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High-throughput proteomics and in vitro functional characterization of the 26 medically most important elapids and vipers from sub-Saharan Africa

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Abstract

Venomous snakes are important parts of the ecosystem, and their behavior and evolution have been shaped by their surrounding environments over the eons. This is reflected in their venoms, which are typically highly adapted for their biological niche, including their diet and defense mechanisms for deterring predators. Sub-Saharan Africa is rich in venomous snake species, of which many are dangerous to humans due to the high toxicity of their venoms and their ability to effectively deliver large amounts of venom into their victims via their bite. In this study, the venoms of 26 of sub-Saharan Africa’s medically most relevant elapid and viper species were subjected to parallelized toxicovenomics analysis. The analysis included venom proteomics and in vitro functional characterization of whole venom toxicities, enabling a robust comparison of venom profiles between species. The data presented here corroborate previous studies and provide biochemical details for the clinical manifestations observed in envenomings by the 26 snake species. Moreover, two new venom proteomes (Naja anchietae and Echis leucogaster) are presented here for the first time. Combined, the presented data can help shine light on snake venom evolutionary trends and possibly be used to further improve or develop novel antivenoms.

Keywords: snakebite envenoming, sub-Saharan Africa, toxicovenomic, in vitro venom characterization, high-throughput assays, cytotoxicity, enzymatic activity of venoms

Introduction

In the deep jungles, on the open savanna, and across deserts, snakes are omnipresent in sub-Saharan Africa, where they play an integral role in the natural ecosystems to which they have adapted over the course of evolution [1]. Some of these snake species are highly venomous, being classified by the World Health Organization as category 1 or 2 snakes of the highest medical importance [2, 3]. Thus, understanding the composition and function of their venoms is not only important for elucidating basic biology and adaptation of species, but also of medical significance. Each year, venomous snakes inflict approximately 500,000 bites in Africa [4, 5], causing major disability and disablement for many rural workers and children [6]. This challenge remains a pressing health care issue, which is further exacerbated by the socioeconomic impact that disability causes for manual laborers [7].

The medically most important snakes of sub-Saharan Africa belong mainly to the Elapidae (e.g., cobras, mambas, and rinkhals) and Viperidae families, although a few species from the Colubridae family (e.g., boas, boa constrictors, and Dispholidus typus) are also known to cause severe envenomings. Victims envenomed by elapid snakes typically display local as well as systemic clinical manifestations. Local manifestation often includes swelling, blistering, and bruising at the anatomic site of the bite, which may evolve into irreversible tissue necrosis and gangrene [5, 8]. In comparison, systemic manifestations may include muscle twitching, spasms, weakness, fatigue, sleepiness, slurred speech, or difficulties in swallowing. These can progress to flaccid paralysis and, in severe cases, fatal respiratory failure, unless mechanical ventilation is provided [5, 9].

Similarly to elapids, envenomings caused by vipers may also result in both local and systemic manifestations. The victims often immediately feel a strong irradiating pain at the site of bite and typically show hot inflammatory erythema, blisters, bruises, and spontaneous bleeding [5]. Systemic clinical manifestations can include temporary loss of vision, fainting, and systemic hemorrhage, which in severe cases can lead to cardiovascular shock [5].

Different toxin families are responsible for the clinical manifestations observed for viper and elapid envenoming. As a first example, venoms from spitting cobras are rich in cytotoxins (CTxs) from the 3-finger toxin (3FTx) family and phospholipase A2s (PLA2s) [10], which interfere with and disrupt the integrity of cellular membranes, resulting in irreversible damage and cell death. In comparison, short- and long-chain α-neurotoxins, another type of 3FTx, found in venoms such as those of the black mamba and forest cobra, block neuromuscular signaling and prevent normal muscle contractions through binding to acetylcholine receptors in neuromuscular junctions [11]. Another example of a class of toxins that interfere with neuromuscular signaling are dendrotoxins. Dendrotoxins belong to the Kunitz-type inhibitors, are...
Proteomic characterization of whole venom by mass spectrometry

In-solution tryptic digestion of the venom proteins

For each of the 26 snake venoms listed in Table 1, the lyophilized whole venom was dissolved in 1× phosphate-buffered saline (PBS), and then 5 μg was vacuum dried and resuspended in 20 μL of 6 M guanidinium hydrochloride containing 10 mM TCEP, 40 mM 2-chloroaceticamide, and 50 mM HEPES (pH 8.5). After adding 40 μL of digestion buffer (10% acetonitrile, 50 mM HEPES pH 8.5), samples were digested with LysC endopeptidase (1:50, w/w) for 3 hours at 37°C. Samples were further diluted with 140 μL of digestion buffer and mixed with trypsin (1:100, w/w). Trypsinized samples were incubated overnight at 37°C, then diluted with 200 μL of 2% TFA to quench trypsin activity. Peptides were desalted on StageTip containing Empore C18 disks, eluted in 60 μL 40% acetonitrile containing 0.1% formic acid (FA), dried in a vacuum centrifuge, and resuspended in 2% acetonitrile containing 1% TFA and iRT peptides (Biognosys, Schlieren, Switzerland).

Liquid chromatography/tandem mass spectrometry analysis

Mass spectrometry data were collected using a Q Exactive mass spectrometer (Thermo Fisher Scientific) coupled to a Thermo EASY-nLC 1200 liquid chromatography (LC) system (Thermo Fisher Scientific). Then, 100 ng of peptides was loaded into a 2-cm C18 trap column (Thermo Fisher Scientific, 164705) connected to a 15-cm reverse-phase analytical column (Thermo Fisher Scientific, ES900). Peptides were separated for 70 minutes with a gradient going from 10% to 60% buffer B (80% acetonitrile, 0.1% FA) over 60 minutes, until spiking to 95% buffer B for the last 10 minutes to wash the column. Full mass spectrometry (MS) spectra were collected at a resolution of 70,000, with an automatic gain control (AGC) target of 3 × 10^5 or maximum injection time of 20 ms and a scan range of 300 to 1,750 m/z. The MS2 spectra were obtained at a resolution of 17,500, with an AGC target value of 1 × 10^6 or maximum injection time of 60 ms, a normalized collision energy of 25, and an intensity threshold of 1.7 × 10^4. Dynamic exclusion was set to 60 seconds, and ions with a charge state >2 or unassigned were excluded.

