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Original Research Article

SELECTED ELEMENTAL COMPOSITION AND AMINO ACID PROFILE OF THE BLACK FOREST COBRA (*NAJA GUINEENSIS*) VENOM

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ABSTRACT

The venom of the black forest cobra (*Naja guineensis*) remains relatively underexplored despite its potential medical and toxicological significance. This study investigated the elemental and amino acid composition of *Naja guineensis* venom to provide insight into its biochemical composition, properties and potential functional roles. Crude venom was obtained from adult *Naja guineensis* and subjected to acid digestion prior to elemental analysis using atomic absorption spectroscopy (AAS). Amino acid composition was determined using high-performance liquid chromatography (HPLC). Elemental analysis revealed the presence of calcium, phosphorus, iron, zinc, and magnesium, with phosphorus occurring at the highest concentration (4650.17 mg/kg). The elevated phosphorus content supports the presence of phospholipase-related activity, which may contribute to the venom's membrane-disrupting properties. Amino acid profiling identified twelve amino acids, including aspartic acid, glycine, threonine, alanine, arginine, methionine, cystine, valine, leucine, phenylalanine, isoleucine, and lysine. Notably, high levels of cystine and phenylalanine were observed, suggesting a structural role in maintaining protein stability through disulfide bond formation, as well as potential involvement in neuroactive pathways. The presence of arginine and lysine further indicates possible roles in enzymatic activity, vasomodulation, and protein synthesis. Overall, the findings demonstrate that *Naja guineensis* venom is a complex biochemical matrix rich in essential elements and amino acids that contribute to its structural integrity and biological activity. These results provide a foundation for further studies on the functional, pharmacological, and therapeutic potential of this relatively understudied species.

Keywords: Naja guineensis, venom, Elemental analysis, Amino acid profiling, atomic absorption spectroscopy, high-performance liquid chromatography

INTRODUCTION

Snake venoms are complex biochemical mixtures composed of proteins, peptides, enzymes, and low-molecular-weight compounds that collectively mediate a wide range of toxic effects (Redureau *et al.*, 2025). Beyond their ecological role in prey immobilization and defense, these venoms have attracted considerable scientific interest due to their pharmacological potential and their contribution to the development of therapeutic agents (Calvete, 2017; El-Aziz *et al.*, 2019; Silva & Isbister, 2020). *Naja guineensis* (also known as the black forest cobra) is one of the recently discovered members of the *Naja melanoleuca* (or forest cobra) species complex, along with *Naja savannula* (Wüster *et al.*, 2018). The venom of these species is a potent mix of neurotoxins, cytotoxins, and various enzymes (Lauridsen *et al.*, 2017), which may serve as a rich source of pharmacologically active compounds that can be harnessed to treat a range of conditions, from chronic pain to blood clotting disorders (Fox & Serrano, 2007; Abd El-Aziz *et al.*, 2019; Gómez-Betancur *et al.*, 2019).

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Elemental analysis of snake venom involves examining the various elements that constitute the venom's complex mixture (Bhargava *et al*., 2022). Understanding the elemental composition of venom provides insights into how these substances affect their targets at a molecular level and how they can be neutralised by antivenoms. The presence of elements such as calcium, zinc, magnesium, and iron in a snake venom may influence enzymatic processes, structural stability, and toxin functionality (Bhargava *et al*., 2022). Similarly, the presence of metal ions may act as cofactors in venom enzymatic reactions or contribute to the conformational stability of venom proteins, thereby enhancing their activity (Cavecci-Mendonça *et al*., 2023). Despite this, elemental profiling of snake venoms has received relatively less attention compared to proteomic analyses (Yusuf *et al*., 2025), particularly in African elapid species.

