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Original article

Biochemical, Hematological Effects and Complications of Pseudosynanceia Melanostigma Envenoming

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Key Words
Stonefishes, pharmacological effects, Pseudosynanceia melanostigma, LD50, haemorrhagic effect

Abstract

Objectives: Venomous fishes have different pharmacological effects and are useful. Among the venomous fish, stonefishes; especially Pseudosynanceia melanostigma has various pharmacological effects on the nervous, muscular and cardiovascular system of humans. In this study, toxicological characteristics, some blood effects, pharmacological and enzymatic properties of Pseudosynanceia melanostigma venom was investigated.

Methods: Crude venom purified by using gel filtration chromatography and the molecular weights of the venom and its fractions were estimated. The approximate LD values of this venom were determinedand the effects of LD50 dose on the blood of rabbits were studied. Hemolytic and Hemorrhagic activity of the venom sample was determined. In this case coagulation tests were performed.

Results: The LD50 of the Pseudosynanceia melanostigma crude venom was also determined to be 194.54 μg/mouse. The effect of two doses of LD50 showed a non-significant differences decrease in RBCs and MCV. In other cases, the results showed significant differences in WBC, Plt, Hb, MCH, MCHC and HCT; also it's showed a significant decrease. WBC count showed a significant increase with two doses of LD50 groups. The prothrombin time and partial prothrombin time were increased after venom treatment. As well as bleeding and clotting time were increased. According to the results, a minimum dose for Haemorrhagic effect 40 μg was obtained.

Conclusion: Venom of Pseudosynanceia melanostigma has inhibitory effect on platelet aggregation that can be used to design and develop of anticoagulant drugs.

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1. Introduction

Venomous fish produce a strong toxin harmful to humans which they deliver by means of a bite, sting, or stab [1]. They carry their venom in venom glands and use various delivery systems, such as spines or sharp fins, barbs, spikes and fangs [2, 3].

Pseudosynanceia melanostigma is a species of stonefish native to the western Indian Ocean and coastal regions of the Persian Gulf. Stonefish (Pseudosynanceia melanostigma), a member of the family Synanceiidae, is considered the most venomous fish. As

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main defense weapons, the stonefish has dorsal spines, that produce a very potent venom. The venom is myotoxic, neurotoxic, vasopermeable, and cardiotoxic [4]. A number of interesting biological activities (haemolytic, oedemic, anticoagulant, blocking of neurotransmitter synthesis and depletion of neurotransmitter store) have been uncovered in the crude venom [5].

More systematic pharmacological studies, including the determination of LDs values and observations of external manifestations of toxicity, were initiated in the late 1950s and early 1960s. In particular, the effects of crude stonefish venoms on the cardiovascular, respiratory and skeletal muscle systems were investigated [6].

The venom from Pseudosynanceia melanostigma had potent haemolytic action, which can cause marked lysis of washed guinea pig erythrocytes, but the venom was less potent in causing lysis of sheep and human erythrocytes; it was also found that haemolysis could be prevented by fresh unheated serum added to cells of the same species, and also that human serum could protect sheep cells from haemolysis by the venom [7].

2. Material and Methods

2.1. Extraction and preparation of Pseudosynanceia melanostigma crude venom

A total of 500 Pseudosynanceia melanostigma fish were caught by local fishermen with nets and delivered live to the laboratory. They were kept at 4°C overnight, and then stored at - 20°C until processed. After the frozen stonefish were allowed to partially thaw, their spines were cut and detached from the surrounding tissues. The crude venom was extracted by suction (using a 2.5 ml syringe) from the venom sacs located in the dorsal spines. The crude venom was incubated at 4°C with sodium chloride 0.15 M, for 24 hoursand. Than, dialysis, lyophilized and stored at -70°C until use. For use, 20 mg of lyophilized venom was resuspended in 2 ml 0.9% NaCl and then centrifuged at 3000 g for 15 min at 4°C. The supernatant (venom extract) with a protein concentration of approximately 2 mg/ml was used for the following tests (7).

