Original Article

Analysis and Identification of Putative Novel Peptides Purified from Iranian Endemic *Echis Carinatus* Sochureki Snake Venom by MALDI-TOF Mass Spectrometry

Nafiseh Nasri Nasrabadi[1,2], Hossein Vatanpour[3], Nasser Mohammadpour Dounighi[4*], Mojtaba Najafi[5], Minoo Ahmadinejad[6], Mohammad Ali Bayatzadeh[4], Giti Pouyanmehr[7]

1. *Student Research Committee, Pharmaceutical Sciences Research Center, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
2. *Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization, Karaj, Iran*
3. *Department of Toxicology, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
4. *Department of Venomous Animals and Anti-venom, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization, Karaj, Iran*
5. *Department of Molecular Genetic and Animal Breeding, Gorgan University of Agricultural Sciences and Natural Resources, Golestan, Iran*
6. *Blood Transfusion Research Center, Institute for Research and Education in Transfusion Medicine, Tehran, Iran*
7. *Ph.D. student of toxicology, Tehran Islamic Azad University, Tehran, Iran*

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Corresponding Author's E-Mail:
nasser_mohammadpour@yahoo.com

ABSTRACT

The Iranian *Echis Carinatus* (IEC) venom is an exclusive natural source of bio-substances for a wide range of purposes in the blood coagulation cascade. The present study for the first time was aimed to assess novel pro-coagulant, anti-coagulant and anti-platelet proteins, named $EC_{1.5\ (a)}$, $EC_{5.1\ (b)}$ and $EC_{4\ (a)}$ from Iranian *Echis Carinatus* (IEC) venom.These peptides were purified by multi-step chromatography methods. Hematological properties were measured using activated clotting tests, platelet aggregation studies, and hemorrhage assessment. Subsequently, these proteins were identified through both their intact molecular mass and peptide mass fingerprint (PMF) using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Multiple sequence alignments were performed by ClustalW, Bioedit software. Molegro Data Modeller (MDM) 3.0 software was used to predict the putative tertiary structure of proteins.$EC_{1.5\ (a)}$, a single-band protein with a molecular mass of 66 and 55 kDa, was observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as a reduced and non-reduced state, respectively. Based on the Mascot results, we considered that $EC_{1.5\ (a)}$ is a metalloproteinase of group II which exhibited potent pro-coagulant activity. It is predicted that the $EC_{1.5\ (a)}$ with hemorrhagic activity, potentially is a metalloproteinase/disintegrin region that constitutes the disintegrin-like domains. Our findings demonstrate that the disintegrin domain of $EC_{1.5\ (a)}$ lacks platelet aggregation inhibitory activity. On the contrary, this factor shows the property of a platelet aggregation inducer. Also, the $EC_{5.1\ (b)}$ was observed as a single-band protein with a molecular mass of 7.5 kDa. $EC_{5.1\ (b)}$ showed both anti-coagulant and anti-platelet properties. Additionally, the structure of the $EC_{5.1\ (b)}$ fraction is expected to be similar to that of phospholipase A_2, while $EC_{4\ (a)}$ structure is potentially very similar to that of Echistatin with 5 kDa molecular mass. We introduce the predicted structure of P-II snake venom metalloproteinase/ disintegrin domains, phospholipase A_2 and Echistatin-like fractions. Further research is therefore needed to determine the complete structure of these novel fractions and elucidate their mechanism of action and future therapeutic applications of cardiovascular and homeostasis disorders.

Keywords: Disintegrin, Hemorrhagic metalloproteinases, Platelet aggregation inducer, Phospholipase A_2, MALDI TOF/MS

1. Introduction

Snake venoms comprise bioactive protein and non-protein mixtures. The proteins of mixtures that contain enzymatic activities, including phospholipase A2, phosphodiesterase, phosphomonoesterase, L-amino acid oxidase, acetyl-cholinesterase, proteolytic enzymes of the serine protease, metalloproteinase classes, arginine esterase, 5'-nucleotidase, hyaluronidase, nucleosidase, glutaminyl cyclase and non-enzymatic proteins, including snaclec, disintegrin, vascular endothelial growth factor, cysteine-rich secretory proteins, kunitz type serine protease inhibitor, and C-type lectin (1, 2). The snake venom molecules that are candidates for perturbing hemostasis are variable, and their pro-coagulant and anti-coagulant properties have been demonstrated in various studies (3-12).

Dysregulation of hemostasis arrangement, one of the body's key organs that contributes to the balance of bleeding and coagulation, is among the most severe clinical symptoms following the envenomation of several genera from all four families, such as *Colubridae*, *Elapidae*, *Viperidae*, and *Atractaspididae*. Identification of substances from snake venom that interferes with coagulation cascade and platelet aggregation mechanisms has contributed immensely to deciphering the details of molecular reactions involved in physiological functions. Additionally, these findings have enabled us to design various novel anti-coagulant, pro-coagulant, platelet aggregation inducers, and inhibitor therapeutic factors, visualizing new perspectives in the treatment of thromboembolic, bleeding, clotting, cardiovascular, and hematological disorders (7, 13, 14).

Further research is needed to delineate the structure-function relationships and to understand the exact mechanism of new anti-coagulants, pro-coagulants, platelet aggregation inducers, and platelet aggregation inhibitors agents. The majority of pro-coagulant proteins of snake venom are divided into four main classes: factor V activators, factor X activators, prothrombin activators, and thrombin-like enzymes, or fibrinogenases. Anti-coagulant molecules from snake venoms are divided into phospholipases A2 (PLA2), fibrin (ogen) olyticsnake venom metalloproteinases (SVMPs), protein C activators, L-amino acid oxidases, C-type lectin-like proteins (snaclecs or SVCLPsC-type lectin-like proteins from Snake Venom.

Snake Venom C-type lectin-like proteins.

C-type lectin-like proteins from Snake Venom. Snake Venom C-type lectin-like proteins), three-finger toxins, and Kunitz-type proteinase inhibitors (4, 14-17). *Echis carinatus* (EC; Scientific name: *Echis carinatus sochureki*; English name: Sind saw-scaled viper; Persian name: Jafari snake) is a member of the *Viperidae* family. *Echis carinatus* is a venomous snake with a wide distribution in the deserts of Iran (18, 19). The venom of EC contains a mixture of proteins and peptides that act against or in parallel with pro-coagulant, anti-coagulant, fibrinolysis activity, and platelet function (4, 14, 15, 20, 21). Achieving the pro- and anti-coagulant fractions from a natural source such as venom can be very valuable in the therapeutic field and anti-venom production technology; moreover, their potential medicine perspectives are beneficial for occlusive arterial or venous thromboembolism. Ecarin (22), Carinactivase (23), and EC-PIII (24) are examples of pro-coagulant fractions purified from *E. carinatus* venom. Multiactivase, a pro-coagulant agent, is purified from *Echis multisquamatus* venom (25). EC-PIII, a novel pro-coagulant factor, is introduced from the venom of EC. Mukherjee et al. (2017) investigated the pro-coagulant and anti-coagulant profiles of EC snake venom (26). Mirakabadi et al. and Vatanpour et al. have examined the anti-coagulant, pro-coagulant, and anti-platelet properties of Iranian *Echis carinatus* (IEC) snake venom (27-29). Moreover, Echistatin purified from the venom of the EC, is an anti-platelet agent (30).

Nevertheless, our knowledge has not shed much light on the possible roles of newly discovered agents, especially agents that affect the homeostasis system,

derived from Iranian endemic snake venom at the physiological level. Considering that there are a lot of geographical distributions of the *Viperidae* family throughout Iran and the importance of medicinal products originating from natural sources, further research must be performed to better understand the incredible versatility of toxins causing homeostasis organization. Our study for the first time focuses on the purification, structure, and functional characterization of pro-coagulant, anti-coagulant, and anti-platelet agents isolated from Iranian endemic EC snake venom using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF/MS).

