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Original Article

Bradykinin-Potentiating Factors of Venom from Iranian Medically Important Scorpions

Goudarzi [1]*, H.R., Salehi Najafabadi [2], Z., Movahedi [1], A., Noofeli [2], M.

1. Central Laboratory, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran
2. Department of Human Bacterial Vaccine, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

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Corresponding Author: hr.goudarzi@rvsri.ac.ir

ABSTRACT

The venom of animals, including snakes, scorpions, and spiders is a complex combination of proteins, peptides, and other biomolecules as well as some minerals. Among the biomolecules of some animal's venom, small peptides that lack disulfide bands known as Non-Disulfide Bridge Peptides (NDBPs) potentiate the bradykinin by preventing the conversion of angiotensin 1 to angiotensin 2 using the mechanism of inhibiting the Angiotensin-Converting Enzyme activity and finally reducing the blood pressure in the victims. This feature of the NDBPs of animal's venom is suggested as the potential of biological drugs. This study aimed to isolate venom components of three species of Iranian medically important scorpions and study the bradykinin potentiating effect of them. The scorpion specimens were collected from the venomous animals and antivenom production department of Razi Vaccine and Serum Research Institute, Karaj, Iran. Moreover, venom extraction was performed by electrical shock (5 volts). The obtained liquid venom of three species specimens was frozen and lyophilized immediately and then preserved in a cool and dried place. The isolation of the venom components for each scorpion was carried out using high-performance liquid chromatography. The obtained ranges of venom fractions (zones) were tested on isolated tissues of guinea-pig ileum and rat uterus using organ bath instrumentation in several replicates. The bioassays resulted in the peptides, including Z_1 and Z_2 regions in the venom fractions of the *Hottentotta saulcyi*, Z_2 in *Odontobuthus doriae*, as well as Z_2 and Z_3 in *Mesobuthus eupeus* demonstrated bradykinin potentiating effect. It is concluded that Bradykinin Potentiating Factors were traceable in the venom of all three scorpion species. Therefore, these venoms have the therapeutic potential to exploit biological-based drugs.

Keywords: Biologic drugs, Bradykinin-potentiating factors, Hypertension, Scorpion venom

Facteurs Potentialisant la Bradykinine du Venin des Scorpions Médicalement Importants en Iran

Résumé: Le venin des animaux, y compris les serpents, les scorpions et les araignées, est une combinaison complexe de protéines, de peptides et d'autres biomolécules ainsi que de certains minéraux. Parmi les biomolécules du venin de certains animaux, de petits peptides dépourvus de bandes disulfures, appelés peptides de pont non disulfure (NDBP) potentialisent la bradykinine en empêchant la conversion de l'angiotensine 1 en angiotensine 2 avec le mécanisme d'inhibition de l'activité de l'enzyme de conversion de l'angiotensine (ACE) et finalement en réduisant la pression artérielle des victimes. Cette caractéristique des NDBPs de venin chez l'animal les suggère comme le potentiel des médicaments biologiques. L'objectif de la présente étude est d'isoler les composants du venin des trois espèces de scorpions médicalement importants en Iran et d'en étudier l'effet de potentialisation de la bradykinine. Les spécimens de scorpion ont été préparés à partir d'animaux venimeux et du département de production l'antivenin de l'Institut de recherche sur le vaccin et le sérum de Razi (RVSRI), et

l'extraction du venin a été réalisée par choc électrique (5 volts). Le venin liquide obtenu de trois spécimens d'espèce a été immédiatement congelé et lyophilisé, puis conservé dans un endroit frais et séché. L'isolation des composants du venin de chaque scorpion a été réalisée par chromatographie liquide à haute performance (HPLC). Les gammes obtenues de fractions de venin (zones) ont été testées sur des tissus isolés de l'iléon de cochon d'Inde et de l'utérus de rat à l'aide d'un instrument à bain d'organe en plusieurs répétitions. Les essais biologiques ont abouti aux peptides comprenant les régions Z1 et Z2 des fractions de venin de Hottentotta sulcyi, Z2 pour Odontobutus doriae et Z2 et Z3 dans les fractions de venom de Mesobuthus eupeus ont démontré un effet de potentialisation de la bradykinine. Il est conclu que les facteurs de potentialisation de la bradykinine (FBP) étaient traçables dans le venin des trois espèces de scorpions. Ces venins ont donc le potentiel thérapeutique d'exploiter des médicaments à base biologique.