2.2. Protein determination

Protein concentration was measured by the method of Lowry et al [8]. Lowry solution consists of a mixture of solution A and B. Solution A: 4 mg/ml NaOH and 20 mg/ml Na2CO3 in water, added 2 g of NaOH and 10 g of Na2CO3 to 400 ml water while stirring until completely dissolved, then adjust volume to 500 ml. Solution B: 10 mg/ml Potassium Sodium Tartrate and 5 mg/ml CuSO4 in water, Add 100 mg Potassium Sodium Tartrate and 50 mg CuSO4 to 8 ml of water in a tube. Shake the mixture until solids are completely dissolved, adjust volume to 10 ml (50:1 mix of solutions A and B). Prepare samples by adding 5, 25 and 50 µl of sample into a glass tube and adjust total volume to 200 µl. Prepare standard by adding 0, 10, 20, 40, 80 and 100 µl of bovine serum albumin (BSA; 1mg/ml) into a glass tube and adjust total volume to 200 µl. To each tube added 2 ml Lowry's solution, vortex, wait for 5 mins. To each tube added 200 µl Folin's Phenol reagent (1:1 mix of commercial reagent and distilled water) while vortexing, wait for 30 mins. Absorbance was measured at 750 nm. Protein determination was calculated by a standard curve using BSA.

2.3. Purification and isolation of Pseudosynanceia melanostigma venom

Lyophilized crude venom of Pseudosynanceia melanostigma (200 mg) was dissolved in 2 ml of sodium chloride (15 mM, pH 6.8) and centrifuged at 14,000 rpm for 15 min at 4°C. Afterwards, the impurities were removed by using a 0.45 µ filter to remove insoluble materials. The solution was applied to a 3×150 cm column packed with Sephadex G-50. The column was equilibrated with sodium chloridebuffer (pH 6.8) and then eluted with the same buffer. Fractions of 9 ml were collected at a flow rate of 60 ml/hour at 4°C. The absorption of each fraction was read at 280 nm. Fractions were pooled and dialyzed overnight at 4°C against distilled water and were concentrated at 4°C. The biochemical and hematological test was done for fractions with the same concentration and fractions [9].

2.4. SDS-PAGE

For determining the molecular weight and the purity of the crude Pseudosynanceia melanostigma and the fractions obtained by using the gel chromatography technique, we performed electrophoresis (SDS-PAGE) according to the method of Laemmli [10].

2.5. Estimation of LD_{50}

The lethal dose 50 (LD50) of the Pseudosynanceia melanostigma venom was determined in mice 18 to 20 g National Institutes of Health (NIH) strain. This test was conducted according to the method by Meier and Theakston [11]. Different doses of crude venom were prepared in physiological serum and were each injected into four mice (2 ml/dose, 0.5 ml/mouse). The doses were chosen was so that no mouse would die at the lower dose, and all mice would die at the higher dose. Mouse mortality within 24 h was recorded and each sample LD was calculated. Upon recording of mortality, the Spearman-Karberstatistical method was used for LD50 calculation [12].

2.6. Hemolytic activity of venom and its fractions

The haemolytic activity of the venom sample was determined using rabbit erythrocyte suspension as described by Garnier. For 1 ml sample of the venom, add sodium chloride 150 mM and 200 µl of 2% erythrocytes, then was kept for 30 min at room temperature. The suspension centrifuged at 3,000 g for 5 min. The percentage of haemolysis was estimated by reading the absorbance at 540 nm in a spectrophotometer. Finally, percent hemolysis was calculated from the following relationship [13]:

Hemolysis = [OD Test - OD Saline] / [OD H2O - ODSaline] ×100

2.7. Hemorrhagic activity

For measuring the activity of venom for hemorrhage under the skin and determinate the minimum hemorrhagic dose, 10, 20 and 40 µl of venom was dissolved in 50 µl normal saline. This concentration of venom injected intradermally into the dorsal area of NIH mice (25 ± 5g, n=3). Two diameters were obtained forthe spot of haemorrhageby measuring the longest diameter of the spot and the diameter perpendicular to the first measurement. The haemorrhagic effect was quantitated as the product of these two diameters. After 24 hours, the hemorrhage spots diameter of mice was investigated with digital caliper. Spots with a diameter greater than 10 mm were recorded as a minimum hemorrhagic dose [14].

