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Research Article

COMPARISON OF ELECTROPHORETIC PATTERNS AND MEDIAN LETHAL DOSE OF VENOM FROM ECHISOF THREE PROVINCES OF IRAN

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ABSTRACT

In this study, the electrophoretic pattern of venom of Echis was studied and compared in the populations of southeast Sistan and Baluchistan (Zabol), southern Khorasan (Cain) and Khuzestan (Aghajari), southwest of Iran. For this purpose, toxicity (mean lethal dose, LD50, and SDS-PAGE and HPLC) methods was used to evaluate venom protein composition.

The results show that the color of lyophilized crud venoms of these three snakes populations are, yellowish cream, and no differences in color was seen. The toxicity level (LD50) of Zabol venom was 28.4 ± 4.5 µg / mouse, which showed no significant difference with the toxicity of Ghaen venom, 24.4 ± 4.15µg / mouse, but the mean toxicity of Aghajari venom was 6.7 ± 0.07, 6 µg / mouse which showed a significant difference in comparison with the two previous venoms at $P \leq 0.05$. The pattern of electrophoresis of venoms from three populations of Zabol, Ghaen and Aghajari show that protein compositions in the molecular weight range of were 21.5, 21.9, 39.5, 82.5, 92.88 KDa. Although the protein densities were different in three venoms, some proteins band either were not obvious or with a lesser densities in electrograms. However, the ratio of some of the protein bands was different, indicating differences in the composition of the three venoms from Echis of three different regions. In the HPLC profile, chromatography peaks clearly indicate that there are significant differences in protein composition and about 14 distinct peaks were observed. Significant differences were observed in the relative intensity of the peaks and the ratio of the peaks to the total area at a given retention time. The results of this study indicate the difference in venom protein composition of Echis snake populations from three different regions of Iran. These observations reveal that venoms extracted from different populations of Echis is even different in closely related populations (Our previous study). Thus studying the protein composition of venom can be an effective complementary tool to identify and may classify the different species of a specie, and antivenin producers should pay particular attention to these differences in the composition of venom in medically important species. These results suggest, that there is a variation inEchis snakes from different geography and climates of Iran, and these factors affect the composition of proteins and the toxicity of venom. Therefore, it is recommended that manufacturers of antivenins would use venoms from Echis of different regions of the country.

INTRODUCTION

Snake venom composions and their application

Although snake venom is made from a complex combination of toxins, enzymes and non-synthetic compounds, the venom of snakes based on toxins and their effect on target cells and tissues is divided into three groups of cytotoxin, neurotoxin and hemotoxin It is also included other types of snake toxins that affect certain types of cells, including cardiotoxin, myotoxin and nephrotoxin.The Viperidae family snakes, including the vipers and the petvipers, produce hematoxinins. Hemotoxin have cytotoxic effects on blood (cellular immune responses) and disrupt the coagulation of the blood. These compounds make red blood cells redundant at once, impair functioning of blood coagulation factors, or cause tissue death and damage the internal organs. Red Blood Cells Reduction the inability of the blood to coagulate naturally causes severe internal bleeding. The accumulation and increase of the number of red blood cells destroyed can affect the normal functioning of the kidney. While some hemotoxins prevent blood coagulation, others

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cause blood platelets or other blood components to stick together. As a result, blood clots block the blood vessels and cause heart failure and heart attack. Since the venom's toxins target specific cells, researchers are evaluating these toxins to make new drugs that can target specific cells. Also, the study of these toxic compounds has produced very effective analgesics and blood diluents. Researchers have used the anticoagulant properties of hemotoxins for the treatment of high blood pressure, blood diseases and heart strokes. Neurotoxins is useful for treatment of brain-related diseases, including stroke. The first venom-based drug that was approved by the US Food and Drug Administration was captopril, taken from the Brazilian viper venom and used to treat high blood pressure. Other drugs taken from snake venoms include Eptifibatide, and Tirofiban, of the venom of African viper, used to treat heart attack and chest pain.

