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Research Article

The first report on isolation of a new highly hemolytic toxin, Scatotoxin, from *Scatophagus argus* venomous bonny spines

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Abstract

Scatophagus argus of family Scatophagidae is a venomous fish. Rough spines insulated from scat possessed potent venom composed of several proteins. Envenomation is associated with local necrosis and severe pain. Following our previous report regarding the hemolytic activity of scat crude venom, this work aimed at purification and evaluation of its hemolytic protein, hereafter designated as Scatotoxin. Specimens were collected from coastal waters of the Persian Gulf, Iran. Proteins were extracted from bone tissue by solubilization buffers and subsequently refolded in a refolding buffer. Purification was performed by reverse-phase HPLC method using a linear gradient protocol. To evaluate the hemolytic activity of Scatotoxin, a quantitative microscopic assay was developed using cell counting by which measurement of activity of the least amount of a sample was achievable. Scatotoxin was isolated in 85% acetonitrile. It is an interesting highly hydrophobic protein. Because hemolysis was observed immediately, scatotoxin is considered a very fast-acting hemolytic agent. Scatotoxin indicated as a 72 kDa protein by SDS-PAGE. The amount of 0.5µg crude venom produced 100% hemolysis and HD50 determined at 0.18µg. HD50 for scatotoxin recorded at 0.003µg. High efficiency of both extraction method and microscopic-scale assay led to the reduction of collected specimens and consequently avoiding harmful effects on the Persian Gulf ecosystem. This issue is ethically important due to decreasing the number of samples too. Among the previously reported hemolytic proteins, Scatotoxin is the first report of a highly hydrophobic protein.

Keywords: *Scatophagus argus*, Hemolysis, Scatotoxin, Hydrophobic toxin

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Introduction

Scatophagus argus (Linnaeus, 1766) also known as green scat or spotted scat, belongs to Scatophagidae family, is a fascinating marine fish which is able to live in fresh water too (Feng *et al*., 2020). Its distribution is in Indo-Pacific region in harbors, brackish estuaries, and lower reaches of freshwater streams, frequently among mangroves (Ru *et al*., 2020). Its rough dorsal and anal spines are venomous and capable of imposing lesions. Envenomation appears within 5–10 min as excruciating and persistent local pain, partial paralysis, erythema, itching, swelling and a throbbing sensation that extends to the limbs, disproportionate to the size of injury. The signs are variable, depending on the size of the fish and the quantity of injected venom (Cameron and Endean, 1970). During the past decades, many marine venomous animals were sacrificed to characterization and evaluation of toxicity or biological activities of their venoms. This note is of significant value concerning ethical issues. Furthermore, the yield and quality of venom extraction methods and also cost effectiveness could be important issues in this kind of projects. Therefore, development of a highly efficient extraction method and also a small-scale assay for characterization of venom activity are necessary to decrease the numbers of collected specimens. Venom extraction from bonny spines in venomous fish is laborious because of the spine rigidity and inflexibility. Beside this, low amount of venom is yielded from each specimen. The conventional methods of venom extraction were not practical for green scat due to the abovementioned issues (Endean, 1961; Saunders and Tökés, 1961; Carlisle, 1962). Following our previous report on hemolytic activity of scat venom (Ghafari *et al*., 2013), this study aimed to isolate and purify the hemolytic protein from scat venom. In this regard, an innovative high yield extraction method was implemented and also a new microscopic-scale hemolysis assay was developed.

Materials and methods

Sample collection

Four *S. argus* specimens with medium length of 25cm were collected by trap from coastal waters of Boushehr province, Persian Gulf, Iran. The specimens were transported to Pasteur Institute of Iran by plane, and were kept alive in an aquarium in appropriate conditions.