Amino acids are the building blocks of proteins, and the specific sequence and structure of these proteins determine the function of the venom's components (Torres *et al.*, 2003). Amino acid composition, therefore, provides further insight into the structural and functional properties of venom proteins (Yusuf *et al.*, 2025). The presence and relative abundance of specific amino acids influence protein folding, stability, and interaction with biological targets (Torres *et al*., 2003). Hence, analysing the amino acid composition of snake venom helps to identify key peptides and proteins responsible for its toxic effects. This knowledge is instrumental in developing synthetic inhibitors and improving the efficacy of antivenoms (Ojeda *et al.*, 2017). The venom of *Naja melanoleuca* complex is particularly interesting due to their complex and potent mix of toxins (Wang *et al.*, 2024). This is even more so for *Naja guineensis*. This species' venom has been less studied compared to other well-known snakes, making it a rich subject for scientific exploration. The black forest cobra's venom contains unique proteins and enzymes that could hold the key to new medical treatments and provide a deeper understanding of venom evolution and function.

Medically, *Naja guineensis* is considered a snake of significant clinical concern due to the composition of its venom. Preliminary findings suggest *Naja guineensis* venom shares key similarities with *Naja melanoleuca*, including a predominance of three-finger toxins (3FTxs) and phospholipase A2 (PLA2) enzymes (Lauridsen *et al*., 2017), which are responsible for its potent neurotoxic and cytotoxic effects, disrupting synaptic transmission and causing local tissue necrosis. This study aimed to characterize *Naja guineensis* venom through elemental and amino acid analyses. By combining atomic absorption spectroscopy (AAS) and high-performance liquid chromatography (HPLC), this work seeks to provide a clearer understanding of the biochemical composition of the venom and its potential implications for toxicology and pharmacology.

MATERIALS AND METHODS

SNAKE COLLECTION

Four adult *Naja guineensis* were collected from locations within Samaru, Zaria, in Kaduna State (Latitude: 11°10′00″ N Longitude: 7°38′00″ E), Nigeria. Zaria lies within the Guinea Savannah ecological zone, which is known to support populations of forest-associated cobra species. Snakes were captured manually by trained personnel using standard snake-handling equipment and transported to the laboratory facility for venom extraction. Species identification was performed based on established morphological characteristics consistent with published taxonomic descriptions (Wüster *et al*., 2018). All procedures involving the snakes were conducted in accordance with institutional and national guidelines for the care and use of experimental animals, and appropriate permits were obtained.

MILKING OF SNAKES

Venom extraction was carried out using the manual milking technique described by Markfarlane (1967), with slight modifications. Briefly, each snake was manually restrained behind the head at the maxillary-mandibular commissure to ensure safe handling and adequate exposure of the fangs. The fangs were then gently positioned over a sterile glass beaker covered with a stretched sterile parafilm membrane, through which the snake voluntarily expelled venom upon stimulation of the venom glands. Care was taken to avoid contamination of the venom samples with blood, saliva, or debris. The freshly collected venom samples from the four snakes were pooled to minimize individual variation, transferred into sterile sample tubes, and immediately frozen prior to lyophilization. The lyophilized venom was thereafter stored in airtight sterile containers at 4°C until further biochemical analyses. The pooled lyophilized preparation was regarded as crude venom throughout the study.

PROTEIN DIGESTION

Protein digestion was performed using lyophilized snake venom. The following steps outline the digestion process:

1. **Reconstitution:** The lyophilized venom sample was reconstituted with 1 ml of distilled water. This ensured that the venom was fully dissolved for the subsequent steps (Ishak *et al.*, 2015).
2. **Digestion Mixture Preparation:** To the reconstituted venom, 3 ml of concentrated nitric acid (HNO_3) and 1 ml of hydrogen peroxide (H_2O_2) were added.

This mixture facilitates the breakdown of complex proteins into simpler forms suitable for further analysis. H_2O_2 decolorizes the sample, to prevent false reading during AAS (Ishak *et al.*, 2015).

3. **Heating:** The sample was then placed in a water bath and heated at a controlled temperature for 45 minutes. This step ensures thorough digestion of the venom proteins.
4. **Cooling:** After heating, the sample was allowed to cool down to room temperature.
5. **Buffer Solution Preparation:** Once cooled, distilled water was added to the digested sample to achieve a final volume of 30 ml. This dilution is critical for maintaining the appropriate concentration for subsequent analyses (Ishak *et al.*, 2015).

