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Original Article

Proteome Analysis of Toxic Fractions of Iranian Cobra (Naja naja Oxiana) Snake Venom Using Two-Dimensional Electrophoresis and Mass Spectrometry

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Abstract

Snake venoms are mostly composed of various proteins and peptides with toxicity and pharmacological effects depending on their geographical sources. *Naja naja oxiana* is one of the most medically important venomous snakes in Iran and Central Asia. The bite of this type of snake can cause severe pain and swelling, as well as neurotoxicity. Without medical treatment, symptoms quickly worsen and death can occur soon. A detailed understanding of venom components can provide new insight into the production of antivenom against toxic agents instead of crude venom. Specific antibodies against toxic fractions are of utmost importance in neutralizing crude venom. Therefore, the proteome profile of these fractions of *Naja naja oxidana venom* was analyzed using fractionation by gel filtration, two-dimensional electrophoresis, mass spectrometry, and data mining. Base on the results, in total, 32 spots were detected and categorized into three protein families, namely three-finger toxin (3FTx), phospholipase, and Cysteine-rich secretory proteins (CRISP). These proteins consist of more than 70% crude venom all with a molecular weight below 25 kDa. The 3FTx as a highly diverse constituent in the venom of *Naja* species was in large quantity in this district. Short-chain neurotoxins, including short neurotoxin, cytotoxin, and muscarinic toxin-like protein, were in abundance, respectively. In conclusion, the recognition of toxic fractions of Naja naja oxiana in this region could be of great help in the production of an effective antivenom against similar compositions. It can also help the medical care department to find out the clinical sign of cobra venom. To the best of our knowledge, this was the first study to report the proteomic of toxic fractions of *Naja naja oxiana* in Iran.

Keywords: Venom proteome, 2D gel Electrophoresis, Mass spectrometry, *Naja naja oxiana*, Chromatography

Analyse de Protéome des Fractions Toxiques du Venin de Serpent de Cobra Iranien (*Naja naja Oxiana*) par Électrophorèse 2D et Spectrométrie de Masse

Résumé: Le venin de serpent en fonction de leur source géographique comprend une variété de protéines et de peptides ayant des effets toxiques et pharmacologiques. *Naja naja oxiana* est l'un des serpents venimeux médicalement importants en Iran et en Asie centrale. La morsure de ce type peut causer de graves blessures et un gonflement, ainsi qu'une neurotoxicité. Sans traitement médical, les symptômes s'aggravent rapidement et la mort peut survenir rapidement. La compréhension des composants du venin dans les détails peut ouvrir une nouvelle vision de la fabrication d'anti-venin contre les agents toxiques au lieu du venin brut. En raison de l'importance des anticorps spécifiques contre les fractions toxiques dans la neutralisation du venin brut, le profil

du protéome de ces fractions de venin de *Naja naja oxiana* a été analysé en utilisant le fractionnement par filtration sur gel, l'électrophorèse bidimensionnelle, la spectrométrie de masse et l'exploration de données. Sur la base des résultats, entièrement 32 taches ont été détectées et classées en trois familles de protéines, la toxine à trois doigts (3FTx), la phospholipase et les protéines sécrétoires riches en cystéine (CRISP). Ces protéines sont constituées à plus de 70% de venin brut, toutes avec un poids moléculaire inférieur à 25 kDa. 3FTx en tant que constituant très diversifié du venin des espèces *Naja* était en grande quantité dans ce district. Les neurotoxines à chaîne courte, y compris la neurotoxine courte, la cytotoxine et la toxine muscarinique, comme la protéine, étaient respectivement en abondance. Pour résumer, la révélation du protéome à partir de fractions toxiques de *Naja naja oxiana* de cette région pourrait fournir une approche de production d'un antivenin efficace contre une composition similaire et aider également le service de soins médicaux à découvrir le signe clinique du venin de cobra. À notre connaissance, il s'agit du premier rapport de protéomique des fractions toxiques de *Naja naja oxiana* d'Iran.
Mots-clés: protéome de venin; Electrophorèse sur gel 2D; Spectrométrie de masse; *Naja naja oxiana*; Chromatographie

Introduction

Snake bite is a serious public health problem in tropical and subtropical areas. Annually, 5.4 million snakebites take place across the globe, accounting for 2.5% of deaths and around 7.5% of amputations, as well as other real disabilities. Although technological advancements are essentially important in the treatment of snake bites, characterization of the components of snake venoms is vital since venom pathogenesis is a multi-factorial issue relying on its composition (Fatima and Fatah, 2014). Medically important venomous snakes can be categorized into three families: Atractaspidae, Elapidae, and Viperidae.