Using proteome Discoverer 2.4, peptide fragmentation spectra (MS/MS) were searched against a database consisting of all Swiss-Prot and TrEMBL protein sequences from the Serpentes suborder available in Uniprot (331,759 entries; downloaded May 2022). The search was performed using the built-in Sequest HT algorithm, which was configured to derive fully tryptic peptides using default settings. Cysteine carboxamidomethyl was set as a static modification and oxidation (M), deamidation (N, Q), and acetyl on protein N-termini were set as dynamic modifications. Label-free quantitation was enabled in both processing and consensus steps, with quantitation being done using Minora Feature Detector. All results were filtered at 1% false discovery rate, and relative protein abundances were estimated by calculating the ratio of the individual protein abundances to the sum of abundances of all proteins detected within a sample.

In vitro functional characterization of the whole venoms

PLA2 enzymatic activity assay

The endpoint PLA2 activity assay was run as described previously [23]. The snake venoms were dissolved at a concentration of 10 mg/mL in assay buffer (10 mM Tris pH 8, 100 mM NaCl, and 10 mM CaCl2), and a 2-fold serial dilution (10 steps) was prepared.

Material and Methods

Venoms and reagents

Chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. High-purity venoms, from a pool of specimens for the 26 snakes in Table 1, were obtained from Latoxan (Portes lès Valence, France), stored at −20°C, and reconstituted in assay buffer just before use. PLA2 substrate 4-nitro-3-(octanoyloxy)benzoic acid (NOB) and SVMP substrate ES010 were purchased from Enzo Life Sciences (Farmingdale, New York, USA). SVMP substrate [γ-tosyl-Gly-Pro-Arg]2-R110 and 96-well plates were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). All substrates for enzymatic assays were dissolved in DMSO to the stock concentration of 100 mM. CellTiter-Glo 3D Cell Viability Assay kit was obtained from Promega (Madison, WI, USA).

Reversed-phase high-performance liquid chromatography

To record the chromatograms, the venoms were separated by reversed-phase high-performance liquid chromatography (RP-HPLC) using an Agilent (Santa Clara, CA, USA) Infinity II as previously described [22]. Briefly, lyophilized venom (10 mg) was dissolved in 1 mL of water containing 0.1% trifluoroacetic acid (TFA; solution A), centrifuged at 14,000 × g for 10 minutes, and transferred to an HPLC vial. For each fractionation round, 100 μL of sample was injected into an RP-HPLC C18 column (250 × 4.6 mm, 5 μm particle size) and eluted at 1 mL/min by applying a gradient toward acetonitrile containing 0.1% TFA (solution B) (0–15% B for 10 minutes, 15–45% B for 60 minutes, 45–70% B for 10 minutes, and 70% B for 9 minutes).

exclusively found in the venoms of mambas, and block ion transport through potassium channels, resulting in involuntary muscle contractions [12, 13]. Snake venoms from most viperid species possess a high fraction of PLA2s (e.g., Gaboon viper, Bitis gabonica), snake venom metalloproteinases (SVMPs) (e.g., carpet viper, Echis ocellatus), and snake venom serine proteinases (SVSPs) (e.g., horned viper, Cerastes cerastes), which all play important roles in the toxicity of these venoms. SVMPs hydrolyze components of the cell wall of capillaries, which first reduces the mechanical integrity and then disrupts the capillary walls, resulting in both local and systemic bleeding [14, 15]. Systemic bleeding can also be caused by SVSPs that interfere with the blood coagulation cascade by decreasing the level of platelets, fibrinogen, and clotting factors [5, 16].

So far, most venom studies on African snakes have included only one or a small handful of species [17–21], and the inclusion of functional data has been somewhat sporadic or absent. These studies have undoubtedly been important for obtaining a first snapshot of venom compositions, which have already enabled further studies within venom evolution, snake biology, and development of (recombinant) antivenom. However, the fact that these studies have been performed in multiple different laboratories using different protocols results in a limited level of data harmonization. To this end, and to elucidate a few so far undescribed venom proteomes, we describe high-throughput methods for proteomics (i.e., venomics) and in vitro functional characterization of snake venoms (i.e., toxicovenomics) in a parallelized manner and characterize sub-Saharan Africa’s 26 medically most important snakes, comprising 18 elapids and 8 vipers (Table 1).

Material and Methods
Venoms and reagents

Chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. High-purity venoms, from a pool of specimens for the 26 snakes in Table 1, were obtained from Latoxan (Portes lès Valence, France), stored at −20°C, and reconstituted in assay buffer just before use. PLA2 substrate 4-nitro-3-(octanoyloxy)benzoic acid (NOB) and SVMP substrate ES010 were purchased from Enzo Life Sciences (Farmingdale, New York, USA). SVMP substrate [γ-tosyl-Gly-Pro-Arg]2-R110 and 96-well plates were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). All substrates for enzymatic assays were dissolved in DMSO to the stock concentration of 100 mM. CellTiter-Glo 3D Cell Viability Assay kit was obtained from Promega (Madison, WI, USA).

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Table 1: List of the 26 venoms from the medically most relevant elapids and vipers from sub-Saharan Africa used in this study. Catalog number and origin are listed.

