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Purification, and characterization of a new pro-coagulant protein from Iranian *Echis carinatus* venom

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ABSTRACT

This work aimed to purify the proteins that cause blood coagulation in the venom of the Iranian *Echis carinatus* snake species in a comprehensive manner. Gel filtration chromatography (GFC), Ion exchange chromatography (IEC), and Size Exclusion High-Performance Liquid Chromatography (SEC-HPLC) were utilized in the purification of the coagulation factors. The prothrombin clotting time (PRCT) and SDS-PAGE electrophoresis were performed to confirm the coagulative fractions. The fraction with the shortest coagulation time was selected. The components of this designated fraction were identified through matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF) following thorough purification. Circular dichroism (CD) was employed to determine the second structure of the coagulation factor. The crude venom (CV) was analyzed and had a total protein concentration of 97%.

Furthermore, the PRCT of the crude venom solution at a concentration of 1 mg/ml was determined to be 24.19 ± 1.05 s. The dosage administered was found to be a factor in the venom's capacity to induce hemolysis. According to CD analysis, the protein under investigation had a helical structure of 16.7%, a beta structure of 41%, and a turn structure of 9.8%. CHNS proved that the purified coagulant protein had a Carbon content of 77.82%, 5.66% Hydrogen, 3.19% Nitrogen, and 0.49% Sulphur. In the present investigation, a particular type of snake venom metalloproteinase (SVMP) has undergone the process of purification and characterization and has been designated as EC-124. This purified fraction shows significant efficacy as a procoagulant. Our findings have shown that this compound has a function similar to factor X and most likely it can cause blood coagulation by activating factor II (FII).

1. Introduction

The hemotoxic properties found in the blood of specific snake species are potent toxins that exhibit cytotoxic properties and interfere with the regular process of blood coagulation. These substances interfere with the functionality of blood coagulation factors, leading to detrimental effects on the internal organs. Certain hemotoxins can inhibit blood coagulation, while others induce the aggregation of blood platelets or other blood constituents [1,2]. The introduction of significant and gradual quantities of blood coagulant toxins into the bloodstream leads to gradual and progressive impairment of the blood's coagulation capabilities from a clinical standpoint. This impairment, known as defibrinating, leads to the failure of blood coagulation [3]. When the concentration of this toxic substance is elevated and it rapidly infiltrates

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the circulatory system, it induces the process of blood coagulation inside the vasculature. The presence of blood coagulation and preventive proteins has been observed in snake venom. The effectiveness of these proteins is influenced by the amount and rate of venom entering the bloodstream [4,5].

The venom derived from *Echis carinatus*, a species native to Iran, has demonstrated several medicinal benefits. Echistatinas and Ecarin are two pharmacological compounds that have been extracted from the venom of the aforementioned snake species [6]. Iranian *E. carinatus* exhibit diminutive physical proportions and are widely acknowledged for their pronounced tendency towards aggressive biting behavior. One notable attribute of this serpent is its extensive geographical range and substantial population, rendering it one of the most perilous reptiles globally [7]. The venom of Iranian *E. carinatus* is composed of a combination of proteins that impact the process of blood coagulation, leading to the manifestation of intense perspiration and significant hemorrhaging. In addition to metalloproteinases, Iranian *E. carinatus* venom also contains serine proteases that can impact the walls of blood vessels and destroy them. The venom of Iranian *E. carinatus* exhibits geographical variations, which can be attributed to the divergence in venom proteins. In 1994, researchers isolated the first phospholipase from the venom of the *Terciopelo* (*Bothrops asper*) using chromatography and electrophoresis techniques. Furthermore, they successfully identified the pharmacological and catalytic constituents of this isolated compound [8].

The venom of Iranian *E. carinatus* is composed of a complex combination of proteins that has a significant impact on the blood coagulation cascade, resulting in the manifestation of distressing perspiration and profound hemorrhaging. One of the proteins present in this group is prothrombin, which undergoes activation and maturation to turn into thrombin. This transformation is facilitated by the prothrombinase proteolytic complex, which consists of a serine proteinase known as factor Xa, cofactors factor V, two calcium ions (Ca^{2+}), and phospholipids [9]. Several exogenous prothrombin activators have been identified within snake venom. Over 50 distinct compounds have shown efficacy in the process of blood coagulation. The primary aim of the coagulation process is to create the enzymatic protein known as thrombin. Fibrinogen is ultimately converted into fibrin by this enzyme. The formation of a complex mesh within the region of injury occurs when fibrin strands interconnect [10]. As previously stated, snake venom contains coagulation and anticoagulation compounds, which can be extracted and utilized through diverse purifying techniques. The present study aimed to isolate and characterize certain blood-coagulating components from the venom of Iranian *E. carinatus*.

2. Material and methods

2.1. Chemicals, venoms and human plasma

Prothrombin-free plasma kit, Factors X-free plasma kit, and calcium chloride were purchased from HYPHEN (BioMed, Paris, France). Bovine serum albumin (BSA) and acrylamide were acquired from Sigma-Aldrich (St. Louis, MO, USA). The following reagents were obtained from Merck Company (Darmstadt, Germany): Coomassie blue G-250, ethanol, orthophosphoric acid, Tris base (Hydroxymethyl Aminomethane), bromophenol blue, sodium dodecyl sulfate (SDS), glycerol, ammonium persulfate, N, N', Methylene Bisacrylamide, Acetonitrile, Trifluoroacetic acid (TFA), ammonium acetate, and hydrochloric acid (HCL). The compound tetraethyl methyl ethylenediamine (TEMED) was acquired from the Fluka company (Switzerland), while the wide range protein marker was obtained from Bio-Rad Company (California, USA). The DEAE Sepharose gel used in this study was acquired from Pharmacia company (Sweden). All other chemicals and reagents used in this study were of analytical grade.

The lyophilized venom of Iranian *E. carinatus* was obtained from the Department of Venomous Animals and Antivenom Production at the Razi Vaccine and Serum Institute, Karaj, Iran. Finally, the study utilized pooled normal human plasma from the Arad laboratory in Karaj, Iran, which had been anticoagulated with sodium citrate and stored at a temperature of −80 °C. This plasma was employed in all subsequent procedures as detailed in the following sections.

2.2. Animals and ethical Issues

Albino male and female mice of the NIH strain, (18 ± 2 g body mass), were obtained from the Venomous Animal and Antivenom Production Department of Razi Vaccine and Serum Research Institute. These mice were housed at room temperature with free access to food and water and fed a standard diet. All experimental procedures were carried out in compliance with the Institutional Animal Ethics Committee (IAEC). The approval number of the ethical committee is IR.SEMUMS.REC.1398.142.

2.3. Venom preparation

The resultant crude extract was mixed with D.D.W and subsequently filtered through a 0.45 μm filter. Following centrifugation at a temperature of 4 °C for 20 min at a speed of 14000 rpm, the supernatant, which constituted the crude extract, was carefully harvested.