Snake venoms are mostly composed of various proteins, peptides, and toxin families, such as serine protease, phospholipase A2, toxins, disintegrins, and numerous others that modify or prevent the functions of their targets. Snake venoms from diverse environmental districts may vastly differ in composition (Gutierrez et al., 2009). This dissimilarity of venom mixtures can lead to variations in their activities and enhance the assessment of antivenoms. Therefore, it is of utmost importance to discover the geographical variation in venom profile and optimize venom formulation in antivenom production (Laustsen et al., 2015). Moreover, venom-based drug discovery and development may possibly be a natural source of new medicines, such as captopril and ziconotide (Harvey, 2014).

Naja is a genus of most medically important snakes known as cobras. The members of this genus are widespread in Africa, Asia, and the North West of Iran (Dehghani et al., 2014). Several studies on Cobra venom revealed that this cocktail includes a range of proteins and peptides with different molecular weights and enzymatic and non-enzymatic activities that belong to different groups including phospholipases A2 (PLA2), cardiotoxins, and neurotoxins. Currently, an increasing number of new proteins is isolated from cobra venom and documented, while some are known as thermo-stable toxins (Binh et al., 2010). The assessment and recognition of the toxic components of each venom can provide an insight into their ancestral relationship and contribute to the preparation of an effective antiserum.

Contrary to traditional chromatography methods, the proteomics field and related procedures have been recently used as a fast and well-organized route for gaining information about venom composition and protein complex (Calvete et al., 2009).

Two-dimensional gel electrophoresis (2DE) is a well-organized technique to separate the components of a protein mixture, such as venom. The flexibility of this extremely capable proteomics tool has made it a strong method to uncover novel proteins or peptides even in a

very close organism (Vejayan et al., 2010). The 2DE was used in a comparative study, along with mass spectrometry, to analyze the composition of a protein mixture. It was firstly used in the venom field in a comparative analysis on the venoms of Dispholidus typus, Crotalus atrox, and Bothrops jararaca. The relationship among genetic, ecological, and phylogeny factors was considered in this technique. A previously conducted study has shown that anti-sera against toxic fractions of *Naja Oxiana* venom can neutralize the crude venom (Akbari et al., 2010). In light of the aforementioned issues, the current study aimed to report proteomics analysis of toxic fractions of Cobra snake in Iran using 2DE and mass spectrometry to gain a better understanding of important constituents.

Material and Methods

Materials. All the chemicals and sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) materials, and Sephadex G50 were purchased from sigma. IPG strips, Oil, BioLyte and all other materials for 2DE were obtained from Bio-Rad. *Naja naja Oxiana* (NNO) venom was prepared from the Department of Venomous Animals, Razi Vaccine and Serum Research Institute, Karaj, Iran.

Size Exclusion Chromatography. Venom fractionation was carried out using Sephadex G50 as a medium to separate Naja naja oxiana (NNO) venom components. The column, 2x 100 cm, equilibrated with PBS pH 7.5, was loaded with 180 mg NNO venom that was already dissolved in the same buffer. Thereafter, the column was washed with the same buffer, 5ml fractions were collected at 40 ml/h flow rate, and their absorbance was measured at 280 nm. According to the chromatogram, the fractions of each peak were pooled, and their amount of protein was analyzed using Quick Start Bradford protein assay (Bio-Rad). All fractions were also run on 15% SDS-PAGE based on the Laemmli method in non-reducing conditions.

Phospholipase A2 Activity. The PLA2 was assayed according to the guidebook of Secretory Phospholipase Assay Kit (ab133089) in all venom fractions. This assay is based on releasing free thiols from hydrolysis of 1, 2-dithioanalog of diheptanoyl phosphatidylcholine that can be detected by DTNB (5, 5'-dithio-bis-(2-nitrobenzoic acid)). To this end, 10 µl of DTNB and 15 µl of assay buffer were added to each well of the microplate as blank. For the test samples, 10 µl of DTNB, 10 µl samples, and 5 µl of assay buffer were added to the related wells. Reactions can be started by the addition of 200 µl substrate to each well. The optical density of all wells was recorded every min at 414 nm for five min by a plate reader. Enzyme activity calculation was assessed according to the manual procedure.

Toxicity Assay. Toxicity of the fractions was assessed by intravenous injection via tail in groups of mice (NIH strain, 18-20 g, Razi Institute). All fractions were firstly lyophilized, and a dilution series was prepared in saline with a concentration factor lower than 1.125. Five mice were injected at each time point. The mortality rate was recorded 12 h after the injection. Lethal Dosage (LD50) Values were assessed using Sperman and Karber method (Reed and Muench, 1938; Hamilton et al., 1977).

Two-Dimensional Gel Electrophoresis. For the first dimension, isoelectric focusing (IEF) was accomplished using immobilized gradient (IPG) strips, 11cm pH 3-11 (Bio-Rad). Strips were rehydrated with 380 µg of toxin fractions samples dissolved in 185 µl of rehydration buffer, including 8M urea, 2% CHAPS, 100mM Dithiothreitol (DTT), 0.5 %(w/v) Bio-Lyte ampholyte, and 0.001% bromophenol blue. Protean IEF cell (Bio-Rad) as a system was used at 20°C with a maximum current of 50 µA/strip. The IEF was performed at 350 V for 1 hour, 1000 V for one more hour, and 5000 V for 80 min.