2.8. Hematologic effects of venom

To investigate the effect of hematologic and coagulation activities of venom and its fractions, the blood samples were collected by test tube containing an anticoagulant (EDTA, sometimes citrate) to stop it from clotting. The sample is then transported to a laboratory. 24 hours after venom injection, blood samples were taken directly from the heart of rabbitsand blood parameters were measured. The blood samples wereanalyzed to determine of hematological parameters such as a red blood cell count (RBC), white blood cells (WBC), the mean corpuscular volume (MCV), the mean corpuscular hemoglobin (MCH), the mean corpuscular hemoglobin concentration (MCHC), platelets (Plt), Hemoglobin (Hb) and hematocrit (HCT) by using an automatic hematological assay analyzer (Nihon Kohden corporation, Japan) [15].

By determining the LD50 (1/2 and 1/3 LD50 dose), the effects of the venom on the blood of rabbits were studied and different scenarios of animal alternately at different times (every 5 minutes) were observed.

2.9. Bleeding and clottingtime

Bleeding time (BT) was performed using Duke method. The rabbits was pricked with a special needle or lancet, preferably on the earlobe, after having been swabbed with alcohol. The prick is about 3-4 mm deep. The rabbitsthen wipe the blood every 15 seconds with a filter paper. The test ceases when bleeding ceases. The usual time is about 2-5 minutes [16].

Clotting time (CT) was determined as described by Harper. The clotting system contained 100 µl of venom, 200 µl of 25 mM calcium chloride, and 200 µl of citrated rabbit plasma [17].

2.10. Prothrombin time and partial thromboplastin time

Tissue thromboplastin was reconstituted according to instructions and was labeled with the time, date and initials of the technician. The thromboplastin reagent was stabile for seven days after reconstitution. The sample was allowed to sit 10-15 minutes and was than inverted gently several times. The reagents were mixed well prior to pipetting any of them reagent at any step in this procedure.

One to two mls of the tissue thromboplastin-CaCl2 reagent (PT reagent) was pipetted into a test tube, and the test tube placed into an incubator at 37°C. The level of the thromboplastin was not allowed to exceed the height of the heat block. Normal rabbit plasm, 100 µl was poured into the test tubes and at least one minute was allowed for the plasma to reach 37°C. Then, 200 µl of the PT reagent was poured into the tube containing the rabbit plasma, and simultaneously stop watch was started.

The solution in the tube was mixed and left in the heat block for a minimum of 7-8 seconds. It was then removed, and its exterior was wiped. The tube was then tilted back and forth gently until a visible clot formed. As the clot formed, the mixture began to gelatinize and turn cloudy. The stop watch was immediately stopped when the clot began to form, and the prothrombin time (PT) in seconds was recorded.

A sufficient quantity of CaCl2 reagent was heated to 37°C for the tests to be performed. Normal rabbit plasma, 100 µl, was poured into a labeled test tube, and 100 µl of partial thromboplastin reagent was added. The plasma/partial thromboplastin mixture was incubated at 37°C for a minimum of three minutes, and CaCl2 100 µl, was forcefully added into the plasma/partial thromboplastin mixture; a stop watch was started immediately.

The mixture in the tube was stirred once after the calcium reagent had been added, and the tube was allowed to remain in the heat block for approximately 20 seconds while being stirred occasionally. After 20 seconds, the tube was removed from the water bath/heat block, and its outside was dried. The tube was then gently tilted back and forth until a visible clot formed, at which time the stop watch was immediately stopped and the Partial thromboplastin-time (PTT) in seconds was recorded [18, 19].

Analyses to determine the means, standard deviations (SDs) and P-values of the test data were performed using the software SPSS. P-value was calculated, and a P-value less than 0.05 was meaningful.

