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COMPARATIVE ANALYSIS OF VENOM PROTEIN PROFILE BY SDS-PAGE OF THREE COMMON SPECIES OF SCORPIONS (SCORPIONES: BUTHIDAE) IN BALOCHISTAN, PAKISTAN

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ABSTRACT

This is the first reported study conducted for partial characterization of venom from three scorpion species (*Odontobuthus odonturus*, *Androctonus finitimus* and *Androctonus bicolor*), collected from different regions of Balochistan, Pakistan. The venom was extracted and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and results revealed the presence of different peptides ranged from 6 kDa to 121 kDa. Peptide bands of 68 kDa, 13 kDa and 6 kDa were found common in all the three venom samples, while peptide bands of 121 kDa, 44 kDa and 23 kDa were shared between *Androctonus finitimus* and *Androctonus bicolor*, similarly 29 kDa of band was common in *Androctonus finitimus* and *Odontobuthus odonturus*. Furthermore, each scorpion species had a minimum of one unique peptide band. The similar electrophoretic characteristics of some peptide components suggested inter-genus and inter-species relationships among different species. Conversely each sample of examined species also represents a distinctive profile that differentiates one species from another thus providing a simple tool for taxonomical studies.

Keywords: scorpions; venom; venom extraction; protein; SDS-PAGE.

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INTRODUCTION

Scorpions (*Scorpiones*), Spiders (*Araneae*) and ticks & mites (*Acarina*) are three most important orders of class Arachnida, which initially arose from marine habitat invaded land and become terrestrial animals by adapting different ways (Coddington *et al*., 2002; Ozkan and Karaer, 2004). Scorpions are the oldest and poisonous Arachnids, inhabit a wide range of allocation (Ozkan and Karaer, 2004; Gomes *et al*., 2010) and can survive in extreme conditions of heat, freezing, draught, starvation (for months) and even complete immersion of water (for days). They are easily identified by the specific characteristics of elongated body, segmented tail with bulbous sac and telson (Williams, 1987). More than 1500 species of scorpions are identified so far (Chowell *et al*., 2006; Del Brutto, 2013), family Buthidae is well known among all due to its hazardous envenomation to humans (Goyffon *et al*., 1982; Shirmardi *et al*., 2010). Scorpion venom provides unique defense against predators as well as plays the role of feeding weapon, subduing prey and dissuading predators during mating (Balozet, 1971; Inceoglu *et al*., 2006). Envenomation of scorpion causes various lethal effects in human extending from inflammation, pain, muscle paralysis, respiratory dysfunction, neurotoxicity, cardiotoxicity and occasionally death (depends upon the age, physiology and genetic makeup of victim) (Bawaskar and Bawaskar, 2012). However, the scorpion venom also possesses variety of therapeutic applications in cancer treatment, (Lorenzo *et al*., 2012; Salem *et al*., 2016; Roxo *et al*., 2019), immunomodulation (Ahras-Sifi *et al*., 2019), anti-bacterial (Wan *et al*., 2017; Mousavi *et al*., 2019), anti-inflammatory responses (Khosravi *et al*., 2017; Elfeky *et al*., 2019) and many more. The activity of venom depends upon the various factors like composition that varies species to species, genetic makeup and geographical distribution of scorpion (Batista *et al*., 2007; Bawaskar and Bawaskar, 2012).

Scorpion venom is a cocktail of several biologically important molecules like GAGs, oligopeptides, amino acids, amines, neurotoxins, nephron-toxin, hemolytic toxins, enzymes (e.g. phospholipase A, phosphodiesterase, Acetylcholinesterase and hyaluronidase), enzyme inhibitors, histamine, serotonin and nucleotide, muco-proteins, simple and low molecular weight proteins, along with water and salt (Ozkan *et al*., 2006; Bawaskar and Bawaskar, 2012; Mozaffari *et al*., 2013). Multiple toxins can be identified in the venom of single species