In Iran, one of the most medically-important species is the saw-scaled viperEchis genus, the Echis venom is a multi-component mixture of substances which contains more than 100 different components (Valenta&Hadi, 2008;Tomeckova, 2017). More than 90 % of the dry weight is composed of proteins comprising a variety of enzymes, nonenzymatic polypeptide toxins, and nontoxic proteins. It containmetalloproteinases (SVMP) and a broad toxin-expression profile including several distinct isoforms of bradykinin-potentiating peptides, phospholipase A2, C-type lectins, serine proteinases and l-amino oxidases. Most significantly, it was identified for a conserved alpha (9) beta(1) integrin-binding motif in several SVMPs, and a new group of putative venom toxins, renin-like aspartic proteases (Tasoulis *et al* 2017).

Researcher's reports on Echis venom has been shown to cause extensive coagulopathy [Howes *et al*. 2005a], indicative of the high proportion of haemostatically-active components within this venom. Its venom isolation and partially characterization showed three SVMPs, designated EoVMP1, EoVMP2 and EoVMP3, from the venom of this species. EoVMP1 was a 24-kDa P-1B SVMP with no haemorrhagic activity, while EoVMP2 was a 56-kDa haemorrhagic P-III SVMP [Howes *et al* 2003, Howes *et al*. 2005b]. EoVMP3 was also a haemorrhagic P-III 65-kDa SVMP [Howes *et al*. 2003].PLA2 (phospholipase A2) is highly neurotoxic substance also occurring in this species venom. PLA2 neurotoxins act presynaptically and can cause the innervation of the facial muscles. Nonprotein compounds contain carbohydrates, metal ions (often part of glycoprotein, metalloprotein enzymes), lipids, free amino acids, nucleosides, and biogenic amines such as serotonin and acetylcholine, polysaccharides, low-molecular-weightsubstances and ions [Wang *et al*, 2001;. Mackessy, 2002). Hemocoagulation and cytotoxic agents may be present in the adder venom, albeit in negligible quantities. Any venomous snake species contains clinically dangerous haemorrhagins and circulating toxins; if injected into the bloodstream they increase capillary permeability and cause haemorrhage. The constituent proteins of snake venom can affect the breakdown of tissue proteins and peptides.

In general, venom has the potential to produce a wide range of molecules that have a great impact on a wide range of drug targets, including the nervous system, kidneys, muscles, heart and blood flow. Scientists believe that there are many mysteries in the venoms of venomous snakes that have not yet been discovered, so research on it can bring a brighter future in the medical world.

Clinical signs of snake bite

They estimate that there will be around 5 million snake's bites a year around the world, which is responsible for 125,000 deaths throughout the year. The snake is particularly abundant in the tropics and the Arctic regions, because in these areas a large number of snakes coexist with humans.

The Viperidae family snakes have a very advanced venom injection system. Venom is regularly produced and stored in venom glands. Before the vipers bite their own victim, their stingscome out of sheet. After biting, the muscles around the venom glands carry some of this lethal compound into the closed canal of the fang. The amount of venom injected is in control of the snake and depends on the size of the victim. After the injection of venom, they usually leave their prey to paralyze and then swallow it. The main part of snake venom is composed of proteins. Protein-based toxins should be injected or absorbed into the tissue or bloodstream in order to have a lethal effect.

Echiscarinatus is one of the most toxic snakes in the Middle East and Africa (Reid &Theakston 1983). The bites of this snake cause bleeding, oliguria (loss of urine output), anvarya (reduced urination) and severe cases,acute renal failure due to intravascular coagulation (Ali *et al*., 2004). Local and systemic bleeding resulting from coagulation in the arteries following bite with this snake is directly related to the presence of metalloenzymes such as gelatinase and sphingomyelinase in venom (Bjarnason& Fox 1994).