2. Materials and Methods

2.1. Chemicals

Bovine serum albumin (BSA), acetonitrile (ACN; high-performance liquid chromatography [HPLC] grade), water (HPLC grade), Tris-buffer, Tris-base, calcium chloride ($CaCl_2$), Na_2CO_3, $CuSO_4$:$5H_2O$, NaOH, Folin reagent, sodium potassium tartrate, Sephadex G-50, ammonium acetate, trifluoroacetic acid (TFA), Foline, diethylaminoethyl (DEAE)-Sepharose, carboxymethyl (CM)-Sepharose, ethylenediaminetetraacetic acid (EDTA), heparin, and other chemicals and reagents used were analytical grade from Merck (Merck Millipore, Darmstadt, Germany). Prothrombin time (PT) and activated partial thromboplastin time (APTT) kits were purchased from Fisher Diagnostics (USA). STA®-Thrombin Kit, Fibri-Prest® 2 Kit, and all other utilized chemicals were of the highest quality available.

2.2. Animals

The Swiss albino mice weighing 18-20 g were obtained from the Venomous Animals and Antivenom Production Department, Razi Vaccine and Serum Research Institute, Karaj, Iranz (Ethical approval number: IR.SBMU.RETECH.REC.1398.620).

2.3. Mass spectrometry

Alpha-cyano-4-hydroxycinnamic acid (CHCA), ACN (MASS grade), TFA, and other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan).

2.4. Biological material and venom extraction

The lyophilized IEC crude venom was obtained from the Department of Venomous Animals and Antivenom Production, Razi Vaccine and Serum Research Institute, Karaj, Iran.

2.5. Protein determination

The Lowry assay was applied to determine the total protein of IEC crude venom and its fractions. It is noted that BSA was considered standard (31, 32).

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The molecular mass of purified fractions/subfractions and crude venom was examined by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (32, 33).

2.7. *In vitro* assessment

2.7.1. Human plasma

The blood samples were collected from healthy individuals (20-30 years) without a history of bleeding or thrombosis with their consent (Ethical approval number: IR.SBMU.RETECH.REC.1398.620). The whole blood was centrifuged at 2,500 rpm for 20 min at 4°C. The platelet-poor human plasma (PPP) was used for pro-coagulant experiments.

2.7.2. Activated clotting time

2.7.2.1. Plasma clotting time

Crude venom/fractions/subfractions samples (50 µl) and plasma (100 µl) were pre-incubated at 37°C for 2 min (34). After that, the samples were added to the plasma. The formation of plasma clots was recorded by chronometer. The plasma clotting time (PCT) test was performed for different concentrations of fractions/subfractions, such as EC_1, EC_2, $EC_{1.5}$, and $EC_{1.6}$.

2.7.2.2. Recalcification time

The recalcification time assay was performed as described previously (35).

2.7.2.3. Prothrombin time

Crude venom samples and purified proteins (50 µl), plasma (100 µl), and PT reagent (50 µl) were pre-incubated for 3 min at 37°C. Afterward, crude venom/isolated proteins and PT reagent were mixed and then shaken for 30 s. Plasma clot formation time was recorded using a chronometer after adding plasma and shaking at 37°C for 5 s.

2.7.2.4. Activated partial thromboplastin time

Crude venom samples and purified proteins (50 µl), plasma (100 µl), and APTT reagent (50 µl) were pre-incubated for 3 min at 37°C. Subsequently, crude venom/isolated proteins, $CaCl_2$ (50 µl), and APTT reagent were mixed. The mixture was shaken for 20 s at 37°C. Plasma clot formation was recorded by chronometer after adding plasma and shaking in the lab lamp lighting.

2.7.2.5. Thrombin clotting time and fibrinogen-clotting activity

Thrombin clotting time (TCT) was determined using a commercial kit (STA®-Thrombin kit [REF 00611]) and a manual coagulation analyzer (PKL PPC 170). Thrombin clotting time was measured after incubating 100 µl plasma at 37°C for 2 min. Thrombin time was recorded by a manual coagulation analyzer after adding 100 µl of mixed thrombin reagent and the sample (crude venom samples/fractions/subfractions). Fibrinogen-clotting activity (FCA) was determined using a commercial Fibri-Prest® 2 Kit (00608,100045), in a water bath based on its instructions. Briefly, 100 µl fibrinogen reagent and 200 µl of diluted human plasma (1:10 concentration/PH=7.4) were incubated at 37°C for 2 min. Next, 200 µl plasma, 100 µl of crude venom samples/fractions/subfractions, and 100 µl fibrinogen reagent were mixed and then shaken in a water bath. The fibrinogen concentration was estimated from a fibrinogen standard DIAGNOSTICA STAGO (100045).

2.7.3. Platelet aggregation assay

2.7.3.1. Human plasma

Blood donors who had taken drugs to interfere with platelet reactions, such as aspirin and other nonsteroidal anti-inflammatory drugs, or thienopyridines, including ticlopidine and clopidogrel, two weeks before the experiment, were excluded from the study. Blood samples were collected from a forearm vein with a vacuum syringe equipped with 8.5-mL plastic centrifuge vacuum tubes containing 1.5 volumes of acid-citrate-dextrose anti-coagulant. The tubes were immediately placed in a water bath at 37°C for 15 min. Washed platelet preparation was carried out according to the modified Preparation of Washed Human Platelets (Platelet aggregation using washed human platelets) method (36, 37). Platelet aggregation was measured in a CHRONO-LOG® Model 700 Whole Blood/Optical Lumi-Aggregometer (Pennsylvania, USA). Assays were performed in magnetic siliconized cuvettes under stirring. Aggregation was started by adding collagen or arachidonic acid agonists (Control), crude venom samples/fractions/subfractions (Sample1), and mixed collagen or arachidonic acid agonists and crude venom samples/fractions/subfractions (Sample2) to washed platelets. The amount of 100% aggregation was recorded with supra-maximal collagen or arachidonic acid agonists concentrations (36, 37).

2.8. *In vivo* assessment

2.8.1. Assessment of hemorrhagic activity

100 µl of the various doses of IEC crude venom (0.01, 0.1, and 1 mg/mL) or purified proteins were subcutaneously injected into the shaved back skin of mice to assess the IEC crude venom and its fractions hemorrhagic activity. After 24 h, the mice were suffocated due to gas inhalation in a particular container (Desiccator). The mice's hemorrhagic points generated subcutaneously were observed and determined. The control group was injected with 100 µl of normal physiological saline solution and ammonium acetate instead of the samples (crude venom/fractions/subfractions), respectively.

2.8.2. Defibrinogenating activity

The defibrinogenating activity of the samples (crude venom/fractions/subfractions) was determined by

injecting mice at a constant volume of 200 µl (using normal saline as a diluent) intravenously into the vein of mice. The control group received a similar injection of normal saline solution. After one hour, animals were anesthetized, and blood samples were collected by cardiac puncture. The blood samples from each animal were placed in a new glass clotting tube without any additives and left at room temperature, and the presence or absence of clots was recorded by gently tilting the tube.

