electrophoresis, the SDS-PAGE gel was stained with Coomassie Brilliant Blue G-250 for 30 min and transferred to a de-staining solution (10% glacial acetic acid and 1% glycerol) for 2 h. Resolved protein bands were visualised using the Molecular Imager PharosFX Plus System (Bio-Rad, California, USA).

2.5. Venom protein extraction using HILIC and trypsin digestion

Fifty micrograms (50 μg) of protein from each venom sample was suspended in 50 mM triethylammonium bicarbonate (TEAB; Sigma) before reduction with 10 mM (DTT; Sigma) in 50 mM TEAB for 40 min at 56 °C. Cysteine residues were alkylated with 30 mM iodoacetamide (Sigma) in 50 mM TEAB for 30 min at 20–25 °C in the dark. After alkylation, two-fold dilutions of the samples were made with binding buffer (100 mM ammonium acetate, 30% acetonitrile, pH 4.5). The protein solution was added to pre-equilibrated MagResyn® HILIC magnetic particles (Resyn Biosciences) prepared according to the manufacturer's instructions and incubated overnight at 4 °C. After binding, the supernatant was removed and magnetic particles washed twice with 95% acetonitrile for 1 min. For digestion, the magnetic particles were suspended in 50 mM ammonium formate (pH 8.0) containing trypsin (Promega) to a final ratio of 1:10 and incubated overnight at 27 °C with constant shaking. Following digestion, peptides were recovered with 1% trifluoroacetic acid (TFA) and incubated at room temperature for 3 min and analysed using liquid chromatography with tandem mass spectrometry (LC-MS/MS).

2.6. Liquid chromatography using Dionex nano-RSLC

Chromatography was performed using a UltiMate™ 3000 RSLCnano System (Thermo Fisher Scientific, USA) equipped with a C_{18} trap column

Table 1
Proteins identified in the venoms of *N. haje*, *N. katiensis,* and *N. nigricollis*.

Protein/peptides identified				
Index	*N. haje*	*N. katiensis*	*N. nigricolis*	Protein family
NP1	*A. superbus* venom factor 1	*A. superbus* venom factor 1	*A. superbus* venom factor 1	CVF
NP39	Ophiophagus venom factor	Ophiophagus venom factor	Ophiophagus venom factor	
NP51	Venom nerve growth factor	Venom nerve growth factor	Venom nerve growth factor	
NP3	Acidic phospholipase A_2 homolog	Acidic phospholipase A_2 homolog	Acidic phospholipase A_2 homolog	PLA_2
NP41	Acidic phospholipase A2 CM-I	Acidic phospholipase A2 CM-I	Acidic phospholipase A2 CM-I	
NP40	Phospholipase A_2 "basic."	Phospholipase A_2 "basic."	Phospholipase A_2 "basic."	
NP7		Basic phospholipase A_2 10	Basic phospholipase A_2 10	
NP43	Phospholipase-B 81	Phospholipase-B 81	Phospholipase-B 81	PLB
NP4	Actiflagelin	Actiflagelin	Actiflagelin	Actin
NP5	Actin, alpha skeletal muscle	Actin, alpha skeletal muscle	Actin, alpha skeletal muscle	
NP11	Cysteine-rich venom protein 1	Cysteine-rich venom protein 1		CRISPs
NP12, NP13, NP15	Cysteine-rich venom protein LEI1	Cysteine-rich venom protein LEI1	Cysteine-rich venom protein	
NP14, NP14, NP15	Cysteine-rich venom protein	Cysteine-rich venom protein	Cysteine-rich venom protein ablominutes	
NP14, NP16		Cysteine-rich venom protein	Cysteine-rich venom protein kaouthin-1	
NP15, NP15, NP17	Cysteine-rich venom protein ablominutes	Cysteine-rich venom protein ablominutes	Cysteine-rich venom protein kaouthin-2	
NP16	Cysteine-rich venom protein kaouthin-1	Cysteine-rich venom protein kaouthin-1		
NP17	Cysteine-rich venom protein kaouthin-2	Cysteine-rich venom protein kaouthin-2		
NP18	Cysteine-rich venom protein pseudechetoxin-like			
NP25, NP26	Haemoglobin subunit alpha	Haemoglobin subunit alpha-D		Globin
NP26, NP27	Haemoglobin subunit alpha-D	Haemoglobin subunit beta		
NP27	Haemoglobin subunit beta			
NP9	Cathelicidin-related peptide Bf-CRAMP	Cathelicidin-related peptide Bf-CRAMP	Cathelicidin-related peptide Bf-CRAMP	AMP
NP10	Cystatin	Cystatin	Cystatin	PI
NP38	Nawaprin	Nawaprin	Nawaprin	
NP30, NP44	Kunitz type serine protease inhibitor	Kunitz type serine protease inhibitor	Serine protease harobin	
NP31, NP44	Kunitz type serine protease inhibitor 2	Serine protease harobin		
NP44	Serine protease harobin			
NP47, NP32	Snake venom 5′-nucleotidase	L-amino-acid oxidase	Snake venom 5′-nucleotidase	LAAO
NP32	L-amino-acid oxidase	L-amino-acid oxidase	L-amino-acid oxidase	
NP33	L-amino-acid oxidase			
NP47	Snake venom 5′-nucleotidase			
NP46	Snaclec bitiscetin subunit alpha	Snaclec bitiscetin subunit alpha	Snaclec bitiscetin subunit alpha	C-type Lectins
NP49	Snake venom vascular endothelial growth factor toxin barietin	Snake venom vascular endothelial growth factor toxin barietin	Snake venom vascular endothelial growth factor toxin barietin	VEGF
NP8, NP19	Bucandin	Cytotoxin 2	Cytotoxin 2	3FTx
NP34, NP8	Long neurotoxin 1	Bucandin	Long neurotoxin 1	
NP35, NP34	Long neurotoxin 2	Long neurotoxin 1	Long neurotoxin 2	
NP36, NP35	Long neurotoxin 1	Long neurotoxin 2	Long neurotoxin 1	
NP37, NP34	Long neurotoxin OH-55	Long neurotoxin 1	Long neurotoxin OH-55	
NP42, NP37, NP45	Alpha-cobratoxin	Long neurotoxin OH-55	Short neurotoxin 1	
NP45, NP42, NP20	Short neurotoxin 1	Alpha-cobratoxin	Cytotoxin 3	
NP20, NP45, NP21	Cytotoxin 3	Short neurotoxin 1	Naniproin	
NP23, NP20, NP22	Cytotoxin 7	Cytotoxin 3	Cytotoxin 4	
NP24, NP21, NP23	Cytotoxin homolog S3C2	Naniproin	Cytotoxin 7	
NP56, NP23, NP24	Cytotoxin 11	Cytotoxin 7	Cytotoxin homolog S3C2	
NP52, NP56, NP52	Weak toxin CM-2	Cytotoxin 11	Weak toxin CM-2	
NP53, NP50, NP53	Weak toxin CM-2a	Three-finger toxin W–V	Weak toxin CM-2a	
NP54, NP52, NP54	Weak toxin CM-10	Weak toxin CM-2	Weak toxin CM-10	
NP55, NP53, NP55	Weak toxin CM-13b	Weak toxin CM-2a	Weak toxin CM-13b	
NP2, NP52	Acetylcholinesterase	Weak toxin CM-10	Acetylcholinesterase	
NP55		Weak toxin CM-13b		