Regarding the genetic and phylogenic variation reported in the Echis populations and the widespread variation in venom composition and the lack of antivenin cross neutralization in the genus Echis (Pook *et al*., 2009) and other snakes (Fry *et al*. 2003, Harrison *et al*., 2003), as well as the effect of diet on the protein composition of snake venom (Barlow *et al*., 2009), the present study was conducted on electrophoretic pattern of venom protein compositions and toxicity in the populations of Echisviper from eastern, Southeast, and Southwest of Iran.

MATERIAL AND METHODS

Gel Electrophoresis by Lamelli 2011

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is used to isolate protein with a relative molecular mass greater than KD 10. Very small proteins (less than KD10) are made due to the ability to attach to the SDS, through gradient gels or using different electrophoresis conditions, such as the Tricine-SDS-page. The main method of LEMMLI SDS PAGE is described here.

Venom sample preparation

a. 10mg crud venom (Powder) + 1ml Distilled water (Stoke) kept at the refrigerator.
b. Preparation of a solution of 3ml ofStoke + 1ml Distilled water.
c. Solution B 20 µl + 20 µl of SDS Reducing Buffer. This solution in a vial was heated about 5 minutes in water (95 ° F).

d. Then, inject 5 µl or 15 µl of the sample from the heated solution (solution C) by Hamilton syringe into the electrophoresis wells.

Protein band detections

To differentiate the protein bands in the venom sample, the Coomassie Brilliant Blue was used to color the gel.

Electrophoresis (SDS page)

The protein pattern of venom samples was examined by SDS-PAGE with 12% gel (Lamelli 2011). Madecrud venom to a concentration of 1.5 mg / ml, each venom sample was injected into wells in two volumes of 5 µl and 15 µl. After the gels staining, they were scanned. by calculating Rf of each of the protein marker bands (as standard) was calculated, using the molecular weights of the marker proteins, the logarithm of the molecular weight was plotted in terms of Rf of each of the protein bands of the marker. Based on this, and calculating the purposedRf band in the samples and placing in the drawn up plot, the molecular weights of the protein bands of the eachvenom was measured.

HPLC Chromatography

Crud venom samples at a concentration of 1 mg / ml was used. The volume of100 µl injected, the column of (Reversed-phase column HPLC, C18) with an acetonitrile gradient with flow rate of 0.5 ml / min at wavelength of 215nm was used. The percentage of proteins present in each venom sample was studied and compared by calculating sub-peak areas between 10 and 57 minutes retention times.

RESULTS AND DISCUSSION

Toxicity (LD50) and pattern of electrophoresis and chromatography of venom from Echis viper

Evaluating the characteristics and electrophoretic pattern of venom extracted from the Echisof three populations of Sistan Baluchistan (Zabol), South Khorasan (Ghaen), and Khuzestan (Aghajari) showed that the color of the lyophilized venoms from Echis of all three populations were yellowish cream and no color differences was observed. The toxicity rate (LD50) of ZabolEchisvenom was 28.4 ± 4.5 µg/mouse, which showed no significant difference with the venom toxicity of Ghaen24.6 ± 1.4 µg/mouse, but the AghajariEchis venom toxicity was6.7± 0.07µg / mouse showed a significant difference with other tow venomsat $P < 0.05$.

Researchers reports on the LD50 from snake venoms from different geographical locations, that some of them are briefly presented here for comparison.

Latifi (1986) measured the LD50 values at the first milking of *Najanajaoxiana*, *Echiscarinatus* and *Pseudocerastespersicus* were 13, 1.8 and 14.4 µg respectively, that was different from the second milking of the same species, 10, 3.9 and 12 µg respectively.