2.9. Purification protocol

IEC crude venom (100 mg) was fractionated using a gel chromatographic Sephadex G-50 column (150×2 cm). It was pre-equilibrated with ammonium acetate buffer (0.05 M, pH 7.4). Fractionation was carried out at a flow rate of 30 ml/min^{-1} under isocratic conditions with the same buffer. The effluent was monitored at 280 nm. We obtained five fractions (i.e., EC_1, EC_2, EC_3, EC_4, and EC_5). Each fraction was collected to measure the anti-coagulant and pro-coagulant capacities. A dose (10 mL of EC_1 fraction with 933 µg/ml), after concentrated by ultrafiltration, was dissolved in 10 ml of 20 mM Tris-base buffer (pH 8.2) and was passed continuously into the ion-exchange column (20×1.6 cm) at a flow rate of 30 ml/h^{-1}. The effluent was monitored at 280 nm by a spectrophotometer (UV752S, Lengguang Co., China). The DEAE-Sepharose column was equilibrated with Tris-base buffer (20 mM, pH 8.2). Then, 0-1.0 M NaCl solution was passed through the chromatography column with a linear gradient. Each fraction was collected to be examined by coagulant tests. The fraction (namely $EC_{1.5}$) with the highest pro-coagulant property was selected for the following purification. The $EC_{1.5}$ and EC_4 were further purified by reversed-phase (RP)-HPLC. These samples were injected into an HPLC system (600, Waters, USA) equipped with a C_4 and C_{18} column (5 µm, 9.4×250 mm, Agilent Inc., USA) for $EC_{1.5}$ and EC_4, respectively. The elution was carried out with a linear gradient of ACN containing 0.1% (v/v) TFA at a flow rate of 0.5 ml/min^{-1} and water containing 0.1% (v/v) TFA at a flow rate of 0.5 ml/min^{-1} over 60 min. Excitation wavelengths of 215 and 280 nm were applied for monitoring the different peptide fraction peaks and then were collected manually for the pro-coagulant activity and hematological assays. The purification profile of $EC_{5.1(b)}$ subfraction had been mentioned in a previous study (32).

2.10. Mass spectrometry and database searching

2.10.1. MALDI-Mass method

The gel was fixed in a solution containing 45% deionized water, 45% methanol, and 10% acetic acid for 2 h. Coomassie blue color was applied for highlighting separated bands on gel electrophoresis overnight. After this time, single bands of $EC_{1.5(a)}$, $E.C_{5.1(b)}$, and $EC_{4(a)}$ were extracted and placed into Eppendorf tubes. In-gel digestion protocol using trypsin was applied to produce peptide segments that had been broken at the lysine-arginine bonds (38). Based on molecular mass data, prediction of the partial structure of $E.C_{1.5(a)}$, $EC_{5.1(b)}$, and $EC_{4(a)}$ was obtained using MALDI-TOF/MS analysis. The obtained m/z values of the peptide ions of the $E.C_{1.5(a)}$, $EC_{5.1(b)}$, and $EC_{4(a)}$ were matched to the Mascot database to identify some matched peptides. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (Applied Biosystems 4800 MALDI TOF/MS, Nd: YAG 200-HZ laser) was used for MS experiments. For MS analysis, the digested sample was spotted on a MALDI plate mixed with CHCA in 50% ACN containing 0.1% TFA (with a 1:2 ratio respectively) as a matrix solution, air dried, and analyzed in reflector positive mode (the mass range was 800-4000 Da). The MALDI-TOF/MS data were interpreted and processed using the Mascot database.

2.11. Alignment

The ClustalW, BioEdit 7.2.6, and BLASTP algorithm (blast.ncbi.nlm.nih.gov/Blast.cgi) methods were used for homology search and analysis of the multiple sequence alignment.

2.12. Bioinformatics analysis

Predicted putative three-dimensional structures of the $E.C_{1.5(a)}$, $EC_{5.1(b)}$, and $EC_{4(a)}$ according to the data

obtained from Mascot analysis were modeled by Molegro Data Modeller 3.0 software.

2.13. Statistical analysis

The general linear model procedure in SAS 9.1 software was applied to examine the significance of the difference in activated clotting time and pharmacological assays of IEC crude venom and its fractions as control. The values were reported as the mean±SD. The p-value of ≤ 0.05 was considered significant.

3. Results and Discussion

3.1. Protein determination

The protein concentrations of IEC crude venom and its fraction/subfractions have been presented in Table 1.

Table 1. Hematologic and pharmacological properties of crud venom and its fractions and subfractions of IEC venom. Results of experiments are reported as the mean±SD of at least triplicate

Sample	Protein	LD_{50} (µgr/mice)	PCT Plasma Clotting Time(s)	Recalcification time (s)	PT (s)	APTT(s)	TCT(s)	fibrinogen-clotting activity g/L	Defibrinogenating Activity	hemorrhagic activity (mm^2)
Venom										
Venom(1mg/mL)	1mg/mL	11.311	6.21±0.02s[1]	5±0.3s[2k]	7.04±0.57s[3op]	11.91±0.06s [4kl]	4.93±0.02s [37ji]	4.70[33]	No Clot[41]	21.39+17/08[49]
Venom(0.1mg/mL)	0.1 mg/mL	-	12±0.5s	8.69±0.4s[j]	10.32±0.45s[i]	12.07±0.15s[kl]	6.14±0.03s[fg]	3.82	No Clot	23.81+16.23
Venom(0.01mg/mL)	0.01 mg/mL	-	20.07±0.57s	28.98±0.55s[h]	12.06±0.55s[h]	18.80±0.12s[j]	6.53±0.04s[f]	1.87	No Clot	9.14+7.19
Control	-	-	No Clot[5]	480.33±0.58s[6a]	10.43±0.11s [7i]	31.9±0.00s [8f]	13.05±0.05s [38d]	2.01[34]	30s[42]	-[50]
Fractions										
$F_1(EC_1)$	933	10.165	4.51±0.27s[9]	5.07±0.06s [10k]	6.70±0.1s [11ip]	11±0.11s [12l]	4.80±0.01s [39jik]	4.70[35]	No Clot[43]	23.53+18.76[51]
$F_2(EC_2)$	470	56	14.17±0.07s	23.38±0.81s[i]	8.99±0.18s [nlmk]	28±0.12s[g]	6.19±0.02s[fg]	1.44	No Clot	8.37+4.52
F_3 (EC_3)	313	-	33.63±2.52s	42.45±0.68s[g]	10.21±0.04s[ij]	26.96±0.12s[gh]	5.59±0.02s[hig]	2.60	No Clot	-
F_4 (EC_4)	28	-	79.34±1.25s	204.64±0.55s[b]	9.91±0.08s [kilj]	30.75±0.12s[f]	6±0.04s [hfg]	1.67	5min	-
F_5 (EC_5)	338	No Toxic	No Clot	>500s No clot	20.48±0.20s[c]	80.01±0.07s[a]	31.22±0.04s[b]	1.15	3min	-
Control	-	-	No Clot[13]	480.33±0.58s [14a]	10.43±0.11s [15i]	31.9±0.00s [16f]	13.05±0.05s [40d]	2.01[36]	30s[44]	-[52]
Subfractions of F_1										
F_{11} ($EC_{1.1}$)	23.75	-	No Clot[17]	50.07±0.18s [18e]	9.16±0.06s [19kmlj]	27.59±0.04s [20gh]	5.11±0.01s [41hji]	1.44[37]	1min[45]	-[53]
F_{12} ($EC_{1.2}$)	35.07	-	No Clot	>500	8.41±0.03s [mn]	26.29±0.01s[h]	4.28±0.01s[ljk]	2.26	3min	-
F_{13} ($EC_{1.3}$)	31.6	-	No Clot	>500	10.79±0.08s[i]	28.28±0.03s[g]	4.52±0.01s[ljk]	2.60	2min	-
F_{14} ($EC_{1.4}$)	8.86	-	No Clot	>500	10.06±0.04s[kij]	27.29±0.02s[gh]	4.58±0.01s[ljk]	2.26	1min	-
F_{15} ($EC_{1.5}$)	62	41.3	7.53±0.07s	9.01±0.03s [j]	6.82±0.10s [p]	12.01±0.02s[kl]	3.69±0.01s[l]	3.08	No Clot	7.18+8.80
F_{16} ($EC_{1.6}$)	37	-	53.04±0.12s	45.53±0.46s[f]	10.06±0.06s[kij]	20.52±0.03s[i]	4.39±0.01s[ljk]	2.60	No Clot	14.73+14.62
F_{17} ($EC_{1.7}$)	10.54	-	No Clot	>500	13.19±0.18s[gf]	27.96±0.05s[g]	8.00±0.04s[e]	1.15	1min	-
Control		-	No Clot[21]	480.33±0.58s [22a]	10.43±0.11s [23i]	31.9±0.00s [24f]	13.05±0.05s [42d]	2.01[38]	30s[46]	-[54]
Subfraction of F_5										
$F_{5.1}$($EC_{5.1}$)	112	-	No Clot[17]	>500[18]	23.12±0.40s[19b]	80.83±0.44s[20a]	29.86±0.34s[41c]	1.15[37]	3min59	-[53]
Control	-	-	No Clot[21]	480.33±0.58s [22a]	10.43±0.11 s[23i]	31.9±0.00s [24f]	13.05±0.05s [42d]	2.01[38]	30s60	-[54]

