Original Article

Distributional Notes, Venom Characteristics, and Antivenom Neutralization Potency of *Bungarus sindanus* Recently Described From Southeastern Iran

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ABSTRACT

Background: Snakebite envenomation affects millions of people worldwide annually, resulting in significant mortality. Based on climatic and geographical conditions, Iran has a wide fauna and distribution of various snake species.

Objectives: Due to changing climatic conditions, displacement of some snake species, and reports of snakebites with symptoms different from those known in Iran, this research was conducted to identify this species in the southeastern region of the Sistan and Baluchistan Province.

Methods: New snake species were collected from these areas after morphological characterization and venom milking. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profile, high-performance liquid chromatography (HPLC) chromatography of venom, determination of toxicity, and cross-neutralization with the Razi Hexavalent snake antivenom (RHAV) were investigated.

Results: Based on morphological characteristics, the samples were identified as kraits of the *Bungarus sindanus* (Boulenger, 1897). The SDS-PAGE profiles of *B. sindanus* venom showed multiple bands at 30 kDa and 60–97 kDa. The median intravenous lethal dose (LD_{50}) of *B. sindanus* was 0.2 µg/mouse (0.01 µg/g). In vivo cross-neutralization tests did not show an adequate effect of commercial antivenom on the venom's clinical performance.

Conclusion: The present study is the first report of a new distribution, bite report, and toxicological and cross-neutralization of *B. sindanus* in southeast Iran, addressing a critical public health concern by examining the emergence of previously unidentified snake species in Iran. The outcomes of this study may have significant implications for understanding the impact of climate change on snake distribution and venom composition, ultimately improving preparedness for snakebite treatment in affected areas.

Keywords: *Bungarus sindanus*, Morphological characterization, Neutralization activity, Toxicity, Iran

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Introduction

Snake envenoming represents a significant medical challenge in numerous developing countries across tropical and subtropical regions. The majority of these incidents occur in Africa, Asia, and Latin America, constituting a global medical emergency (Gutierrez et al., 2010; Van et al., 2014). It is estimated that a minimum of 1.2 million individuals are affected by snakebites annually, resulting in approximately 20,000 fatalities, which has led the World Health Organization (WHO) to classify it as a neglected tropical disease (WHO, 2007). Improving access to effective antivenom treatments and strengthening the healthcare infrastructure in affected regions could significantly reduce the mortality rate associated with snakebite envenoming. Increasing awareness of snake envenoming as a global health concern may also promote international collaboration, facilitating the development of more effective prevention strategies and treatment modalities (GBD 2020, Gutierrez et al., 2010).

Biodiversity hotspots have been proposed as a model for determining global conservation priorities for high species richness, endemism, number of rare or threatened species, and severity of threats, and the need to update this information is important (Myers, 1988). Iran is located at 25°–40°N and 44°–63°E in the Palearctic zoogeographical region and has a strategic position as a bridge between the Oriental and African realms, which are among the richest venomous snakes distributed in Central Africa, eastern and southern parts of India, and southern Asia (Hosseinian, 2019; Vaghefi et al., 2019). The distribution of species and biodiversity of the families of venomous snakes, Viperidae and Elapidae, have been studied worldwide. Iran is a region with a high species distribution and is known as an area with conservation priorities (Kazemi et al., 2023; Dehghani et al., 2014; Yousefi et al., 2020). According to a WHO report (WHO, 2018), Iranian venomous snakes of highest medical importance include members of Viperidae, *Echis carinatus*, *Macrovipera lebetina*, *Pseudocerastes persicus*, *Gloydius halys,* and *Montivipera albicornuta*, and Elapidae: *Naja oxiana*.

In recent decades, climate change in Iran has probably led to longer periods of temperature increase in the southern parts, as well as changes in air humidity and the frequency of floods. In general, the combination of these consequences results in a climate with long dry periods, which leads to flooding and changes in the fauna and flora of the region (Vaghefi et al., 2019; Zarei et al., 2019).