2.4. Determination of lethal dose

To assess the toxicity of the venom, various doses of crude venom (5.63, 7.04, 8.8.11, 13.75, 17.18, and 21.48) were prepared in 2.5 ml of physiological serum. Each dose was intravenously injected into the tails of four mice at a volume of 0.5 ml per mouse. The cumulative survival and death rates were recorded within 24 h. The LD_{50} value was determined using the Spearman- Karber's statistical method [11]. LD_{50} value was calculated with the subsequent formula:

$$M = x100 \pm d/n \left(\sum r - n/2\right)$$

$$M = \log LD_{50}$$

In this formula, M is log LD_{50}, x100 = log (the dose that had 100% lethality), n = the number of mice used in each dose, r = the number of dead mice in each dose, and d = log (factor of dose intervals).

2.5. Preparation of human normal citrated plasma

Pooled normal citrate plasma was prepared by sampling from 20 healthy human donors. The ratio of blood to sodium citrate 3.2% in coagulation tests is 1–9. After blood collection, the tubes were inverted 8 times to ensure complete blood citration. Citrated blood was centrifuged at 3500 rpm for 15 min to separate its plasma. Pooled normal plasma (PNP) was standardized by pooling plasma from different individuals and 0.7 ml was aliquoted into 1.5 mL plastic tubes and immediately frozen at 80 °C for coagulation studies [12].

2.6. Plasma Recalcification time (PRCT)

PRCT is a simple coagulation test that evaluates both internal and external coagulation pathways, in which 100 μl of 0.025 M calcium chloride is added to 300 μl of pooled normal plasma (calcium-free citrated plasma), and the coagulation time is recorded. With the difference that in modified PRCT, to measure the effect of venom on coagulation time, 10 μl of different concentrations of 0 (control), 1, 0.1, and 0.01 mg/ml of *Echis carinatus* venom was also added to Calcium chloride and its acceleration effect on coagulation time is evaluated [13].

2.7. Purification protocol

2.7.1. Gel filtration

A solution of crude venom (150 mg in 3 mL of D.D.W) was loaded on a Sephadex G-50 column with dimensions of 1.5 m × 2 cm (Pharmacia, Sweden), and received the clear venom filtrate application. The column was equilibrated with 0.05 M ammonium acetate solution before being applied. The column was eluted with an equilibration buffer at a flow rate of 30 mL/h, and fractions of 3 mL were retrieved. The Biochrom Libras22 spectrophotometer was used to measure protein elution at 280 nm. The individual peaks were subjected to analysis for PRCT and Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The fraction that exhibited procoagulant activity was pooled [14].

2.7.2. Anion exchange chromatography

The sample demonstrating the highest level of pro-coagulant activity (F1) was applied to a DEAE-Sepharose column (Pharmacia-Sweden). The column dimensions were 1.6 × 22 cm. Before loading the sample, the column was equilibrated with a 20 mmol/L Tris buffer solution at a pH of 8.1. The fractionation process was conducted with a flow rate of 30 mL/h, and fractions of 3.0 mL were collected. The column was eluted with a linear gradient (0–0.5 M) of NaCl to remove unbound proteins and bound proteins. The elution profile was monitored using a spectrophotometer at a wavelength of 280 nm [15]. To determine their PRCT, the individual peaks were analyzed. The sample was filtered with Amicon Ultra-15 Centrifugal Filters (MWCO 3 kDa) to perform dialysis, in comparison to pure water.

2.7.3. Size exclusion-high performance liquid chromatography (SEC-HPLC)

The protein concentrate obtained in the previous step (0.8 mg/ml) was injected into SEC-HPLC using Waters Ultrahydrogel 2000 (7.8 × 300 mm, 5 μm, pore size- 100 Å) column. This column was pre-equilibrated with ammonium acetate in water. An isocratic elution took place for 30 min in the column. The protein was eluted at a flow rate of 0.7 ml/min and monitored at a wavelength of 280 nm. The isolation and collection of 5 peaks were accomplished using SEC-HPLC. The peaks were analyzed with the PRCT test and the purity of the protein was confirmed by 12% SDS-PAGE. Protein concentration was determined by the method of Bradford [16]and standardized with bovine serum albumin.

2.8. MTT assay

In a 96-well plate, 5×10^4 cell/well Hu02 cells were incubated overnight with various concentrations (100, 10, 1, and 0.1 μg/ml) of crude venom, and the final purified fraction, complete medium as a negative control for 24 h. A 5 mg/mL stock solution of "MTT" (1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenyl formazan, Thiazolyl blue formazan) was prepared in sterile PBS, and 10 μL added to each well containing 100 μL culture medium. After 4 h, the MTT-containing medium was removed, and 100 μL of sterile DMSO was added to solubilize the formazan crystal. The solutions were analyzed in an ELISA plate reader (BIOTEK Inc, USA), measuring the absorbance at 570 nm [17]. The percentage of cell viability was calculated from the absorbance values of the test wells and the control wells using the following Equation:

$$\%Cell\ viability = [(Mean\ absorbance\ in\ test) / (Mean\ absorbance\ in\ control)] \times 100$$

2.9. Hemolysis assay

The determination of the potential hemolytic effects of the crude venom and final purified fraction on human red blood cells (RBC) was conducted through the use of a hemolytic assay. Fresh samples of blood were primarily obtained from a volunteer with blood type O. A suspension of human erythrocytes, consisting of 20% (vol/vol), was prepared in PBS. This suspension was then diluted 1:20 in PBS, and 100 μL of the resulting solution was added in triplicate to 100 μL of a 2-fold serial dilution series of the crude venom and purified fractions in a 96-well plate. Triton-X 100 (1%) was utilized as a positive control for 100% lysis of RBCs, while sterile 0.9% NaCl solution was used as a negative control. Following an incubation period of 1 h at 37 °C, the plate was centrifugated at 3500 rpm for 10 min. Subsequently, 150 μL of the supernatant was transferred to a new 96-well plate to measure the absorbance at 414 nm using a microplate reader (STAT FAX 2100, BioTek, Winooski, USA). The percentage of hemolysis was then calculated according to the provided formula [18].

$$\text{Hemolysis } (\%) = \frac{\text{Means OD sample} - \text{Means negative control}}{\text{Means OD positive control} - \text{Means OD negative control}} \times 100$$

2.10. CHNS chemical analysis

The PerkinElmer 2400 analyzer, which is manufactured in the United States, was utilized in the CHNS study. The initial step in the PerkinElmer 2400 elemental analyzer involves introducing the sample into the combustion column, where it undergoes combustion at the temperature of 1150 °C in the presence of oxygen gas. The sample is converted into a gaseous state by this process. The regeneration column, which operates at 850 °C, is filled with helium as the carrier gas. Subsequently, the oxygen that is already present in the system is taken in. The gaseous compounds representing the elements are collected by absorbent columns and subsequently identified by the TCD detector at different time intervals. The outcome was established based on the proportion of elements that were present [19].