Ethical issues

Several ethical issues considered in our study according to our previous paper (Ghafari *et al*., 2013). Briefly, clove powder (*Caryophyllus aromaticus*) was used as anesthetizing agent and subsequently the time period for anesthetizing and recovery were shortened too. We avoided decapitating the specimens and after spine removal, the injured location was disinfected. After collection of spines, to avoid killing the fish, they were maintained in a large pool in Pasteur Institute of Iran.

Venom preparation and extraction

The bonny spines were prepared based on Ghafari *et al*. (2013). An innovative method was used for protein extraction from spines based on urea reagent (Rudolph and Lilie, 1996). This idea originated from archeological methods for protein extraction from bony fossils (Cleland *et al*., 2012). This method was developed in our study based on biotechnological methods (Rudolph and Lilie, 1996).

This method consists of two stages, including simultaneous bone demineralization and protein solubilization and refolding processes. In the first stage, bonny structure was demineralized and simultaneously the proteins were extracted from bone tissues by incubating the trimmed spines in a solubilization buffer (containing Urea (8M), NaCl (0.2M), Tris-Cl (50mM), EDTA (2mM) at pH 8.5) at 4°C for 72 hours. In the refolding process, the extracted proteins were refolded by refolding buffer (containing Tris (50mM), NaCl (9.6mM), KCl (0.4mM), EDTA (1mM), Triton (0.5%), Urea (2M) , DTT (1mM) at pH 8) at 4°C for 48 hours. Refolding stage is necessary since structural conformation of the extracted proteins was affected by urea reagent in solubilization stage. Subsequently, filtration (0.2µm- Takara bioscience Co., Japan) and dialysis were performed by an ultra-filter (5kDa cut off- Thermo Co., USA) to remove micro-particles and the released chemical materials, respectively. The sample was then lyophilized by a freeze dryer (Alpha 1-2 LD plus, Martin Christ Gefriertrocknungsanlagen Co., Germany).

Protein determination

Protein concentration was determined by BCA method (Smith *et al.*, 1985) according to manufacturer instruction (iNtRON Biotechnology Co., South Korea). Optical density was measured at 562nm using a microplate spectrophotometer (EPOCH, BioTek Co., USA).

SDS-PAGE

Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out according to standard method (Laemmli, 1970). The venom samples were loaded onto a 12% polyacrylamide gel.

Isolation of hemolytic protein

The extracted crude venom was purified using HPLC system (Knauer Wissenschaftliche Gerate Co., Berlin, Germany) equipped with a C18 column (5µm 100Å - 250 × 4.6mm, Beckman Coulter Co., USA). TFA (0.05%) in ultra-pure water and acetonitrile containing TFA (0.05%), was designated as solution A and B and were used for eluting the fractions. The column was eluted by a linear gradient of solution B from 80 to 95 percent for 45 minutes at 0.5 ml/min. The eluted peaks were monitored at 214nm. The collected fractions were lyophilized by a freeze dryer (Alpha 1-2 LD plus, Martin Christ Gefriertrocknungsanlagen Co., Germany). The lyophilized powder was

solubilized in 200µL ultra-pure water and stored at -20°C.

Micro-hemolytic assay

For visual inspection of hemolysis, a new microscopic assay was developed. Fresh human blood from a healthy volunteer was drawn by venous puncture in heparinized tubes. Plasma and buffy coat were removed by centrifugation (Sigma, 1-14) for 5 min at 664 g at 25°C, and the erythrocytes were washed three times with phosphate-buffered saline (pH 7.4). A suspension of erythrocytes was made at 0.01% dilution in PBS. From this suspension, 10µL RBC placed on a neubauer slide as negative control. A suspension containing RBC (5µL), each of samples (1µL), and PBS (4µL) was used for this assay. This assay performed with distilled water and $CaCl_2$ (2mM) separately to control their effects on the erythrocytes. The results were observed with a microscope (Bell photonic Co., Italy) at 40X magnification and documented using a digital camera (Canon G12 - Japan).