(*continued on next page*)

Table 1 (*continued*)

Protein/peptides identified				
Index	*N. haje*	*N. katiensis*	*N. nigricolis*	Protein family
NP2		Acetylcholinesterase		
NP6, NP48	Alpha- and beta-fibrinogenase OhS1	Alpha- and beta-fibrinogenase OhS1	Snake venom metalloproteinase-disintegrin-like mocarhagin	SVMP
NP48, NP48, NP57	Snake venom metalloproteinase-disintegrin-like mocarhagin	Snake venom metalloproteinase-disintegrin-like mocarhagin	Zinc metalloproteinase-disintegrin-like BfMP	
NP57, NP57, NP58	Zinc metalloproteinase-disintegrin-like BfMP	Zinc metalloproteinase-disintegrin-like BfMP	Zinc metalloproteinase-disintegrin-like MTP9	
NP58, NP58, NP59	Zinc metalloproteinase-disintegrin-like MTP9	Zinc metalloproteinase-disintegrin-like MTP9	Zinc metalloproteinase-disintegrin-like NaMP	
NP59, NP59, NP60	Zinc metalloproteinase-disintegrin-like NaMP	Zinc metalloproteinase-disintegrin-like NaMP	Zinc metalloproteinase-disintegrin-like atragin	
NP60, NP60, NP61	Zinc metalloproteinase-disintegrin-like atragin	Zinc metalloproteinase-disintegrin-like atragin	Zinc metalloproteinase-disintegrin-like atrase-A	
NP61, NP61, NP62	Zinc metalloproteinase-disintegrin-like atrase-A	Zinc metalloproteinase-disintegrin-like atrase-A	Zinc metalloproteinase-disintegrin-like mikarin	
NP62, NP62, NP28	Zinc metalloproteinase-disintegrin-like mikarin	Zinc metalloproteinase-disintegrin-like mikarin	Hemorrhagic metalloproteinase-disintegrin-like kaouthiagin	
NP28	Hemorrhagic metalloproteinase-disintegrin-like kaouthiagin			
NP29	Hyaluronidase-1	Hyaluronidase-1		SVH