2.11. MALDI-TOF MASS spectrometry

2.11.1. In gel digestion

Tryptic digestion in the pro-coagulant protein gel was conducted by the reported protocol. Briefly, the protein band was excised with a clean scalpel and transferred to a clean microcentrifuge tube. The piece of gel was stained with 100 mM acetonitrile/ammonium bicarbonate (1:1 v/v). Then 50 μl of trypsin buffer (trypsin in 10 mM ammonium bicarbonate containing acetonitrile) was added to the cover gel and incubated overnight at 37 °C. Following digestion, the resulting peptides were extracted with extraction buffer (formic acid/acetonitrile with a volume ratio of 1:2) and concentrated for MALDI-TOF MS analysis [20].

2.11.2. MALDI-TOF MS analysis

Matrix-assisted laser desorption/ionization mass spectrometry (Nd: YAG 200-HZ laser, Applied Biosystems 4800 MALDI TOF/TOF, Nd: YAG 200-HZ) was used for MS experiments. For MS analysis, the digested sample on the MALDI plate was mixed with alpha-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile containing 0.1% trifluoroacetic acid (1:2 ratios, respectively) as the matrix solution was air-dried and analyzed in positive reflectance mode (mass range was 4000-800 Da). The data were interpreted and processed using Data Explorer version 4.0 (Applied Biosystems) software and Mascot Distiller v.2.8. The Metazoa (animal) taxonomy was selected in the Swissprot database for protein identification. Furthermore, specific parameters such as trypsin proteolysis have been selected. The likelihood of protein matching was determined using the MASCOT protein indexes.

2.12. Determining the second structure by circular dichroism (CD)

The secondary structure of the coagulation protein was determined by performing a CD spectrum analysis. Using CD spectroscopy, the mean residue molar ellipticities of the protein were determined. This was

achieved using the Jasco J-810 spectropolarimeter (Jasco, Japan). The measurements were conducted at a temperature of 25 °C, using a scanning speed of 200 nm/min and performing five scans. A 0.2–0.5 mg/mL of protein solution DDW was placed into a 1 mm quartz cell, and its spectra were scanned from 190 to 350 nm [18]. Subsequently, the data acquired from circular dichroism spectroscopy was analyzed to determine the proportionate ratio of secondary structural elements.

2.13. The coagulation of Factor II, X free plasma

The determination of coagulation factor function in one-stage clotting assays relies on the capacity of diluted patient plasma to restore the clotting time of a plasma sample that possesses normal levels of all clotting factors except for the specific factor under investigation, wherein it is completely absent. The evaluation of the results is done by comparing the graphical plot of a parallel line bioassay with that of a standard plasma. The procedure involves a coagulation test where all clotting factors are present, except for factors II, and X. The concentrations of factor X and factor II are negatively correlated with the associated clotting time [21]. This examination involved the use of commercial plasmas that had deficiencies in factor II, or factor X. The quantification of the activity of a specific coagulation factor in plasma was performed using a reference curve that was established using multipoint calibration, as conducted by HYPHEN BioMed (France).

2.14. Simulation of 3D structure of EC-124 and bioinformatics studies

Protein PilotTM Version 5.0.2.0, 5346 (Sciex) software was employed to analyze the raw data. The analysis was performed against the taxonomic FASTA file of each species, which was obtained from UniProt (Serpentes_Tax_id8570). The analysis parameters used gel-based ID and focused on biological modifications. The fixed modifications of demethylation and deamidation were designated, while the variable modification of oxidized methionine was defined. The proteins that were discovered underwent validation using the following filters: a protein score threshold of greater than 20, a peptide score threshold of greater than 10, a scored peak intensity (SPI) threshold of greater than 70%, and a false discovery rate (FDR) threshold of 1%. The abundance of each particular venom was estimated relative to the overall spectral intensity of all detected proteins using the mean spectral intensity (MSI).

The Protein PilotTM search methodology relied on the Percolator node to accurately distinguish accurate peptide spectrum matches from inaccurate ones. This discrimination was achieved by employing the false discovery rate (FDR), which facilitated the enhancement of the

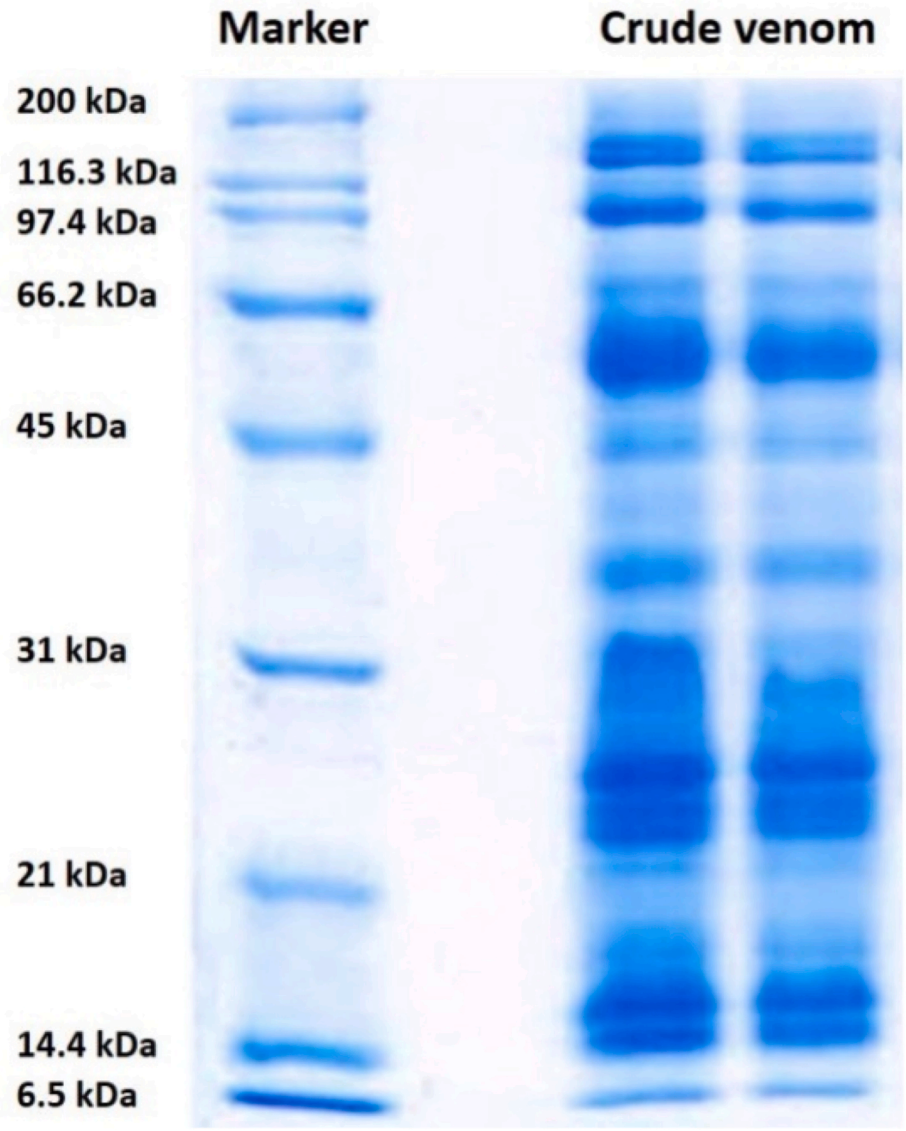

Fig. 1. Electrophoresis of proteins isolated from crude venom on SDS-PAGE.