able to produce potent synergic effects in victims. The peptides (20-75 amino acids) and proteins (120-370 amino acids) components of venom have a variety of fundamental roles (El-Hennawy, 1992; Batista *et al*., 2007; Dias *et al*., 2018). Even the single family of Buthidae has revealed 400 toxic peptides in venom by means of protein analysis (Shirmardi *et al*., 2010). Peptides/proteins isolated from scorpion's venom are considered to be the investigatory research tool by the researchers, as they account for many of the therapeutic applications of the venom. The classical approaches for the investigation of peptide components of scorpion's venom were mainly about function-to-structure studies, which were based on auspicious physiological effects of these components on the body (Pimenta *et al*., 2001). Such classical approaches contributed in understanding of the structures of various toxins included disulfide bridge peptides (De Lima *et al*., 2007). Whereas the modern approaches investigate structure-to-function assessments of scorpion's venom peptide, which contributes into pharmacological characterization of peptide components (Rates *et al*., 2008). The biochemical investigations about the identification of peptides and their sequencing by using high out-put methods have attracted the researchers. As peptides in venom are potent, highly selective and relatively safe to use in therapeutic medicines when isolated and used in appropriate concentration. Therefor the prospect for venom peptide identification and sequencing seem very optimistic.

For better understanding about the pathophysiology of scorpion's sting and taxonomic protein profiling, the researchers are evaluating the diversity and fundamental medicinal applications of scorpion's venom by adapting different techniques (Salama and Sharshar, 2013; Cid-Uribe *et al*., 2020), like High Performance Liquid Chromatography, mass spectroscopy, gel electrophoresis, gel filtration and many other techniques have been in practice to study proteomics in venom (Mladic *et al*., 2020; Šedo *et al*., 2020). Here, we report the occurrence of three species of scorpion from different localities of Balochistan. The present study was designed to characterize the protein/polypeptides by means of SDS-PAGE from electrically extracted venom of three commonly found scorpion species. The approximate molecular weights of the electrophoretic separated venom proteins/peptides were also determined and compared to assess the interrelationship between all the examined species.

MATERIALS AND METHODS

Site of Scorpion collection: The scorpions were collected from different areas of Balochistan, Pakistan with the help of experienced local people during the period of July, 2018 to October, 2018 and March, 2019 to September, 2019. The three species i.e. *Odontobuthus odonturus* (Pocock, 1897), *Androctonus finitimus* (Pocock, 1897) and *Androctonus bicolor* (Ehrenberg, 1828) were collected from infested areas of Loralai, Chaman, Uthal and Splinji, most of them are rocky areas. O. *odonturus* were collected during the day time under the stones. A. *finitimus* and A. *bicolor* were found near water sources during late hours of night. The scorpions were easily caught by using forceps and placed them in tightly capped plastic jars and brought to the Biochemistry laboratory at University of Balochistan, where the animals were kept in plastic boxes containing 5cm of thick sand layer and stones for providing them natural habitat. A small shallow container was kept in one corner of the box as a water source. The lid of each box was modified for proper ventilation. The scorpions were fed with earthworms and raw meat twice in a month.

Sample preparation: Crude venom was extracted from each scorpion by using an electrical stimulation method as described by (Ozkan and Filazi, 2004). Extraction of venom was carried out by fixing each scorpion on a plane surface via sticky tape and the stinger of scorpion was held in the Eppendorf tube with the help of biceps. The scorpion's tail was first immersed in concentrated Sodium Chloride solution for improving the conductance, then electricity was applied for a few seconds to the telson (and at the best suitable place for maximum venom recovery) via the electrode connected to 25V of battery. The extracted venom droplet was collected in the Eppendorf tube and diluted with distilled water in 1:1 ratio. The diluted venom was centrifuged at 14000rpm for 15 minutes. Supernatant was separated and stored at -20ºC for further characterization. The protein content of each venom sample was determined by Bradford assay (1976) and measured the absorbance of each sample at 595 nm.

Protein characterization by Sodium dodecyl sulfate polyacrylamide gel electrophoresis: All the three samples of venom were subjected to SDS-PAGE by the method reported by Laemmli (1970) and Amersham Pharmacia (2013). Tris-glycine discontinuous buffer system (i.e. 10% (w/v) resolving gel, 4% (w/v) stacking gel) and Tris/glycine/SDS running buffer was used for SDS-PAGE analysis. Stored venom of each sample was diluted 1:4 ratio with gel loading buffer and kept on preheated water bath at 100ºC for 5 minutes and loaded, along with the protein marker (PageRuler # 26616) of known molecular weight ranging from 10 kDa to 180 kDa. The separation gels were stained with 0.025% Coomassie Brilliant Blue R250. Stained gel was photographed and molecular weights of separated bands were determined by comparing the sample bands with reference to protein marker by using GelAnalyzer 2010a software. A similarity index between the examined species was calculated on the basis of presence and absence of bands by using the following formula;

$$S = 2a/2b + b + c$$

Where;

a = represents the common bands present in sample 1 and sample 2.

b = represents the number of unique bands present in sample 1 but not in sample 2.

c = represents the number of unique bands present in sample 2 but not in sample 1.