Mots-clés: Médicaments Biologiques, Facteurs de potentialisation de la bradykinine, Hypertension, Venin de scorpion,

INTRODUCTION

There are about 170000 species of venomous animals in the world, the most well-known of which are snakes, scorpions, spiders, and a group of bees. It is estimated that the total venom of these animals contains more than 40,000,000 proteins and peptides (An European project supported by the Seventh Framework Program, 2011). Some of the venomous compounds are non-enzymatic proteins binding to specific ion receptors and channels in the injured body and acting as an agonist or antagonist which may lead to neurotoxic disorders, cardiotoxicity, or tissue necrosis effects (McCleary and Kini, 2013). Although venoms are recognized as pathogens in the body, they can also be considered as healing substances (Biswas et al., 2012). The products derived from creatures have been used since ancient times, and the traditional medicine and ancient medical texts refer to the use of toxins for the treatment of various diseases. A classic example of the successful relationship between medical science and toxinology is the discovery and development of a blood pressure-lowering drug (De Lima et al., 2010). For the first time in 1960, a scientist named Ferrera identified and extracted peptides from *Bothrops jararaca* snake, which enhanced the bradykinin effect involving in lowering blood pressure. Further studies on these peptides led to the production of a hypertension drug called captopril. This drug, which is widely used worldwide, reduces blood pressure by inhibiting angiotensin-converting enzymes (Ferreira, 1965). Scorpion venom contains a complex combination of mucopolysaccharides, hyaluronidase, phospholipase, serotonin, histamine, inhibitor enzymes, peptides, and proteins. These polypeptides are classified into two groups of peptides with and without Disulfide Bridge. The first group contains peptides linking about 30-70 amino acids by 3-4 disulfide bonds. This group mainly includes peptides that affect cell membrane channel activity (Miyashita et al., 2007). The second group peptides lack the cysteine residue in their sequences, and therefore, lack the disulfide bond. The molecular weight of this group of peptides is in the range of 2500-3000 Daltons. One of the characteristics of this peptide group is the presence of proline residues in their C-terminal end. This group has milder toxic effects than the first group and has antimicrobial, hemolytic, and bradykinin potentiating effects (Zeng et al., 2005). Studies have shown that the proline amino acid residue in the C-terminal fragment plays an important role in enhancing the effect of bradykinin. Accordingly, recent peptides due to structural and pharmacological properties are considered as one of the most important issues in the biosynthesis of antihypertensive drugs (Zhijian et al., 2006). Studies on *Tityus serrulatus* scorpion venom showed that the bradykinin-

potentiating factors (BPFs) could have effects on blood pressure through the inhibition of Angiotensin-Converting Enzyme activity and bradykinin receptor synthesis (Ferreira et al., 1993). The idea of the possible existing of BPFs in the venom of Iranian medically important scorpion was considered based on the reported clinical signs of victims who were stung by a scorpion in Iran for the secondary reduction of blood pressure (Radmanesh, 2001).

The current study investigated the effects of BPFs isolated from three scorpion species (*i.e., Hottentotta saulcyi*, *Odontobuthus doriae, Mesobuthus eupeus*) based on the bioassay of venom components using isolated tissue contractions in organ bath instrumentation.

MATERIAL AND METHODS

Species of scorpions. The scorpion species under study were *Odontobuthus doriae, Hottentotta saulcyi,* and *Mesobuthus eupeus* that provided from Razi Vaccine and Serum Research Institute, Kraj, Iran. The scorpion species were identified by Dr. Shahrokh Navidpour, head of the scorpion reference laboratory of Iran.

Extraction and clarification of venom. The extraction of the venom was performed by a weakly electroshock (5 volts). The obtained venom was frozen in a -50 °C freezer and then dried by a freeze-dryer instrument. In order to separate impurities, such as mucoproteins, epithelial cells, and possible contaminants, 30 mg of venom was dissolved in 3 ml of distilled water. Subsequently, it was centrifuged at 11000 rpm for 30 minutes at 4 °C. The supernatant was later filtered through a 0.45 syringe filter and finally lyophilized.