Based on reports of snakebites with distinct clinical symptoms, including progressive neuromuscular paralysis and high mortality rates from species known in Sistan and Baluchistan Province, southeast Iran, an extensive investigation was promptly conducted to identify the species. Following the collection of specimens in this area and their subsequent transfer to the Venomous Animal Laboratory of the Razi Vaccine and Serum Research Institute, the morphological characteristics of the newly discovered snake were found to be consistent with those of the genus *Bungarus* sp. (Elapidae). The *Bungarus* sp. is recognized as one of the most medically significant snake species with high mortality rates in South Asia. This krait is a diurnal species that exhibits aggressive behavior, striking rapidly if inadvertently provoked. Its envenomation effect is primarily neurotoxic, resulting in systemic paralysis and respiratory failure. Symptoms include abdominal pain followed by bilateral ptosis, muscle weakness, and paralysis; however, local inflammation (pain and swelling) at the bite site is typically absent (Oh et al., 2019). Given that details regarding the venom protein have not been elucidated with respect to the pathogenesis and treatment of envenomation, characterization, toxicity assessment, and neutralization studies of the venom were conducted in the present investigation. As *N. oxiana* is the sole Elapidae species in the region that is included in commercial antivenoms, it was comparatively examined. It is anticipated that this preliminary research on the venom of the newly reported species in the region will contribute to the reduction and control of snakebites, as well as the development and improvement of therapeutic antivenoms.

Materials and Methods

The three *Bungarus* sp. snakes collected from Eshkastak Village (26°32’21.3”N, 61°39’21.4”E), Nikshahr (26°14’14.6”N, 60°13’24.5”E), and Sarbaz Village (26°37’53.3”N, 61°15’39.0”E), Sistan and Baluchistan Province (southeastern Iran). Live specimens were transferred to the venomous animal department of the Razi Vaccine and Serum Research Institute, in separate racks. Snakes were kept according to guidelines for the production, control, and regulation of snake antivenom immunoglobulins, Annex 5, TRS No. 1004 (WHO, 2018).

Morphological characteristics

Morphological traits included the number of ventral and dorsal DS1 (one-head length behind the head), DS2 (rows of scales at mid-body), and DS3 (five ventral scales anterior to the anal plate) (Boulenger, 1897), and subcaudal scales, the patterns of the upper labial and

lower labial scales, postocular, preocular, subocular, and temporal scales, the anal plate, the ventral body, and the ventral tail surface pattern, which are important morphological characteristics (Van, 2014).

Preparation of the venom sample

After venom milking from *Bungarus* sp. and *N. oxiana* (bite-membrane method), the venom samples were centrifuged at 5000 × g for 10 min at 4 ˚C, and the supernatant was lyophilized using a Christ Alpha 1-2 freeze-dryer. The lyophilized venom was stored at -20 ˚C until use.

Characterization of the venom

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

The 20 µL crude venom samples of *Bungarus* sp. and *N. oxiana* (1 mg/mL) were loaded onto a gel (4% stacking, 12% running gel) under non-reduced conditions (Laemmli, 1970). The electrophoresis was performed at a constant voltage of 100 V. The gel was stained with 0.12% (W/V) Coomassie Brilliant Blue G-250 (Sigma-Aldrich) using the "blue-silver" method (Candiano et al., 2004). The Bio-Rad Precision plus Unstained Protein Standard was used to assess the molecular weights of the protein bands.

High-performance liquid chromatography (HPLC)

The crude venom of *Bungarus* sp., *N. oxiana*, and Razi Hexavalent snake antivenom (RHAV) were fractionated by the reversed-phase HPLC (RP-HPLC) gradient method using a C18 column (C18 Agilent USA -ZORBAX 300SB, 4.6×250 mm). After filtration through a 0.22 µm membrane, 100 µL of crude venom solution (1 mg/mL) was injected. The gradient method was performed using mobile phases A (deionized water + 0.1% TFA) and B (acetonitrile + 0.1% TFA): 0–1 min B 5%, 2–20 min B 20%, 21–45 min B 60%, at a flow rate of 1 mL/min. The chromatogram was recorded at a wavelength of 215 nm and processed using ChemStation software (Agilent).

The interaction of venom and antivenom and RP-HPLC analysis

The interaction of venom with antivenom was performed according to the potency test and conducted at a ratio of 1/10 of the venom-to-antivenom protein concentrations, then incubated at 37 ˚C for 30 min. The mixture was then centrifuged at 10000×g for 12 min, and the supernatant was collected for HPLC analysis. HPLC analysis of the supernatant was performed using the same method as for crude venom and antivenom.