number of peptides that were confidently identified while maintaining a predetermined false discovery rate. The obtained results have undergone a filtration process, wherein only peptides with high confidence (FDR ≤0.01) have been taken into account for the identification outcomes [22].

2.15. Statistical analysis

The experiments were repeated three times and the results were reported as mean ± standard deviation. SPSS software v.26 was used to analyze the results. To accomplish this goal, ANOVA and Tukey's tests were utilized. The significance threshold was determined by a p-value <0.05.

3. Results

3.1. Determination of crude venom (CV) properties

The protein concentration inside the crude venom of Iranian *E. carinatus* was determined to be 97% 0.11% SDS-PAGE analysis was performed to assess the protein bands present in the crude venom, and the results are shown in Fig. 1. The value of PRCT for the crude venom sample with a concentration of 1 mg/ml was 24 ± 1.0.

3.2. Separation of pro-coagulant Factor from venom by gel filtration chromatography

Gel filtration chromatography was used to fractionate the crude venom, and the resulting fractions were then evaluated (Fig. 2A). Fraction 1 (F1) showed the most prominent peak, as depicted in Fig. 2A. The protein content in each of the acquired fractions was determined using the Bradford method. Fraction 1 was found to have the highest protein concentration, measuring 1.60 mg/ml, as expected. The same concentrations of all fractions (all samples were standardized and concentrated based on fraction 5) were used in PRCT (Table 1). The results show that fraction 1(F1) has the best procoagulation performance. Therefore, a serial diluted of this fraction was used to measure CT_{50} (Table 2). CT_{50} is the sample concentration that results in a 50% decrease in plasma coagulation time compared to the control plasma. The concentration of the F1 sample, specifically 0.025 mg/ml, was obtained as an estimated CT_{50} value for this specific fraction. Subsequently, the SDS-PAGE gel was stained with Coomassie Blue G-250, resulting in the visualization of protein bands as depicted in Fig. 2B.

3.3. Ion exchange chromatography of F1 fraction

Based on the findings derived from the preceding stages, the initial fraction (F1), which exhibited the highest level of activity in the PRCT, was chosen for subsequent purification by ion exchange chromatography. The findings were acquired from the graph displaying three distinct peaks (Fig. 3A). Fractions 11, 12, and 13, obtained from F1 by anion chromatography, were evaluated using the Bradford method to determine their protein content. The F12 fraction had the highest protein level, measuring 0.099 mg/ml. To accurately compare the results of the PRCT for each peak, it was necessary to standardize the concentration of all peaks acquired using anion chromatography based on peak F13, which was determined to be equivalent to a value of 0.076 mg/ml. An experiment was conducted to assess coagulation using the PRCT on standardized samples. The fraction denoted as F12, which exhibited a coagulation time of 16.4 s, was selected as the experimental sample for the subsequent phase of the study, based on the acquired data (Table 3). Fig. 3B displays the electrophoretic pattern of three fractions acquired using anion chromatography, alongside a conventional protein marker featuring a molecular weight range of 6.5–200 kDa.

Table 1
Concentration values and PRCT test results (at 0.41 mg/ml) for different crude venom fractions.

Fractions name	Concentration (mg/ml)	PRCT (sec)
F1	1.60	9
F2	0.81	68
F3	0.52	77
F4	0.09	152
F5	0.41	158

3.4. Size exclusion-high performance liquid chromatography (SEC-HPLC)

The purification of coagulant proteins was conducted utilizing the SEC-HPLC, and the resultant spectrum is depicted in Fig. 4A. Five fractions (peak) were observed in this spectrum. The percentage and concentration of each peak were determined by measuring the area under the peak and injecting a 100 μl sample at a concentration of 0.08 mg/ml (equivalent to 80 μg of the F12 fraction). To establish an accurate comparison among the PRCT results, the concentrations of all the fractions acquired using size-exclusion high-performance liquid chromatography (SEC-HPLC) were standardized to match the concentration of the F122 peak, which was 7.5 μg/ml. A PRCT coagulation test was performed on the concentrated samples. The F121–F125 fractions had clotting periods of 55, 78, 106, 75, and 118 s, respectively. The resulting bands were evaluated using Coomassie Blue G-250 staining (Fig. 4B) after the SDS-PAGE process was conducted. It is evident that, except for the F125 fraction, the remaining fractions exhibit distinct banding patterns. In this study, the F124 fraction or EC-124 (a 22 kDa coagulant protein) was selected for MALDI -TOF MS analysis. Finally, the clotting time was measured in the purified fraction from crude venoms of *E. carinatus* or EC -124, with a duration of 12 s at a concentration of 100 μg/ml. This observation indicates the existence of pro-coagulants that are predominantly active in this particular venom, as shown in Table 4.

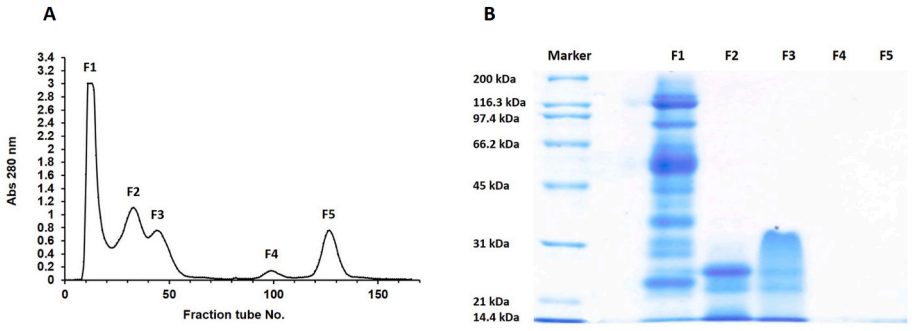

Fig. 2. (A) The spectrum obtained from gel filtration chromatography of crude venom with five fractions, **(B)** Electrophoretic pattern of crude venom, and fractions obtained from gel filtration chromatography. F1–F5: fractions 1 to 5, CV: crude venom.