The IPG strips were equilibrated in two steps. At the first step, the IPG strips were equilibrated for 20 min with a reduction buffer containing 6M urea, 2% SDS, 0.375M Tris-HCl (pH 8.8), 20% glycerol, and 2% DTT. In the second step, the strips were equilibrated for 20 min with equilibration alkylation buffer including

3% iodoacetamide, 6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), and 20% glycerol. Equilibrated strips were run by loading over on 12% SDS-PAGE gels using Payapajohesh system at 100 V for 3-4 h. The gels were stained with Coomassie Brilliant Blue (CBB) R-250. The molecular weight of the mass of spots was estimated via sinaclon prestained protein marker.

Mass Spectrometry and Database Searching. For mass spectrometric analyses, protein spots were cut out from CBB-stained 2D gel and gel-digested with trypsin. In-gel tryptic digestion was performed after reduction with DTE and S-carbamidomethylation with iodoacetamide. Gel pieces were washed twice with 50% (v/v) aqueous acetonitrile containing 25 mM ammonium bicarbonate and dried in a vacuum concentrator for 20 min. Promega Sequencing Grade Modified Trypsin was dissolved in 50 mM acetic acid supplied by the manufacturer. Thereafter, it was diluted five times with 25mM ammonium bicarbonate to give a final trypsin concentration of 0.02 mg/ml. The gel pieces were rehydrated by the addition of 10 ml of trypsin solution, and after 10 min, 25mM ammonium bicarbonate solution was added to cover the gel pieces. The digests were incubated overnight at 37°C.

A 1ml aliquot of each peptide mixture was applied to ground steel matrix-assisted laser desorption/ionization (MALDI) target plate, followed immediately by an equal volume of a freshly-prepared 5 mg/ml solution of 4-hydroxy-a-cyano-cinnamic acid (Sigma) in 50% aqueous (v/v) acetonitrile containing 0.1% trifluoroacetic acid (v/v). Positive-ion MALDI mass spectra were obtained using a MALDI-TOF/TOF instrument (Bruker ultraflex III, Elmsford, New York) in reflectron mode, equipped with an Nd: YAG smart beam laser. The MS spectra were acquired over a range of 800-5000 m/z. Final mass spectra were externally calibrated against an adjacent spot containing six peptides (des-Arg1-Bradykinin, 904.681; Angiotensin I,1296.685; Glu1-Fibrinopeptide B, 1750.677; ACTH (1–17 clip), 2093.086; ACTH (18–39 clip), 2465.198; ACTH (7–38 clip), 3657.929.). Monoisotopic masses were obtained using a SNAP averaging algorithm (C4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583) and an S/N threshold of 2. For each spot, the ten strongest precursors, along with an S/N greater than 30, were selected for MS/MS fragmentation. Fragmentation was performed in LIFT mode without the introduction of a collision gas. The default calibration was used for MS/MS spectra which were baseline subtracted and smoothed (Savitsky-Golay, width 0.15 m/z, cycles 4). Monoisotopic peak detection used a SNAP averaging algorithm (C4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583) with a minimum S/N of 6. Bruker flexAnalysis software (version 3.3) was applied to perform spectral processing and peak list generation. Tandem mass spectral data were submitted to database searching (NCBI nr database) using a locally-running copy of the Mascot program (Matrix Science Ltd., version 2.4) through the Bruker ProteinScape interface (version 2.1).

Search criteria were as follows: Trypsin; Fixed modifications, Carbamidomethyl (C); Variable modifications, Oxidation (M) and Delaminated (NQ); Peptide tolerance, 100 ppm; MS/MS tolerance, 0.5 Da; Instrument, MALDI-TOFTOF. The obtaine results were filtered to accept only peptides with an expect score of 0.05 or lower. Homology search and multiple amino acid sequence alignments were carried out using the BLAST (http://expasy.org/tools/blast/) and CLUSTALW (http://services.uniprot. org/clustalw/) programs, respectively.

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Results

Purification of *Naja naja Oxiana* Venom. Column chromatography was carried out to prepare the fractions of *Naja naja Oxiana* venom using Sephadex G-50. The chromatogram indicated a well-separated four fractions, F1-F4 (Figure 1). The first two fractions with a molecular weight above 25 kDa were nontoxic, while fractions three and four, which have the major large quantity of crude venom with low molecular

weight, were toxic. All fractions were analyzed on 15% SDS-PAGE under non-reducing conditions. The electrophoresis analysis of crude venom revealed a range of molecular weight mass of components between 5 to more than 116 kDa. The first and second fractions are included in proteins mostly around and over 25 kDa. The third and fourth fractions are composed of proteins with molecular weight lower than 25 kDa (Figure 2). The lethal toxicity of the third fraction was estimated at about 59 µg/mice and 1.8 µg/mice for the fourth fraction, while the rest two fractions with heavier ingredients were recognized as nontoxic. Consequently, fractions three and four were used in 2DE analysis to realize the number and identity of their proteins.