In vivo toxicity test (determination of Lethal Dose 50). The serial dilutions of Lyophilized crude venom of each scorpion were selected from 100% live to 100% dead doses with an incremental factor of 1.25; additionally, they were injected via an IV route into Balb/c mice (20 gr). The dilution series was prepared in saline solution and injected into 4 groups of Balb/c mice (20 gr). The data were analyzed using Spearman-Karber and Reed-Muench's statistical methods (Reed and Muench, 1938; Hamilton et al., 1977).

Formula 1. Spearman-Karber method for calculating the Lethal Dose (LD_{50})

$$\log\ (100\%\ \text{lethal Dose}) \pm \frac{\text{incremental factor}\ (1.25)}{n}\ \Sigma\ \text{killed samples} - \frac{n}{2} = \text{A and B}$$

$$\text{antilog A} = \alpha$$

$$\text{antilog B} = \beta$$

Notice: Correct amount (α or β) is in the Doses range = LD_{50}

Formula 2. Reed-Muench method for calculating LD_{50}

$$\frac{50\% - < 50\%}{> 50\% - < 50\%} \times \log 1.25 = \alpha$$

$$\text{Log of immediately Dose less than 50\% lethal} + \alpha = \beta$$

$$\text{antilog}\ \beta = LD_{50}$$

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In order to determine the molecular weight range of the components of three crude venoms, vertical electrophoresis was employed using 14% separating gel and 4% stacking gel staining with Coomassie Brilliant Blue (G250, Lader, Sinagen Co.)

Reversed-phase high-performance liquid chromatography. A reversed-phase high-performance liquid chromatography (RP-HPLC) (Amersham Bioscience; ÄKTA prime plus) was used to separate the components of the venom. The chromatographic conditions consisted of columns C18 (Agilent; 4.6 × 250 mm), HPLC grade water (A) and acetonitrile (B) with 0.05% trifluoroacetic acid as mobile phases at the gradient of 5-80% acetonitrile and a flow of 1 ml/min. The volume and concentration of injected venom into the device at a time was 2 mg per 200 µl. The regions

(zones) of fractions were determined and collected after obtaining the chromatographic repeatability.

Dose-Response. Different values of bradykinin from 1-10 ng/ml were applied to the isolated tissue to determine the effective dose based on measuring the mean contraction of the muscle. Subsequently, the effect of bradykinin enhancement was investigated by different fractions obtained from the scorpion venom.

Preparation of tissues.

A) Guinea-pig ileum: A guinea pig weighing 200-400 gr after 24 h of starvation was used in this study. The animal was anesthetized with ether in a short time, subsequently, two parts of its terminal ileum were surgically removed. The tissue was rapidly transferred into a Tyrode's solution containing 1 µg/ml of atropine sulfate. Following that, blood vessels and other probable contents were removed from the tissue. Organ bath temperature was set at 37 °C, and then, the fractions of each scorpion venom were added 10 sec before adding synthetic bradykinin to the wells. The isolated tissue was washed three times with Tyrode's solution after each test.

B) Rat uterus: To prepare the rat uterus tissue, 10 ng diacylbestrol was firstly added in 0.5 ml sterile sesame oil and then injected intraperitoneally to the virgin female rat weighed 200-250 gr. The injection of diacylbestrol was performed according to the protocol to ensure that the ovarian tube was empty from oocytes and tissues. After 20 h, the animal was anesthetized with ether in a short time, and two parts of the uterus were surgically removed immediately. The tissue was rapidly transferred into the De Jalon solution containing 1µg/ml atropine sulfate. After removing the connective tissue and blood vessels, the tissues were installed in organ bath wells. The other conditions of the test were as those mentioned above.

RESULTS

Toxicity test. The lyophilized crude venom toxicity (LD_{50}) of each three Iranian medically important scorpions was evaluated and the results are presented in Table 1 The mean values were obtained from both Spearman-Karber and Reed-Muench methods.