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus (subgenus)</th>
<th>Snake</th>
<th>Catalog No.</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elapidae</td>
<td>Dendroaspis</td>
<td>D. angusticeps</td>
<td>L1307</td>
<td>Tanzania</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D. jasenoi</td>
<td>L1308</td>
<td>Cameroon</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D. polylepis</td>
<td>L1309</td>
<td>Kenya, South Africa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D. viridis</td>
<td>L1310</td>
<td>Ghana</td>
</tr>
<tr>
<td></td>
<td>Hemachatus</td>
<td>H. haemachatus</td>
<td>L1311</td>
<td>South Africa</td>
</tr>
<tr>
<td></td>
<td>Naja (Afronaja)</td>
<td>N. ashei</td>
<td>L1375</td>
<td>Kenya</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N. katinensis</td>
<td>L1317</td>
<td>Burkina Faso</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N. mossambica</td>
<td>L1376</td>
<td>South Africa, Tanzania</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N. nigrinicta</td>
<td>L1368</td>
<td>South Africa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N. nigrilobus</td>
<td>L1327</td>
<td>Cameroon, Tanzania, West Africa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N. nubia</td>
<td>L1342</td>
<td>Egypt</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N. pullida</td>
<td>L1321</td>
<td>Kenya</td>
</tr>
<tr>
<td></td>
<td>Naja (Boulangerina)</td>
<td>N. melanoleuca</td>
<td>L1318</td>
<td>Cameroon, Ghana, Uganda</td>
</tr>
<tr>
<td></td>
<td>Naja (Craesus)</td>
<td>N. anchietae</td>
<td>L1374</td>
<td>Namibia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N. annulifera</td>
<td>L1314</td>
<td>Sub-Saharan Africa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N. haje</td>
<td>L1315</td>
<td>Egypt, Mali</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N. nivea</td>
<td>L1328</td>
<td>South Africa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N. senegalensis</td>
<td>L1350</td>
<td>Mali</td>
</tr>
<tr>
<td>Viperidae</td>
<td>B. arietans</td>
<td>B. gabonica</td>
<td>L1104</td>
<td>Burundi, Tanzania</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B. nasicornis</td>
<td>L1106</td>
<td>West Africa, Burundi</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B. rhinoceros</td>
<td>L1105</td>
<td>Ghana</td>
</tr>
<tr>
<td>Cerastes</td>
<td>C. cerastes</td>
<td>C. cerastes</td>
<td>L1107</td>
<td>Egypt, Tunisia</td>
</tr>
<tr>
<td>Echis</td>
<td>E. leucogaster</td>
<td>E. leucogaster</td>
<td>L1109</td>
<td>Mali</td>
</tr>
<tr>
<td></td>
<td>E. ocellatus</td>
<td>E. ocellatus</td>
<td>L1114</td>
<td>Cameroon, Mali, Ghana</td>
</tr>
<tr>
<td></td>
<td>E. pyramidum</td>
<td>E. pyramidum</td>
<td>L1110</td>
<td></td>
</tr>
</tbody>
</table>

Then, 100 μL/well of each dilution was added to a 96-well plate, together with 100 μL/well of NOB (final concentration 0.25 mM). The plates were shaken at 300 rpm for 2 minutes and then incubated at 37°C for 40 minutes. The plates were then centrifuged (3,000 × g, 4°C, 3 minutes) before the absorbance was recorded at 405 nm using a VICTOR Nivo plate reader (PerkinElmer, Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin-streptomycin, and 1× RPMI supplement [25] under standard conditions (37°C, 5% CO2, and 85% humidity). For the cell viability assay, cells were seeded at 4,000 cells/well in 100 μL medium and incubated overnight under standard conditions. Snake venoms were dissolved in PBS at a concentration of 1 mg/mL and added to each well followed by a 24-hour incubation. The rate values were then plotted against the venom concentration and a non-linear fitting with a sigmoidal dose-response equation was used to determine the EC50 values (the venom concentration at which half of the maximum RFU rate proportional to product conversion was observed) using GraphPad Prism 9 software.

**SVSP and SVMP enzymatic activity assay**

To measure SVSP and SVMP activities, enzymatic assays were performed. The hydrolysis reactions were performed in 96-well plates with a final volume of 100 μL per well. The snake venoms were dissolved in PBS for SVSP or assay buffer (10 mM Tris pH 8, 100 mM NaCl, 10 mM CaCl2) for SVMP assays at a concentration of 10 mg/mL, and 10 dilution steps of a 2-fold serial dilution were prepared. To start the reaction, 50 μL of 2 μM SVSP substrate R110 or 10 μM SVM substrate ES010 was mixed with 50 μL of each snake venom concentration of the serial dilution. Fluorescence data were recorded using a VICTOR Nivo plate reader at 25°C. For the SVSP assay, an excitation wavelength of 480 nm and an emission wavelength of 530 nm with 11 kinetic cycles and an interval of 90 seconds were used. For the SVMP assay, an excitation wavelength of 320 nm and an emission wavelength of 405 nm with 16 kinetic cycles and an interval of 90 seconds were used. The reactions were run in duplicate and a blank containing no venom was included.

The rate of relative fluorescence units per second (RFU/s) recorded for each venom concentration was the slope calculated from the linear fitting on its time–response curve. The rate values were then plotted against the venom concentration and a non-linear fitting with a sigmoidal dose-response equation was used to determine the EC50 values (the venom concentration at which half of the maximum RFU rate proportional to product conversion rate was observed) using GraphPad Prism 9 software.

**Cell viability assay**

The N/TERT keratinocyte [24] cell line was cultured in Dulbecco’s modified Eagle’s medium (DMEM:F12, Thermofisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin-streptomycin, and 1× RMPI supplement [25] under standard conditions (37°C, 5% CO2, and 85% humidity). For the cell viability assay, cells were seeded at 4,000 cells/well in 100 μL medium and incubated overnight under standard conditions. Snake venoms were dissolved at a concentration of 1 mg/mL and then 2-fold diluted in 8 dilution steps in sterile PBS. The venom dilutions were then further diluted 1:1 in medium to the maximum concentration of 1 mg/mL and added to each well followed by a 24-hour incubation. Thereafter, the CellTiter-Glo luminescent cell viability assay [26] was used to analyze the cytotoxicity of the 26 snake venoms. The assay was performed in triplicates, with no venom as negative control (maxi-
mum viability of the cells). IC₅₀ values (the venom concentration inducing 50% loss of cell viability) were determined using non-linear fitting with the dose–response inhibition equation on the venom dose curves analyzed using GraphPad Prism 9 software.