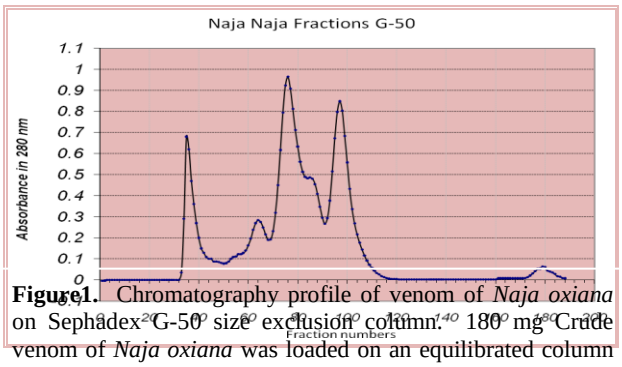

Figure1. Chromatography profile of venom of *Naja oxiana* on Sephadex G-50 size exclusion column. 180 mg Crude venom of *Naja oxiana* was loaded on an equilibrated column with PBS pH=7.5. The column was eluted with the same buffer at a flow rate of 40 ml/h and 5 ml of collected fractions.

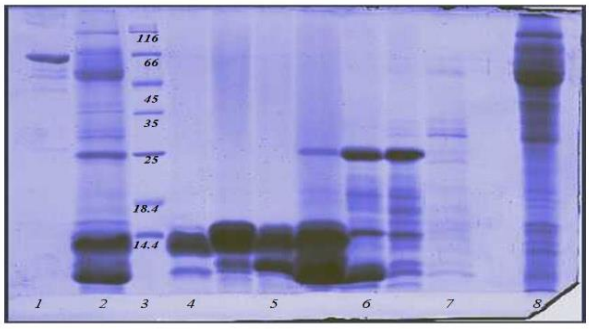

Figure 2. 15 % SDS-PAGE analysis of the non-reducing conditions of crude *Naja* venom and mixed fractions. Lane 1: Albumin, lane 2: crude venom, lane3: protein marker, lane 4: fraction 4, lane 5: fraction 3, lane 6: fraction 2, lane 7: fraction 5, lane 8: fraction on

Phospholipase A2 Activity. Regarding the results of absorbance at optimized dilution in equal conditions for all samples, enzyme activity was in maximum quantity in fraction three. Enzyme activity was about 0.83 U/ml in the third fraction and 0.04 U/ml in the fourth one. This signifies the main toxicity effect of the third fraction derived from PLA2 activity (Figure 3).

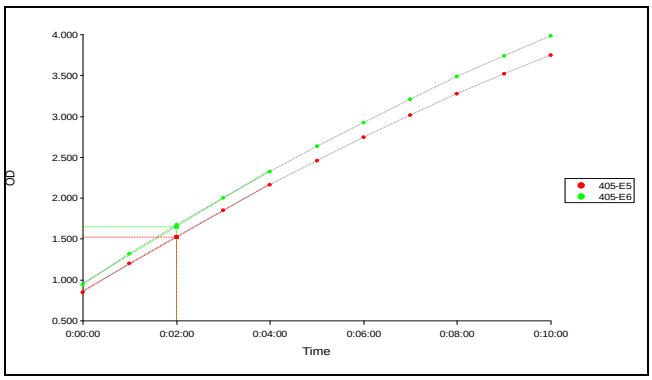

Figure 3. The curve of kinetic assay of Phospholipase A2 in the fourth fraction through hydrolysis of 1, 2-dithioanalog of diheptanoyl phosphatidylcholine, and detecting by DTNB at 405 and 415 nm

Two-Dimensional Gel Electrophoresis. The 2DE method was applied on freeze-dried fractions three and four (Figure 4-5). All spots were recognized by MALDI-TOF-TOF through in-gel trypsin digestion. Figure 4 displays the protein profile of fraction three in 2DE that is composed of 19 spots. The distribution of components from fraction three in 2DE can be divided into three zones, all lower than 25 kDa molecular weight. Zone one includes two spots with a molecular weight below 11 kDa and an acidic isoelectric point (PI). Zone two is composed of mass proteins between 7-17 kDa with a neutral PI. Zone three is the denser group with 12 spots that are more basic using 7-25 kDa in their mass. Proteins in spots 1 and 11 were detected as weak toxin s4C1 belonging to the three-finger toxin (3FTx) family with a molecular weight of 7.9 kDa and accession number P01400. The 2nd, 13th, and 17th spots were revealed as Cytotoxin Vc-5, a member of 3FTx, a short-chain subfamily with accession number Q9PS34. Spots 3-6 are all classified as Acidic PLA2 that are correlated with the phospholipase family with great