Table 1. Toxicity of the lyophilized crude venom of three scorpion species

Species	LD_{50} ±SD (µg/mouse)
Odontobuthus doriae	14±0.57
Mesobuthus eupeus	98±1.43
Hottentotta saulcyi	75±1.22

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The electrophoretic pattern of the three crude venoms demonstrates that the molecular weights of the protein and peptide components are ≤ 9 kDa (Figure 1).

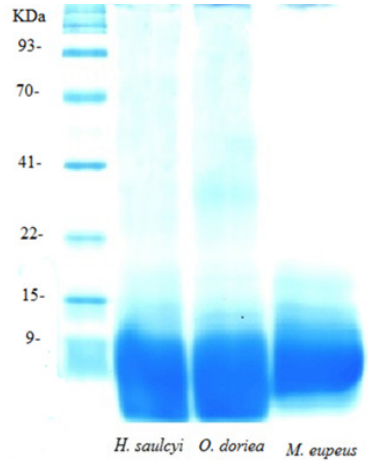

Figure 1. The electrophoretic pattern of crude venoms for scorpions (i.e., *Odontobuthus doriae, Hottentotta saulcyi, and Mesobuthus eupeus*) using 14% separating gel and 4% stacking gel staining with Coomassie Brilliant Blue (G250, Lader, Sinagen Co.).

Reversed-phase high-performance liquid chromatography. The following chromatograms (Figures 2-4) were repeatedly obtained. Based on these chromatograms, different regions (zones) were isolated, and the relevant fractions were collected and then freeze-dried. The regions Z1-Z6 in *H. saulcyi*, Z1-Z4 in *O. doriae*, and Z1-Z5 in *M. eupeus* scorpion were selected for bioassays.

Zones including toxic fractions. The venom of the Buthidae family species is strongly neurotoxic. These toxins are almost bounded to acetylcholine receptors. The attached toxin prevents the transmission of neural

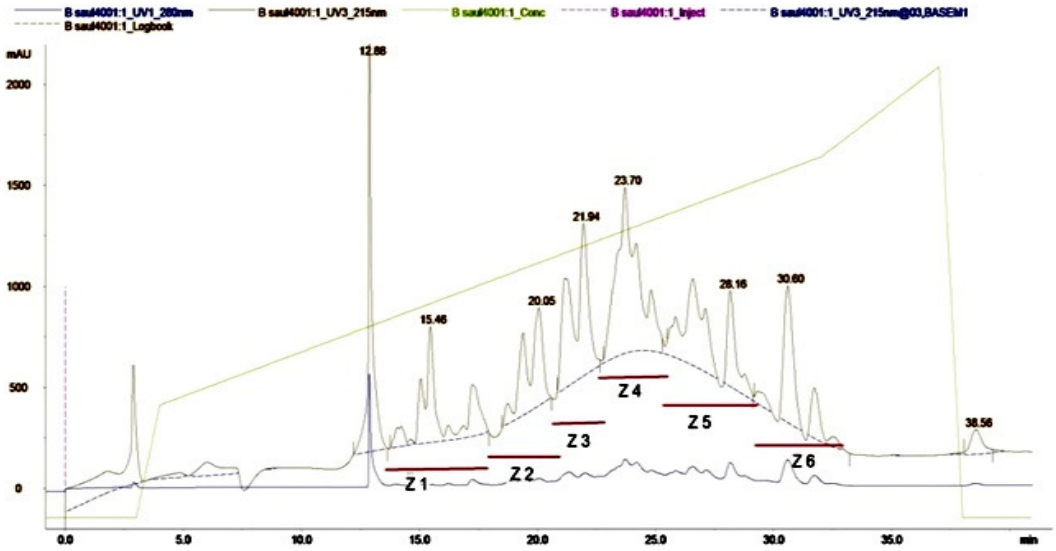

Figure 2. Chromatogram of crude venom fractions of *Hottentotta saulcyi* using reverse-phase high-performance liquid chromatography, Amersham Bioscience; ÄKTA prime plus, columns C18 (Agilent; 4.6×250 mm).