Determination of venom toxicity

To evaluate the toxicity of *Bungarus* sp. and *N. oxiana* crude venom, a mouse assay recommended by the (WHO Guideline, 2018) was performed, and the LD_{50} value (or the amount of venom required to kill 50% of the test population) was determined. Different serial concentrations of crude venom solutions in physiological saline (0.9% NaCl) were injected intravenously into groups of mice (n=5 per group). The numbers of dead and surviving mice were recorded 24 h after venom administration. The Spearman-Kaerber method was used to determine the median lethal dose (LD_{50}), and 95% confidence intervals (CIs) were calculated.

The antivenom neutralization potency against *Bungarus* sp. venom

The neutralization potency of the hexavalent snake antivenom (RHAV) was determined by estimating the half-maximal effective dose (ED_{50}) value, which represents the minimum amount of antivenom required to protect 50% of the test population injected (WHO Guideline, 2018). Specifically, according to the lethal dose of the venom, various volumes of the antivenom were incubated for 30 min at 37 ˚C with a predefined challenge dose of venom (5× LD_{50}), and then intravenously administered to mice (18–22 g; 5 mice per group). Additionally, the median effective ratio (ER_{50}), which is the ratio of the amount of venom (mg) to the volume dose of antivenom (mL) at which 50% of mice survived, and the neutralization potency (P) of antivenom, calculated as the amount of venom completely neutralized per unit volume of antivenom (mg/mL), were determined using SPSS software, version 27.

Results

From July to April 2023, samples were collected from Eshkastak village (26°32'21.3"N, 61°39'21.4"E), Nikshahr (26°14'14.6"N, 60°13'24.5"E), and Sarbaz (26°37'53.3"N, 61°15'39.0"E) of Sistan and Baluchistan Province, Iran. The specimens were then transferred to the Venomous Animal Laboratory of the Razi Vaccine and Serum Research Institute for morphological and toxicological studies.

Morphological finding

Based on their morphological characteristics, the specimens were identified as *Bungarus sindanus* (Boulenger, 1897). The snake was black with narrow white stripes and approximately 1 m long, and the head was distinct from the neck with a short snout, small eyes, round pupils, white upper lips, and a pointed tail. The loreal scales were absent. The morphological traits included 225 ventral scales, 15:17:16 dorsal scales, and 51 subcaudal scales, 7+7 upper labial scales, 7+7 lower labial scales, 2 postocular, 1 preocular, 0 subocular, and 2+1 temporal scales, a single anal plate, and no loreal scale observed. Seventeen dorsal rows of scales at mid-body (DRS) and the absence of a loreal scale are important morphological characteristics of the identified specimens (Figure 1).

Characterization of the venom

SDS-PAGE

The SDS-PAGE profiles were obtained for *B. sindanus* and *N. oxiana*, revealing considerable qualitative and quantitative differences. Proteins with molecular weights less than 31kDa were dominant in both species. The *B. sindanus* venom showed two bands in the range of high molecular weight toxins (>45 kDa), while the *N. oxiana* venom exhibited six bands (Figure 2 and Table 1).

The SDS-PAGE profiles of *B. sindanus* venom showed thick and high-intensity bands under 30 kDa and within 60–97 kDa. However, no protein bands were observed within the range of 31-50 kDa.

High-performance liquid chromatography (HPLC)

The RP-HPLC profiles of *B. sindanus* and *N. oxina* venoms are shown in Figure 3. The different chromatographic patterns of crude venom from *B. sindanus* and *N. oxina* revealed several significant peaks, with the highest-intensity peaks observed between 6 and 30 min. The RP-HPLC chromatogram of antivenom showed two peaks. The chromatogram of the antivenom also demonstrated a higher-intensity peak corresponding to $F(ab')_2$ antibody fragments, while the preceding peaks were related to the preservative (Figure 3C).

The interaction of venom and antivenom and RP-HPLC analysis

No changes in the HPLC profile of *B. sindanus* venom were observed after interaction with antivenom $F(ab')_2$ antibody fragments. The lack of suppression of venom peaks indicates non-neutralization of venom components by the antivenom. Only a decrease in the height of the first major peak was observed following the interaction between the venom and antivenom.