similarity to other Naja species. Alpha-elapitoxin-Nno2a, a member of the long-chain subfamily of 3FTx, was in spots 9 and 14. The other form of 3FTx was spot 10, known as Muscarinic toxin-like protein 2; moreover, weak neurotoxin 10 in 12 and 18 spots was a member of 3FTx. Spots 13F3b and 16 with an accession number of E2IU04 belong to 3FTx. Spot 19 was recognized as a protein of the cysteine-rich secretory protein (CRISP) family. In Figure 5, we found the protein content of fraction four which included a range of components with PI>7 and molecular weight below 20 kDa. In the fourth fraction, in total, 20 spots were analyzed (Figure 5). Those spots were in the two protein families with a variety of subfamilies. Short neurotoxin 1 was identified in spots 1, 9, 10, and 14-20 from the 3FTx family, all with an accession number of P01427. Spots 2-4 were from PLA2 with an accession number of Q5G290. Alpha-elapitoxin-Nno2a was a member of the 3FTx family in spots 5-7. Tables 1 and 2 present the protein list of database match with peptide sequences generated by trypsin digestion of spots from Naja oxiana venom fractions three (F3) and four (F4) that were fractionated by 2DE, respectively.

Table 1. List of identified proteins by MALDI-TOF/TOF of the third fraction of *Naja*

Spot no.	protein	Accession number	Mass (Da)	Matched peptide	Protein family /species	Calculated PI
1	Weak toxin S4C11	P01400	7995	LTCLICPEK R.GCAATCPEAKPR.E R.EIVECCSTDK.C	snake 3FTx / *Naja melanoleuca*	8.58
2	Cytotoxin Vc-5	Q9PS34	7116	MFMVAAPHVPVK	snake 3FTx/ *Naja oxiana*	9.41
3	Acidic phospholipase A2 2	P15445	14135	GGSGTPVDDLDR ISGCWPYFK GDNNACAASVCDCDR	Phospholipase A2/ *Naja naja*	4.93
4	Acidic phospholipase A2 (fragment)	Q5G290	14290	LAAICFAGAPYNDNNYNIDLK	Phospholipase A2/ *Naja sagittifera*	4.83
5	Acidic phospholipase A2 2	P15445	14135	GGSGTPVDDLDR GDNNACAASVCDCDR LAAICFAGAPYNDNNYNIDLK	Phospholipase A2/ *Naja naja*	4.93
6	Acidic phospholipase A2 natratoxin	A4FS04	13977	GGSGTPVDDLDR	Phospholipase A2/*Naja atra*	4.93
9	Alpha-elapitoxin-Nno2a	P01382	8596	TWCDAWCGSR TPIPITSETCAPGQNLCYTK	snake 3FTx/*Naja oxiana*	7.63
10	Muscarinic toxin-like protein 2	P82463	7750	GCAATCPIAENR	snake 3FTx/ *Naja kaouthia*	8.19
11	Weak toxin S4C11	P01400	7995	LTCLICPEK GCAATCPEAKPR	snake 3FTx/ *Naja melanoleuca*	8.58
12	Weak neurotoxin 10	Q802B2	10540	GCAATCPEAKPR	snake 3FTx/ *Naja sputatrix*	9.17
13	Cytotoxin Vc-5	Q9PS34	7116	YVCCNTDR MFMVAAPHVPVK MFMVAAPHVPVKR	snake 3FTx/ *Naja oxiana*	9.41
14	Alpha-elapitoxin-Nno2a	P01382	8596	TWCDAWCGSR TPIPITSETCAPGQNLCYTK VIELGCAATCPTVESYQDIK	snake 3FTx family, Long-chain/*Naja oxiana*	7.63
16	Three-finger toxin	E2IU04	7417	YVCCNTDR MFMMSDLTIPVKR	snake 3FTx */ Naja atra*	9.15
17	Cytotoxin Vc-5	Q9PS34	7116	YVCCNTDR MFMVAAPHVPVK	snake 3FTx *Naja oxiana*	9.41
18	Weak neurotoxin 10	Q802B2	10540	GCAATCPEAKPR	snake 3FTx/ *Naja sputatrix*	9.17
19	Cysteine-rich venom protein natrin-1	Q7T1K6	27834	EIVDLHNSLR MEWYPEAASNAER VLEGIQCGESIYMSSNAR VLEGIQCGESIYMSSNAR	CRISP/ *Naja atra*	8.26

Table 2. List of identified proteins by MALDI-TOF/TOF of the fourth fraction of *Naja Oxiana*