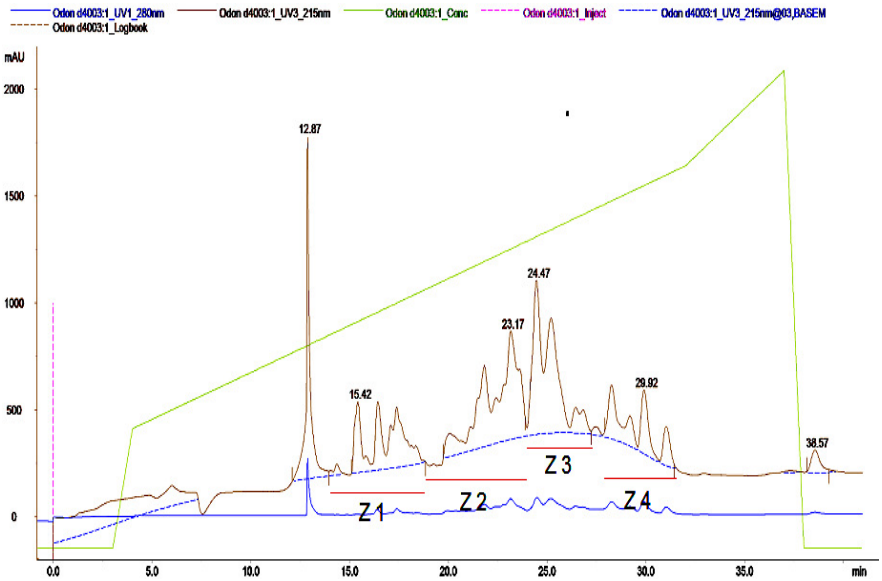

Figure 3. Chromatogram of crude venom fractions of *Odontobuthus doriae* using reverse-phase high-performance liquid chromatography, Amersham Bioscience; ÄKTA prime plus, columns C18 (Agilent; 4.6×250 mm).

impediments during neuromuscular involvement and results in sequential contractions. The areas of Z3 and Z4 in the venom of *H. saulcyi*, Z3 in *O. doriae*, as well as Z4 and Z5 in *M. eupeus* illustrate toxic effects. However, in future studies, the aforementioned zones can be purified and each fraction should be analyzed separately.

Dose-response results. The results of the dose-response study are shown in Figure 6. The dose threshold was 1ng/ml with the first dose through a change in muscle tension. However, for a bioassay

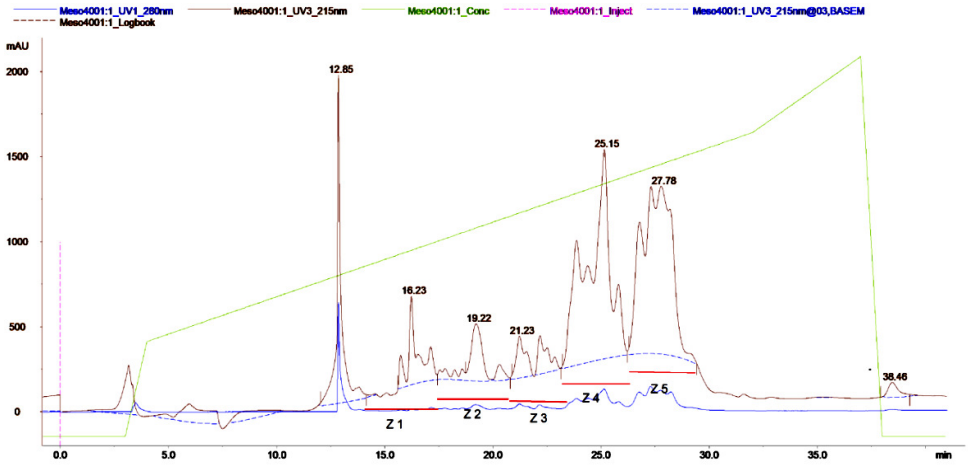

Figure 4. Chromatogram of crude venom fractions of *Mesobuthus eupeus* using reverse-phase high-performance liquid chromatography, Amersham Bioscience; ÄKTA prime plus, columns C18 (Agilent; 4.6 × 250 mm).