Subfraction of (EC1.5) F_{15}										
($EC_{1.5(a)}$) $F_{15\,(a)}$	47.8	-	8.37±0.53s [25]	9.01±0.03s [2 6j]	6.94±0.05s [27op]	13±0.02s [28k]	4.00±0.05s [43lk]	3.23 [39]	No Clot [47]	6.18+7.60 [55]
($EC_{1.5(b)}$) $F_{15(b)}$	12	-	No Clot	>500s	10.50±0.29s [i]	32.00±0.58s [f]	-	-	-	-
Control	-	-	No Clot [29]	480.33±0.58s [30a]	10.43±0.11 s [31i]	31.9±0.00s [32f]	13.05±0.05s [44d]	2.01 [40]	30s [48]	- [56]
Subfraction of $F_{5.1}$										
($EC_{5.1(a)}$) $F_{5.1(a)}$	8	-	No Clot [25]	>500s [26]	19.18±0.38s [27d]	36.00±0.58s [28d]	-	-	-	- [55]
($EC_{5.1(b)}$) $F_{5.1(b)}$	52	-	No Clot	>500s	26.21±0.15s [a]	61.00±0.58s [b]	38.97±0.49s [41a]	1.15 [39]	3min [47]	-
Control	-	-	No Clot [29]	480.33±0.58s [30a]	10.43±0.11s [31i]	31.9±0.00s [32f]	13.05±0.05s s [44d]	2.01 [40]	30s [48]	- [56]

Values are means ± S.E. Different letters within the same column represent significant differences ($p \leq 0.05$).
[1].Test (Clotting Activity): Plasma (PPP) + (Venom (1,0.1,0.01 mg/ml)[2].Test (Plasma Recalcification Time): Plasma (PPP) + CaCl2+ (Venom (1,0.1,0.01 mg/ml)[3].Test (PT): Plasma (PPP) + Thromboplastin-D + (Venom (1,0.1,0.01 mg/ml)[4].Test (APTT): Plasma (PPP) + Reagent aPTT + CaCl2 + (Venom (1,0.1,0.01 mg/ml)[5].Control (Clotting Activity): Plasma (PPP) + Water[6].Control (Plasma Recalcification Time): Plasma (PPP) + CaCl2+Water[7].Control (PT): Plasma (PPP) + Thromboplastin-D + Water[8].Control (APTT): Plasma (PPP) + Reagent APTT +CaCl2+ Water[9].Test (Clotting Activity): Plasma (PPP) + (Purified Fractions)[10].Test (Plasma Recalcification Time): Plasma (PPP) + CaCl2+ (Purified Fractions)[11].Test (PT): Plasma (PPP) + Thromboplastin-D + (Purified Fractions)[12].Test (APTT): Plasma (PPP) + APTT Reagent +CaCl2 + (Purified Fractions)[13].Control (Clotting Activity): Plasma (PPP) + Ammonium acetate [14].Control (Plasma Recalcification Time): Plasma (PPP) + CaCl2+ Ammonium acetate[15].Control (PT): Plasma (PPP) + Thromboplastin-D + Ammonium acetate[16].Control (APTT): Plasma (PPP) + APTT Reagent +CaCl2 + Ammonium acetate[17].Test (Clotting Activity): Plasma (PPP) + (Purified SubFractions)[18].Test (Plasma Recalcification Time): Plasma (PPP) + CaCl2+ (Purified SubFractions)[19].Test (PT): Plasma (PPP) + Thromboplastin-D + (Purified SubFractions)[20].Test (APTT): Plasma (PPP) + APTT Reagent +CaCl2 + (Purified SubFractions)[21].Control (Clotting Activity): Plasma (PPP) + Tris Base[22].Control (Plasma Recalcification Time): Plasma (PPP) + CaCl2+ Tris Base[23].Control (PT): Plasma (PPP) + Thromboplastin-D + Tris Base[24].Control (APTT): Plasma (PPP) + APTT Reagent +CaCl2 + Tris Base[25].Test (Clotting Activity): Plasma (PPP) + (Purified SubFractions)[26].Test (Plasma Recalcification Time): Plasma (PPP) + CaCl2+ (Purified SubFractions)[27].Test (PT): Plasma (PPP) + Thromboplastin-D + (Purified SubFractions)[28].Test (APTT): Plasma (PPP) + APTT Reagent +CaCl2 + (Purified SubFractions)[29].Control (Clotting Activity): Plasma (PPP) + Acetonitrile[30].Control (Plasma Recalcification Time): Plasma (PPP) + CaCl2+ Acetonitrile[31].Control (PT): Plasma (PPP) + Thromboplastin-D + Acetonitrile[32].Control (APTT): Plasma (PPP) + APTT Reagent +CaCl2 + Acetonitrile[33].Test (Fibrinogen-clotting activity): Plasma (PPP) + ((Venom (1,0.1,0.01 mg/ml)+Fibrinogen Reagent[34].Control (Fibrinogen-clotting activity): Plasma (PPP) + Fibrinogen Reagent + Water[35].Test (Fibrinogen-clotting activity): Plasma (PPP) + (()+Fibrinogen Reagent + Fractions[36].Control (Fibrinogen-clotting activity): Plasma (PPP) + Fibrinogen Reagent + Ammonium acetate[37].Test (Fibrinogen-clotting activity): Plasma (PPP) + Fibrinogen Reagent + (Subfractions)[40].Control (Fibrinogen-clotting activity): Plasma (PPP) + Fibrinogen Reagent + Tris – Base[41].Test (DefibrinogenatingActivity): Clot Reaction Time blood heart from mice heart after injection. (Venom 1,0,1,0.01 mg/ml)[42]. Control (Defibrinogenating Activity): Clot Reaction Time blood heart from mice heart after injection. Normal Salin (Venom 1,0,1,0.01 mg/ml)[43]. Test (DefibrinogenatingActivity): Clot Reaction Time blood heart from mice heart after injection. (Fractions)[44]. Control (DefibrinogenatingActivity): Clot Reaction Time blood heart from mice heart after injection. (Ammonium acetate)[45]. Test (DefibrinogenatingActivity): Clot Reaction Time blood heart from mice heart after injection. (Subfractions)[46]. Control (DefibrinogenatingActivity): Clot Reaction Time blood heart from mice heart after injection. (Tris – Base) [47]. Test (Defibrinogenating Activity): Clot Reaction Time blood heart from mice heart after injection. (Subfractions)[48]. Control (Defibrinogenating Activity): Clot Reaction Time blood heart from mice heart after injection. (Acetonitrile)[49].Test (hemorrhagic activity (mm2) : The size of the hemorrhagic lesion (Venom 1,0,1,0.01 mg/ml) in mice[50].Control (hemorrhagic activity (mm2) : The size of the hemorrhagic lesion (Normal salin) in mice[51].Test (hemorrhagic activity (mm2) : The size of the hemorrhagic lesion (Fractions) in mice[52].Control (hemorrhagic activity (mm2) : The size of the hemorrhagic lesion (Ammonium acetate) in mice[53].Test (hemorrhagic activity (mm2) : The size of the hemorrhagic lesion (Subfractions) in mice[58].Control (hemorrhagic activity (mm2) : The size of the hemorrhagic lesion ((Tris – Base)) in mice[54].Test (hemorrhagic activity (mm2) : The size of the hemorrhagic lesion (Subfractions) in mice[55].Control (hemorrhagic activity (mm2) : The size of the hemorrhagic lesion (Acetonitrile) in mice[56].Control (hemorrhagic activity (mm2) : The size of the hemorrhagic lesion (Sodium Acetate) in mice