Spot no.	protein	Accession number	Mass (Da)	Matched peptide	Protein family/ Species	Calculated PI
1	Short neurotoxin 1	P01427	7336	VKPGVNLNCCR	snake 3FTx / *Naja oxiana*	8.72
2	Acidic phospholipase A2 6	Q5G290	14290	LAAICFAGAPYNDNNYNIDLK	phospholipase A2/ *Naja sagittifera*	4.83
3	phospholipase A2 6	Q5G290	14290	LAAICFAGAPYNDNNYNIDLK	phospholipase A2 / *Naja sagittifera*	4.83
4	phospholipase A2 6	Q5G290	14290	LAAICFAGAPYNDNNYNIDLK	phospholipase A2/*Naja sagittifera*	4.83
5	Alpha-elapitoxin-Nno2a	P01382	8596	TWCDAWCGSR TPIPITSETCAPGQNLCYTK	snake 3FTx / *Naja oxiana*	7.63
6	Alpha-elapitoxin-Nno2a	P01382	8596	TWCDAWCGSR CCSTDDCNPHPK	snake 3FTx / *Naja oxiana*	7.63
7	Alpha-elapitoxin-Nno2a	P01382	8596	TWCDAWCGSR CCSTDDCNPHPK	snake 3FTx/*Naja oxiana*	7.63
8	Muscarinic toxin-like protein 2	P82463	7750	WHMIVPGR WHMIVPGR GCAATCPIAENR SIFGVTTEDCPDGQNLCFK	snake 3FTx/*Naja kaouthia*	8.19
9	Short neurotoxin 1	P01427	7336	TCSGETNCYK VKPGVNLNCCR	snake 3FTx/ *Naja oxiana*	8.72
10	Short neurotoxin 1	P01427	7336	TCSGETNCYK VKPGVNLNCCR	snake 3FTx/*Naja oxiana*	8.72
14	Short neurotoxin 1	P01427	7336	VKPGVNLNCCR LECHNQQSSQPPTTK	snake 3FTx/*Naja oxiana*	8.72
15	Short neurotoxin 1	P01427	7336	KWWSDHR VKPGVNLNCCR	snake 3FTx /*Naja oxiana*	8.72
16	Short neurotoxin 1	P01427	7336	LECHNQQSSQPPTTK TCSGETNCYK VKPGVNLNCCR	snake 3FTx /*Naja oxiana*	8.72
17	Short neurotoxin 1	P01427	7336	VKPGVNLNCCR	snake 3FTx /*Naja oxiana*	8.72
18	Short neurotoxin 1	P01427	7336	VKPGVNLNCCR	snake 3FTx/*Naja oxiana*	8.72
19	Short neurotoxin 1	P01427	7336	VKPGVNLNCCR	snake 3FTx/*Naja oxiana*	8.72
20	Short neurotoxin 1	P01427	7336	TCSGETNCYK VKPGVNLNCCR	snake 3FTx /*Naja oxiana*	8.72

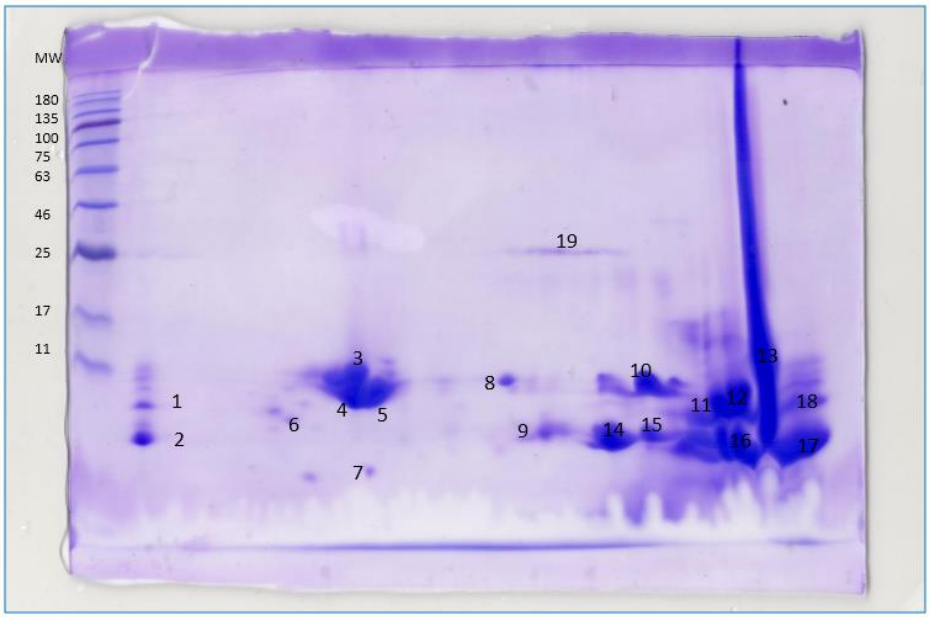

Figure 4. Protein profile of the third fraction of N.naja oxiana venom. 400 µg of the freeze-dried fraction was loaded on an 11-cm IPG strip (pH3-10 from left to right) then running by isoelectric focusing as the first dimension, followed by 12% SDS-PAGE as the second dimension that was stained with coomassie blue.