**Thromboelastography assay**

Thromboelastography (TEG) was run for all viperid venoms (Bitis arietans, Bitis gabonica, Bitis nasicornis, Bitis rhinoceros, Cerastes cerastes, Echis leucogaster, Echis ocellatus, and Echis pyramidum) according to a protocol adapted from Seneci et al. [27] using a TEG 5000 thromboelastogram (Haemonetics, Boston, Massachusetts, USA). Solutions of 72 μL 25 mM CaCl₂, 72 μL 0.25 mM phospholipids (Rossix, Möln达尔, Sweden, catalog no. #PL052), 20 μL Tris-HCl buffer (50 mM Tris + 150 mM NaCl, pH 7.4), and 7 μL crude venom at 1 or 0.1 mg/mL in PBS (final concentration of ~20 μg/mL and ~2 μg/mL, respectively) were mixed in TEG disposable cups (Haemonetics). Last, 189 μL of citrated human plasma was added, and the samples were immediately run for at least 30 minutes. Negative controls were run by replacing venom with 7 μL PBS. TEG traces (3 replicates per venom concentration per species) were exported as TIFF files and processed in Adobe Photoshop 2022 (Adobe, San Jose, CA, USA).

**Results and Discussion**

**Venom composition**

The venom proteomes of the 26 medically most important elapids and vipers from sub-Saharan Africa (Table 2) were determined using a bottom-up proteomics approach, involving the enzymatic digestion of whole venoms, separation and analysis by LC-MS/MS, assignment of the identified proteins to their respective protein families, and calculation of protein family abundances as a percentage of total identified proteins (mol/mol) (Fig. 1, Supplementary Table S1). In addition, the RP-HPLC chromatograms of the venoms are shown in Supplementary Fig. S1.

**Elapidae**

The elapids included in this study belong to the genera Naja, Hemachatus, or Dendroaspis (Table 2). Of these, the true cobra lineage (Naja sp.) is by far the most widespread and diverse group throughout the African continent. To reflect their evolutionary and ecological diversity, African true cobras can be further divided into the 3 subgenera Afronaja, Boulinggerina, and Uraeus [28].

The subgenus Afronaja includes all African spitting cobras (Naja ashei, Naja katienza, Naja mossambica, Naja nigrinicta, Naja nigrinola, and Naja pallida), with representative species found from Egypt (N. nubia) to South Africa (N. mossambica and N. nigrinicta) [29]. Despite the widespread distribution and different habitat preferences of Afronaja species, their toxin arsenal is remarkably conserved both inter- and intraspecifically [29]. In terms of protein abundance, the proteomic analysis shows that the bulk of their venom consists of 3FTxs (~79%, of which 75% are CTxs) and PLA₂s (~17%), which is in accordance with previous studies [29, 30]. Notably, N. nubia diverges from the general Afronaja venom profile and contains a considerable amount of short neurotoxins (sNTxs, ~10%). Envenomings by N. nubia therefore often result in both cytotoxic and neurotoxic clinical manifestations compared to the mostly cytotoxic manifestations seen in envenomings caused by the rest of Afronaja species.

The only member of the subgenus Boulinggerina included in this study was the forest cobra (Naja melanoleuca) [31], native to the forests and savannahs of central Africa, where it feeds on reptiles, amphibians, birds, small mammals, and even fish [32, 33]. Its venom was shown to have a high content of 3FTxs (~64%), of which most were CTxs (~27%), and also a considerable amount of PLA₂s (~28%), which is in agreement with previous studies [19]. Additionally, the venom contained a substantial proportion of long neurotoxins (lNTxs, ~16%), the fourth highest among all snakes in this study, which could explain the neurotoxic manifestations reported after envenoming [34]. One toxin family in which the abundance differed from an earlier study was the SVMPs, where Lauridsen et al. [19] reported an abundance of 9.7%, whereas we only found 0.7%. Plausible explanations for this discrepancy could be a combination of variation between venom batches as a consequence of intraspecific venom variability, differences in the units used to quantify relative protein abundance (mol/mol in this study vs. wt% in Lauridsen et al. [19]), and/or the use of different proteomic methods (no decomplexing step prior to mass spectrometry in this study). Notably, the venom profile of N. melanoleuca differed from the other 2 Naja subgenera, containing less CTxs than Afronaja and more PLA₂s than Uraeus.

The Uraeus subgenus (Naja anchietae, Naja annulifera, Naja haje, Naja nivea, and Naja senegalensis) consists of species with predominantly neurotoxic effects [34]. Like Afronaja, this lineage is widespread across the continent from Morocco (N. haje) to South Africa (N. anchietae, N. annulifera, and N. nivea) and has a highly diverse diet, which includes amphibians, reptiles, birds, and other snakes [35]. The venoms in this subgenus were predominantly composed of 3FTxs (~95%), most of which were CTxs as in the Afronaja subgenus, despite the mainly neurotoxic characteristics of Uraeus envenomings. This was not the case for N. haje, which contained mainly sNTxs (40%), lNTxs (38%), and CTxs (16%), although a previous study has reported a higher abundance of CTxs (36%). N. senegalensis also contained significant amounts of sNTxs (12%) and lNTxs (23%). Moreover, all Uraeus species except N. anchietae contained some neurotoxins (at least ~6%), in line with the neurotoxic clinical manifestations associated with envenomings caused by these snakes. For snakes where proteomics data were available, our data corroborate the previously reported findings for N. senegalensis [37] and N. nivea [38]. For N. annulifera [39], a previous study reported an abundance 11.18% SVMPs, while our data showed only 0.6%, which, as mentioned previously, could be due to intraspecific venom variability and/or different methods for proteomics and quantification. In contrast to the other Naja species, Uraeus cobras all showed very low levels of PLA₂s (~0.15%), which were up until recently thought to be almost ubiquitous in snake venoms [40]. Of note, the proteomic venom composition for N. anchietae is presented here for the first time, strengthening a previous theory that low levels of PLA₂s are a feature of all snakes [19].