RESULTS

The protein electrophoretic bands pattern of all three examined species of scorpions using SDS-PAGE exhibited distinctive protein profiles as shown in (Table 1) and (Fig 1). The SDS-PAGE analysis revealed the presence of a total of fourteen different polypeptide bands with different intensities. The molecular weights (MWs) of different bands ranged from 6 kDa to 121 kDa in all venom samples. Out of which, the bands with MW of 68 kDa, 13 kDa and 6 kDa were found common. Furthermore, the three polypeptide bands i.e. 121 kDa, 44 kDa and 23 kDa were shared between *Androctonus finitimus* and *Androctonus bicolor* but these bands were absent in *Odontobuthus odonturus*. Similarly, a band of 29 kDa was seen in both *Androctonus finitimus* and *Odontobuthus odonturus* but this band was not observed in *Androctonus bicolor*. However, the variability among the intensity of shared bands represents the concentration of protein in each venom sample. SDS-PAGE also showed a unique band of 27 kDa, which was observed only in *Androctonus bicolor*, while the two bands of 70 kDa and 33 kDa were only detected in *Androctonus finitimus*. Four polypeptide bands of 108 kDa, 51 kDa, 37 kDa and 17 kDa were specifically noticed in venom of *Odontobuthus odonturus*.

The similarity index was carried out by Dice's similarity coefficient 'S' (Dice, 1945), which is based on the number of separated bands expressed by SDS-PAGE. The obtained results indicated the highest similarity ratio between the species of *Androctonus bicolor* and *Androctonus finitimus* (i.e. 0.75), followed by the similarity between *Androctonus bicolor* and *Odontobuthus odonturus* (i.e. 0.47), While *Androctonus bicolor* and *Odontobuthus odonturus* revealed the lowest similarity ratio among all the examined species (i.e. 0.4).

Table 1: Protein profile of crude venoms extracted from different scorpion's species of Balochistan.

Protein bands visible in gel electrophoresis (in kDa)	O. *odonturus* (Lane-1)	A. *finitimus* (Lane-2)	A. *bicolor* (Lane-3)
121		+	+
108	+		
70		+	
68	+	+	+
51	+		
44		+	+
37	+		
33		+	
29	+	+	
27			+
23		+	+
17	+		
13	+	+	+
6	+	+	+
Total no of bands	**8**	**9**	**7**

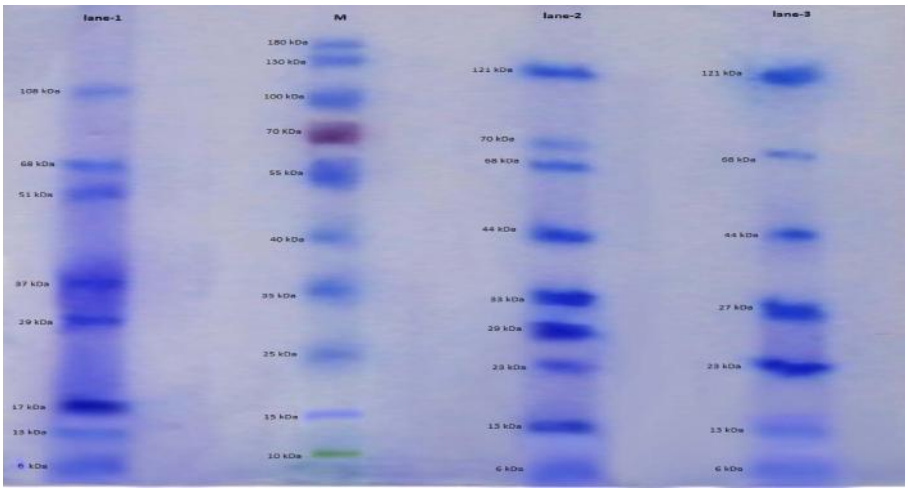

Fig. 1. SDS-PAGE protein profile of crude venom extracted from three different species.
M: protein standard, lane-1: venom of *Odontobuthus odonturus*, lane-2: venom of *Androctonus finitimus*, lane-3: venom of *Androctonus bicolor*