Results

Protein analysis

The extracted venom of *S. argus* was subjected to SDS-PAGE analysis and showed 6 separated bands in the gel. Major and minor protein bands observed between 10 to 250 kDa (Fig. 1).

Protein isolation by RP-HPLC

RP-HPLC of *S. argus* venom on C18 column in a new gradient protocol resulted about 15 fractions during the first 22 min (Fig. 2). The aliquots of each peak were then used for hemolytic activity. We attained three major fractions eluted in about 85-85.83% of acetonitrile (Fig. 3A). SDS-PAGE profile of these fractions showed a 72 kDa protein (Fig. 3B).

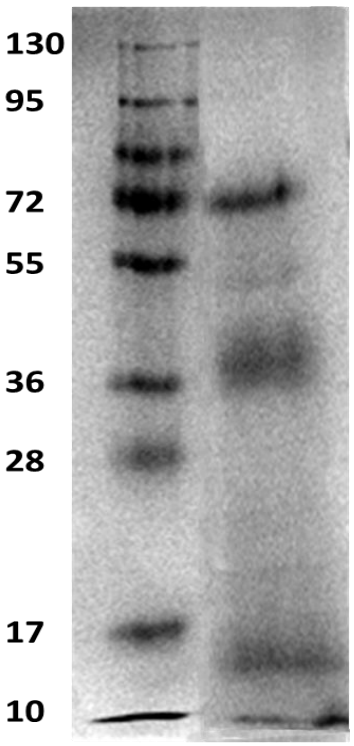

Figure 1: Electrophoretic profile of *S. argus* venom. The venom was analyzed by SDS-PAGE using 12% polyacrylamide gel and stained with coomassie brilliant blue. From left to right: Lane 1. Molecular weight marker (10–250 kDa); Lane. 2 *S. argus* venom.

Morphological evaluation of hemolysis

A microscopic method was performed as hemolytic test and immediate interaction between the erythrocytes and crude venom and also the purified fractions were observed by a microscope at 40X magnification and documented using a digital camera (Figs. 4 and 5).