Post-digestion preservation was not required due to the presence of HNO_3 in the sample, which inherently preserved the integrity of the reconstituted venom (Pappas, 2012).

ATOMIC ABSORPTION SPECTROSCOPY (AAS)

Following protein digestion, the sample was subjected to Atomic Absorption Spectroscopy (AAS) using Surgifield SM0042 atomic absorption spectrophotometer (Surgifield Medical, Davon Ex England, United Kingdom) to analyze the metal ion content. AAS is a technique used to measure the concentrations of metal ions in the digested venom sample (Welz & Sperling, 1998). The process involved the following steps:

1. **Sample Introduction:** The digested sample was introduced into the AAS instrument, and each 1 ml of the 30 ml buffer solution was diluted to 25 ml.
2. **Metal Ion Analysis:** The instrument's flame or graphite furnace atomised the sample, allowing for the quantification of specific metal ions based on their absorption of light at characteristic wavelengths.
3. **Data Collection:** The concentrations of Ca, P, Fe, Zn, and Mg were measured and recorded.

AMINO ACID ANALYSIS (AAA)

Amino Acid Analysis (AAA) was conducted to determine the amino acid composition of the digested venom sample using High-Performance Liquid Chromatography (HPLC) technique as described by Cohen & Michaud (1993)

1. **Filtration:** Before HPLC analysis, the digested sample was filtered using an Agilent filter. This filtration step was crucial to remove any particulates that could interfere with the HPLC system or clog the column (Cohen & Michaud, 1993).
2. **HPLC Setup:** The HPLC system was calibrated and prepared for amino acid analysis, ensuring accurate separation and quantification of amino acids in the sample (Cohen & Michaud, 1993).
3. **Sample Injection:** The filtered sample was injected into the HPLC system.
4. **Separation and Detection:** The HPLC column separated the amino acids based on their chemical properties, and the detector identified and quantified each amino acid present in the sample (Cohen & Michaud, 1993).
5. Amino acids are absorbed and emitted at different wavelengths; some are absorbers, others are emitters, and few are found to be both absorbers and emitters (Middendorf *et al.*, 2000). The data encompasses retention times (RT), relative areas, and quantified amounts for various amino acids present in the sample. The chromatogram peaks correspond to different amino acids detected in the sample. Each peak's retention time helps identify the amino acid, while the area under each peak correlates with the concentration. The concentration of each amino acid is calculated and expressed in Pmol/µl.

RESULTS

ELEMENTAL CONTENTS OF *NAJA GUINEENSIS* VENOM

The results show that *Naja guineensis* venom contains calcium, phosphorus, iron, zinc, and magnesium in significant amounts. The most abundant of these elements was phosphorus (4650.17 mg/kg), the concentration of which was more than 6 times that of the second most abundant, Calcium (678 mg/kg). The element with the least concentration was iron (369.45 mg/kg). The concentrations of other elements, zinc and magnesium, are 437.70 mg/kg and 506.70 mg/kg, respectively (Table I).

TABLE I: ELEMENTS AND THEIR CONCENTRATION IN *NAJA GUINEENSIS* VENOM

Elements	Concentration (mg/kg)
Calcium	678
Phosphorus	4650.17
Iron	369.45
Zinc	437.70
Magnesium	506.70

TABLE II: AMINO ACID COMPOSITION OF *NAJA GUINEENSIS* VENOM

S/N	Amino Acid	Retention Time (mins)	Area (m^2)	Amount (Pmol/µL)
01	Aspartic acid	1.435	1.09883	107.6191
02	Glycine	7.335	0.03705	244.2298
03	Threonine	7.888	0.04891	747.9855
04	Alanine	8.046	1.48866	143.3287
05	Arginine	10.124	0.28937	290.8145
06	Methionine	11.200	40.19258	46.4519
07	Cystine	11.267	2.53831	896.3922
08	Valine	12.465	0.01627	635.6608
09	Leucine	13.547	0.96362	97.1849
10	Phenylalanine	13.610	0.14033	1719.8472
11	Isoleucine	14.361	15.5219	10
12	Lysine	15.974	0.97615	199.6444