Species	LD50	Reference
Echiscarinatus	0.5655 µg/g	Parveen et al. 2017
Echiscarinatus	22 µg /mouse	Christensen 1979
Echiscarinatusmultisquamatus (Iran)	11.1 µg /mouse(8.89- 13.89µg /mouse)	Salmanzadehetal. 2013
Echiscarinatusmultisquamatus (Australia)	3.26 µg /g	Thomas 1999
Echiscarinatus (Iran)	5±1.1	Latifi 2000
Echis (Southeast Iran, Zabol)	28.04±4.5µg /mouse	Present study
Echis (East Iran, Ghaen)	24.6±4.1µg /mouse	Present study
Echis (Southwest Iran, Aghajari)	6.7±0.07µg /mouse	Present study

LD50 scores for various Echis snakes from different geographical location (Thomas, 1999)

Species (country)	Common name	Intra-venous mg/kg	Intra-peritoneal mg/kg	Venom Yield mg
Echiscarinatusmultisquamatus (Iran)	Saw-scaled viper			3.26
Echiscarinatussochureki (Pakistan)	Saw-scaled viper			2.98
Echiscarinatus	Saw-scaled viper	0.151	2.15	4.48-5
Echiscoloratus	Carpet viper		0.575	0.263
Echisocellatus (Nigeria)	Saw-scaled viper			2.71
Echispyramidium (Egypt)	Saw-scaled viper			0.65
Echispyramidium (Kenya)	Saw-scaled viper			0.94
Echispyramidium (Saudi Arabian)	Saw-scaled viper			1.67

According to the results and available reports, the toxicity of snake venom is not only affected by the age and size of the snake, nutrition, fasting state, frequency of milking, stress, season, temperature, breeding in the snake (Glenn *et al*. 1972, Thomas 1999, Christensen 1979, Barlow *et al*., 2009, Latifi 1986 & 2000, Parveen *et al*., 2017 and Salmanzadeh *et al*. 2013), but also depends on the geographic distribution of the snake.

The electrophoretic patterns of venom of Echis from three regions of Zabol, Ghaen and Aghajari were shown in two volumes of 15 µl and 5 µl (Figure1), the protein composition of these three venomswere in the molecular weight (MW)range of 19.02– 128.64KDa. Theprotein with molecular weights of 21.5, 21.9, 39.5, 82.5,92.88 KDa were present in venom of these three population. The protein with molecular weight 92.88KDain the venom of Aghajari was more than Zabol and Ghaen venoms, either this protein was very little or notpresent in two later venoms, whereas the density of protein with 21.9 KDaMW in Ghaen venom was more than the other two venoms and this protein almost was not seen in two venom of Zabol and Aghajari. Density of proteins with82.5 KDa and 21.5KDa MW in Zabol and Aghajarivenoms were more, but in the Ghaenvenom was very low. The density of protein 39.5KDa MWin the venom of Ghaen and Zabolwasmore than Aghajari venom. Although the protein densityof 21.5 KDa MW in the venoms of all three populationswas noticeable, the density of the bands in the Zabol venom and Aghajariwas greater than that of GhaenEchis. It seems that the protein compounds present in venoms from three vipers of Zabol (southeast of Iran), Aghajari (southwest of Iran) and Ghaen (East of Iran) are different, although this claim is only based on the venoms electrophoretic pattern.

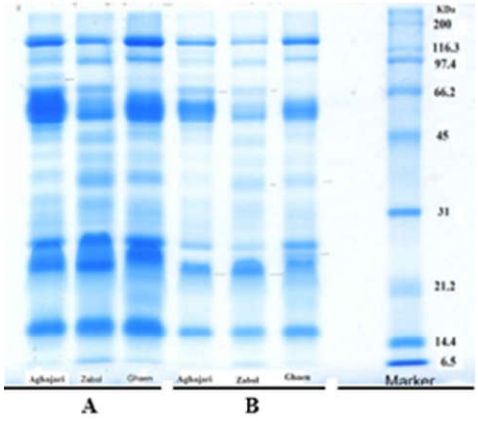

Figure 1 Electrophoreticpattern of crud venom From Echis from Aghajari, Zabol and Ghaen populations (SDS-Page12% Gel), A: 15µl and B: 5µl venom injection volume