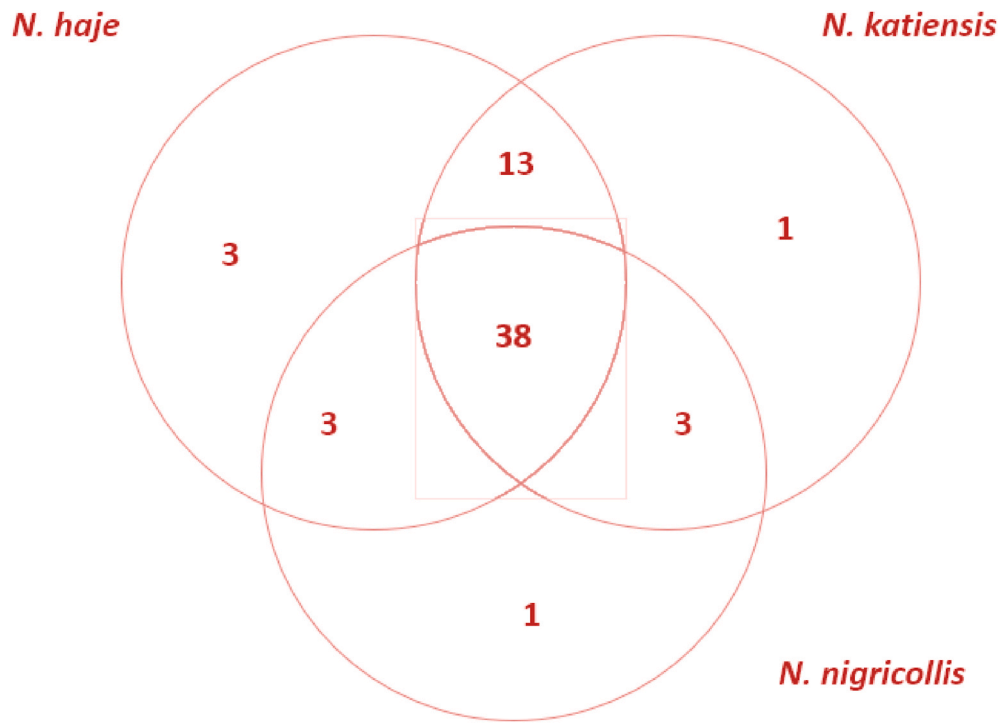

Fig. 2. Venn diagram showing proteins, which are common and uncommon in the venom samples of *N. haje, N. katiensis,* and *N. nigricollis* as revelaed by mass spectrometry.

(5 mm × 300 μm; Thermo Fisher Scientific) and a CSH C_{18} analytical column (1.7 μm, 25 cm × 75 μm; Waters). The solvent system used consisted of the following: Solution A: H_2O w/0.1% formic acid, solution B: acetonitrile with 0.1% formic acid. The samples were loaded on the trap column using the loading solvent at a flow rate of 2 μL/min from a temperature-controlled autosampler set at 7 °C. Loading was performed for 5 min before the sample was eluted from the analytical column. The flow rate was set at 250 nL/min and the gradient was generated as follows: 5–35% solution B for 60 min and 35–50% solution B for 60–75 min. Chromatography was performed at 40 °C, and the outflow was delivered to the mass spectrometer through a stainless-steel nano-bore emitter.

2.7. Liquid chromatography mass spectrometry (LC-MS) analysis

LC-MS analysis was performed on a Fusion mass spectrometer (Thermo Scientific, San Jose, CA) equipped with a nanospray ion source (Nanospray Flex™ Ion Sources, Thermo Fisher Scientific) coupled to a Dionex Ultimate 3000 RSLC nano-HPLC system. Peptides recovered from the on-bead HILIC digestion for each venom sample was introduced through a stainless-steel emitter. Data were collected in positive mode with a spray voltage set to 1.8 kV and ion transfer capillary set to 280 °C. Spectra were internally calibrated using polysiloxane ions at m/z 445.12003 and 371.10024. The first MS scan was performed using the orbitrap detector set to a resolution of 120,000 over an m/z range of