Table 2
PRCT test results for different dilutions of F1 fraction.

Dilution Factor	Conc. (mg/ml)	PRCT1 (Sec)	PRCT2 (Sec)	PRCT3 (Sec)	PRCT4 (Sec)	PRCT5 (Sec)	PRCT6 (Sec)	PRCT (Ave)	SD
1	1.6	9	10	9.6	8.59	8.64	7.8	8.94	0.72
1/2	0.8	21.96	28.09	27.07	25.31	25.26	22	24.95	2.32
1/4	0.4	33	29.73	34	29.29	29.99	34.33	31.72	2.10
1/8	0.2	48	54	44.26	44.9	41.02	56.63	48.14	5.52
1/16	0.1	56.91	60.06	60.04	55.67	56.43	66	59.19	3.49
1/32	0.05	70	67	80	66	69	72	70.67	4.61
1/64	0.025	90	104	106	84	86	91	93.50	8.48
1/128	0.0125	120	141	167	140	116	135	136.50	16.62
1/256	0.00625	195	202	152	175	187	171	180.33	16.57
Control	0	214	152	178	180	194	218	189.33	22.59
Crude Venom	1	24.2	25.48	22.9	–	–	–	24.19	1.05

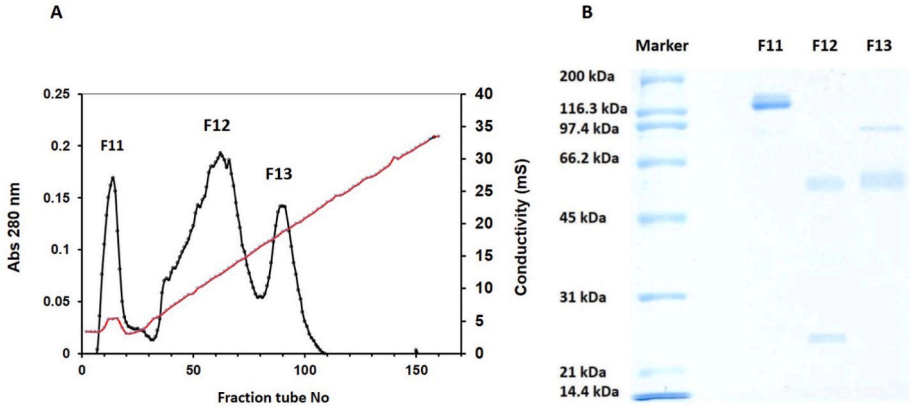

Fig. 3. (A) Spectrum resulting from purification of F1 fraction by anion chromatography. **(B)** Electrophoretic pattern of fractions obtained from ion exchange chromatography. F11–F13: fractions 11 to 13.

Table 3
Concentration values and PRCT test results (at 0.076 mg/ml) for different F1 fractions obtained from ion exchange chromatography.

Fractions name	Concentration (mg/ml)	PRCT (sec)
F11	0.085	220
F12	0.099	16.4
F13	0.076	57
Control	–	200

3.5. MALDI-TOF analysis

In the previous step, the F124 fraction with a 75-s coagulation time and an appropriate electrophoretic pattern were selected for future experiments. The protein band of approximately 22 kDa was carefully cut for the digestion of trypsin. After digestion, the resultant peptides were eluted using an extraction buffer, extracted, and concentrated for Peptide Mass Fingerprint (PMF) analysis by MALDI-TOF MS (Fig. 5). The MASCOT search result confirmed that the protein band was around 22 kDa in SDS-PAGE. A list of proteins identified in *E. carinatus* venom after in-gel trypsin digestion and MALDI-TOF-MS analysis is shown in the Supplementary Table 1. Disintegrin metalloproteinase/disintegrin

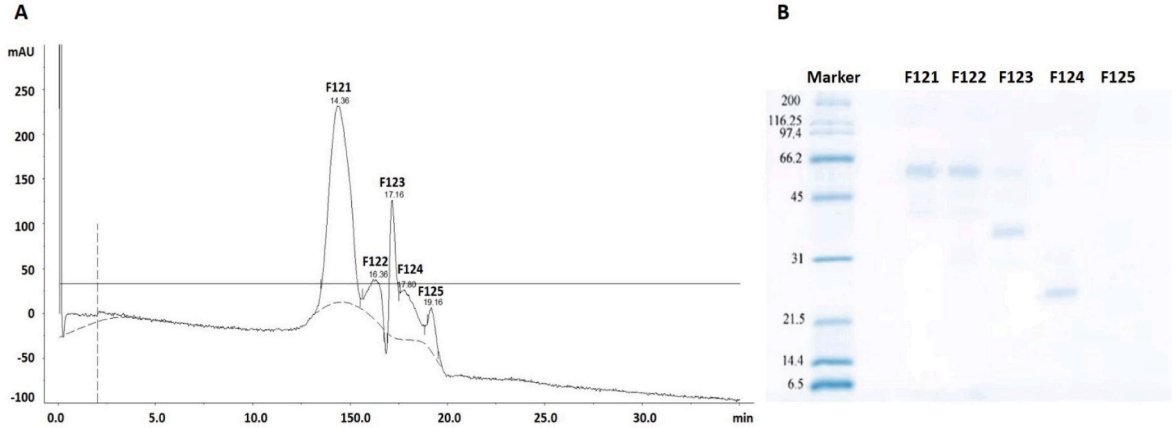

Fig. 4. (A) SEC-HPLC spectrum after F12 sample injection. **(B)** Electrophoretic band pattern of fractions obtained from SEC-HPLC (F121–F125: fractions obtained from SEC-HPLC).

Table 4
Concentration values and PRCT test results (at 7.5 μg/ml) for different F12 fractions obtained from SEC-HPLC.

Fractions name	Concentration (μg/ml)	PRCT (sec)
F121	35.5	55
F122	7.5	78
F123	24.6	106
F124	7.5	75
F125	7.5	118
Control	–	239

Echistatin was the most similar component of the purified coagulant fraction. Also, the purified peptide was 98% similar to Venom peptide 2a and disintegrin multi-squamatin (Supplementary Table 1).

3.6. Determination of the secondary structure of the procoagulant factor

The findings from the CD assay, as depicted in Fig. 6, aimed to ascertain the secondary structure of the protein acquired, which possesses a molecular weight of 22 kDa peptide. The results indicate that EC-124 comprises 16.7% helix structure, 41% beta structure, and 9.8% turn structure. Furthermore, an occurrence of random structure up to 32.5% was detected in this peptide.

3.7. CHNS analysis

The CHNS analysis revealed that the EC-124 contains 73.82% w/w Carbon, 5.66% w/w Hydrogen, 3.19% w/w Nitrogen, and 0.49% w/w Sulphur.