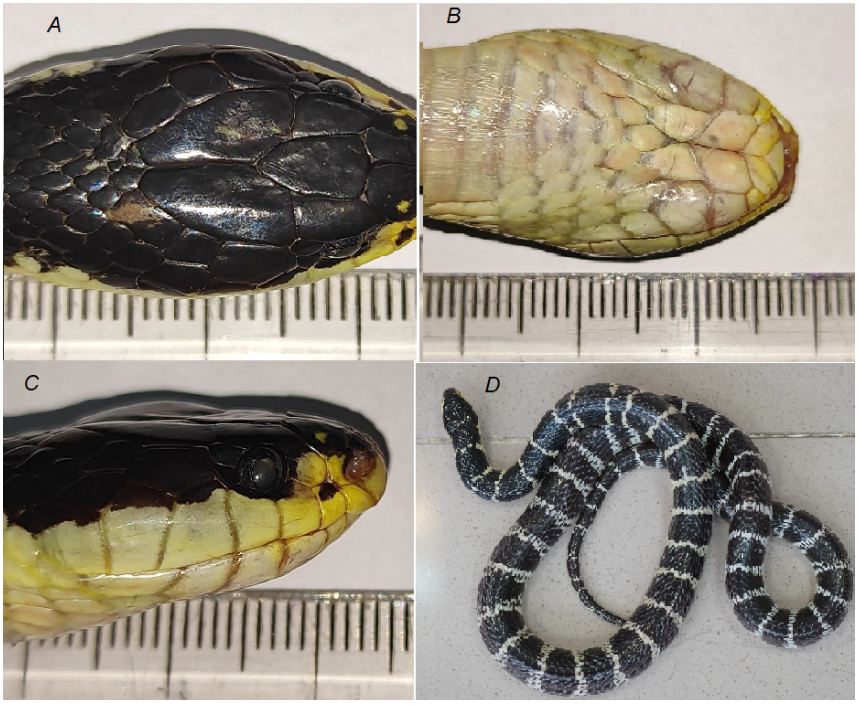

Figure 1. *B. sindanus* from Sistan and Baluchistan Province, Iran

A) Dorsal view of the head, B) Ventral view of the head, C) Lateral view of the head, D) Dorsal view of the body

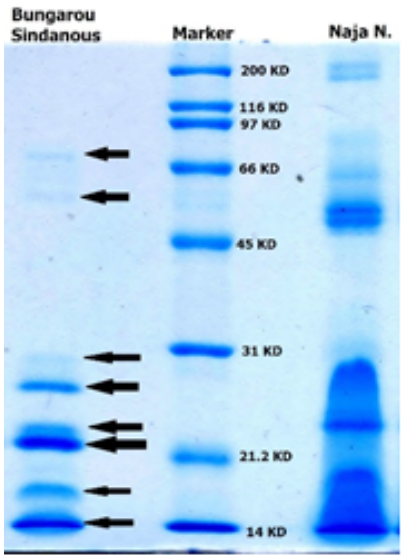

Figure 2. SDS-PAGE of crude venom of *B. sindanus* and *N. oxiana* under non-reduced conditions (4% stacking, 12% running gel), with standard protein markers

Toxicity of venom

The median intravenous lethal doses (LD_{50}) of *B. sindanus* and *N. oxina* venoms were measured. The results showed that the LD_{50} values of these snake venoms differed significantly. The median lethal dose for *B. sindanus* was 0. 2 µg/mice (0.01 µg/g), while the toxicity of *N. oxina* venom was 7.8 µg/mice (0.39 µg/g).

The antivenom neutralization potency against *Bungarus* sp. venom

The neutralization activity of RHAV immunoglobulin against *B. sindanus* and *N. oxina* venoms is shown in Table 2. The ED_{50} of RHAV toward the Iranian *B. sindanus* venom was 13 mL, the median effective ratio (ER_{50}) was 0.001 mg/mL, and the neutralization potency (P) of the antivenom against the venom was 5±0.8 mice LD_{50} (1 µg). Half-maximal effective dose (ED_{50}) of RHAV for Iranian *N. oxiana* venom was 1 mL, the ER_{50} was 0.4992 mg/mL, and the neutralization potency (P) of the antivenom was 64±4.1 mouse LD_{50} (499.2 µg).

Discussion

Although reptile species are widely distributed throughout Iran, their distribution patterns are changing owing to climate change and habitat destruction. Under climate change, some venomous species may expand their distribution ranges, and snakebite risk will increase in human- populated areas of the country. (Vaghefi et al., 2019; Zarei et al., 2019). The herpetofauna of Iran includes 81 species of snakes belonging to seven families and 34 genera (Dehghani et al., 2023, Rastegarpoysni et al., 2008, Zare khormizi et al., 2024). The highest diversity is related to Viperidae, which includes Crotalinae (one genus, one species) and Viperinae (seven genera, ten species). The Elapidae family, including Elapinae (3 genera, 3 species) and Hydrophiinae (1 genus, 10 species), is also very important in the region (Dehghani et al., 2023, Rostampor et al., 2024, Yousefi, 2020).