3. Results

3.1. Protein determination

The amount of protein Pseudosynanceia melanostigma crude venom about 33.1 mg/ml was determined.

3.2. Purification and isolation of venom

The crude venom was fractionated by gel filtration and six peaks (F1 to F6) were obtained (Fig. 1). Only fraction F6 showed coagulant activity.

3.3. SDS-PAGE

The crude venom of Pseudosynanceia melanostigma and all fractions were analyzed by SDS-PAGE to estimate their protein composition (Fig. 2). The molecular weights of protein from the venom ranged from 20 to 120 kDa. The fraction 6 contained one major protein band of 20 kDa.

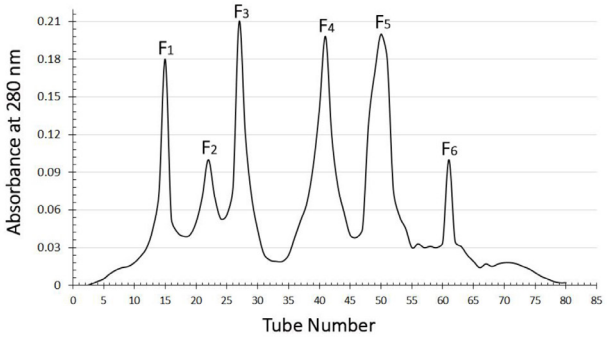

Figure 1 Sephadex G-50 chromatography of Pseudosynanceia melanostigma venom.

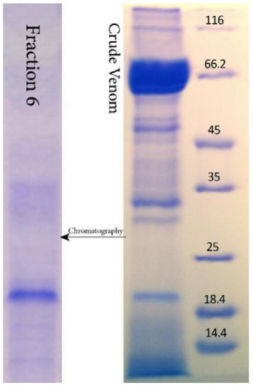

Figure 2 SDS-PAGE of Pseudosynanceia melanostigma crude venom and fraction 6

3.4. Hemolytic effect

By investigation of effect on erythrocytes cells of rabbits and mice, the hemolytic effect on rabbit blood was 91.67%. This effect on mice blood was 89% (table 1) which related to venom concentration of 5 mg/ml.

Table 1 Hemolytic effect on mice and rabbit erythrocytes at different concentrations of Pseudosynanceia melanostigma venom

Venom Concentration (mg/ml)			
0.5	2	3.5	5
Haemolysis% (mice)			
19	37	62	84
21	44	65	88
23	46	71	95
21 ± 2	**42.33 ± 4.72**	**66 ± 4.58**	**89 ± 5.57**

Venom Concentration (mg/ml)			
0.5	2	3.5	5
Haemolysis% (rabbits)			
27	33	69	90
24	31	71	92
27	36	73	93
26 ± 1.73	**33.33 ± 2.52**	**71 ± 2**	**91.67 ± 1.53**

3.5. Estimation of LD_{50}

LD50 was calculated according to the following formula:
M = X100 ± d/n (r-n/2)
M = Log LD50
X100 = Log 230 = 2.3617
n =4
t0.05 = 2.20

(For 4 + 4 + – 1 degrees of freedom) M = 2.3617 ± 0.097/4 (0 + 1 + 4 – 4/2) M = 2.3617 ± 0.0727
M = 2.289
LD50 = Antilog 2.289
LD50 = 194.54µg
Determination of the LD50 range:

Min= 194.54µg
Max= 271.9µg

Table 2 The mortality rate of mice after 24 hours with injection of different concentration of Pseudosynanceia melanostigma venom

Death after 24 hours	Venom concentration in 5.0 ml	Mice (n=4)
----	150 µg	Mice (Group 1)
+---	190 µg	Mice (Group 2)
++++	230 µg	Mice (Group 3)
++++	270 µg	Mice (Group 4)

P<0.05,
+: Dead mice
-:Live mice

3.6. Haemorrhagic effects of venom

According to the results, a minimum dose forhaemorrhagic effect 40 µg was obtained (Fig 3.). Results were recorded as the mean in Table 3. A minimum haemorrhagic dose for fraction 6 was 11 ± 0.68.