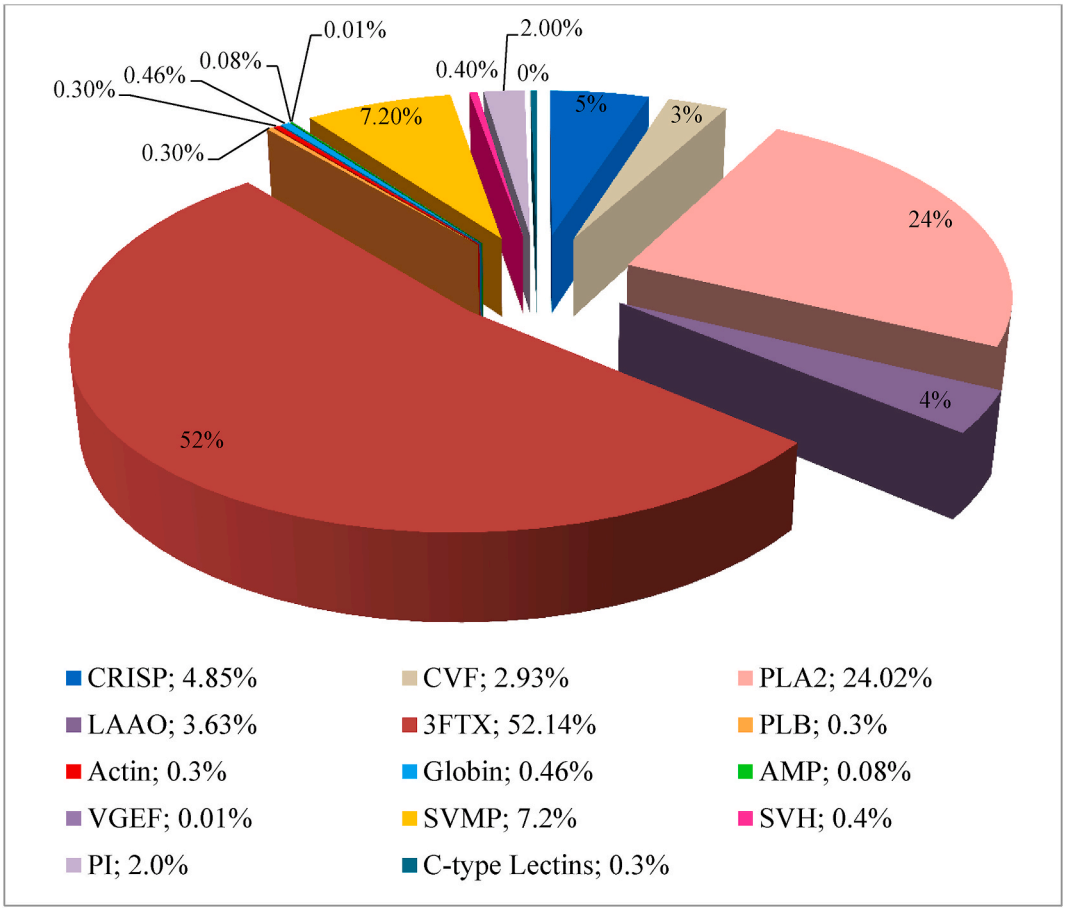

Fig. 3. Relative distribution of protein families identified in the venom proteome of *N. haje*. SVH: snake venom hyaluronidase, SMVP: snake venom metalloproteinase, 3FTx: three-finger toxins, VEGF: vascular endothelial growth factor, AMP: antimicrobial peptide, PLA_2: phospholipase A_2, PLB: phospholipase B, CRISPs: cysteine rich secretory proteins, PI: protease inhibitor, LAAO: L-amino acid oxidase, CVF: cobra venom factor.

350–1650 with an AGC target at 3E5 and maximum injection time of 40 ms. Data were acquired in profile mode. The second MS scan was performed using monoisotopic precursor selection for the ion with charges of +2 to +7 with error tolerance set at ±10 ppm. Precursor ions were excluded for 60 s after fragmentation. Precursor ions were selected for fragmentation in the HCD mode using the quadrupole mass analyser with HCD energy set to 30%. Fragment ions were detected in the orbitrap mass analyser at a resolution of 30,000. The AGC target was set to 5E4 and the maximum injection time was set to 80 ms. Data were acquired in centroid mode.

2.8. Database search and data analysis

The raw files generated by the mass spectrometer were imported into Proteome Discoverer v1.4 software (Thermo Fisher Scientific, USA) and processed using the Sequest and Amanda algorithms. Database interrogation was performed against a concatenated database created by concatenating all 'snake protein' entries in the Uniprot-Serpentes database with the cRAP contaminant database (https://www.thegpm.org/crap/). The precursor mass tolerance was set to 10 ppm and fragment mass tolerance was set to 0.02 Da. Deamination (NQ), oxidation (M), and acetylation of the N-terminal protein were allowed as dynamic modifications and thiomethyl of C was used as a static modification. Peptide validation was performed using the Target-Decoy PSM validator node. The search results were imported into Scaffold Q+ for further validation (www.proteomesoftware.com). Peptide identification, by peptide-spectrum match approach against UniProt-Serpentes database, and subsequent assembly, matching, and identification of protein sequences were performed using X! Tandem and Sequest search engines. The search engines were incorporated into Scaffold Q+, version 4.10.0 at 99% protein threshold, 95% peptide threshold, and two-peptide minimum criterion. The relative abundance of each protein/peptide present in the venom samples of *N. haje*, *N. katiensis*, and *N. nigricolis* was quantified by plotting a graph of the identified proteins against their relative abundance using Stata statistical software (Stata® USA) version 16, 2019.

2.9. Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD024627 and 10.6019/PXD024627, at https://www.ebi.ac.uk/pride/archive/projects/PXD024627.