The known *Elapinae* species in Iran include *N. oxiana* and *Walterinnesia morgana*, and the most important medicinal species responsible for most snakebite cases belong to the genus *N. oxiana* in the eastern and southeastern parts of Iran (Dehghani et al., 2023, Safaei-

Table 1. Molecular weight (kDa) of SDS-PAGE profile results of *B. sindanus* and *N. oxiana* crude venoms

Snake Species	Molecular Weight (kDa)								
Band No.	1	2	3	4	5	6	7	8	9
B. sindanus	78	59	30	27	22	21	16	14	
N. oxiana	200	116	97	66	45	31	21	14	6

Table 2. Neutralization potency of Iranian *B. sindanus* and *N. oxiana* venom lethality by RHAV

Venom	i.v. Mouse LD_{50} (µg/g)	ED_{50} (µL)	ER_{50} (mg/mL)	Neutralization Potency (P) (µg/mL)
B. sindanus	0.01	13	0.001	1
N. oxina	0.39	1	0.4992	499.2

Mahroo et al., 2015). Recently, snakebite envenomations with different symptoms have been reported in the Sistan and Baluchistan region (southeast Iran). By examining the morphological characteristics, a kraits species, *B. sindanus* (Elapidae) Boulenger, 1897, was identified in southeast Iran. In the present study, the venoms of the Iranian *B. sindanus* and *N. oxiana* snakes were analyzed by SDS-PAGE, RP-HPLC chromatograms, toxicity, and neutralizing potency of Razi Hexavalent Antivenom.

Kraits of the genus *Bungarus* (Daudin, 1803), belonging to the family Elapidae, comprise 16 recognized species — *B. andamanensis*, *B. bungaroides*, *B. caeruleus*, *B. candidus,* B. ceylonicus, *B. fasciatus*, *B. flaviceps*, *B. lividus*, *B. magnimaculatus*, *B. multicinctus*, *B. niger*, *B. persicus*, *B. sindanus*, *B. slowinskii*, *B. suzhenae,* and *B. walli*. These species are distributed throughout the tropical regions of South and Southeast Asia, including India, Sri Lanka, Bangladesh, Indonesia, Taiwan, southern China, Thailand, Vietnam, Pakistan, and Afghanistan (Alirol et al., 2010, Slowinski, 1994). Among them, the common kraits *B. caeruleus* and *B. sindanus* are reported from India, Pakistan, and Afghanistan (Sunagar et al., 2021; Pillai et al., 2012). Abtin et al. (2014), described a new species of Krait (Elapidae: Bungarus) from Sistan and Baluchistan, region along the Iran–Pakistan border, related to the allopatric species *B. sindanus* and *B. caeruleus*, and designated it as *B. persicus*. However, morphological examination of the specimens in the present study revealed characteristics consistent with *B. sindanus* (Boulenger, 1897).

In the SDS-PAGE profile of *B. sindanus* isolated from Sistan and Baluchistan, multiple bands below 30 kDa and between 60 and 97 kDa were observed. The SDS-PAGE profile of *B. sindanus* venom isolated from Rajasthan and Maharashtra, India showed minor intraspecific differences of <10 kDa and >50 kDa (Sunagar et al., 2021). These minor differences were also evident in specimens isolated from Iran and Maharashtra, India. It appears that this difference in protein profiles is due to the difference in the relative abundance of 3FTx, PLA2, and β-bungarotoxin proteome components in *B. sindanus*, which have also been observed in samples isolated in Pakistan and India (Sunagar et al., 2021, Oh et al., 2019). The venom of *N. oxiana*, the only species of the Elapidae family present in the RHAV antivenom, showed nine bands in the range of 6-200 kDa, which is a completely different pattern from that of *B. sindanus*. The difference in the bands was especially evident in the bands above 45 kDa.