3.8. Toxicity of crude venom and EC-124

The LD_{50} of crude venom was 12.3 μg/mice when tested by the i.v. administration while the toxicity of EC-124 was very low, and its LD_{50} was 0.6 μg/mice. Therefore, the purification of the final procoagulant fraction (EC-124) has significantly reduced the toxicity in mice. Also, the analysis of the results of the MTT assay showed that the IC_{50} of the crude venom is significantly lower than the EC-124 fraction against the Hu02 cell line. Therefore, the toxicity of crude venom is significantly higher than the EC-124 against these cells ($P < 0.001$). The MTT assay results showed that the IC_{50} in crude venom and EC-124 is 39.67 and 223.65 μg/ml, respectively (Fig. 7A).

3.9. Hemolysis activity of crude venom and EC-124

The hemolysis test was performed for all fractions at each stage, and finally, the fraction with the least hemolysis compared to the crude venom was performed and entered the next stage of fractionation. Specifically, the results of the hemolysis test for crude venom and EC-124 fraction showed that the hemolysis percentage of crude venom was significantly higher than the EC-124. Fig. 7B shows the hemolysis percentage of these two compounds in their different dilutions. As it is known, there is a significant difference in the hemolysis percentage of human red blood cells between the highest ($P < 0.01$) and lowest ($P < 0.005$) concentrations of both compounds.

3.10. The coagulation of Factor II, X-deficient plasma

Table 5 presents the study's findings examining the coagulation of plasma lacking in Factor II and X in the presence of snake venom. The addition of 22 kDa peptide (EC-124) to fresh human plasma led to a decrease in the time required for re-calcification and plasma coagulation, and it was found that the decrease in PRCT time also depends on the peptide dose. Based on these observations, it was found that EC-124 like factor X (prothrombinase), can activate coagulation factor II (thrombin) and thereby activate fibrinogen and form a fibrin clot. Considering that snake venom causes a simultaneous reduction of both PT and PTT test results and affects both external and internal coagulation pathways, therefore, the site of its effect is the common coagulation pathway (X, V, II, and I). Considering the enzyme properties of factors X and II, the similarity of the poison to one of these two factors was doubted, and therefore commercial plasmas without these two factors were purchased. Factor X activates prothrombin (factor II) in the presence of platelet phospholipid, calcium (factor connecting FX to platelets), and factor V (factor connecting FX to FII), so it is also known as

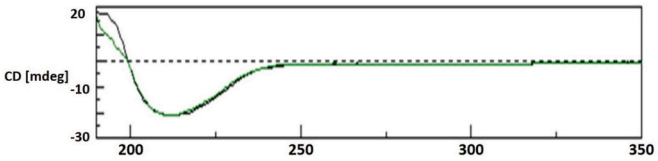

Fig. 6. CD spectrum was obtained for the determination of the secondary structure of the EC-124 protein from the crude venom.

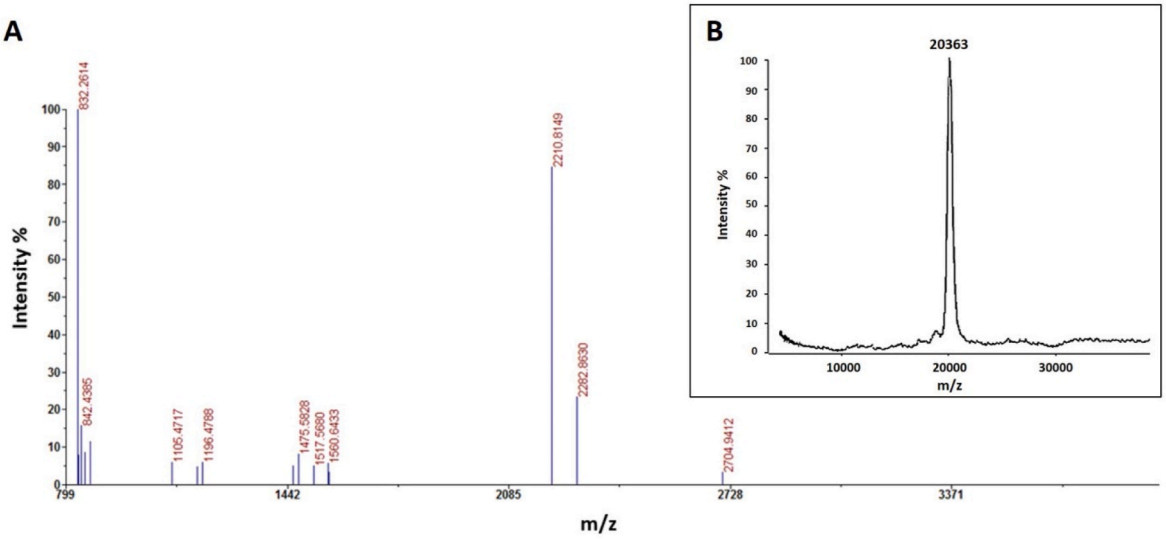

Fig. 5. (A) The graph was obtained from the MALDI-TOF raw data (list of peptide masses and intensities) of the EC-124 protein. (B) The one peak graph of estimated protein after analysis of these data by Mascot Distiller v.2.8 (Instrument mode: MALDI-TOF/TOF mode).

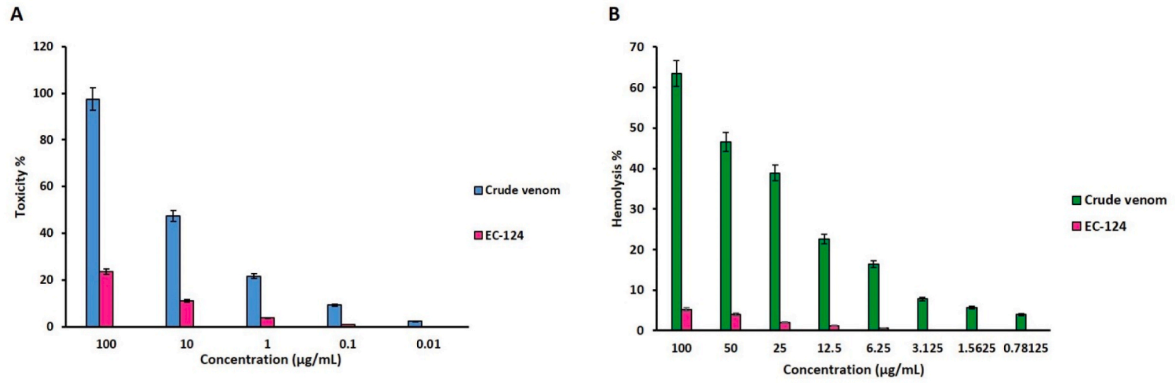

Fig. 7. (A) Toxicity/Concentration and (B) Hemolysis/concentration charts for crude venom and EC-124 obtained by MTT assay (on Hu02 cell line) and Hemolysis test.