3.2. Isolation, purification, and characterization of $EC_{1.5(a)}$, $EC_{5.1(b)}$, and $EC_{4(a)}$

The chromatogram indicated five well-separated fractions from EC_1 to EC_5, obtained by Sephadex G-50 (Fig. SD1 (Supplementary Data 1). Activated clotting time assays, including PCT, PT, APTT, TCT, and FCA, as well as aggregation activity, hemorrhagic activity, and defibrinogenating activity were estimated for all the fractions. Further purification was continued using anion exchange chromatography DEAE-Sepharose on EC_1 and EC_5 fractions. In this step, seven fractions were obtained ($EC_{1.1}$-$EC_{1.7}$) from EC_1 and one fraction ($EC_{5.1}$) from EC_5 (Fig.1 A, Fig. SD2 A, respectively). Out of seven fractions, $EC_{1.5}$ and $EC_{1.6}$ showed pro-coagulant activity with more and less potency, respectively. The $EC_{1.5}$ was pooled and dialyzed; afterward, it was applied to a C_4 RP-HPLC column. Our findings indicated two peaks as named $EC_{1.5(a)}$ and $EC_{1.5(b)}$ (Fig.1 B). $EC_{1.5(a)}$ showed the pro-coagulant property. Our hematological results are summarized in Table 1. $EC_{5.1(b)}$ and EC_4 were applied to a C_{18} RP-HPLC column (Fig. SD2 B, Fig. 1 C, respectively).

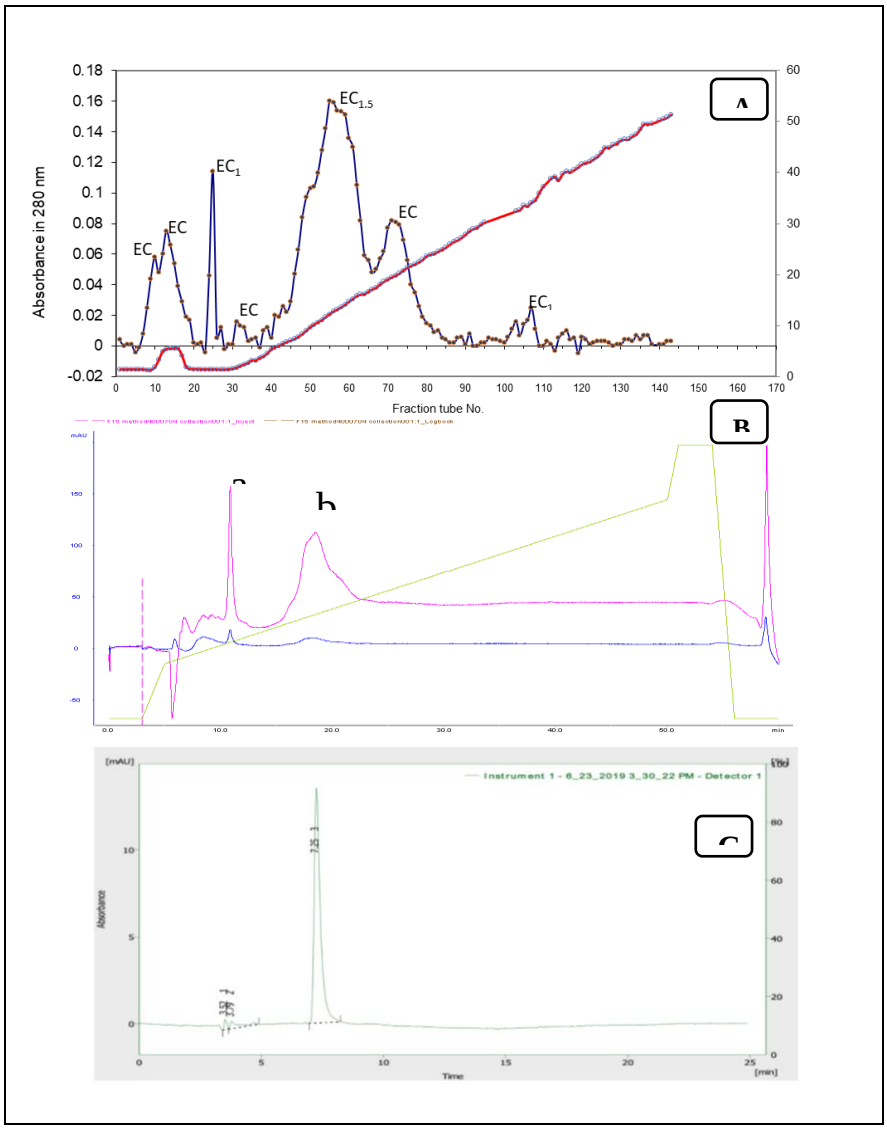


Figure 1. (A): Purification of EC_1 by DEAE-Sepharose chromatography. Ion exchange chromatography profile. (B): Further purification of Subfraction $EC_{1.5}$. HPLC chromatography of $EC_{1.5}$ Subfraction obtained from DEAE-Sepharose chromatography. (C): Further purification of fraction EC_4.HPLC chromatography of EC_4 fraction obtained from Size exclusion chromatography (G50-Sephadex chromatography)

3.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The molecular mass profile of samples (crude venom/fractions) varied from 6.5 to 200 kDa (Fig. SD3 A). The $EC_{1.5(a)}$, $EC_{5.1(b)}$, and $EC_{4(a)}$ subfractions, obtained from the final step of purification (HPLC Chromatography), indicated a single band in SDS-PAGE electrophoresis (Fig. 2 A, B, Fig. SD3 B).

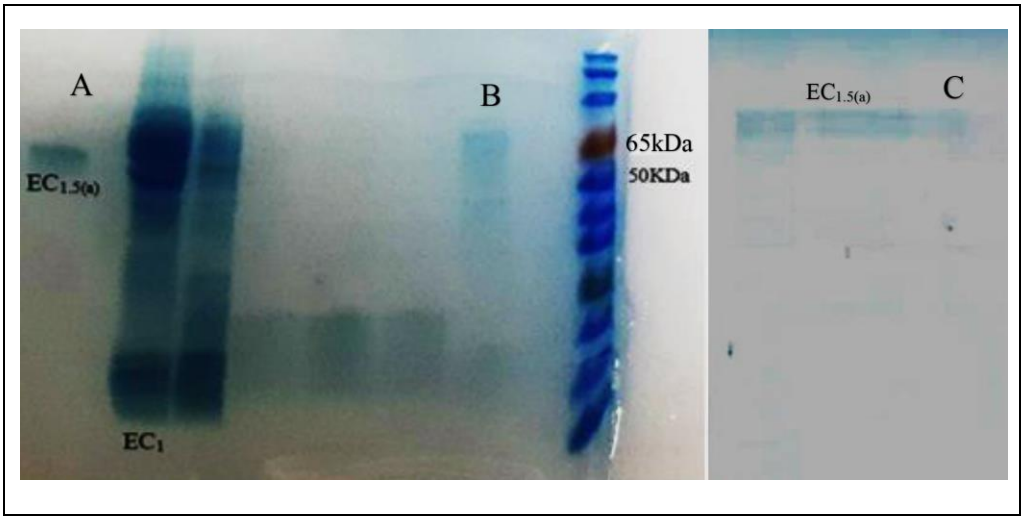


Figure 2. (A):12.5% SDS-PAGE profile of the EC_1 fraction and $EC_{1.5(a)}$ Subfraction of IEC venom obtained from Size exclusion chromatography and HPLC Chromatography, respectively (non-reduced state) B: 12.5% SDS-PAGE profile of $EC_{1.5(a)}$ Subfraction (Reduced state). C: 12.5% SDS-PAGE profile of $EC_{1.5(a)}$ Subfraction (Reduced state) (more amount)