The differences in the RP-HPLC chromatogram patterns of *B. sindanus* and *N. oxiana* crude venom are quite

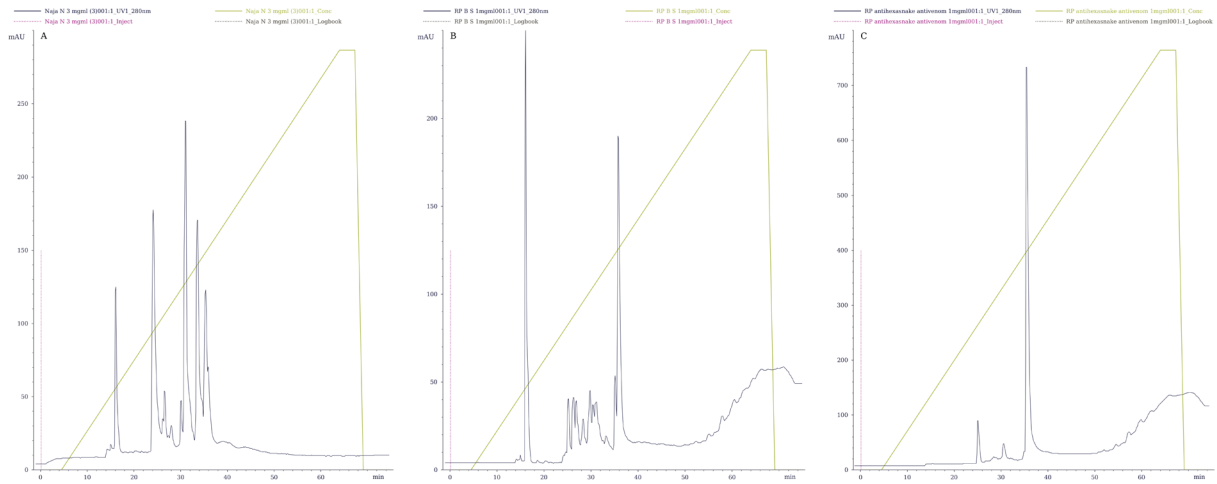

Figure 3. RP–HPLC profiles of crude venoms from (A) *N. oxiana* and (B) *B. sindanus*, and of RHAV (C), obtained using a C18 column (4.6×250 mm) with mobile phases A (deionized water + 0.1% TFA) and B (acetonitrile + 0.1% TFA) at a flow rate of 1 mL/min

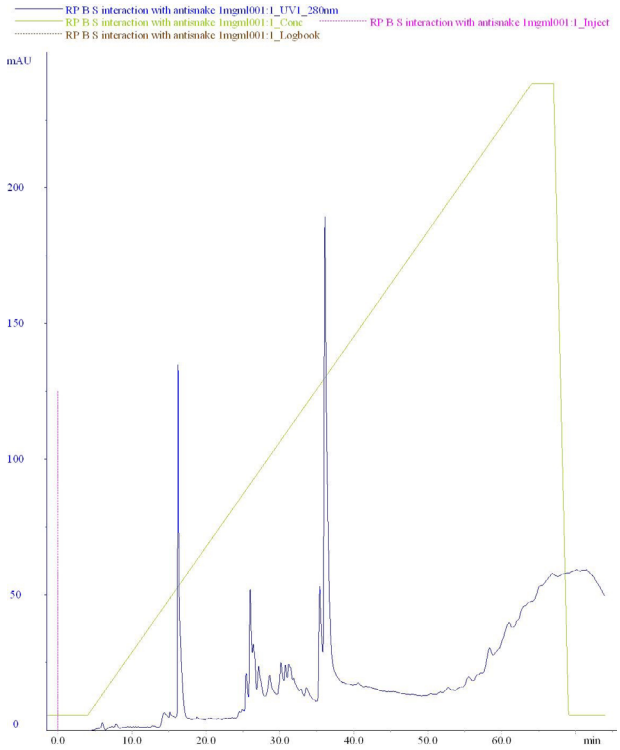

Figure 4. RP-HPLC profile of the interaction between RHAV and *B. sindanus* venom

clear. The most abundant toxins in *B. sindanus*, as identified by LCMS/MS of the RP-HPLC fractions, included three-finger toxins (3FTx), phospholipase A2 (PLA2), beta-bungarotoxin A-chains, and Kunitz-type serine protease inhibitors (KSPI) (Oh et al., 2019, Senji Laxme et al., 2019). In contrast, *N. oxiana* venom predominantly contains 3FTx, PLA_2, metalloproteinases, L-amino acid oxidases, cobra venom factor (CVF), and cysteine-rich secretory proteins (CRISPs) (Manuwar et al., 2020; Samianifard et al., 2024; Tan et al., 2015). These compositional differences likely underlie the distinct clinical manifestations observed following bites from these two species (Manuwar et al., 2020, Samianifard et al., 2024, Tan et al., 2015). These differences lead to different clinical symptoms in snakebites.