According to the results of chromatography (HPLC) of venoms from Echis vipers from three population of Zabol, Ghaen and Aghajari, 74 peaks were detected. The comparison of area of evaluated peak with the total area of peaks at different retention times in chromatogram 2-4,show certain remarkable differences. The evaluated area at the peak at the retention time of 55 min was similar in the samples of Aghajari and Ghaen venoms, but was almost twice as high as in the Zabol sample. Comparison area of peak atretention time of 47 min in the Ghaensample (5.9), which was almost identical to that of the Aghajarivenom (4.5), while at the same time it was estimated 2.4 for Zabol. Evaluated area for peaks at the retention time 44 minutes, for three venom samples were different, so that in Zabol sample 1.1, Ghaen venom 2.2 and Aghajari 11, which showed a significant differences. At the retention time of approximately 34 min in the peak area evaluated for Ghaen venomwas 11.2 and almost close to the Aghajarivalue (13.6), but it was 1.6 to 2 times lesser than to Zabol sample (21.8). At retention time of approximately 21 minpeak area forZabol venom was 9.6, which was 1.8 times more thanGhaen venom (5.2) and 1.2 times more than Aghajari venom (7.8). In general, results reveal the difference in protein composition of all three venom samples.

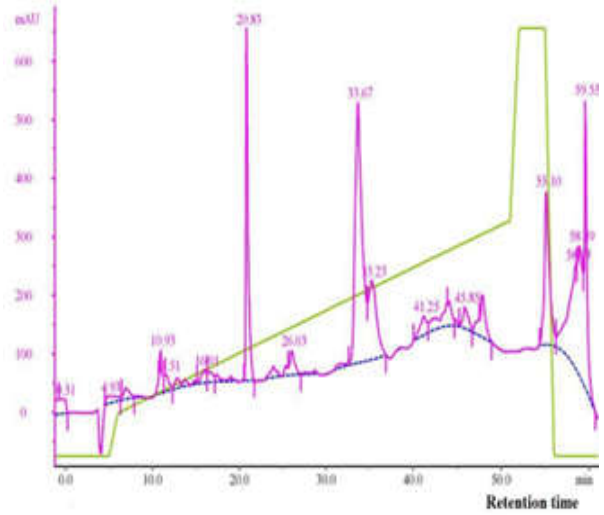

Figure 2 Chromatogram of crud venom from ZabolEchis (Reversed –page HPLC column)

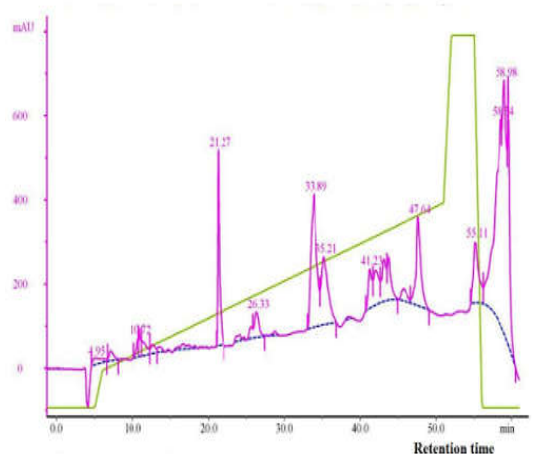

Figure 3 Chromatogram of crud venom from GhaenEchis (Reversed –page HPLC column)