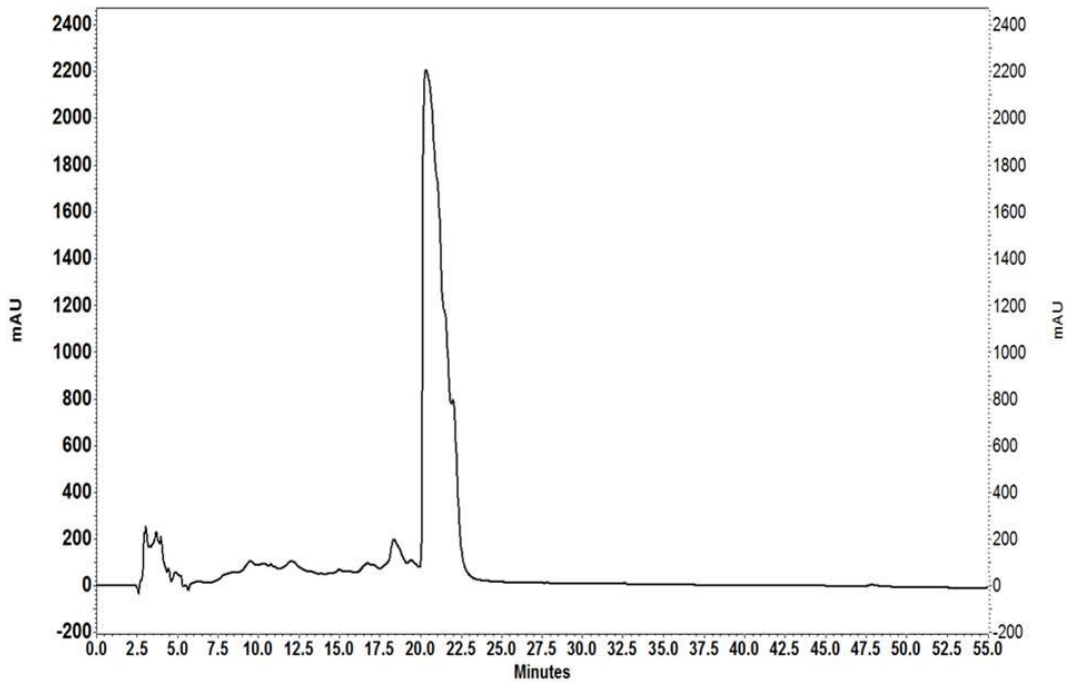

Figure 2: RP-HPLC chromatogram of scat venom. All 15 fractions eluted at high concentration of acetonitrile ranged from 80 to 85.83%.

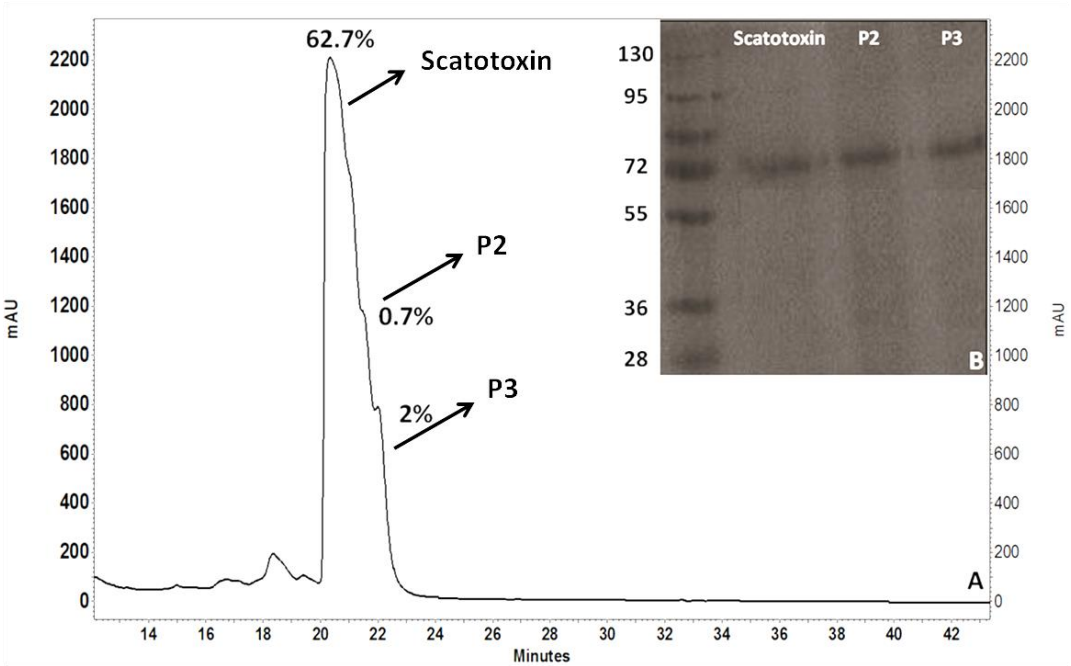

Figure 3: Magnified RP-HPLC chromatogram of scat venom. 3A. Three major peaks were eluted from column at high concentration of acetonitrile ranged from 85 to 85.83%. 3B. The purified fractions were about 72 kDa in SDS-PAGE. Among the peaks, only Scatotoxin had hemolytic activity.