DISCUSSION

Despite all the harmful and beneficial aspects of scorpion's venom, negligible amount of epidemiological data related to scorpion's sting has been published so far in overall Pakistan and particularly in the province of Balochistan. Due to scarcity of published data, only one case report was found so far in which the author illustrated the multi-organ dysfunction in a 30 years old female bitten by a yellow scorpion (most probably *Leiurus quinquestriatus*) in Lasbela district of Balochistan (Khan and Ullah, 2017). The incidents of scorpion's envenoming are high in remote towns and villages, but most of the time victims manage to treat the symptoms by local means of traditional herbs. The hypothetical reason behind the un-availability of published data about scorpion envenoming and epidemiology in Balochistan is either the lack of awareness of healthcare departments or has never been shared by national literatures.

Balochistan is the largest province of Pakistan in terms of land area and is a rich source of diverse scorpion's fauna. In our study the three species of scorpion i.e. *Odontobuthus odonturus* (Pocock, 1897), *Androctonus finitimus* (Pocock, 1897) and *Androctonus bicolor* (Ehrenberg, 1828) are first time reported from different localities of Balochistan: Loralai, Chaman, Uthal and Splinji, within the period of July-2018 to Oct-2018 and March-2019 to September-2019 and their venom protein/polypeptides were characterized by SDS-PAGE analysis. All the collected species of scorpions belong to the family Buthidae. The collection area, body length and color of each scorpion species are shown in Table 2.

Table 2: Parameters observed in collected species.

No	Specie name	Area of collection	Color	Body length
1.	*Odontobuthus odonturus* (Pocock, 1897)	Loralai	Yellow	7-9 cm
2.	*Androctonus finitimus* (Pocock, 1897)	Chaman	Yellow	9-9.5 cm
3.	*Androctonus bicolor* (Ehrenberg, 1828)	Uthal, Splinji	Black	7.5-8.5 cm

Description of species

Odontobuthus odonturus: This species of scorpion is first reported by (Pocock, 1897). The adult size reaches up to 8.5-9cm. The color of the scorpion is light to dark yellow (Fig 2). This species is also reported in southeast Iran (Jalali and Rahim, 2014; Nejati *et al*., 2014) and Sargodha Pakistan (Yaqoob *et al*., 2017).

Fig. 2. *Odontobuthus odonturus*

Androctonus finitimus: It is first described by (Pocock, 1897). It is also a member of the fat-tailed scorpion. These scorpions are very aggressive and fast moving. The adult size ranges up to 9.5cm. In Pakistan it is reported in Sindh and Punjab provinces (Kovařík and Ahmed, 2013; Ahsan *et al*., 2018). It is yellow brown in color while the telson with the last two segments of the tail is black in color (Fig 3). The venom of A. *finitimus* is of medical importance (Riaz *et al*., 2019).

Fig. 3. *Androctonus finitimus*

***Androctonus bicolor*:** This species of scorpion is described by (Ehrenberg, 1828) and its common names are black fat-tail and man killer scorpion. The length of the adult animal is about 8.5 cm. A. *bicolor* has black

colored body (Fig 4) and is almost similar morphology to that of A. *crassicauda*, with the slightest difference in Pedi-palp. The Pedi-palp of A. *bicolor* is thin as compared to that of A. *crassicauda*. It is the world's most venomous scorpion. Their venom has a variety of pharmacological applications (Al-Asmari *et al*., 2015; Al-Asmari *et al*.,2016).

Fig. 4. *Androctonus bicolor*

The venom was extracted via electrical stimulation method, as it is the more convenient way of venom recovery in terms of best yield of toxic compounds, less chances of injury to the scorpion and capability of consecutive extraction of venom from same scorpion for several times (Oukkache *et al*., 2013; Díaz-García *et al*., 2019). The venom regeneration time was different for all the three examined species i.e. Within the interval of 15 days *Odontobuthus odonturus* didn't yield any venom after 4-5 consecutive extractions, while the venom yield of *Androctonus finitimus* and *Androctonus bicolor* was at minimum on their 2-3 consecutive extraction. Our observation is a little differed by the observation made by Yaqoob *et al*., (2016); in which the authors reported the time of minimum venom regeneration was about 5-6 consecutive extraction with 15 days' lapse in the *Mesobuthus tamulus* and *Odontobuthus odonturus* scorpion's species. However, the difference in observation may be due to the difference in weather conditions and feeding type, as well, the chances of venom used by the animal before coming to the captivity are also not negligible as the scorpions were collected in the time of year in which they show maximum activity. Furthermore, another study reported by Díaz-García *et al*., (2019) in which author has considered the 15 days' interval not sufficient to re-obtain full quality and quantity of the venom, as the scorpion's venom is very precious to the animal and it involves high metabolic and energy consuming processes.