In vivo experiments in mouse models have shown that *B. sindanus* venom is one of the most venomous snakes in India and is more than 11 times more potent than *B. caeruleus* snake venom (Sunagar et al., 2021). Ecology and environment are factors that affect the composition and potency of snake venom (Sunagar, 2014). The toxicity of the *B. sindanus* population from Rajasthan (northwest India) was 0.018 µg/g; that from Maharashtra (west India) was 0.02 µg/g (Parveen1et al., 2017). Also, Oh et al. (2019) reported high toxicity with LD_{50} values of 0.04 µg/g (intravenous) and 0.15 µg/g (subcutaneous); in the current study, the toxicity of Iranian *B. sindanus* was determined to be 0.01 µg/g. The toxicity of *N. oxiana* snake venom was determined to be 0.39 µg/g. Therefore, Iranian *B. sindanus* venom showed higher toxicity than *N. oxiana* snake venom Oh et al. (2017).

Since, to date, no polyvalent or monovalent antivenom has been developed specifically against *B. sindanus* venom, the neutralization activity of antivenoms produced against venoms from other Elapidae snakes, such as *B. caeruleus* and *N. oxiana*, has been investigated. The potency test of commercial antivenom (PSVPL) showed effective activity against *B. caeruleus* (0.744 mg/mL), while demonstrating ineffective neutralizing potency against *B. sindanus* (0.15 mg) (Sunagar et al., 2021). This finding was consistent with a neutralization study of commercial polyvalent snake antivenom produced by VINS, in which the neutralization activity against *B. caeruleus* from India and *B. sindanus* from Pakistan was 0.48 mg/mL and 0.25 mg/mL, respectively (Oh et al., 2017; Oh et al., 2019). Although *Bungarus* sp. (*B. caeruleus*) snake venom has been used in VINS and PSVPL antivenoms, these preparations have not shown adequate effectiveness in neutralizing *B. sindanus* venom. This reduced efficacy is likely due to differences in the protein structures between *B. caeruleus* and *B. sindanus* (Sunagar et al., 2021).

According to the distribution and fauna of Iran's snakes, the families Viperidae and Elapideh (*N. oxiana*) are used in the formulation of the RHAV immunoglobulin. In the present study, the neutralization activity of RHAV against the venoms of *N. oxiana* and Iranian *B. sindanus* was evaluated and reported to be 0.4992 and 0.001 mg/mL, respectively. The insignificant changes observed in the HPLC profile of scorpion venom after interaction may be attributed to the lack of binding between the venom and the antibody fragments of the antivenom. Furthermore, the absence of notable suppression in the venom peaks may indicate the limited ability of the antivenom to neutralize specific venom components.

Since *B. sindanus* specimens were collected from the eastern border region between Iran and Pakistan, this finding suggests an expansion of the population range of this genus within the Elapidae family from Southeast Asia westward. However, the neutralizing activity of the antivenom against the venom of *B. sindanus* was found to be severely limited. Our findings highlight the importance of accurate species identification and the urgent need for regionally specific and effective antivenoms in Iran.

This study also underscores the critical necessity of implementing preemptive strategies to address human-wildlife interactions as the geographical distributions of venomous reptiles undergo changes. Policymakers and healthcare professionals must anticipate and prepare for potential increases in snakebite incidents in populated areas. The development of comprehensive public awareness programs and the improvement of access to antivenom treatments are essential for protecting public health, particularly as climate change continues to alter the natural habitats of reptiles across Iran.

Ethical Considerations

Compliance with ethical guidelines

All animals used in our experiment were treated humanely and in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. The Animal Care Committee of Razi Vaccine and Serum Research Institute, Karaj, Iran, approved all animal experimentation procedures.

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Authors' contributions

Conceptualization and supervision: Nasser Mohammadpour Dounighi and Hedieh Jafari; Methodology, investigation, data analysis and writing: Abbas Zare Mirakabadi, Hadi Rabiei, Mehdi Kheirollahpour, Mohammad ali Bayatzadeh, Hedieh Jafari, Morphological identification: Ali Salemi.

Conflict of interest

The authors declared no conflict of interest.

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