The results show that *Naja guineensis* venom contains aspartic acid, glycine, threonine, alanine, arginine, methionine, cystine, valine, leucine, phenylalanine, isoleucine and lysine. Phenylalanine is the most abundant amino acid in the venom, the concentration of which (1719.8472 mg/kg) was almost twice that of the second most abundant amino acid, Cystine (896.3922 mg/kg), followed by threonine (747.9855 mg/kg). The three amino acids with the least concentration are Leucine, Methionine and Isoleucine with concentrations of 97.1849 mg/kg, 46.4519 mg/kg and 10 mg/kg, respectively Table II.

DISCUSSION

The high concentration of Phosphorus is particularly noteworthy, as phosphorus compounds are known to play critical roles in the venom's enzymatic activity and toxicity. This aligns with findings from previous studies, such as Wexler & Anderson (2005), which highlighted the significance of phosphorus in venom phospholipases, crucial for their membrane-degrading activities. Phosphorus-containing compounds are closely associated with enzymatic activity in snake venoms, particularly in relation to phospholipase-mediated membrane disruption (Kini, 2003). Phospholipase A_2 (PLA_2) enzymes, which are widely distributed in cobra venoms, depend on specific ionic environments for optimal catalytic activity, including the presence of divalent cations such as calcium (Kini, 2003). In addition, metal ions such as zinc and calcium are known to stabilize enzyme conformation and enhance catalytic efficiency, especially in metalloproteinases and related venom enzymes (Fox & Serrano, 2007). These observations suggest that the elemental composition of the venom contributes directly to its functional activity.

The presence of high levels of phenylalanine in the venom may imply high antimicrobial and/or anticancer activity of the venom. Phenylalanine helps shape venom protein, improve folding and enhance membrane interaction. In a PLA_2-derived venom peptide pair that differed by only leucine → phenylalanine at one position, the phenylalanine-containing peptide showed stronger antibacterial and antitumor activity, suggesting that introducing phenylalanine can enhance membrane-active, cytotoxic properties, thereby improving certain antimicrobial or anticancer activities when present at key positions (Almeida *et al*., 2024). Schifano & Caputo (2021) reported that systematic replacement of hydrophobic residues including with phenylalanine showed that amino acid composition and hydrophobicity delicately tune activity; some changes enhance activity, others abolish it. Phenylalanine is also a precursor to key neurotransmitters such as dopamine, norepinephrine, and epinephrine, hence, it plays an important role in neurochemical processes (Fernstrom & Fernstrom, 2007). Although direct evidence linking phenylalanine abundance to specific venom activities in elapid species remains limited, its presence may reflect the broader neuropharmacological potential of venom components. Similarly, the detection of amino acids such as arginine and lysine suggests potential roles in enzymatic activity and protein–receptor interactions. Arginine, in particular, is involved in nitric oxide synthesis and has been associated with vascular regulation, which may influence physiological responses during envenomation (Villar-Briones & Aird, 2018).

The presence of high levels of cystine and phenylalanine aligns with the findings of Tadokoro *et al*. (2020), which emphasised the importance of these amino acids in the structural and functional properties of venom proteins. Cystine forms disulphide bonds that contribute to the stability and potency of venom proteins, as noted by Munawar *et al*. (2018). They pointed out that the majority of the peptides found in snake venom are stable molecules that can withstand the severe proteolytic environment found in the venom gland. These peptides' natural stability aids in their capacity to bind to target receptors inside their victim (after envenomation). The detection of multiple amino acids with both absorbance and fluorescence properties underscores the complex nature of the venom's composition. The relatively high levels of cystine are particularly significant, as cystine residues form disulfide bonds that are

critical for maintaining the structural integrity and biological activity of venom peptides. This characteristic is especially prominent in three-finger toxins (3FTxs), which dominate elapid venoms and rely on conserved disulfide frameworks for stability and function (Kini & Doley, 2010). These structural features enable venom peptides to resist proteolytic degradation and maintain high affinity for molecular targets such as nicotinic acetylcholine receptors, thereby contributing to their potent neurotoxic effects (Doley & Kini, 2009; Lauridsen *et al*., 2017).