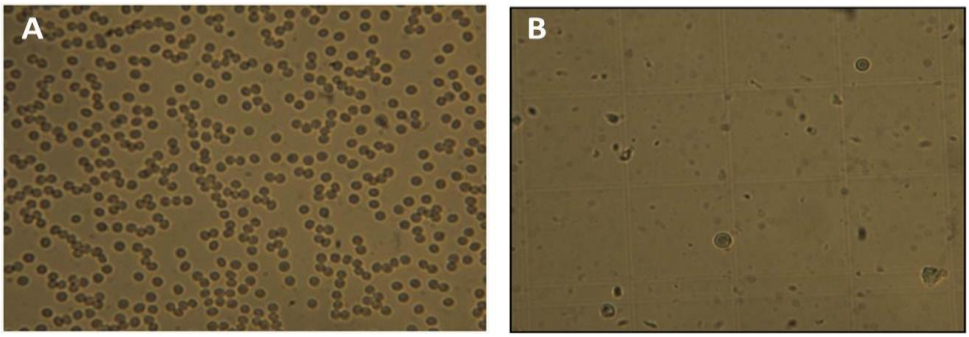

Figure 4: Evaluation of the effect of scat venom on human erythrocytes. Intact erythrocytes before hemolysis assay (4A). Hemolysis of erythrocytes after incubation with scat venom (4B). The results were observed by a microscope (Bell, photonic) at 40X magnification.

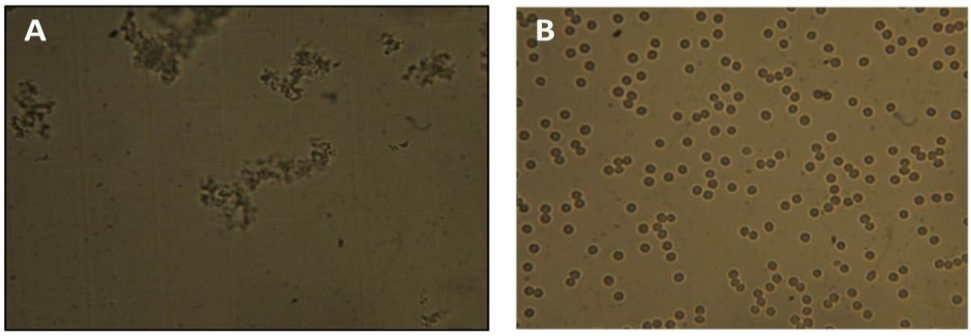

Figure 5: Evaluation of the effect of CaCl2 and distilled water during incubation with erythrocytes. Aggregated erythrocytes after incubation with 2mM CaCl2 (5A). The erythrocytes after incubation with distilled water. No immediate hemolytic effect showed on erythrocytes (5B).

Microscopic scale hemolytic assay

The amount of 0.5µg crude venom produced 100% hemolysis and HD50 identified at 0.18µg. Hemolysis was dose dependent and slope of hemolysis gradually increased up to 0.5µg (Fig. 6). HD50 for Scatotoxin recorded at 0.003µg and hemolysis was dose dependent up to 0.05µg (Fig. 7).

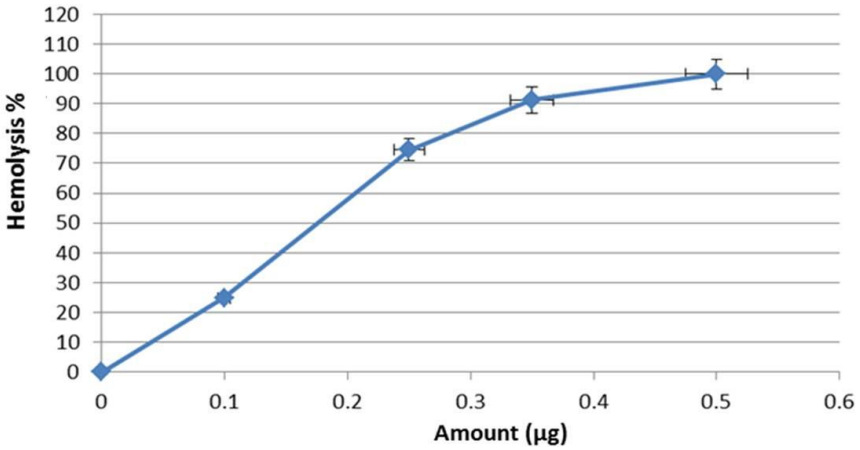

Figure 6: Micro-hemolytic assay for *S. argus* venom on human erythrocytes. The immediate interaction between the erythrocytes (0.01%) and venom was observed by a microscope. The amount of 0.5µg crude venom produced 100% hemolysis and HD50 identified at 0.18µg. Hemolysis was dose dependent and slope of hemolysis gradually increased up to 0.5µg.