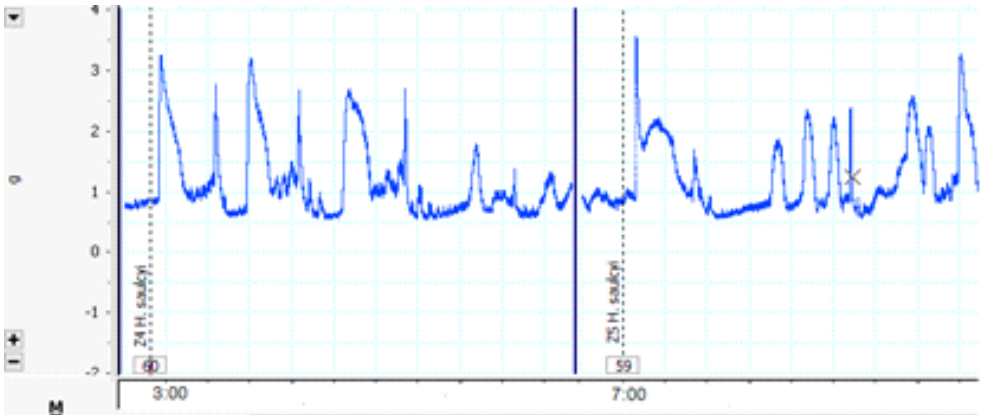

Figure 5. Effect of toxic fractions on isolated tissue causing severe oscillations in muscle contraction.

experiment, it is necessary to obtain a mean dose. In this assay, the midpoint of the muscle stretching on a curve was determined as 4ng/ml which produced a tensile of 2.3±0.1 g.

Bioassays (Bradykinin potentiating effects). The obtained zones, including nontoxic fractions, were tested on the guinea-pig ileum and rat uterus tissues in six replicate using organ bath instrumentation. The Z1 and Z2 regions in *H. saulcyi* venom fractions, Z2 in *O. doriae*, and Z2 and Z3 in *M. eupeus* venom showed obviously the bradykinin potentiating effect based on measuring the contraction of the isolated tissues.

Contraction graphs of the isolated ileum and uterus tissues with initially added venom fractions as shown in Figures 7 and 8, respectively, revealed no changes in the amount of muscle tension; however, after 10 seconds when bradykinin was added to the wells (4ng/ml), the amplification pattern was visible, compared to the time when the venom fractions were only added. The positive amounts of potentiation unit (PU) (the ratio of the increase in tension amount to the tension rate resulting from the dose-response) demonstrated significantly existing BPFs in the venoms of scorpions under study (Table 2). The potentiating

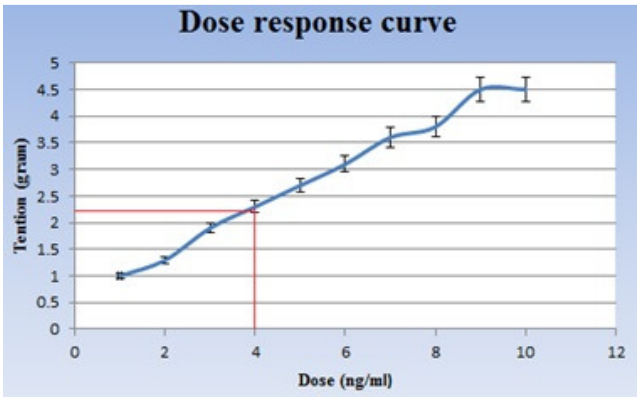

Figure 6. Dose-response curve of bradykinin acetate on isolated tissue of the guinea-pig ileum.

Table 2. Mean of potentiation unit resulted from the venom fractions of each scorpion

Scorpion	Fraction Zones	Potentiation Unit on Guinea-Pig Ileum±SD	Potentiation Unit on Rat Uterus±SD
Hottentotta saulcyi	Z_1	0.26±0.029	0.22±0.024
	Z_2	0.44±0.039	0.35±0.035
Odontobuthus doriea	Z_1	0.52±0.045	0.30±0.032
Mesobuthus eupeus	Z1	0.57±0.047	0.39±0.037
	Z3	0.61±0.039	0.48±0.032