The presence of low molecular weight toxin peptides (<15 kDa) was reported by a number of researchers (Gomes *et al*., 2010), who used a variety of techniques to separate these peptides from crude venom of Buthidae scorpions. The Buthidae included the vast group of scorpions, widely distributed in all over the world. The members of the Buthidae family have always been considered of great medical importance among all the other families of scorpions. In the present study three medically important Buthidae scorpions were collected and their SDS-PAGE analysis was carried out to reveal the difference between the protein concentration of each species, as well as their interrelation was also observed.

In the reported study SDS-PAGE analysis of venom extracted from *Odontobuthus odonturus* revealed 8 bands, in which 2 bands were below 15 kDa, however, another similar study on the venom of *Odontobuthus odonturus* reported the presence of one such low molecular weight polypeptide band (about 8.5 kDa) (Yaqoob *et al*., 2017). The presence of a 6 kDa low molecular weight peptide in our analysis, corroborate with the results of Ozkan *et al*., (2011), who reported the presence of 6 kDa peptide in the venom of *Leiurus abdullahbayrami* Buthidae scorpion. As the peptides of 6 and 13 kDa were found common in all the examined species, the finding of present study doesn't agree with the narrative about the venom composition of Buthidae scorpion, as described by Smith *et al*., (2012) that the predominant low molecular weight peptide in venom of Buthidae scorpions are above the range of 7 kDa. Furthermore, our findings are also supported by another research conducted by Zafar *et al*., (2013), who reported the presence of low molecular weight peptides within the range of 2 kDa to 8 kDa in venom of *Odontobuthus odonturus* by HPLC analysis.

Androctonus considered one of the most dangerous groups of scorpions in all over the world. They often called fat-tailed scorpions by having the characteristically fat metasoma. In Latin the word "*Androctonus*" originated from Greek which means "man killer". The two species of *Androctonus* i.e. *Androctonus finitimus* and *Androctonus bicolor* have been reported in our study. The electrophoretic analysis of venom extracted from *Androctonus finitimus* has showed 9 bands, in which 2 bands are below 15 kDa i.e. 13 kDa and 6 kDa. This result is almost similar to another study which reported total of 8 bands in which two are the peptides with molecular weight of 12 kDa and 8.5 kDa in venom of *Androctonus finitimus* by using 12% of resolving gel (Yaqoob *et al*., 2017). Electrophoretic analysis of *Androctonus bicolor* revealed 7 bands within the range of 121 kDa to 6 kDa. These findings however differ with the findings of another study reported by Salama and Sharshar, (2013): These authors studied the protein profile of venom extracted from 8 different scorpion's species by maceration of telson and reported the presence of 6 bands in venom of *Androctonus bicolor* by SDS-PAGE profiling ranging between 200-18 kDa with no low molecular weight peptides being reported.

The absence of low molecular weight peptide is may be due to the presence of unwanted substances of hemolymph origin in the venom, extracted by telson's maceration method as already discussed by Oukkache *et al*., (2013). The presence of low molecular weight peptide in venom of *Androctonus bicolor* extracted by electrical stimulation in current study provides the strong evidence for this account.

The peptides of low molecular weight (i.e. 13 kDa and 6 kDa) expressed in all the three examined species are responsible for toxicity of their venom. The obtained results agreed with the suggestion made by Rodríguez-Ravelo *et al*., (2013) that the peptides within the molecular range of 14 kDa to 19 kDa indicates phospholipase enzyme. This enzyme aid toxicity to the venom, as well as the presence of hyaluronidase enzyme represented by the band with molecular weight of 45 kDa, which is very closed to the 44 kDa band, identified in *Androctonus finitimus* and *Androctonus bicolor* proteomic profile of reporting work (Figure 1 & Table 1). Additionally, the low molecular weight peptides ranging between 6.5-8.5 kDa are suspected to act on sodium channels (de la Vega and Possani, 2005; Rodríguez-Ravelo *et al*., 2013) thus employing the medicinal importance of all the three examined species in proliferations, invasiveness and migrative properties of cell. Another peptide of 68 kDa is also found common in all the three reporting species, this finding corroborate by finding of Ozkan and Ciftci., (2010); who reported the presence of the common band of 68 kDa in all the 8 venom samples of *Mesobuthus gibbosus* which is also a Buthidae scorpion, thus may be proving the inter-genus similarity between Buthidae scorpions. As the present study reported the SDS-PAGE analysis of three different species, the presence of multiple peptides with same molecular weights (68 kDa, 13 kDa & 6 kDa) in different samples of venom may possibly have different amino acid compositions, which needs further investigations. Furthermore, the presence of unique bands in all the three different venom samples of examined species may contribute to the future studies of taxonomic protein profiling.