The rinkhals (Hemachatus haemachatus) is a spitting elapid classified in their own monotypic genus despite greatly resembling true cobras in morphology and general biology. This species is native to southeastern Africa, where it can be found in several ecosystems (e.g., savannah, woodland, and shrubland) and is known to prey mainly, although not exclusively, on amphibians [35]. According to our proteomic data, its venom composition is similar to those of the Afronaja, spitting cobras, which convergent evolutionarily the ability to spit venom. In fact, H. haemachatus venom was shown to have a high content of 3FTxs (~73%) and PLA₂s (~20%). Of the 3FTxs identified, the most abundant were CTxs or CTx homologs, with a small amount of sNTxs. This correlates with the cytotoxic and neurotoxic clinical manifestations of H. haemachatus envenomings [41]. The predominance of 3FTxs and PLA₂s in this species is in line with a previous study by Sánchez.
et al. [8]. However, there are some minor discrepancies, as this previous study detected a larger amount of SVMPs (7% vs. 1.4%).

All 4 members of the Dendroaspis genus (mambas) were included in this study, namely, Dendroaspis angusticeps, Dendroaspis polylepis, Dendroaspis jamesoni, and Dendroaspis viridis. Widespread throughout the African continent, these species constitute one of the few predominately arboreal elapid lineages worldwide, cruising through the canopy of rainforest and woodland regions [42, 43]. As an exception, D. polylepis is often (but not always) more ground-dwelling than its congeners, being commonly found in open savannas and rocky hills [44, 45]. Overall, mambas mainly prey on birds and small mammals such as rodents and bats [42, 46], although their diet and ecology are poorly known.

Signature components of mamba venoms are the presynaptic neurotoxins called dendrotoxins, which belong to the Kunitz-type protease inhibitor family [12, 13]. Dendrotoxins are especially abundant in D. jamesoni and D. viridis, whereas D. angusticeps and D. polylepis have rather similar and show a high abundance of SVMPs (27% and 24%), SVSPs (17% and 12%), disintegrins (15%), and C-type lectins (CTLs)/snaclecs (14% and 32%). SVMPs dominate the venom of B. arietans (62%), whereas B. rhinoceros venom is particularly rich in PLA2s (39%) and SVSPs (18%). All these 4 species have been analyzed previously by Calvete et al. [51], and overall, our data correlate relatively well with this previous study with some

### Viperidae

The Viperidae family is represented by the genera Bitis, Cerastes, and Echis in this study. Of these, Bitis is the most geographically widespread and taxonomically diverse viperid genus in Africa, with 18 currently recognized species (commonly referred to as African adders) found from Morocco to South Africa [43]. More specifically, B. rhinoceros is found in eastern Africa from Guinea to Togo, while B. gabonica and B. nasicornis occur from Nigeria to central, eastern, and southern Africa [2]. Last, B. arietans occurs across open woodlands, grassland, and semiarid habitats throughout sub-Saharan Africa, southern Arabia, and Morocco [48]. Large-sized African adders like those included in this study are mostly generalist predators feeding on small mammals, birds, lizards, and occasionally toads [49, 50].

The venom compositions of B. gabonica and B. nasicornis are rather similar and show a high abundance of SVMPs (27% and 24%), SVSPs (17% and 12%), disintegrins (15%), and C-type lectins (CTLs)/snaclecs (14% and 32%). SVMPs dominate the venom of B. arietans (62%), whereas the rhinoceros venom is particularly rich in PLA2s (39%) and SVSPs (18%). All these 4 species have been analyzed previously by Calvete et al. [51], and overall, our data correlate relatively well with this previous study with some
Figure 1: Composition of the whole venoms of the 26 medically most important elapids and vipers from sub-Saharan Africa. Toxins are grouped according to protein families and expressed as a percentage of total identified proteins (mol/mol). SVMP disintegrins have been classed as SVMPs, while non-SVMP disintegrins have been classed as disintegrins (as per UniProt definitions). CTL, C-type lectin; CTx, cytotoxin; lNTx, long neurotoxin; PLA2, phospholipase A2; sNTx, short neurotoxin; SVMP, snake venom metalloproteinase; SVSP, snake venom serine proteinase; 3FTx, 3-finger toxin.

The only member of the Cerastes genus included in this study is the Saharan horned viper (*Cerastes cerastes*). This species is distributed throughout North Africa and further eastward as far as southwestern Israel and southwestern Saudi Arabia [56]. Like many other viper lineages, *C. cerastes* is an ambush predator, relying on camouflage to lunge at small rodents and lizards by surprise [57]. Our proteomics analysis of *C. cerastes* venom shows a high abundance of SVSPs (27%) as opposed to a relatively low abundance of CTLs (4%), which is in agreement with other studies [58–60] (Fig. 1, Supplementary Table S1). On the other hand, the largest discrepancy compared to previous reports is observed for SVMPs, which were found to only constitute 8.6% of venom proteins in this study compared to 30–60% reported in literature, and disintegrins, which were found to constitute 43% of venom proteins in this study compared to approximately 10% previously reported in literature [58–60]. It is important to note that some SVMPs contain disintegrin (P-II class) or disintegrin-like (P-III class) domains [53], and therefore, mismapping of the peptides is possible but unlikely in this particular case since all such peptides mapped exactly to known *C. cerastes* disintegrins in the UniProt database.

The final Viperidae genus included in this study is Echis. The family members included are *E. ocellatus*, *E. pyramidum*, and *E. leucogaster*, of which no proteomic data have been reported previously for the latter. *E. ocellatus* and *E. pyramidum* are distributed throughout northern Africa, while *E. leucogaster* occurs in West Africa, isolated areas of the western Sahara, and throughout Algeria [61]. The diet of *Echis* snakes is widely varied, including invertebrates, such as scorpions and centipedes, small mammals, birds, lizards, and amphibians [62]. Our proteomics data show that the venom of *E. pyramidum* and *E. leucogaster* mainly consists of SVMPs with

variations observed regarding PLA2s (*B. gabonica*, *B. nasicornis*, and *B. rhinoceros*), disintegrins (*B. arietans*, *B. gabonica*, and *B. nasicornis*), SVMPs (*B. rhinoceros*), and CTLs (*B. nasicornis*) (Fig. 1, Supplementary Table S1). This discrepancy can be due to several reasons, such as using different methods to generate and analyze the data, as well as intraspecific variation in venom composition [52]. Furthermore, the complexity of some snake venom components (e.g., SVMPs exist in a size range from 20 to 100 kDa [53]) may lead to inconsistencies between studies. This emphasizes the importance of using the same proteomic approach for cataloguing venom composition of different snakes to enable comparison. Large amounts of rhinocerase 2, a SVSP homolog that contains a H57R mutation [54], was found in the venom of *B. rhinoceros*. Interestingly, this H57R mutation was also found in peptides from *B. gabonica* and *B. nasicornis*, yet in smaller amounts. Such SVSP homologs have previously been detected in *B. gabonica* [55], but this is the first time they have been found in *B. nasicornis*.