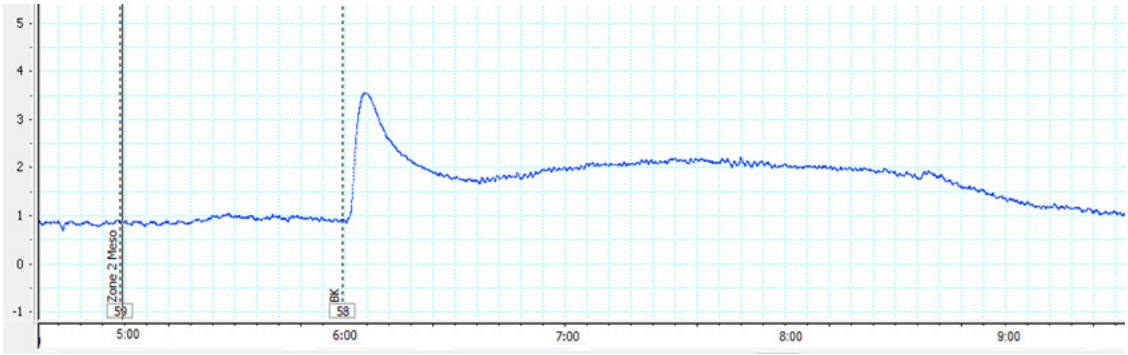

Figure 7. The pattern of the contraction curve for the isolated guinea-pig ileum. It was initially added by venom fractions of *Mesobuthus eupeus* scorpion. After 10 seconds, the addition of the bradykinin (mean dose) increased the contraction to a maximum of 3.6 g. The time to reach the balance lasted for 35 sec.

property of venom fractions occurred at both types of isolated tissues though the amounts of PU in the guinea-pig ileum was bigger than those in the rat uterus. However, this assumption should be more assessed in the future.

DISCUSSION

During the last decades, studies on the venom of scorpions have attracted the attention of toxicologists. Accordingly, increasing knowledge in this field has been remarkable over the years. The intriguing achievements resulting from the beneficial efforts of researchers in this field are simultaneously known as applied and theoretical tendencies. In addition to isolating and studying the structure of toxic agents involved in humans and animals, studies have focused on the pharmacological effects of peptides in scorpion venom. It is worth noting that the investigations on low molecular weight peptides and biological activity in snake venom had begun about three decades before such studies on the scorpion venom, especially on peptides, had bradykinin-potentiating activity. Due to

the divergence of the first and second structures of the non-disulfide-bridged peptides in the scorpion venom, their classification is very difficult based on the amino acid sequences or structural similarity. Therefore, in the relevant sources, the classification is carried out based on comparing pharmacological activity, peptide length, and structure similarity. Zeng et al. (2005) classified these peptides into peptides without disulfide bridges separated from scorpion venom. It should be noted that the same study was previously performed to unify the nomenclature of short-chain peptides (Tytgat et al., 1999). Studies on the BPFs were developed from scorpion venom in a recent decade. Ferreira et al. (1993) isolated the peptide T as novel bradykinin potentiating peptide (BPP) from *Tityus serrulatus* scorpion venom. Moreover, Meki et al. (1995) separated peptide K12, a BPP from an Egyptian scorpion venom named *Buthus occitanus*. In a more advanced study, Zeng et al. (2000) cloned and characterized a novel cDNA sequence encoding the precursor of a venom peptide (BmKbpp) related to a BPP from Chinese scorpion named *Buthus martensii* (Sosnina et al., 1990). Salman et al. conducted a study (2016) on the venom of a spider species which