3. Results and discussion

3.1. 1D SDS-PAGE of the crude snake venoms

The protein banding pattern of the snake venoms was revealed by staining with Coomassie Brilliant Blue. Although the snakes belong to the same genus, the electrophoretic profile showed a difference in the molecular weights of the proteins (Fig. 1). This indicated that the protein profiles of the venoms were heterogeneous, with molecular weights of the proteins ranging from 7 to 225 kDa. The venom proteins of *N. haje* and *N. katiensis* displayed a similar protein banding pattern, while that

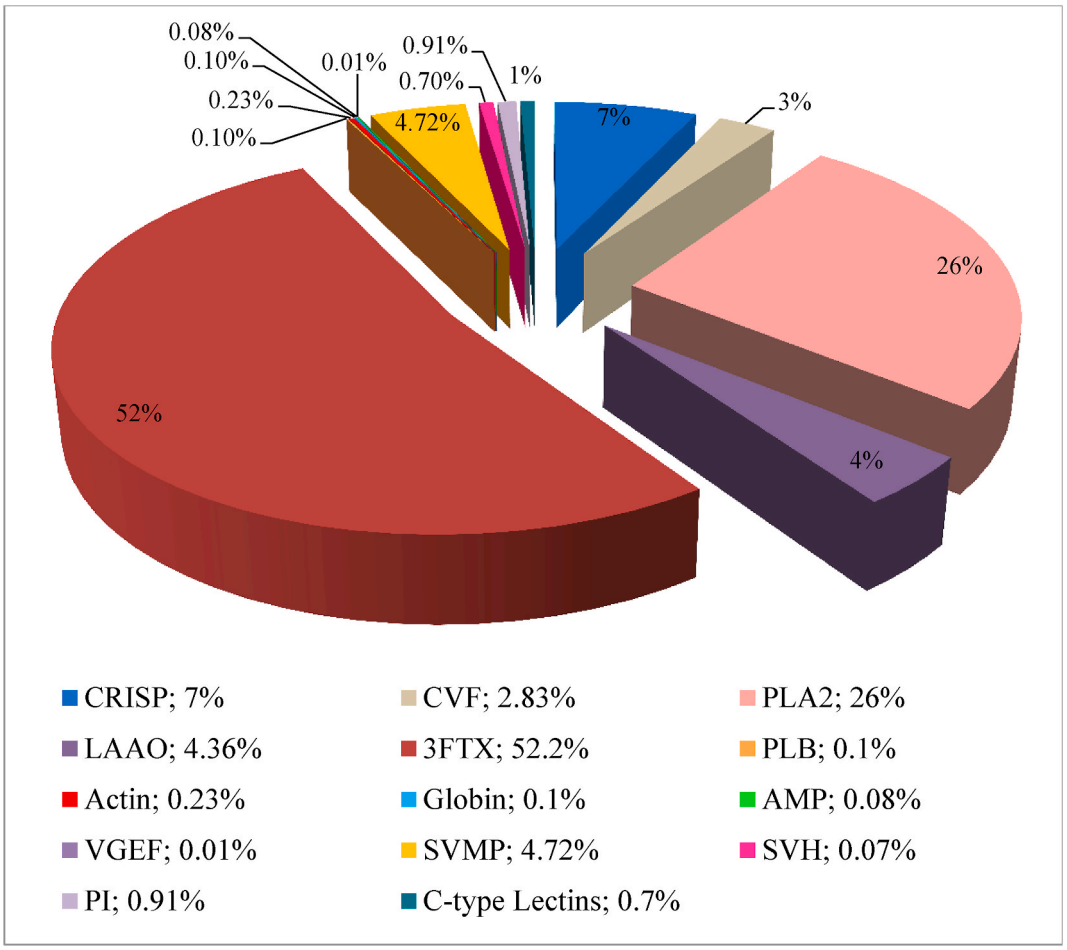

Fig. 4. Relative distribution of protein families identified in the venom proteome of *N. katiensis*. SVH: snake venom hyaluronidase, SMVP: snake venom metalloproteinase, 3FTx: three-finger toxins, VEGF: vascular endothelial growth factor, AMP: antimicrobial peptide, PLA_2: phospholipase A_2, PLB: phospholipase B, CRISPs: cysteine rich secretory proteins, PI: protease inhibitor, LAAO: L-amino acid oxidase, PI: protease inhibitor, CVF: Cobra venom factor.

of *N. nigricollis* was slightly different, with clustered proteins in the regions of <7 kDa, 9–10 kDa, and 32 kDa. The variation observed in the analysed venoms confirms the fact that slight differences may exist in the protein compositions of the venoms of closely related species (Fry et al., 2008; Tasoulis and Isbister, 2017; Gutierrez et al., 2017).

3.2. Proteomic characterisation using LC-MS/MS

Following LC-MS/MS analysis, 57, 55 and 46 proteins were identified in the crude venom extracts of *N. haje*, *N. katiensis*, and *N. nigricollis* respectively (Table 1). The identified proteins had a molecular mass of 5–185 kDa (supplementary file, Table A1) and share toxins belonging to two major protein families; 3FTx and PLA_2. This correlates with previous reports on other African spitting cobras that 3FTx and PLA_2 represent the majority of the toxins in the venom (Petras et al., 2011; Katali et al., 2020). In total, 62 venom proteins were identified (supplementary file, Table A1), of which 38 were common in the three spitting cobras (Fig. 2). In a related study, the venomics of Southern African *Naja mossambica* and *Naja nigricincta nigricincta* revealed that the majority of the identified proteins were common in both snakes (Katali et al., 2020). It is established that the venom proteomes of African spitting cobras displayed resemblance in their compositions despite the differences in taxa or location (Petras et al., 2011). The high similarity among the protein components of Nigerian *N. haje, N. katiensis*, and *N. nigricollis* venoms (Fig. 2) suggests that antivenom raised against these species may cross-neutralise the venom of other cobra species indigenous to Nigeria.