Arginine and lysine are involved in enzyme activity and protein synthesis. Arginine is particularly noteworthy for its role in nitric oxide synthesis, which is vital for various physiological processes, including vasodilation and neurotransmission (Villar-Briones & Aird, 2018). Phenylalanine and methionine are essential for the production of neurotransmitters and other bioactive molecules. Phenylalanine's high concentration as detected suggests its significant presence in the venom, which may be linked to its involvement in the biosynthesis of important neuroactive compounds. However, no literature was found to support this in the venom of Elapids.

The high concentration of arginine in the venom suggests its importance in vasodilation, immune response modulation, and neurotransmission and its potential use in developing treatments for conditions involving impaired nitric oxide production, such as cardiovascular diseases (Martí & Reith, 2021). Lysine, another amino acid detected in significant quantities, is essential for various metabolic processes, including calcium absorption and collagen formation. This aligns with research by Singh *et al.* (2011) which underscores lysine's role in enhancing the body's immune response and tissue repair mechanisms.

The property of cysteine is being harnessed in the pharmaceutical industry to design stable and effective protein-based drugs (Hashim *et al*., 2014). This is particularly relevant in designing drugs that require long-term stability and activity, such as enzyme replacement therapies and biologics used in chronic disease management.

Phenylalanine is a precursor to important neurotransmitters such as dopamine, norepinephrine, and epinephrine (Dinu & Apetrei, 2020). The neuroactive properties of phenylalanine and its role in neurotransmitter synthesis can be leveraged in developing treatments for neurological disorders, including Parkinson's disease and depression. The therapeutic potential of venom components is supported by extensive research indicating their efficacy in modulating neurotransmission and providing neuroprotective effects. The presence of phosphorus in high concentrations further supports the venom's enzymatic capabilities, particularly in membrane degradation and cell lysis.

Phosphorus-containing enzymes, such as phospholipases, are known to disrupt cellular membranes, leading to cell death. This property is significant for developing anti-cancer therapies, as highlighted by recent studies exploring snake venom components for their cytotoxic effects on cancer cells (Vyas *et al.*, 2013).

Taken together, the combined elemental and amino acid data point to a venom system that is both structurally stable and functionally versatile. The interplay between metal ions and amino acid composition likely underpins key aspects of venom activity, including enzyme function, toxin stability, and interaction with biological membranes and receptors. These findings are consistent with broader venom research, which demonstrates that venom composition is closely linked to toxicological function and ecological adaptation (Calvete, 2017).

From a pharmacological perspective, the results reinforce the growing recognition of snake venoms as valuable sources of bioactive compounds. Several venom-derived molecules have been successfully developed into therapeutic agents, particularly in the management of cardiovascular and haematological disorders (Silva & Isbister, 2020). The structural stability associated with cystine-rich peptides, alongside the presence of amino acids involved in metabolic and signalling pathways, highlights the potential of venom-derived compounds in drug development.

However, certain limitations should be considered. The use of pooled crude venom may not account for individual variability, including differences related to age, sex, or geographic origin, which have been shown to influence venom composition (Calvete, 2017). In addition, while elemental and amino acid analyses provide valuable biochemical insights, they do not directly identify specific toxin families or functional activities. Future studies should therefore incorporate proteomic and functional assays to better correlate composition with biological effects.

CONCLUSION

This study provides a foundational biochemical characterization of *Naja guineensis* venom, demonstrating that it is elemental and amino acid composition contributes significantly to its structural integrity and biological activity. These findings expand the limited knowledge on this species and provide a basis for further toxicological and pharmacological investigations.

The key findings indicated that the venom, being protein in nature, enabled successful protein digestion process which in turn, enabled detailed examination of the venom's biochemical properties. Elemental detection using AAS revealed significant concentrations of calcium, phosphorus, iron, zinc, and magnesium, with phosphorus showing the highest concentration.

CONFLICT OF INTEREST

The work was self-sponsored, hence the authors have no conflict of interest to declare.

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