The final Viperidae genus included in this study is Echis. The family members included are *E. ocellatus*, *E. pyramidum*, and *E. leucogaster*, of which no proteomic data have been reported previously for the latter. *E. ocellatus* and *E. pyramidum* are distributed throughout northern Africa, while *E. leucogaster* occurs in West Africa, isolated areas of the western Sahara, and throughout Algeria [61]. The diet of *Echis* snakes is widely varied, including invertebrates, such as scorpions and centipedes, small mammals, birds, lizards, and amphibians [62]. Our proteomics data show that the venom of *E. pyramidum* and *E. leucogaster* mainly consists of SVMPs with
abundances of ~41% and ~42%, respectively. In contrast, E. ocellatus mainly consists of PLÀ2s with an abundance of ~42%, followed by ~26% SVMPs (Fig. 1, Supplementary Table S1). This differs from previous studies, where SVMPs were also reported as the major component of E. ocellatus venom with abundances of ~70% [63, 64]. Again, different methods used to analyze the venom composition and the origin of the snakes milked to obtain the venoms may be underlying reasons for the observed differences. SVMPs of Echis venom comprise less than 2% of the whole venom (Fig. 1, Supplementary Table S1), which is in accordance with previous reports [63, 64]. In agreement with a previous study showing that the genetic variability between E. pyramindum and E. leucogaster is very low [65], our proteomics data show that the venom composition of E. leucogaster is quite similar to that of E. pyramindum. Strikingly, the amount of disintegrins was found to be 11% for E. ocellatus and less than 0.01% for E. pyramindum and E. leucogaster (Fig. 1, Supplementary Table S1). Extensive, likely diet-driven, interspecies venom variation has been documented in Echis representatives at the transcriptome and proteome levels [63] and in functional toxicity studies [66–68]. It is plausible that this interspecies variation can explain the differences between our results and previous analyses of E. pyramindum and E. ocellatus venom.

### In vitro functional characterization of whole venoms

To evaluate and compare functional activities of sub-Saharan Africa’s 26 medically most relevant elapid and viper venoms, we determined the concentrations of snake venom resulting in 50% product conversion at a fixed substrate concentration (EC_{50} values) in PLÀ2, SVSP, and SVMP enzymatic activity assays, as well as the concentrations of snake venom reducing the cell viability by 50% (IC_{50} values) in a cell viability assay. Lower EC_{50} or IC_{50} values indicate more potent activity of the analyzed toxins in the whole venom.

### PLÀ2 enzymatic activity

Secreted PLÀ2s are one of the major components of many animal venoms. These 13- to 15-kDa enzymes need Ca^{2+} ions to catalyze the hydrolysis of phospholipids [69]. However, it is noteworthy that some PLÀ2s have lost their enzymatic activity during evolution [70]. In the noncatalytic PLÀ2s, the catalytic residue D49 is mutated to another amino acid (e.g., lysine, serine, asparagine, glutamine, or arginine), resulting in a conformational change of the Ca^{2+} binding loop that prevents the reaction by hindering Ca^{2+} coordination, which is essential for catalysis [71, 72]. Despite sharing a 40–99% amino acid sequence identity and highly conserved 3-dimensional structures, snake venom PLÀ2s display a wide variety of pharmacologic activities, including neurotoxic, myotoxic, cytotoxic, anticoagulant, and hemolytic effects [73].

Among the 18 snake species of the Elapidae family included in this study, the subgenus Afromaja shows the highest PLÀ2 activity with EC_{50} values of 15–38 μg/mL (Fig. 2A, Table 2). The subgenus Bouleggerina and genus Hemachatus have moderate PLÀ2 activity, with EC_{50} values of 80–90 μg/mL, while the subgenus Uraeus exhibits low PLÀ2 activity with EC_{50} values above 200 μg/mL. Finally, Dendroaspis venoms display the weakest PLÀ2 activity (EC_{50} values over 1 mg/mL), which is in agreement with previous findings [18, 74]. The PLÀ2 activity of elapid snake venom can therefore be ranked in the following order: Afromaja > Bouleggerina > Hemachatus > Uraeus > Dendroaspis. This is in agreement with a previous publication on PLÀ2 activity of the 3 Naja subgenera [40] and correlates to the relative abundance of PLÀ2s in our proteomics data, except for N. melanoleuca, which exhibits the highest PLÀ2 abundance among the elapids but slightly lower activity than the 7 snakes from the Afromaja subgenus (Fig. 2A, Table 2).

Within the 8 snakes from the Viperidae family, B. gabonica and B. nasicornis show the lowest EC_{50} values for PLÀ2 activity (~40 μg/mL, Fig. 2B, Table 2), which is comparable to that of the subgenus Afromaja from the Elapidae family. The high PLÀ2 activity of B. gabonica and B. nasicornis venom is in agreement with a previous publication [51] but differs from our proteomics data, which show a low PLÀ2 abundance (Fig. 2C). Notably, 2 other species from the Bitis genus, B. arietans and B. rhinoceros, show weak PLÀ2 activity, likely due to high abundances of mutated PLÀ2s in their venom (Fig. 2C). Similarly, the 3 species from the Echis genus exhibit high relative abundances of PLÀ2 but weak enzymatic activity, which can be explained by high amounts of noncatalytic PLÀ2s found in Echis venoms (Fig. 2C). This result is consistent with a previous study reporting high myotoxic activity, but low enzymatic activity, of S49 PLÀ2s in E. ocellatus and E. pyramindum venoms [75]. The Cerastes genus displays the second highest PLÀ2 activity, with an EC_{50} value of 144 μg/mL. In general, our data demonstrate that there is a high correlation between PLÀ2 activity and PLÀ2 relative abundance for most of the viperid snake venoms.