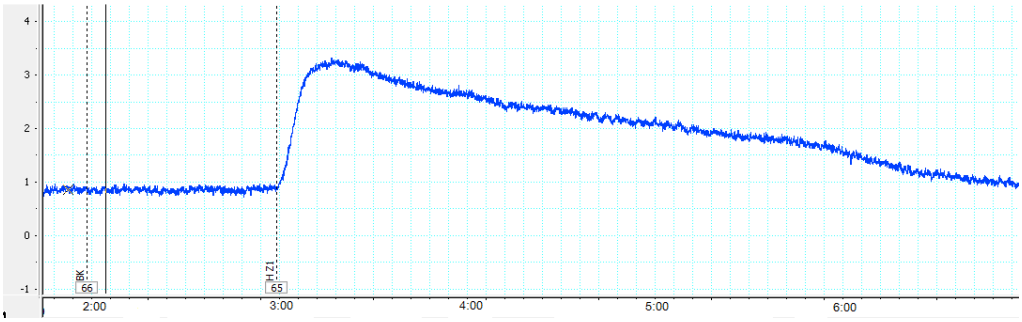

Figure 8. The pattern of the contraction curve for the isolated rat uterus. It was initially added by venom fractions of *Mesobuthus eupeus* scorpion. After 10 seconds, the addition of the bradykinin (mean dose) increased the contraction to a maximum of 3.3 g. The time to reach the balance lasted for 35 sec.

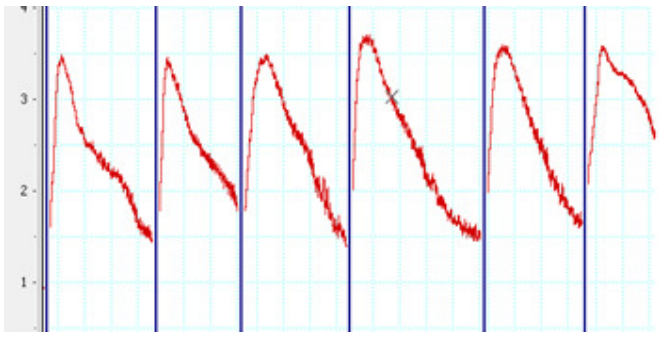

Figure 9. Contraction graphs and potentiating effect of venom fractions on isolated guinea-pig ileum

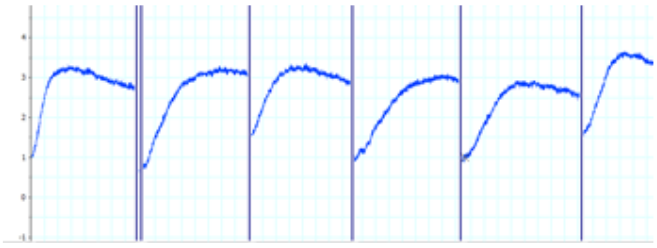

Figure 10. Contraction graphs and potentiating effect of venom fractions on isolated rat uterus

belonged to the class Arachnida, the same as scorpions, and investigated the effect of BPPs obtained from the *Latrodectus tredecimguttatus* on the inhibition of carboxycathepsin. In addition, they studied its role in the preparation of black widow spider venom kininase. Surprisingly, there have been improvements in recent years in the studies on various pharmacological aspects of isolated BPPs from scorpion venom. These studies investigated hepato and nephroprotective effects of BPF (Salman, 2009), effect of a single dose of a BPF on total protein and albumin in serum (Bekheet et al., 2013), prevention of hepatic and renal toxicity, as well as protective effect of BPF against kidney damage in laboratory animals (Bekheet et al., 2011). In the present study, the venoms of three species of Iranian scorpions, namely *M. eupeus*, *H. saulcyI,* and *O. doriae* were studied to detect and measure the bradykinin-potentiating activity of their fractions. The mentioned scorpion specimens are native species in Iran, and there have been no studies investigating these scorpion venoms so far by Iranian or other researchers. As a result, the Iranian scorpions are valuable sources for the isolation and investigation of the biological factors along with various pharmacological effects, such as BPP. Probably, even in the zones with toxic effects, peptides with bradykinin-potentiating activity are present which dominate by toxic elements. Accordingly, further studies are recommended to be conducted to separate and purify the target peptides. Moreover, it is of utmost importance to conduct molecular studies and amino acid analysis.

The findings in this study revealed the nontoxic fractions of crude venom of all three Iranian medically important scorpions, including *M. eupeus*, isolated tissues of guinea-pig ileum, and rat uterus using organ bath instrumentation. Therefore, BPFs were the clear indications of the venom of the mentioned scorpion species. However, this assumption should be assessed by more accurate purification and also in-vivo experiments in the future. It can also be concluded that these venoms are potentially a natural source of therapeutic agents to achieve biological-based drugs.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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