Proteins were identified using Multi-Dimensional Protein Identification Technology (MuDPIT) incorporated on Scaffold Proteome software version 4.10.0 at 99% protein threshold, 95% peptide threshold, 0.5% false discovery rate (FDR), and two-peptide minimum criterion. Peptides and proteins were searched against the UniprotKB- Serpentes-database. SVH: Snake venom hyaluronidase, SMVP: Snake venom metalloproteinase, 3FTx: three finger toxins, VEGF: vascular endothelial growth factor, AMP: antimicrobial peptide, PLA_2: phospholipase A_2, PLB: phospholipase B, CRISPs, cysteine rich secretory proteins, LAAO: L-amino acid oxidase, CVF: Cobra venom factor. We characterised proteins using Sequest and X!Tandem incorporated Scaffold Proteome Software 4.10.0: 2019/100. NP: Naja Protein, NP1-NP62 represents each of the proteins detected in the analysed venoms. To obtain the relative abundance of the individual proteins, a graph of the identified proteins was plotted against their relative abundance, as shown in Fig. 6.

In a parallel comparison, *N. haje* and *N. katiensis* had 13 proteins in common; *N. haje* and *N. nigricollis* had 1 protein in common, while *N. katiensis* and *N. nigricollis* had 3 common proteins (Fig. 2). There were 3 exclusive proteins in the venom of *N. haje* and 1 exclusive protein in *N. katiensis* and *N. nigricollis* (Fig. 2). Interestingly, this study identified 3 uncommon proteins in the venoms of the Nigerian spitting cobras: actiflagelin (3NOJ_WALAE) from *Walterinnesia aegyptia*, cystatin (CYT_NAJKA) from *Naja kaouthia,* and cathelicidin (CAMP_BUNFA) from *Bungarus fasciatus*. The function of these proteins in snake venom is unknown. However, they are valuable molecules with potential applications in biomedicine (Koh et al., 2006; Peigneur and Tytgat, 2018; Harvey, 2014; Abd El-Aziz et al., 2019). Based on biological functions,