3.4. *In vitro* assessment

3.4.1. Activated clotting time

3.4.1.1. Plasma clotting time; recalcification time; prothrombin time; activated partial thromboplastin time

PCT, RT, PT, and APTT tests were applied for samples (crude venom/fractions/subfractions) that were purified from several stages of chromatography. The fractions (EC_1, EC_2) with the highest pro-coagulant capacity and the fraction (EC_5) with the anti-coagulant capacity were candidates for further modified, improved, and reproducible purification. Here, EC_1, EC_4, and EC_5 fractions are discussed. The hematological results are summarized in Table 1. The PCT assay was performed for different prepared concentrations of EC_1, EC_2 fractions, and subfractions of EC_1: $EC_{1.5}$ and $EC_{1.6}$. The dose-dependent profile of PCT is shown in Fig. SD 4, SD 5, SD 6, and SD 7. In RT, PT, and APTT assays, EC_1, $EC_{1.5}$, and $EC_{1.5(a)}$ indicated a significant decrease compared to the control (P=0.0001), while $EC_{5.1(b)}$ showed a significant increase in comparison to the control (P=0.0001). In RT, PT, and APTT assays, no significant differences were found between samples and control in EC_4 (P>0.05).

3.4.2. Thrombin clotting time and fibrinogen-clotting activity

Thrombin clotting time and FCA were performed for samples (crude venom/fractions/subfractions) (Table 1). EC_1, $EC_{1.5}$, and $EC_{1.5(a)}$ effectively decreased, whereas EC_5 and $EC_{5.1(b)}$ increased the thrombin time compared to the control. It was also found that EC_1, $EC_{1.5}$, and $EC_{1.5(a)}$ increased, while EC_5 and $EC_{5.1(b)}$ decreased the fibrinogen concentration compared to the control *in vitro* assessment (Table 1). In the TCT assay, EC_1, $EC_{1.5}$, and $EC_{1.5(a)}$ showed a significant decrease in comparison to the control (P=0.0001).

3.4.3. Inducing the aggregation effect of IEC crude venom, EC_1 fraction, and $EC_{1.5(a)}$ subfraction on washed platelets

EC_1 and $EC_{1.5(a)}$ caused aggregation of human-washed platelets. EC_5, $EC_{5.1(b)}$, and EC_4 fractions hindered platelet aggregation induced by collagen and arachidonic acid agonists (Fig. 3, 4).

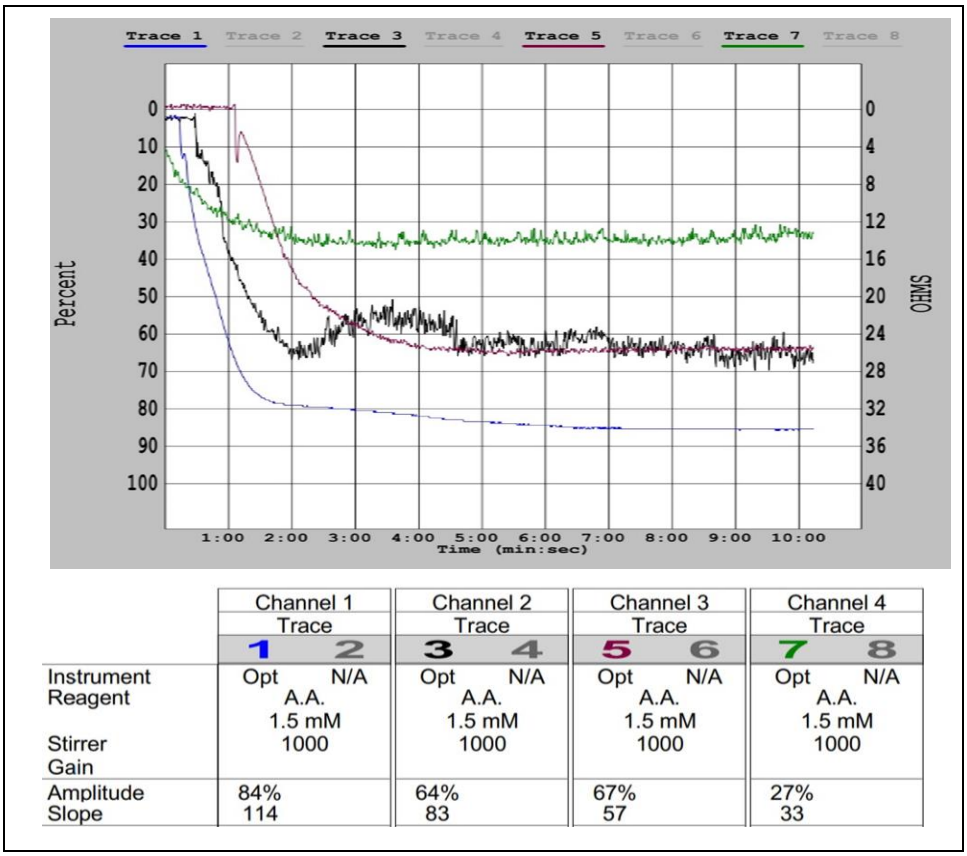


Figure 3. Inducing effect aggregation of crude EC venom, EC_1 Fraction and $EC_{1.5(a)}$ subfraction on washed platelets. Channel1. Washed platelets + Agonist (Arachidonic acid) + (Normal saline); Channel 2. Washed platelets + ($EC_{1.5(a)}$ Subfraction) + (Normal saline); Channel 3. Washed platelets + (EC_1 Fraction) + (Normal saline); Channel 4. Washed platelets + (Crude Venom(1mg/ml)) + (Normal saline)

3.5. *In vivo* assessment

3.5.1. Assessment of hemorrhagic activity

IEC crude venom samples (0.01, 0.1, and 1 mg/mL) and its fractions (EC_1, EC_2)/subfractions ($EC_{1.5(a)}$, $EC_{1.5}$, and $EC_{1.6}$) with determined protein concentrations (Table 1) created local hemorrhagic (Fig. 5). The dimension hemorrhagic lesion as a result of crude venom/fractions/subfractions is tabulated in Table 1.

3.5.2. Defibrinogenating activity

The results of the defibrinogenating activity of the samples (crude venom/fractions/subfractions) are presented in Table 1. In *in vivo* defibrinogenating assessment, one hour after the administration of these fractions, the concentration of fibrinogen in the mice plasma decreased in a concentration's protein-dependent manner (EC_1-EC_4, $EC_{1.5}$, $EC_{1.6}$, and $EC_{1.5(a)}$), leading to an increase in the coagulation time of the blood taken from mice's heart.