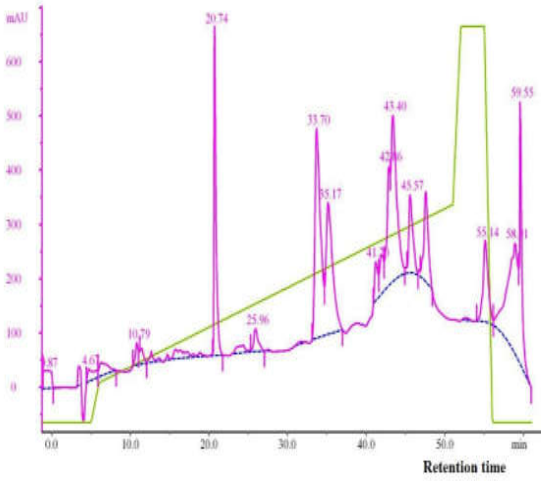

Figure 4 Chromatogram of crud venom from AgajariEchis (Reversed –page HPLC column)

The *Echiscarinatus* venom contains a combination of various metalloproteinase enzymes, fibrinogenolytic and hemorrhagic metalloproteinase enzymes (Salmanizadeh *et al*., 2013). The study of electrophoretic profile of venom using SDS-PAGE (12.5%) by Rabat *et al*. (2017) showed that one of the main protein bands of raw venom was 30 kDa, which is similar to previous reports (Babaei *et al*. 2013; Biqui and Mirkabadi 2013) and three other bands of less than 17, 31-24, and 52-76 kDa, which are relatively similar to our results and also the obtained data from Pakistan's Snake venom (Shaikh 2014). It seems that in addition to the time of collection of venom, natural selection plays an important role in the composition of venom in individuals of the same species (Feroze *et al*., 2009, Magro *et al*., 2001). Gelatinase and fibrinogenolytics of the metalloproteinase family are known to be harmful enzymes in the *Echiscarinatus*, which has been shown to be a damaging factor in traumatic lesions.

Tasoulis *et al*. (2017) reported on thevenom's proteins of various species of snakes. In their study 132 species of snake: 42 species of 360 (12%) Elapidae (elapids), 20 species of 101 (20%) Viperinae (True vipers), 65 species of 239 (27 Crotalinae, ptervipers) and five species of non-front fangedsnakes were identified. Approximately 90% of the total

venom compositions included 8 proteins for Elapids, 11 proteins for Viperins and 10 protein families for Crotalins. There were four dominant protein families, which included phospholipase A2 (the most common protein in all fanged snakes), metalloproteazes, serine proteases, and three-finger toxins (3 FTx: The 3FP protein domain has no enzymatic activity and is typically between 60-74 amino acid residues long, most of nurotoxines.). The presence of six secondary proteins has also been reported: cysteine-rich secretory proteins, L-amino acid oxidases, kunitz peptides, C-type lectins/snaclecs, disintegrins andnatriuretic peptides.Elapids venom consists of three-finger toxins and phospholipase A2, and viper venom contains metalloproteases, phospholipase A2, and serine proteins. Although 63 families of proteins have been identified, more than half of them were found in 5% of the species of snakes studied, and their frequency was low.Firoze *et al*. (2008) in anelectrophoresis study of the Echiscarinatusvenom's protein pattern from Pakistan's Punjab, proteins with molecular weights of 4300, 5500, 11000, 14000, 52000, 63000,67000, 75000and 100,000 was reported. They have believed that these proteins could be specific to the *Echiscarinatus* species. The author suggested that changes are due to genetic factors, as well as changes in the composition of the venom resulting from natural selection, not from changes in diet, and the composition of venom may also vary over time in the same individuals.

In general, the results of this study indicate the difference in venom protein composition of Echis populations from three different regions of Iran. These observations, according to other researchers, suggest that venom composition among different populations of Echis is also different even in closely relatedtaxa. Thus studying the protein composition of venom can be an effective complementary tool to identify and classify the different species, and antivenin producers should pay particular attention to these differences in the venom composition of medically important species.

CONCLUSION

According to the results of this research, it can be admitted that there is a remarkable variation in Echis venom composition from different geography and climates of Iran, and these factors affect the toxicity of venom. Therefore, it is recommended that manufacturers of antivenin consider the use of venoms from snakes of different geographical area.

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