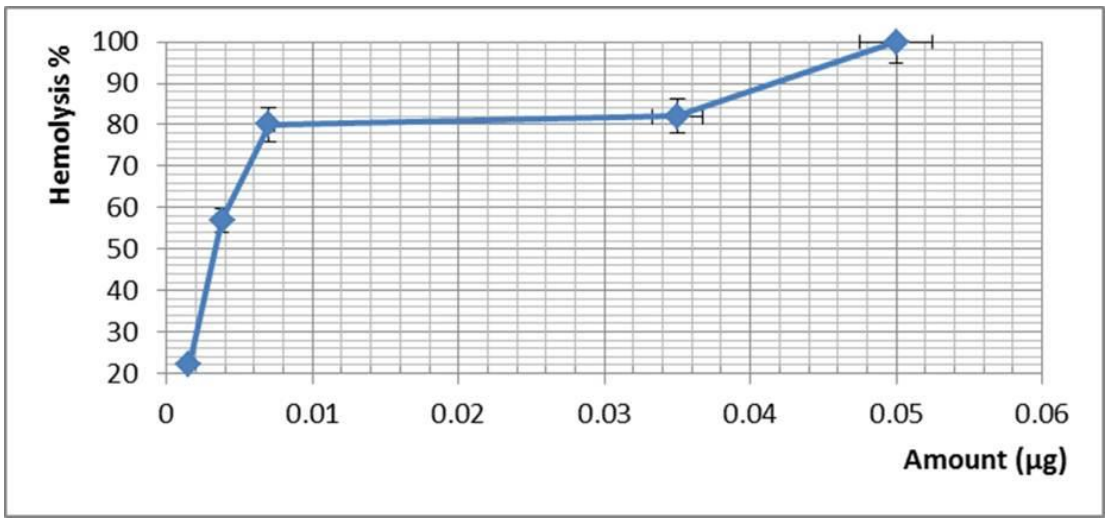

Figure 7: Microscopic hemolytic assay for Scatotoxin on 0.01% human erythrocytes. HD50 recorded at 0.003µg and hemolysis was reached to 100% at 0.05µg.

Discussion

Discovery of bioactive molecules from venomous creatures, especially from marine animals, is of significant value because of their potential to present as a pharmaceutical agent. Among the venomous fish, scat venom has been less considered regarding the characterization of its proteins. Venom extraction from bonny venomous spines is an important step in this kind of research. Alongside this issue, a high yield protein extraction method is critical. Conventional extraction methods use acetone, normal saline, and ammonium acetate which yield low protein levels. Accordingly, in this study, we tried to use an innovative method to improve extraction efficiency regarding quantity and quality.

This new idea triggered by a protein extraction method from bony fossils in archeology using urea reagent (Cleland *et al*., 2012). During demineralization of bonny spines in extraction stage, maintaining the conformation of extracted proteins is necessary for reaching active molecules. Concerning this issue, we used an innovative high yield method for extraction and refolding of proteins from bonny spines. Our reagent was the same as the solubilization buffer that routinely used for solubilizing inclusion bodies containing compact recombinant proteins. For refolding the solubilized proteins, we used a buffer which is the same as the buffer used for refolding of recombinant proteins (Rudolph and Lilie, 1996).