3.6. MALDI-TOF MS and bioinformatics analysis

Identifications of the $EC_{1.5(a)}$ (hemorrhagic toxin and aggregation inducer), $EC_{5.1(b)}$ (anti-coagulant), and $EC_{4(a)}$ (anti-platelet) isolated from the venom of IEC were revealed by the MALDI-TOF/MS data analysis in Mascot format datasets. The outcomes from MALDI-TOF/MS are displayed in Fig. 6 (A, B, C, D, E, H, K). The positive matrix factorization (PMF) analysis was achieved for $EC_{1.5(a)}$, $EC_{5.1(b)}$, and $EC_{4(a)}$ (Fig. 6 (F, G, I, J, L). The results of PMF confirmed 10 peptides out of 15 obtained peptides, which were

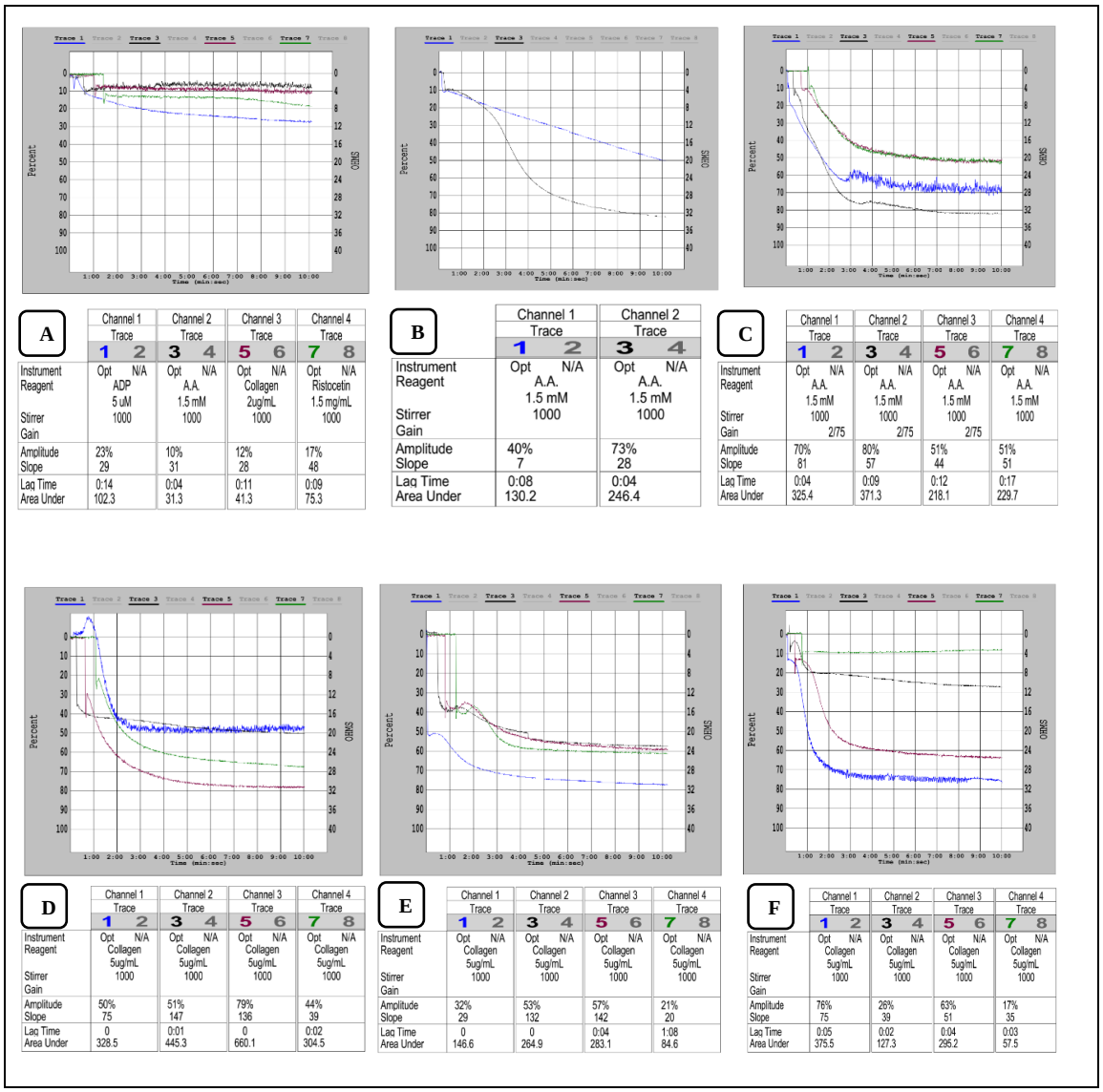


Figure 4. Effects of fractions on the platelet aggregation in washed human platelets suspension. A) 1. Washed platelets + F_5Fraction + (Normal saline)2. Washed platelets + (F_4Subfraction) + (Normal saline) 3. Washed platelets + ($F_{4(b)}$ Subfraction) + (Normal saline) 4. Washed platelets + ($F_{5.1(b)}$Fraction) + (Normal saline) B) 1. Washed platelets + + (F_5 Fraction+ aa) 2. Washed platelets + + F_2 Fraction + aa) C) 1. Washed platelets + ($F_{2.4}$Subfraction+aa) 2. Washed platelets + ($F_{2.4.2}$Subfraction+aa) 3. Washed platelets + ($F_{5.1(b)}$ Fraction+aa) 4. Washed platelets + (F_4Fraction +aa) D) 1. Washed platelets +(F_4Fraction) + Collagen 2. Washed platelets + (Venom1mg/ml+ Collagen) 3. Washed platelets + (F_1 Fraction+ Collagen) 4. Washed platelets + (F_5Fraction + Collagen) E) 1. Washed platelets + ($F_{4(b)}$Fraction+Agonist (Collagen)) 2. Washed platelets + (F_4Fraction+aa))3. Washed platelets + (F_4Fraction+Agonist (Collagen)) 4. Washed platelets + ($F_{5.1(b)}$Subfraction+Agonist(Collagen))F) 1. Washed platelets + Collagen+ (Normal saline) Control 2. Washed platelets + ($F_{4(b)}$+ aa) 3. Washed platelets + ($F_{2.4.2\,(b)}$ Fraction+ Collagen) 4. Washed platelets + ($F_{5.1(b)}$Subfraction + Collagen)

matched to $EC_{1.5(a)}$ (Table SD1). The results of PMF confirmed 4 peptides out of 6 obtained peptides,