### SVSP enzymatic activity

SVSPs are members of the S1 peptidase family, which catalyze the cleavage of covalent peptide bonds of proteins via the conserved catalytic triad His57–Asp102–Ser195, in which serine serves as the nucleophile amino acid at the active site [76]. These 26- to 67-kDa enzymes affect the coagulation cascade, as well as the fibrinolytic and kallikrein–kinin systems, and cause hemostatic imbalances in victims [77]. In this relation, SVSPs can be classified as procoagulant, anticoagulant, platelet aggregating, or activator of fibrinolysis [78].

Our proteomic data show negligible amounts of SVSPs in the elapid venoms (Fig. 3B), and therefore, EC_{50} values of SVSPs were determined only for viperid venoms (Fig. 3A, Table 2). C. cerastes shows the lowest EC_{50} value (25 μg/mL), which correlates with the high SVSP abundance in its venom (27.5%, Fig. 3B). Within the genus Bitis, the SVSP activity of B. gabonica and B. nasicornis is moderate, with EC_{50} values of ~100 μg/mL, whereas B. arietans and B. rhinoceros demonstrate weak SVSP activity with EC_{50} values between 500 and 1,000 μg/mL (Fig. 3A, Table 2). Although B. rhinoceros exhibits the second highest SVSP abundance among the 8 vipers included in this study, its EC_{50} value is in the high range. This could be because, as mentioned before, an SVSP homolog with a catalytic site mutation [54] was found in our proteomic analysis. Finally, the genus Echis shows the weakest SVSP activity, with EC_{50} values above 1 mg/mL, which agrees with their low SVSP abundance (below 2%, Fig. 3B).

### SVMP enzymatic activity

The Zn^{2+}-dependent SVMPs are one of the most abundant toxins in viperid venoms [5] mainly responsible for inducing systemic hemorrhage after envenomings with these snakes. There are 3 major classes of SVMPs: P-I contains only a metalloproteinase (M) domain, P-II contains an M domain and a disintegrin (D) domain, and the most complex P-III class is composed of an M domain, a D domain, and a cysteine-rich (C) domain.

All elapids show high EC_{50} values of at least 100 μg/mL, except N. annulifera, with a value of 80 μg/mL. This is in agreement with previously published data showing that SVM are in the second most abundant protein family in N. annulifera venom after 3FTxs.

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Characterization of 26 African snake venoms

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Figure 2: PLA2 enzymatic activity of whole venoms of elapids (A) and vipers (B) at different venom concentrations. Absorbances at 405 nm were normalized by subtracting values of the negative control (absence of venom). Error bars: SD from 2 independent measurements. (C) Relative abundance of enzymatically active PLA2s (D49) and enzymatically inactive PLA2s (mut) in 26 sub-Saharan snake venoms. A heatmap displaying EC50 values for each snake venom is plotted above the corresponding abundance.
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Figure 3: (A) SVSP enzymatic activity of viper whole venoms at different venom concentrations. RFU, relative fluorescence unit. Error bars: SD from 2 independent measurements. (B) Relative abundance of SVSPs in 26 sub-Saharan snake venoms. A heatmap displaying EC50 values for each snake venom is plotted above the corresponding abundance.

SVMPs in the Dendroaspis species have a low abundance but a high activity in D. jamesoni and D. viridis (EC50 values of 241 and 136 μg/mL, respectively). This is in agreement with an earlier study in which SVMP-dependent anticoagulant activity was observed in Dendroaspis species despite the low SVMP abundance [79]. Among the 8 viperids included in this study, B. arietans show the lowest EC50 value (3.3 μg/mL), while the 3 venoms from the Echis subgenus show high amounts of SVMPs (Fig. 4C), with EC50 values between ~2 and 14 μg/mL (Fig. 4B, Table 2). B. arietans has an EC50 value in the same range as the Echis venom, whereas all other Bitis species have EC50 values around 40–50 μg/mL.

Cell viability

CTxs and PLA2s are known to, either individually or synergistically, interfere with and disrupt the integrity of cellular membranes, leading to irreversible damage and cell death [25, 80]. In snake venoms, cytotoxins are mainly found in the genera Naja and Hemachatus of the Elapidae family [8], while PLA2s are found in all venomous snake families, including Elapidae and Viperidae [81]. Therefore, the cytotoxicity of all elapid and viperid venoms included in this study was evaluated using an immortalized human keratinocyte cell line, which has been reported to be sensitive to snake venom cytotoxins and PLA2s [25].

Treatment of the cells with venoms resulted in a concentration-dependent inhibition of cell viability (Fig. 5). As expected, the viper venom were more potent (IC50 2.0–6.5 μg/mL) than venoms from the elapids (IC50 >100 μg/mL). Among the Elapidae, 4 species from the Naja genus (i.e., N. senegalensis, N. melanoleuca, N. nigricincta, and N. naja) show IC50 values close to those of the Viperidae (below 10 μg/mL), while the Dendroaspis genus demonstrates IC50 values above 100 μg/mL. These results are in alignment with a high abundance of cytotoxins in the Naja genus and PLA2s in the Viperidae family, as well as a very low abundance of these 2 toxin families in the Dendroaspis genus.

Thromboelastography

The blood coagulation cascade is a primary target for many snake venom toxins due to its pivotal role in maintaining homeostasis, and most venomous snake families possess toxins in their venoms that can interfere with this system. This is particularly evident (although not exclusive to) vipers, whose venoms are generally dominated by proteins that cause coagulopathies (e.g.,...
Figure 4: SVMP enzymatic activity of the whole venoms of elapids (A) and vipers (B) at different venom concentrations. RFU, relative fluorescence unit. Error bars: SD from 2 independent measurements. (C) Relative abundance of SVMP subfamilies in 26 sub-Saharan snake venoms. SVMP disintegrins are included in the SVMP category. A heatmap displaying EC50 values for each snake venom is plotted above the corresponding abundance.
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SVMPs, SVSPs, and disintegrins) [82, 83]. Thus, we assessed the coagulotoxic effects of the venoms of all viper species included in this study via TEG by incubating whole venom with human plasma and physiologic cofactors of coagulation (i.e., calcium and phospholipids).