Table 5
The Coagulation of Factor-II vs Factor-X deficient plasma.

Tests	Methodology	Result
Negative Control	Step 1: 300 µl Factor X deficient plasma Step 2: 120 s incubation at 37 °C Step 3: Addition of 90 µL 0.025 M calcium +10 µl Ammonium acetate then immediately record the clotting time	No Clot
Venom	Step 1: 300 µL Factor X deficient plasma Step 2: 120 s incubation at 37 °C Step 3: Addition of 90 µl 0.025 M calcium + 10 µl EC-124 then immediately record the clotting time	Clot in 49s (Ave)
Negative Control	Step 1: 300 µl Factor II deficient plasma Step 2: 120 s incubation at 37 °C Step 3: Addition of 90 µl 0.025 M calcium + 10 µl Ammonium acetate then immediately record the clotting time	No Clot
Venom	Step 1: 300 µl Factor II deficient plasma Step 2: 120 s incubation at 37 °C Step 3: Addition of 90 µl 0.025 M calcium + 10 µl Ec-124 then immediately record the clotting time	No Clot

prothrombinase factor. The prothrombinase complex is responsible for the activation of prothrombin to thrombin, which subsequently facilitates the conversion of soluble fibrinogen to an insoluble fibrin clot. To investigate the idea that EC-124 creates a procoagulant response through its similarity to FXa activity, prothrombin treatment was performed with EC-124 and it was found that the peptide was not capable of activating FII-deficient plasma. Still, it can coagulate FX-deficient plasma by mimicking the function of factor Xa. As a result, this shows that this peptide's function and probably structure are similar to coagulation factor X.

3.11. Bioinformatics analysis

The theoretical molecular mass of 20363 predicted by ProtParam (http://www.expasy.ch/tools/protparam.html), was calculated from their amino acid sequences, indicating that disulfide bonds have been formed. Molegro Virtual Docker was used to obtain the electrostatic potential of 20 kDa. Negatively and positively charged amino acids are shown in red and blue, respectively. The Grand Average of Hydropathicity (GRAVY) is −0.644. The net charge observed is −3.4, which indicates the pI: 6.16 This can be due to the presence of 19 positively charged residues (Arg & Lys) and 22 negatively charged residues (Asp & Glu). (web.expasy.org/cgi-bin/protparam/protparam and pepcalc.com). The sequence of a 20 kDa procoagulant peptide was obtained from the UniProt database. MolegroVirtual Docker 6.0 performed additional principal analysis. Based on sequence and structural similarity, three important functional domains were identified in a 22 kDa procoagulant. The primary amino acid sequence of the peptide is CESGPCCRNCKFLKEGTICK.

The multiple sequence alignment was analyzed using the Clustal W, Bioedit V7.2, and BLASTP algorithm (blast.ncbi.nlm.nih.gov/Blast.cgi) methods. The acquired data was later utilized to construct the phylogenetic analysis and consensus sequences (Supplementary Fig. 1).

The functional domain of a 22 kDa peptide was also determined. Fig. 8 shows that the domain composition consisted of family zinc metalloprotease, Disintegrin, Metallo-peptidase family M12B Reprolysin-like, Metallo-peptidase family M12, ROK family, Peptidase M66, Matrixin, Astacin, and Myo-inositol oxygenase (Supplementary Fig. 2).

4. Discussion

The qualities of the crude venom from Iranian *E. carinatus* were assessed by examining many parameters, including protein content, coagulation time, and hemolysis characteristics. The protein concentration inside the crude venom of Iranian *E. carinatus* was determined to be 97%. Gel Chromatography, Ion-Exchange Chromatography, and SEC-HPLC were used in this study to comprehensively purify the coagulation factor that was found in the crude venom.

The optimal proportion of the PRCT test has been chosen in each phase and then inputted into the next step. Consequently, the purification process of the coagulation factor was systematically conducted by utilizing diverse chromatographic techniques. In prior research, the majority of the studies focused on the isolation of anticoagulant agents or the partial purification of coagulation agents. In contrast, our study entirely concentrated on the purification of coagulation agents.

In this research, it was found that snake venom was able to significantly reduce PRCT time, and therefore its pro-coagulation effects were proven. On the other hand, snake venom had the ability to coagulate in plasma lacking factor X, but it did not coagulate in plasma lacking factor II. This finding shows that the snake venom compensates for the absence of factor X and has the same function as factor X and acts as a prothrombinase, but it loses its function in plasma lacking factor II. Other studies have also shown that the Venoms derived from Viperid and Crotalid snakes are frequently known to contain activators of Factor II. Procoagulant agents possess the capacity to generate pharmacological substances that facilitate the production of blood clots, hence offering therapeutic advantages for patients afflicted with hemophilia or other bleeding diseases.

Employing the mentioned purification techniques, we successfully isolated an active fraction with a molecular weight of approximately 22 kDa. Notably, unlike the crude venom, this purified fraction did not demonstrate any hemolytic activity across all tested concentrations. In most of the past studies on snake venom, the effect of hemolytic activity

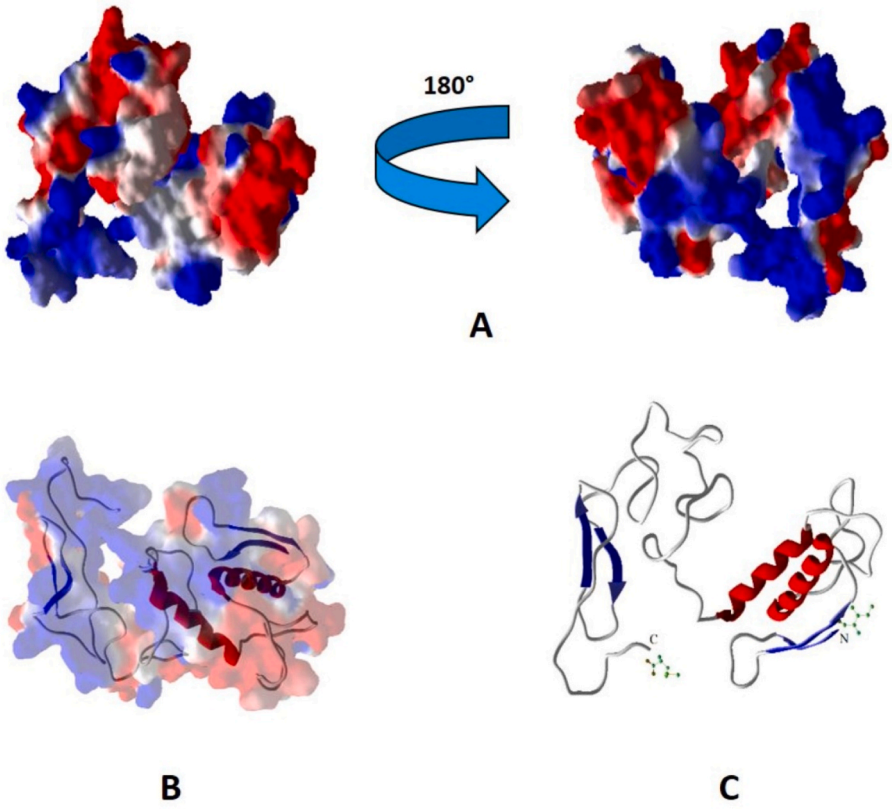

Fig. 8. Structural analyses of estimated 20363 Da procoagulant peptide. (A). The net charge of residues (Red: negative, Blue: positive), (B) Schematic representation and surface of estimated procoagulant peptide structure, (C) The ribbon model of 20363 Da peptide.