Table 3 Effect of Pseudosynanceia melanostigma venom on hemorrhage under the skin in mice

Control	Group 1	Group 2	Group 3
0 µg/50 µl	10 µg/50 µl	20 µg/50 µl	40 µg/50 µl
0 mm	3.1 ± 0.51 mm	3.8 ± 1.4 mm	10 ± 0.94 mm

P < 0.001

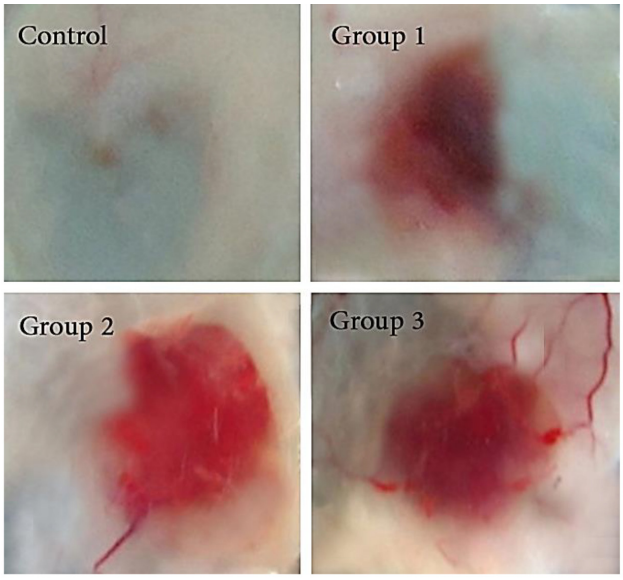

Figure 3 The haemorrhagic effect of different concentrations of Pseudosynanceia melanostigma venom

3.7. Hematologic effects of venom

The effect of crude venom of Pseudosynanceia melanostigma on hematological parameters of male rats exposed to two doses of LD50 are presented in table 4. The results showed a non- significant differences decrease ($P < 0.01$) in RBCs and MCV in first and second groups compared with the control group. The results showed significant differences in WBC, Plt, Hb, MCH, MCHC and HCT between the first and second groups, also it showed a significant decrease ($P < 0.01$ and $P < 0.001$) in first and second groups compared with the control group. The result showed a significant increase ($P < 0.01$) in the WBC count treatment with two doses of LD50 groups compared with the control group.

Table 4 Effect of Pseudosynanceia melanostigma venom on blood parameters

%HCT	MCHC (g/dl)	MCH (pg)	MCV (fl)	Hb (g/dl)	Plt (10^3/mm^3)	WBC (10^3/mm^3)	RBC (10^6/mm^3)	Parametr / Group
40 ± 3.8	31 ± 0.07	21 ± 0.05	69 ± 0.1	12.4 ± 1.5	340 ± 1.42	9 ± 0.8	5.2 ± 2.4	**Control**
30.2 ± 0.61***	21.2 ± 0.08**	16.3 ± 0.05**	63 ± 0.04**	7.45 ± 0.8***	61 ± 0.72**	36.2 ± 0.43**	4.6 ± 0.54**	**Group 1 1/2 LD_{50}**
34 ± 0.25***	25.8 ± 0.04**	18.4 ± 0.09**	66.2 ± 0.08**	8.2 ± 0.41***	79 ± 0.9**	28.9 ± 0.2**	4.9 ± 0.3**	**Group 2 1/3 LD_{50}**

**P < 0.01; ***P < 0.001; pg=pictogram; fl=femtoliter; g/dl=gram/deciliter

Comparing the coagulation time of normal plasma (without venom) with those obtained for crude venom and fraction 6 were found to increase the plasma clotting time. Regarding Table 5, by conducting the PT, PTT, BT and CT test on mice plasma, they were shown that crude venom and fraction 6 increase the coagulation time.