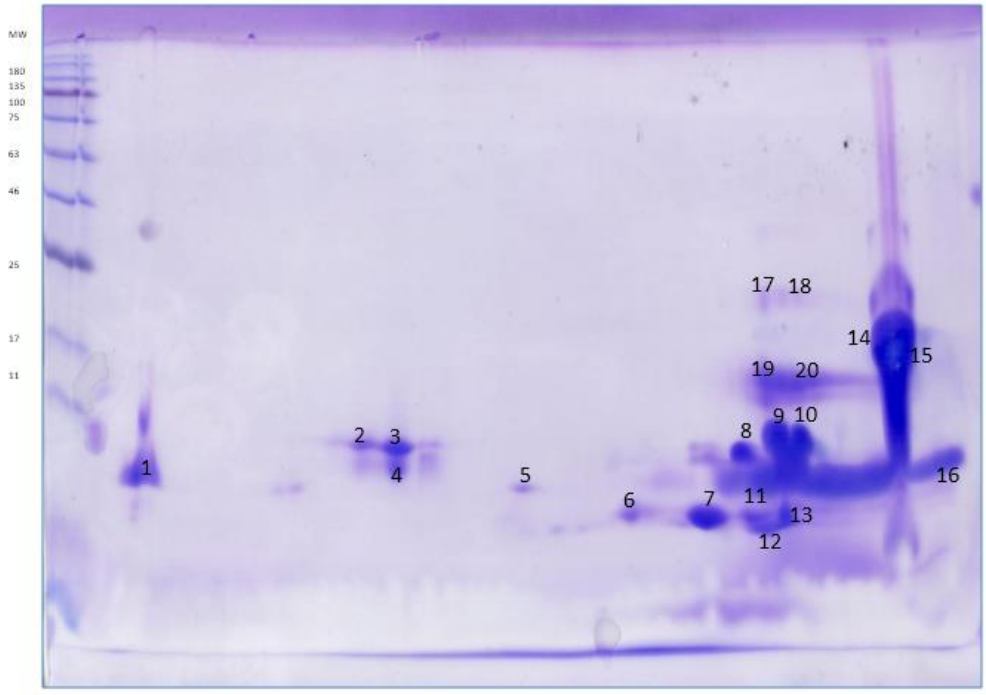

Figure 5. Protein profile of the fourth fraction of *N.naja oxiana* venom. 250 µg of the freeze-dried fraction was loaded on an 11-cm IPG strip (pH3-10 from left to right) then running by isoelectric focusing as the first dimension, followed by 12% SDS-PAGE as the second dimension that was stained with coomassie blue.

Discussion

Researchers have been long involved in the field of venoms using diverse techniques, such as 2DE and mass spectrometry (Malih et al., 2014), chromatographic fractionation of all ingredients, mass spectrometry (Georgieva et al., 2011), or attached protocol using two last methods together (Lomonte and Calvete, 2017). In the present study, the composition of toxic fractions of Iranian cobra venom (*Naja naja Oxiana*) was demonstrated using 2DE and mass spectrometry containing a varying molecular weight lower than 25 kDa. The proteome analysis of these two fractions demonstrated that toxic fractions from the Iranian *Naja* included three Protein families, according to their abundance known as 3FTx, phospholipase, and CRISP with mostly non-enzymatic activity.

The 3FTx represents the major amount of *Naja* venom. This protein family has a similar configuration containing finger-like structures with conserved cysteine amino acid in their sequences (Dutta et al., 2017). The members of the 3FTx family can be classified into subfamilies based on their biological effects, such as cytotoxicity, neurotoxicity, as well as their diverse effects, such as neurotoxicity, cardiotoxicity, cytotoxicity, and platelet aggregating activities. In the present study, a range of 3TFx was identified as Alpha-elapitoxin, a weak neurotoxin, Muscarinic toxin, and Cytotoxin that were previously reported as thermo-stable toxins (Binh et al., 2010). These results were in agreement with those obtained in other studies on other cobra *Naja sumatrana* (Yap et al., 2014), *Naja atra* (Huang et al., 2015), and *Naja naja* (Sintiprungrat et al., 2016) in different geographical areas in Asia and *Naja haje* (Malih et al., 2014) in Africa.

Therefore, understanding of 3FTs abundance of each *Naja* venom in an area due to possible variation in structure and quantity could help antivenom producers to manage their products as specific as possible. A previously conducted study provided some data regarding the toxic fractions of Iranian cobra (Akbari et al., 2010). Based on the results of the stated study, they were composed of 78% crude venom and reported nearly the same as Indian *naja naja* (Dutta et al., 2017) and possibly the other *naja* venoms with lethal toxicity 64.2 and 1.65 µg/mouse respectively. An antiserum against these toxic fractions could proficiently neutralize the crude venom. Moreover, other studies indicated that proteins with high molecular weight can be immunogenic in *naja oxiana* (Talebi Mehrdar et al., 2017), naja *kautia* (Kulkeaw et al., 2007), although they are obviously not toxic.