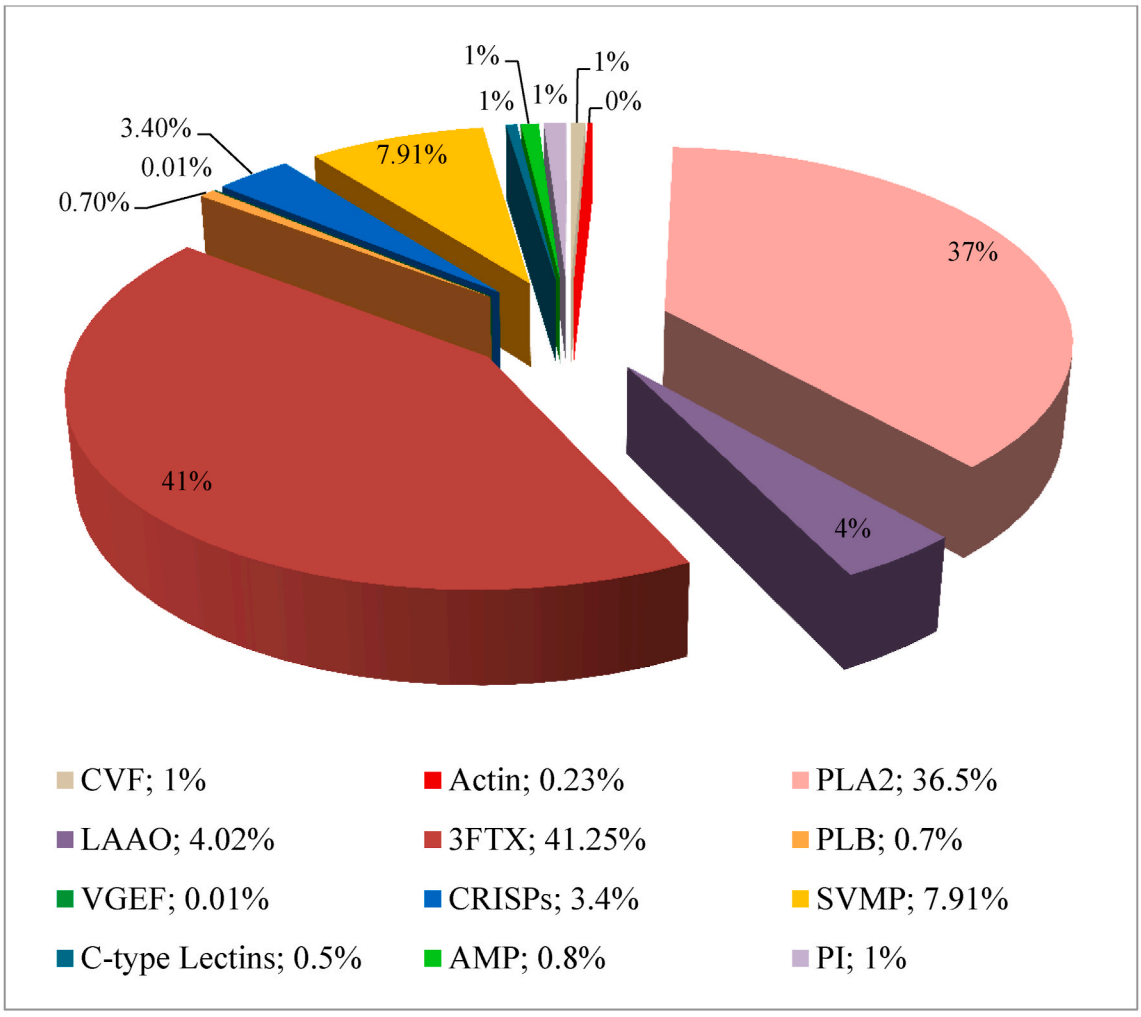

Fig. 5. Relative distribution of protein families identified in the venom proteome of *N. nigricollis.* SVH: Snake venom hyaluronidase; SMVP: Snake venom metalloproteinase; 3FTx: three-finger peptide toxins; VEGF: vascular endothelial growth factor; PLA_2: phospholipase A_2; PLB: phospholipase B; CRISPs; cysteine rich secretory proteins; CVF: Cobra venom factor; LAAO: L-Amino Acid Oxidase.

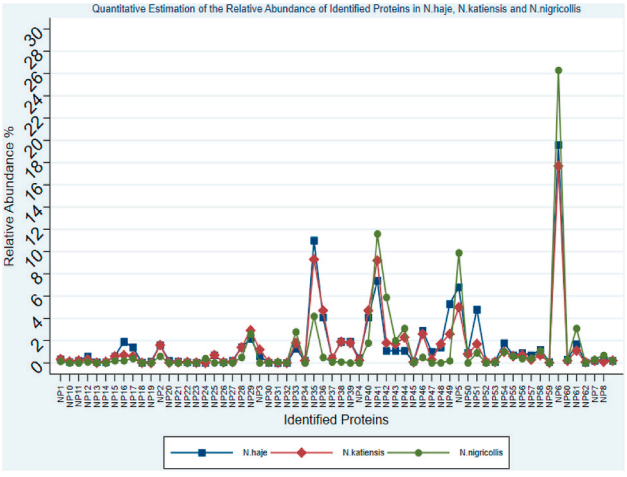

Fig. 6. The relative abundance of the individual proteins identified in the venom proteomes of *N. haje*, *N. katiensis,* and *N. Nigricollis*. NP: Naja Protein, NP1-NP62 represents each of the proteins detected in the analysed venoms.

actiflagelin has been reported to activate sperm motility (Abd El-Aziz et al., 2018). Cystatin is an inhibitor of cysteine proteases and has been reported to inhibit angiogenesis by downregulating the expression of vascular endothelial growth factor and basic fibroblast growth factor (Xie et al., 2013; Brenik et al., 2019). Cathelicidin is known to be an antimicrobial agent (De Barros et al., 2019).

3.3. Relative abundance of the protein families

The relative abundance of protein families identified in this study was expressed as percentages in the pie charts (Figs. 3–5). The results showed that 3FTx was highly abundant in the venoms of all three spitting cobras with a representation of 52% in *N. haje* and *N. katiensis* (Figs. 3 and 4), followed by 41% *N. nigricollis* (Fig. 5). The second most abundant protein family identified in this study was PLA_2 with a representation of 37% in *N. nigricollis*, 26% in *N. haje* and 24% in *N. katiensis*. This result suggest that envenomation by *Elapidae* would most likely induce cytotoxicity in the victims, particularly haemotoxic effects, due to the presence of 3FTx toxins. Our findings are in agreement with the commonly described protein family distribution pattern in *Elapidae*, where more than 75% of their total venom composition consist of two major protein families; PLA_2s and 3FTxs (Tasoulis and Isbister, 2017; Laustsen et al., 2015; Guitierrez et al., 2006, 2009). Snake venom metalloproteinase (SVMP) represented 5–8% of the total proteins identified whereas cysteine-rich secretory proteins (CRISPs) represent

between 3 and 7%. In addition, L-amino acid oxidase (LAAO) represented 4% of the total proteins identified in the venoms of these cobra species. Snake venom hyaluronidases (SVH), vascular endothelial growth factor (VEGF), antimicrobial peptide (AMP), phospholipase B (PLB), globins, C-type lectins, actin, protease inhibitors, and cobra venom factor (CVF) were also detected, albeit, at a very low abundances ranging from 0.01% to 2.93% of the venom proteomes (Figs. 3–5). The proteins identified in the venoms of these snakes belonged to 14 protein families in *N. haje* and *N. katiensis* and 12 protein families in *N. nigricollis* (Table 1).