Table 5 Effect of Pseudosynanceia melanostigma venom on hemostatic factors

CT sec	BT sec	PTT sec	PT sec	
186 ± 0.8	228 ± 1.1	27 ± 1.6	9.5 ± 0.85	**Control (plasma+dH_2O)**
234 ± 1.4	270 ± 1.04	34 ± 1.2	16 ± 1.05	**Crude Venom**
241 ± 2.1	283 ± 0.56	39 ± 1.3	19 ± 0.54	**Fraction 6**

*p < 0.05

4. Discussion

Marine animal venoms are considered and studied less compared to other animals such as snakes. In general, there is little information about these important biological resources. The limited information is essentially due to the difficulty of extracting venom, instability and diversity of marine compounds.

Stonefish venom is a complex consisting of various enzymatic and non-enzymatic compounds with diverse pharmacological properties. One of the criteria and indicators of animal venom toxicity is the concentration of its protein, so that high concentration of protein can be an indicator of the strength and variety of its pharmacological properties [20, 21]. Assessment of venom and obtain the median lethal dose of crude venom compared to other species of stonefish revealed that the median lethal dose of Pseudosynanceia melanostigma is similar to the stonefish S. horrid [22].

Behavioral changes in rats poisoned by intravenous injection of venom can be compared with changes reported in cases exposed to other venomous fish, with the difference that some of the behavioral symptoms are more intense. The effects of crude venom of Pseudosynanceia melanostigma in rats was similar to the symptoms reported in stonefish S. verrucosa, including sudden death along with signs such as convulsions, muscle imbalance, paralysis, bristle and tremble [23]. Symptoms such as bristle, jerking, rotational movements and subsequent posterior limb paralysis and sudden death are among the different signs observed by other researchers [24].

One of the most important activities of crude venom and its fractions is hemolytic effect or the effect on washed erythrocytes of animal species and almost all venomous fish have this kind of activity in their venom [20]. The venom of Pseudosynanceia melanostigma showed hemolytic effect on the washed erythrocytes of rats and rabbits. This effect on rabbit erythrocytes was more intense. Such results are comparable with other studies on other species of venomous fish, especially stonefish [25, 26].

One of the features of venom in venomous fish, especially in order Scorpaeniformes, is the hemorrhagic activity

or subcutaneous bleeding, which was investigated in the present study. This activity is induced as a result of the destruction of the basement membrane of blood vessel walls caused by enzymes [27, 28].

On the other hand, the venom and its fraction No. 6 showed high hemorrhagic activity. The results are indicative of dose-dependent nature of this activity; statistical analysis confirms this result. Both crude venom and its fraction had hemorrhagic and anticoagulant activities; when these two activities happening together, anticoagulant ability is considered as an enhancer and can predispose to hemorrhage.

Take a look at results indicates that the hemoglobin and hematocrit percentage was significantly decreased; we concluded that the venom could lead to intravascular hemolysis, therefore extensive destruction of erythrocytes. The obtained result corresponds to the findings of some researchers [29].

Platelets also are useful in the toxicological assessments. In the present study, platelet count has been decreased compared to the control group. This could be the reason for the reduction of platelet aggregation, and reflected in the bleeding spots in internal organs and necrotic spots on the skin surface.

According to the existing literature and studies carried out, despite the relatively large information about inhibitory effect on platelet aggregation due to venom of other vertebrates and invertebrates, such as a variety of snakes and arachnids, there is very little information on the characteristics of the venom of venomous fish, especially stonefish family. Therefore, this issue requires further research to clarify this aspect of the venom activity and to open the door for designing and developing anticoagulants.

According to the results obtained in the haemostatic agent of the venom and coagulation tests, it can be concluded that this venom is hepatotoxic venom that confirms previous results and indicates the anticoagulant properties of venom and its effective fractions. As well, the hemorrhagic effect of the venom is determined through this method.

The results of the present research show that the venom of this species of stonefish and it's specifically fractions have haemostatic effect and can be used as an anticoagulant for the design and construction of anticoagulant drugs in the future.

Ethics committee approval

The present study was approved by the Ethics Committee of Razi vaccine and serum research institute, Karaj, Iran.

Conflicts of Interest

The authors declare that they have no conflicts of interest to disclose.

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