The results of the present study pointed out that the short neurotoxin subfamily is a dominated part of both fractions, especially in fraction four with more than 60% abundance and an LD50 with almost the same result as cobra venom from Pakistan, even though it was rich in long neurotoxin (Wong et al., 2016). Fewer short-neurotoxins were found in the venom of cobras from India and Sri Lanka (Sintiprungrat et al., 2016). Further studies even found this dissimilarity in another cobra proteomic venom (*Naja kaouthia*) from different geographical areas where it is rich in long-term neurotoxicity from Thailand, while in Malaysia and Vietnam, the majority fraction of the venom is cytotoxins. It is noteworthy that these differences, even at the transcription level, showed that *Naja kauthia* of Thailand has a long neurotoxin that was a short form of the same species of Malaysia (Tan et al., 2017). This means that even with an efficient antivenom, the dose effectiveness should be corrected and a single source in antiserum production cannot sufficiently cover the vast area (Tan et al., 2015).

In another study, the antigenic epitope analysis of this venom showed a series of sequences from toxic fractions, including short neurotoxin and elaptotoxin which are involved in immunized serum confirming the critical role of these fractions (Kazemi-Lomedasht et al., 2019). Another 3FTx that was revealed in our analysis was cytotoxin Vc-5 which is one of the dose-dependent effective members of cobra toxin with cytolysis activity. In general, two groups of cytotoxins have been characterized in *naja* genus, groups P and S, based on their residues. Form S is mainly active in

muscle cell depolarization, while form P is characterized by the highly hemolytic activity of cells. These toxins were previously categorized as Vc1 and Vc5, respectively, with 60-62 amino acids as amphipathic proteins (Dubovskii et al., 2005). Here we have type P that is the potent shape of cytotoxin and also epitopes with immunizing serum (Kazemi-Lomedasht et al., 2019) that were different from *naja* from India (Dutta et al., 2017).

The obtained results also showed the other protein family belonging to the CRISP family. These proteins have a molecular weight of approximately 20-30 kDa. These proteins, with no proteolytic, hemorrhage, and coagulant activity, all include a conserved 16 cysteine residue outline, creating eight disulfide bonds (Modahl and Mackessy, 2019). Although their function is not fully recognized, their pharmacological activities inhibit smooth muscle contraction, block cyclic nucleotide-gated ion channels, and cause hypothermia in animals. In this study, two CRISP were identified that were similar to *Naja atra and naja hanna* in fraction three, while it has been reported in different quantity of constituted venom of *naja* in India and Pakistan (Sintiprungrat et al., 2016). Recently, the new effects of CRISP on vascular, blood, and lymphatic permeability to rattlesnake venom has highlighted the importance of studies on this protein family (Suntravat et al., 2019).

The third family was PLA2 that was demonstrated mostly as acidic PLA2 in both fractions considered in this study with three accession numbers, all belonging to the D49 subfamily. The PLA2 from snake venom can be categorized into various isoforms with an enormous range of biological activities. The D49 subfamily of PLA2 includes a conserved residue in Asp-49. Characterizing of PLA2 showed that the acidic form of this enzyme does not have any fatal effect even in higher doses (Tan et al., 2017); moreover, it was already reported of *naja naja* from Pakistan (Wong et al., 2016). Consequently, the main function of the acidic isoform of PLA2 needs further studies. When the transcription level is low (Tan et al., 2017) and the expression amount is around 12% of the total crude content of venom (Laustsen et al., 2015), it may point to indirect and supplementary effects of this isoform on the supplementary effect of other toxins (Mukherjee, 2010).

Conclusion

To the best of our knowledge, this study was the first to report snake venom proteomics analysis of medically important species from Iran. The present study analyzed the toxic fractions of *Naja oxiana* venom from Iran. This was carried out using a proteome method combining 2DE, mass spectrometry, and data mining through fractionation by size-exclusion chromatography. The results of the analysis indicated that toxic fractions constitute more than 70% weight of crude venom of *Naja oxiana*. These toxic fractions were composed of three protein families, among which the 3FTs was dominant. All *Naja* species were similar in containing 3FTs, which was demonstrated as short neurotoxin here, in the majority of crude venom. Anti-venom manufacturers, particularly in Asia, are involved in the production of anti-venoms which are of paramount importance in medical application. Therefore, a detailed analysis of the components of snake venom, especially the toxic fractions, can provide a better insight into the toxicological components in each geographical region, in comparison with other regions with similar snake species. These data can fill the void of knowledge that existed in regards to the toxic components of *Naja oxiana* venom from this area. This information can be of great help to antivenom manufacturers in formulation optimization and better treatments. This information may also be useful for the use of recombinant antigens in antivenom production in the future.

Authors' Contribution

Study concept and design: A. N. and M. S.
Acquisition of data: A. N.
Analysis and interpretation of data: A. N. and M. S.
Drafting of the manuscript: A. N.

Critical revision of the manuscript for important intellectual content: A. N., M. S. and F. T.
Administrative, technical, and material support: A. N., M. S., F. T. and N. M. D.

Ethics

It is declared that all ethical considerations were taken into account in the preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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