Erdeş *et al*., (2014) determined the peptide profile of venom extracted from the scorpion *Leiurus abdullahbayrami* (Buthidae) by electrical stimulation and have detected the presence of multiple protein bands ranging between 150-10 kDa on 10% Glycine SDS-PAGE with two major bands of 10 kDa and 70 kDa. Likewise, Ozkan *et al*., (2011) have detected the presence of several bands ranging between the molecular weight of 3-188 kDa by using 4-12% gradient gel electrophoresis, in the venom of *Leiurus abdullahbayrami* extracted by electrical stimulation and observed the presence of 4 different bands with molecular weight 4 kDa, 6 kDa, 31 kDa and 46 kDa.

It is notable from above giving evidences that the electrophoretic pattern analyzed by the current work is more or less similar to the other studies carried out by electrophoretic protein profiling. However, the slight difference in the approximate bands sizes, positions and intensities are negotiable in terms of extraction methods, analytical methods and sample handling with comparison to other published data (Salama and Sharshar, 2013; Zafar *et al*., 2013; Yaqoob *et al*., 2017). Furthermore, the presence and/or absence of some bands are certainly due to the environmental conditions, feeding habits, age and sexual status of the scorpion (Abdel-Rahman *et al*., 2009). The geographical variations could also be a reason as already reported and discussed by Badhe *et al*., (2007) that SDS-PAGE profiling of venom extracted from *Mesobuthus tamulus* indicated a significant difference in venom composition of same specie collected from different geographical regions of India. However, the geographical and environmental variability in venom of the same species are of great reputation for effective anti-venom production (Abdel-Rahman *et al*., 2009).

The similarity indices among different species of reporting work represented the inter-species and inter-genus differences. Inter-species variations are expressed by the highest similarity (i.e. S=0.75) between *Androctonus bicolor* and *Androctonus finitimus* regardless of their morphological differences and the inter-genus variation are represented by the moderate similarity indices (i.e. S=0.47 and S=0.4) between the *Odontobuthus odonturus* with that of *Androctonus finitimus* and *Androctonus bicolor* respectively. These inter-species and inter-genus variations and similarities have evolved during the course of complex evolutionary processes.

As very little published data is present worldwide regarding the protein profiling by SDS-PAGE of the venom of reported species, the current study provides a crucial insight about the diversity of these species in province of Balochistan Pakistan. Just like *Leiurus abdullahbayrami* (one of the most studied scorpion in terms of medical significance) the venom of reported specie also contains both short and long chain neurotoxins thus provide a comprehensive knowledge about the significance of these species.

Finally, it is vital to survey and investigate the existing species of scorpions in province Balochistan, the comparison of the proteomic profile of the venom and their quantitative and qualitative variations can provide help in taxonomic studies. Additionally, the current analysis can open a window for further investigations in the field of therapeutic applications by development of anti-venom as well as countless other medicinal approaches by investigating the amino acid composition of bioactive peptides and their biological activities against serious life-threatening diseases.

Conclusion: To the best of authors' knowledge, the current study is the first contribution towards the presence of precious scorpion's fauna in the province of Balochistan, Pakistan. By using electrophoretic analysis of the venom extracted from three medically important scorpion species of Buthidae family (i.e. *Androctonus finitimus*, *Androctonus bicolor* and *Odontobuthus odonturus*), partial Protein characterization of venom was provided and also inter-specie and inter-genus comparison between the species. Although, the scorpion's venom consists of a wide range of active toxicological and immunological peptides, it is also composed of short peptides that offer an excellent resource for their use in pharmacology, especially for cancer treatment and impairment of tumor growth.

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