In our previous studies (Ghafari *et al*., 2013; Ghafari *et al*, 2015) the yield of extraction was about 1 to 2 mg protein/specimen while the current method yielded 25 to 30 mg protein/specimen in the same size and weight of collected spines. This amount was approximately 18 fold greater than that of our previous report. In this new extraction method, the proteinaceous nature of crude extract was not affected by the reagents used for extraction. SDS-PAGE confirmed that six separated proteins or peptides in the range of 10-

250 kDa were successfully extracted from spines. *S. argus* crude venom caused hemolysis of erythrocytes suspension at HD50 and HD100 of 0.18 and 0.5 µg, respectively while, Scatotoxin caused hemolysis of erythrocytes suspension at HD50 and HD100 of 0.003 and 0.05 µg, respectively. Hemolysis was dose dependent for both crude venom and hemolytic fraction.

Comparison of HD50 value for melittin, a potent hemolytic peptide from bee venom (Zarrinnahad *et al*., 2018), with Scatotoxin showed that its activity was approximately equal to melittin on human RBCs. Regarding this issue, it can be hypothesized that Scatotoxin may possess anticancer and antimicrobial activity similar to melittin (Mahmoodzadeh *et al*., 2015; Akbari *et al*., 2019). This hypothesis originated from this fact that a hemolytic toxin like melittin invades the membrane of bacteria or cancerous cells and causes necrosis (Mahmoodzadeh *et al*., 2015; Shams Khozani *et al*., 2019).

Hemolysis seems to be an important factor in venom toxicity of marine animals (Moghadasi *et al*., 2020). Almost all piscine venoms possess this activity (Chhatwal and Dreyer, 1992; Garnier *et al*., 1995; Sivan *et al.*, 2007), however some of them possess no hemolytic activity (Memar *et al*., 2016).

Ghadessy *et al*. (1996) reported a protein from a stone fish, Stonustoxin (SNTX), with hemolytic activity. This toxin has a molecular weight of 148 kDa and comprises of two subunits, termed α (71 kDa) and β (79 kDa). Ueda *et al.* (2006) purified a hemolytic lethal factor, Neoverrucotoxin (neoVTX), from a stonefish species *Synanceia verrucosa*. They reported that it is a dimeric protein with a molecular mass of 166 kDa.

Concerning hydrophobicity, some other hemolytic proteins with high hydrophobicity have early been reported from venomous animals including *Hemiscorpius lepturus* scorpion (Borchani *et al*., 2011), *Loxosceles* spp. (Brown spider) venom (Swanson and Vetter, 2006, McDade *et al*., 2010; Lane *et al.,* 2011; Gehrie *et al*., 2013), and *Apis mellifera meda* (Taghizadeh Dezfuli *et al*., 2014; Pashaei *et al*., 2019). According to our results, among 15 fractions that were isolated from crude venom, three major fractions had similar molecular weight of 72 kDa and consequently eluted from the column. For visual inspection of erythrocyte hemolysis, an innovative micro-hemolytic assay was used. The first major peak (scatotoxin) had significant instant hemolytic activity and the other ones were inactive. It is speculated that they are inactive isoforms of scatotoxin. As Scatotoxin eluted at 85% of acetonitrile, it is an interesting highly hydrophobic protein. The area percent of Scatotoxin showed that the hemolytic protein was 71 percent of total proteins in the crude venom. Hemolysis was immediately observed at a maximum time of 30 second thus it can be suggested that scatotoxin is a very fast acting hemolytic agent. This study is pending to monitor in vitro anticancer activity of scatotoxin on several cancer cell lines as a pilot study.

High efficiency of both extraction method and microscopic-scale assay led to reducing the collected specimens and consequently avoiding harmful effects on the Persian Gulf ecosystem. This issue is ethically important due to decreasing the number of samples. Among the documented hemolytic proteins, scatotoxin is the first report of a highly hydrophobic hemolytic protein. In conclusion, our novel extraction method was highly efficient for providing high amount protein from the low numbers of bonny spines while maintaining its biological activity.

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