All venoms were tested and presented similar activity, at ~2 μg/mL and ~20 μg/mL, except B. arietans, which showed inconclusive results at ~2 μg/mL. Both anti- and procoagulant effects were observed on a broadly genus-specific basis. More specifically, venoms from all Bitis species, except B. nasicornis, displayed an overall strong anticoagulant activity, with no visible clot formation (Fig. 6).

Unlike Bitis, C. cerastes venom produced a detectable, stable clot almost immediately after assay initiation (Fig. 6). Interestingly, other researchers have reported that the venom of this species exerts both pro- and anticoagulant effects in a concentration-dependent manner, whereby low venom concentrations (~200 μg/mL), as used in our study, enhanced blood clotting, in agreement with our observations, while higher amounts disrupted coagulation [84].

Last, an even stronger procoagulant activity than seen for C. cerastes was observed for the 3 Echis representatives included in this study (Fig. 6). This is not surprising, as a signature trait of most Echis species is the presence of exceptionally potent prothrombin activators (all part of the SVMP family) in their venoms, which results in uncontrolled formation of fibrin clots due to excessive production of thrombin [85–88]. This agrees with our proteomic data, in which Echis venoms show high amounts of SVMPs for all 3 species.

**Conclusion**

In this study, we systematically analyzed and compared the proteomics and in vitro functional activity of multiple snake venoms and provide toxicovenomic profiles of 26 of sub-Saharan Africa’s medically most important elapids and vipers. To the best of our knowledge, the venom composition of N. anchietae and E. leucogaster is presented here for the first time. Overall, our data show that the elapid venoms contained large amounts of neurotoxic and cytotoxic 3FTxs and PLA2s, whereas the viper venoms were dominated by hemotoxic and/or cytotoxic PLA2s, SVMPs, and SVSPs, as expected based on clinical manifestations observed for elapid and viperid envenoming [5].

The high-throughput, label-free, quantitative proteomics approach presented here comes with some limitations. During mass spectrometry, proteins are identified through mapping of the peptide sequence to a database. It is important to keep in mind that peptides from highly similar isoforms may be difficult to map back to their parent proteins, resulting in false positives (e.g., detection of a disintegrin instead of an SVMP). Additionally, the lack of a comprehensive database may thus result in false negatives; if the sequence of a protein is not present in the database, it cannot be detected. In the UniProt reference database used here, there was a noticeable lack of SVMPs from the 26 snakes, apart from
Spontaneous control

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Overlaid thromboelastography traces showing the ability of the different venoms (2 μg/mL for all species except \textit{B. arietans}, 20 μg/mL) to clot plasma relative to a spontaneous control in 1 hour. Time is plotted horizontally and amplitude (clotting strength) is plotted vertically. Eight representatives of the Viperidae family, which possess procoagulant (\textit{C. cerastes}, \textit{E. leucogaster}, \textit{E. ocellatus}, and \textit{E. pyramidum}) and anticoagulant (\textit{B. arietans}, \textit{B. gabonica}, \textit{B. nasicornis}, and \textit{B. rhinoceros}) venom, are depicted. Blue traces represent spontaneous clot controls and red traces represent samples (\(n = 3\)).}
\end{figure}

The coagulotoxic effect of the viper venoms included in this study was assessed by TEG, showing a procoagulant effect for venoms from \textit{C. cerastes} and the genus \textit{Echis}. In contrast, all \textit{Bitis} species showed strong anticoagulant activity except \textit{B. nasicornis}, which also showed an anticoagulant activity but to a lesser extent. Finally, cell viability of a keratinocyte cell line was inhibited by addition of snake venoms from all snake species, except the ones from the genus of \textit{Dendroaspis}. This is not surprising, given that \textit{Dendroaspis} venoms are known to be highly neurotoxic, cause very little tissue damage, and display very low enzymatic activities [18, 74].

Some inconsistencies were observed between the relative abundance of certain toxin families and whole venom activity in their respective \textit{in vitro} functional assays. For example, our data show that the venoms of \textit{N. annulifera}, \textit{D. viridis}, and \textit{D. jamesoni} had high SVMP activity despite low abundance of such proteins. When it comes to \textit{in vitro} assays for characterizing toxin functions, a limitation of the present study is the lack of assays to assess the activity of neurotoxins, as several species included herein (e.g., mambas and most \textit{Uraeus} cobras) possess predominately neurotoxic venoms [18, 34, 74].

Overall, this study provides a foundation for further studies on snake biology and evolution, for which we recommend an integrated approach combining genomics, transcriptomics, and proteomics to provide information on gene expression and other molecular mechanisms linked to phenotypic diversity [1]. Moreover, the toxicovenomic profiles elucidated in this study may aid
in the development of effective antivenoms through better understanding of the behavior of snake venoms and their roles as drug targets.

Additional Files

Supplementary Fig. S1. RP-HPLC chromatograms of the whole venoms of 26 sub-Saharan snakes.

Supplementary Table S1. Composition of the whole venoms of 26 sub-Saharan snakes.

Data Accessibility

The data sets supporting the results of this article, including results from the in vitro functional activity assays, are available in the GigaScience GigaDB repository [92]. Results from the proteomics characterizations have been deposited to the ProteomeXchange Consortium via the PRIDE [91] partner repository with the dataset identifier PXD036116.

Abbreviations

CTTs: C-type lectins; CTxs: cytotoxins; FA: formic acid; LC: liquid chromatography; INTxs: long neurotoxins; MS: mass spectrometry; NOB: 4-nitro-3-(octanoyloxy)benzoic acid; PBS: phosphate-buffered saline; PLA2s: phospholipase A2s; RFU/s: relative fluorescence units per second; RP-HPLC: reversed-phase high-performance liquid chromatography; sNTxs: short neurotoxins; SVMPs: snake venom metalloproteinases; SVSPs: snake venom serine proteinases; 3FTxs: 3-finger toxins; TEG: thromboelastography; TFA: trifluoroacetic acid.

Competing Interests

All authors declare no conflict of interest.

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Authors’ Contributions


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