3.4. The relative abundance of individual proteins

From the estimated relative abundance of the individual proteins identified in the venom of these snakes, alpha and beta fibrinogenase (NP6) constituted the most abundant protein in *N. nigricollis* (26%), *N. haje* (19%), and *N. katiensis* (18%) respectively. Acidic phospholipase A_2 (NP41) was the second most abundant individual protein, with a representation of 12% in *N. nigricollis*, 9% in *N. katiensis* and 7% in *N. haje* respectively. This was followed by long neurotoxin (NP35) which constituted 11% in *N. haje*, 9% in *N. katiensis*, and 4% in *N. nigricollis*. Actin alpha skeletal muscle (NP5) represented 10% of the individual proteins in *N. nigricollis*, 6.5% in *N. haje*, and 5% in *N. katiensis* (Fig. 6). This suggests that diverse biochemical and toxicological effects would be provoked in Naja-envenomed victims, notably neurotoxic and fibrinogenolytic effects.

3.5. Toxicological effects of the proteins and toxins

The major toxic proteins identified in the venoms of these Nigerian *Elapidae* have both enzymatic and non-enzymatic activities associated with their toxicity. The toxin protein classes (supplementary file, Table A.2) included phospholipase A_2 (4 proteins), phospholipase B (1 protein), acetylcholinesterase (1 protein), L-amino acid oxidase (1 protein), snake venom 5′ endonuclease (1 protein), and alpha and beta fibrinogenase (1 protein).

Phospholipase A_2 present in the venoms of these snakes (with a molecular mass ranging between 13 and 15 kDa) exert a wide range of biochemical activities, including neurotoxic and myotoxic effects (Harris and Scott-Davey, 2013). These toxins exert neurotoxic and proinflammatory effects by modulating presynaptic terminals and sensory nerve endings (Camara et al., 2003; Harris and Scott-Davey, 2013; Sribar et al., 2014). The presynaptic effects of phospholipase A_2 are characteristic of beta neurotoxins that target the motor nerve terminals at the neuromuscular junction (Sribar et al., 2014; Gutierrez et al., 2017; Ferraz et al., 2019).

Serine protease inhibitors exert ion channel blocking activities (Boldrini-Franç;a et al., 2020). Two isoforms of these peptides have been detected in the analysed venoms of Nigerian spitting cobra species.

L-amino acid oxidases are among the well-studied protein families in snake venom (Kunalan et al., 2018). These toxins are responsible for inducing apoptosis, haemorrhage, platelet aggregation, and cytotoxic effects in snakebite victims (Carmago et al., 2008; Aird, 2002; Guo et al., 2012; Stabeli et al., 2004; Ferraz et al., 2019).

Another toxin with enzymatic activities, identified in the venom proteomes of these snake species, was acetylcholinesterase (AChE). AChE is a membrane-bound protein that induces neuromuscular paralysis (Frobert et al., 1997; Cousin and Bon, 1999). This enzyme terminates signal transduction through the rapid hydrolysis of acetylcholine and α-and β-fibrinogenases linked to fibrinogenolytic activity, and hemostasis-impairing-toxin-exerting coagulopathy.

Some non-enzymatic proteins with reported toxic and pharmacological activities such as cysteine-rich secretory proteins (CRiSPs), cytotoxins and vascular endothelial growth factor were also identified in the venoms of the cobra species analysed in this study. They synergistically contribute to the wide range of clinical manifestations associated with envenoming (Gutierrez et al., 2017; Ferraz et al., 2019). Snaclec, which is a well-known anticoagulation and pro-coagulation agent (Morita, 2005; Clemetson, 2010; Vaiyapuri et al., 2012), was among the non-enzymatic proteins identified in this study. Long and short neurotoxins are a class of snake venom toxins belonging to the 3FTx family. Four isoforms of these proteins were identified in this study. They bind strongly to muscular and neuronal acetylcholine nicotinic receptors, inhibit neuronal and neuromuscular transmission, and lead to paralysis and respiratory failure (Sribar et al., 2014; Gutierrez et al., 2017; Ferraz et al., 2019).

Author contributions

Emeka John Dingwoke, Fatima Amin Adamude, Abdullahi Balarabe Sallau: Conceptualization. Abdullahi Balarabe Sallau, Sani Ibrahim, Mujitaba Suleiman Abubakar: Supervision. Emeka John Dingwoke, Fatima Amin Adamude:Designed the analysis. Emeka John Dingwoke, Fatima Amin Adamude Gadija Mohamed, Ashwil Klein: Investigation. Fatima Amin Adamude, Emeka John Dingwoke: Data analysis. Fatima Amin Adamude, Emeka John Dingwoke: Writing original draft preparation. All authors critically reviewed and edited the final manuscript version.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2021.03.014.

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