was determined through the utilization of blood agar plates, thereby substantiating the venom's possession of hemolytic characteristics. However, in this study, we used the exact method of measuring the percentage of hemolysis with the microdilution method to observe even the smallest percentage of hemolysis.

Salmanizadeh et al. (2013) evaluated the hemostatic effects induced by the primary anticoagulant constituents found in the venom of *E. carinatus*. A total of 10 sub-fractions were obtained from the venom of *E. carinatus* through the utilization of gel chromatography and ion exchange chromatography techniques. Subsequently, three distinct sub-fractions were intravenously injected into mice in the capacity of anti-coagulant sub-fractions. A comparative analysis was conducted to evaluate the effects of three sub-fractions on blood coagulation time. The results indicate that the blood coagulation time following the injection of these sub-fractions is significantly prolonged compared to the normal blood coagulation time. Furthermore, the coagulation time observed after the injection of these sub-fractions is also longer than the coagulation time observed after the injection of crude venom [23].

In our study, the initial approach for the purification of the final venom was the utilization of the preparative reverse-phase high-performance liquid chromatography (RP-HPLC) method, due to the acetonitrile-caused inactivation of the PRCT test, we separated it with a different column of HPLC. Consequently, size-exclusion chromatography was utilized, leading to a notably efficient purification of the fractions. Size-exclusion chromatography (SEC-HPLC) is a commonly employed methodology that facilitates the separation of molecules by exploiting their hydrodynamic volume [24]. One notable characteristic of SEC-HPLC is its stringent prevention of any interactions occurring between analytes and the stationary phase, distinguishing it from alternative separation techniques. The capability of SEC-HPLC to effectively separate physiologically active proteins stems from its ability to operate under circumstances that closely resemble physiological environments. However, it should be noted that the resolution of this technology is relatively reduced in comparison to RP-HPLC. SEC-HPLC is confined to the analysis of materials with minimal complexity, such as for quality control of protein drugs [25,26]. In the comprehensive separation of intricate protein mixtures, SEC-HPLC is commonly used as a prefractionation or final polishing technique [27].

The final part of the experiments performed in this study was to determine the nature of the purified coagulation protein from the *E. carinatus* venom. After performing several purification steps, fraction F124 with a coagulation time of 75 s and a suitable electrophoretic pattern was selected as the best coagulant in the snake venom. In a parallel project on fraction F-121, the separation of the target protein was also done at the same time. F-121 had a coagulation time of 55 s. The protein band of about 22 kDa was carefully excised for trypsin digestion. After digestion, the resulting peptides were eluted with extraction buffer, extracted, and concentrated for PMF analysis by MALDI-TOF MS. In MALDI-TOF-MS analysis of *E. carinatus* venom, Disintegrin metalloproteinase/disintegrin echistatin was the most similar component of purified coagulant fraction. Also, the purified peptide was 98% similar to Venom peptide 2a and disintegrin multi-squamatin. Disintegrins have a platelet aggregation inhibitory activity, which are used as anticoagulants [28]. In a classification, the Disintegrins family is divided into 7 groups, where RVV-X (Russell viper venom factor X activator) is considered an activator of factor X. The classification was based on the disintegrin-like domain of the human metalloproteinase (ADAM-10) [29]. According to this classification and the similarity of the function of F-124 to factor X, it can be assumed that the identification of the 22 kDa protein band (related to F-124) by MALDI-TOF and reporting it as a disintegrin seems correct.

Also, the results of CD analysis to determine the second structure of the obtained protein with a molecular weight of 22 kDa showed that this protein has a 16.7% helix structure, 41% beta structure, and 9.8% turn

structure. 32.5% random coil structure was observed in this protein. This was the first study carried out to determine the second structure of the coagulation factor of Iranian *E. carinatus* venom.

5. Conclusion

The venom of Iranian *Echis carinatus* contains a diverse array of compounds that exhibit dual functionality in the process of blood coagulation. The specific research objectives can determine the isolation and purification of coagulation and anticoagulant components from the venom of this snake. This study aimed to extract and identify specific blood coagulant fractions derived from the venom of Iranian *E. carinatus*. Methods for isolating and purifying coagulation components found in Iranian *E. carinatus* venom have been presented in this study, which is both effective and reproducible. The purified coagulation factors were identified, and characterized in this study by evaluating the function and structural properties. This could potentially result in the development of more effective coagulation agents that are closer to being tested in clinical trials. The venom derived from snakes belonging to the Viperidae family, such as the Iranian *E. carinatus*, is a valuable reservoir of novel chemicals with diverse applications in the medical field. The findings of our study indicate that a multi-step purification process can be employed to obtain coagulation components in their pure form. MALDI-TOFMS and CD techniques are highly effective in revealing the composition and secondary conformation of this particular coagulation factor. In the end, this protein showed a strong procoagulant activity with a dose-dependent effect on citrated mice plasma, shortening significantly the PRCT test. However, correctly identifying the gene sequence of this coagulation factor and cloning and producing the recombinant form of it in the appropriate host, can provide a platform for conducting animal studies and clinical trials.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

CRediT authorship contribution statement

Sayeneh Khodadadi: Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Hadi Rabiei:** Visualization, Validation, Methodology, Investigation, Data curation. **Soroush Sardari:** Data curation, Formal analysis. **Hosein Mahboudi:** Data curation, Formal analysis, Methodology. **Mohammad Ali Bayatzadeh:** Data curation, Formal analysis. **Nader Vazifeh Shiran:** Data curation, Formal analysis. **Maryam Sardabi:** Data curation. **Mohammad Reza Akbari Eidgahi:** Formal analysis, Data curation. **Hamid Madanchi:** Writing – review & editing, Visualization, Validation, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization. **Nasser Mohammadpour:** Conceptualization, Data curation, Funding acquisition, Supervision, Visualization, Writing – review & editing.

Declaration of competing interest

The authors confirm that this article content has no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101701.

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