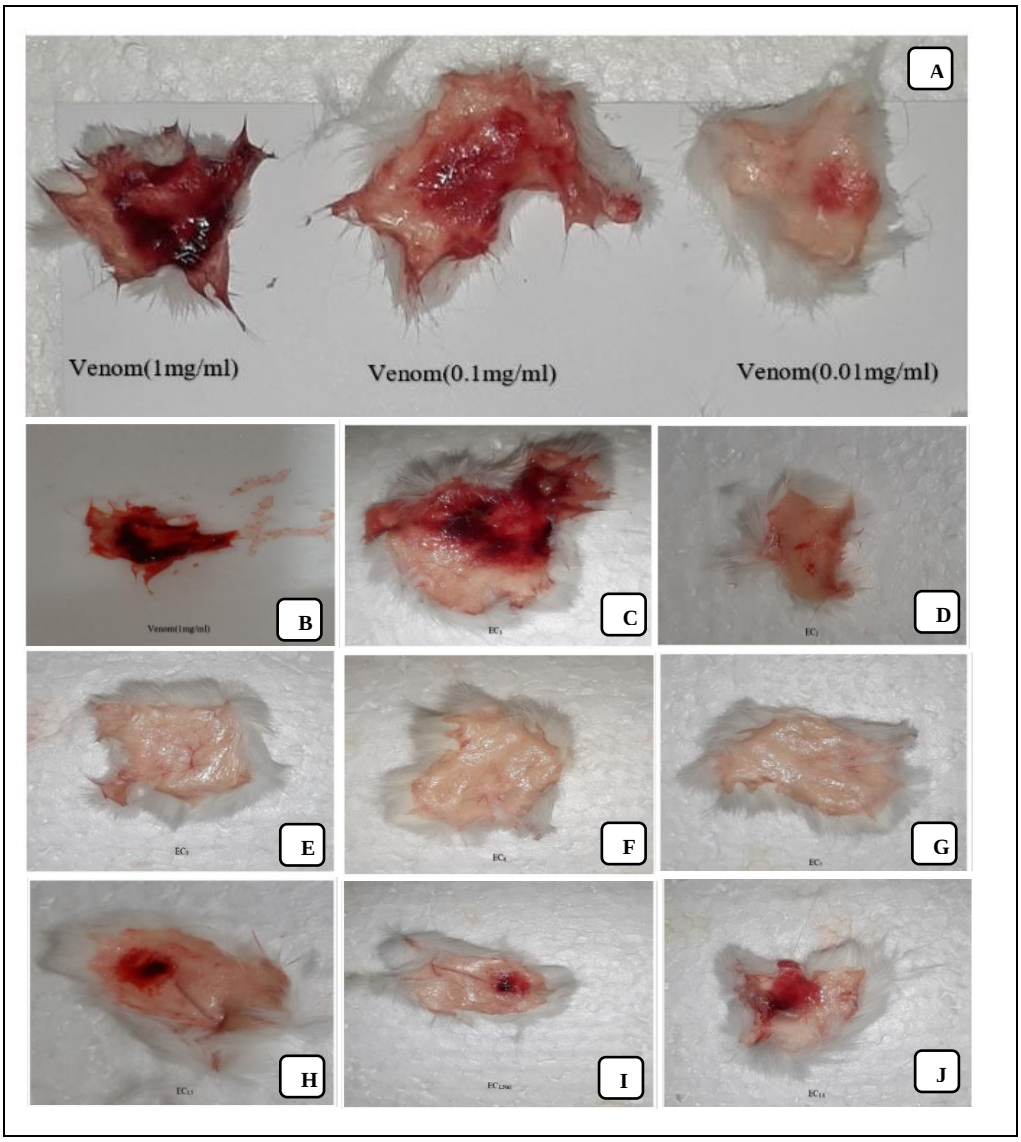


Figure 5. Hemorrhagic point of mice. A (Crude Venom(1,0.1,0.01mg/ml)), B (Crude Venom(1mg/ml)), C (EC_1), D (EC_2), E (EC_3), F (EC_4), G (EC_5), H ($EC_{1.5}$), I ($EC_{1.5(a)}$), J ($EC_{1.6}$)

which were matched to $EC_{5.1(b)}$ (Table SD 2). The results of PMF confirmed 3 peptides out of 6 obtainedpeptides, which were matched to $EC_{4(a)}$ (Table SD 3).

3.7. Alignment

The highly considerable homology of the purified peptides with Viperidae venom protein families in protein databases was shown by multiple sequence alignment (Fig. SD 8,9).

3.8. Bioinformatics analysis

The nearest 3D structures of $EC_{1.5(a)}$, $EC_{5.1(b)}$, and $EC_{4(a)}$ resulting from bioinformatics analysis according to the data resulting from Mascot analysis are illustrated in Fig. SD8.

As reported in the study by Mukherjee in 2017, EC venom contains both pro-coagulant and anti-coagulant proteins (26). In the current study, the novel pro-coagulant, anti-coagulant, and anti-platelet agents were purified from the venom of Iranian endemic ECby multi-step chromatography. This procedure was performed based on a novel platform for obtaining homogeneous IEC snake-venom pro-coagulant, anti-

coagulant, and anti-platelet fractions, named $EC_{1.5(a)}$, $EC_{5.1(b)}$, and $EC_{4(a)}$ to a high degree, which were observed as a single-band protein of 66, 7.5, and 5 kDa on the gel electrophoresis, respectively. The pro-coagulant potential has been reported in various snake venoms (39). According to the results of PMF analysis (Table 1 SD), the protein sequence coverage of $EC_{1.5(a)}$ was calculated at 100%, 100%, 98%, 63%, 53%, 47%, 38%, 35%, 34%, 10%, and 8% homologous in sequence to Zinc metalloproteinase-disintegrin-like uracoina-1 (Fragment), Disintegrin (Fragment), Disintegrin EO5B, Disintegrin multisquamatin, Disintegrin isoform D-3, Disintegrin VLO4, Disintegrin pyramidin-A, Disintegrin leucogastin-A, Disintegrin pyramidin-B, Disintegrin

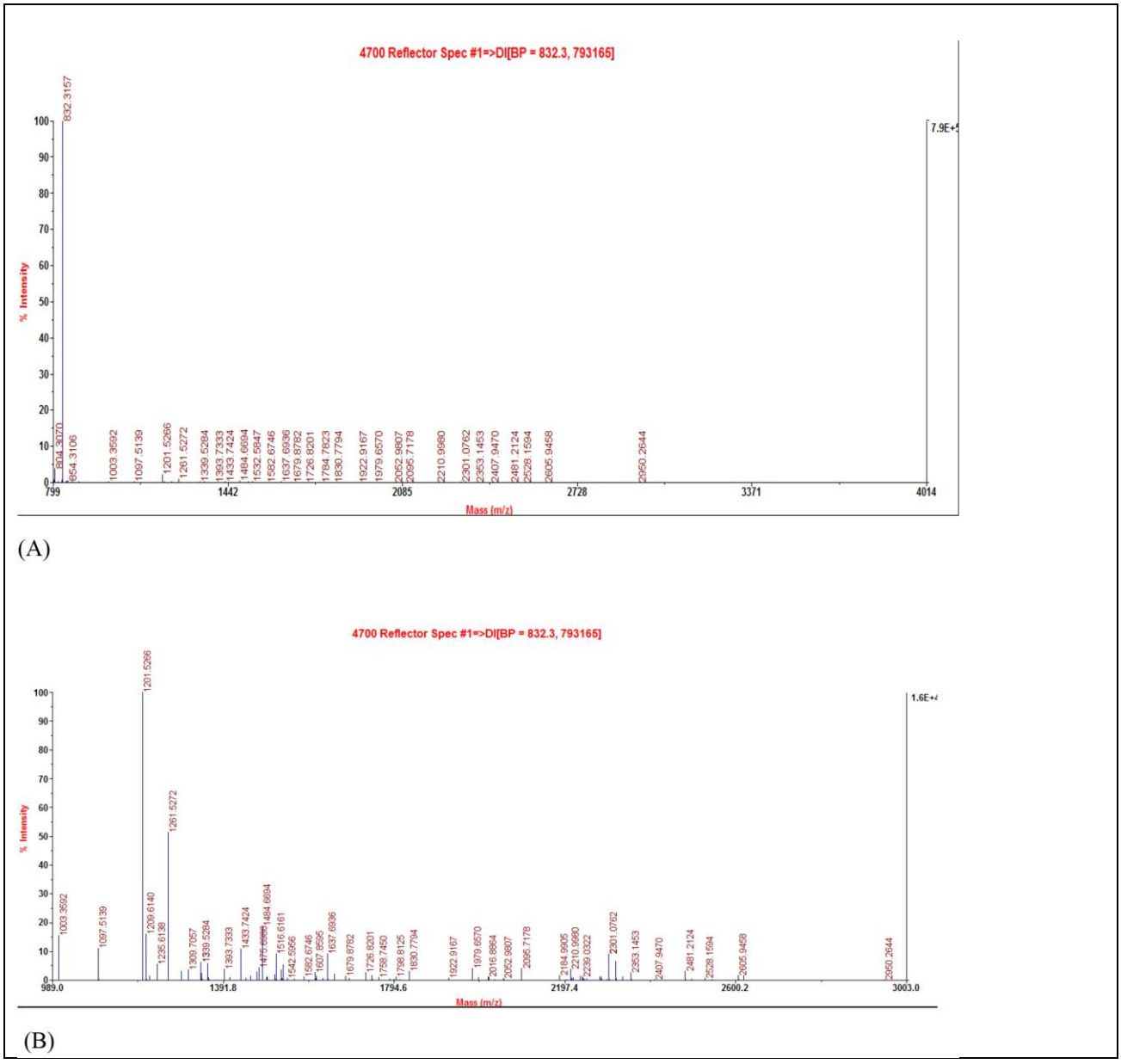


Figure 6. (A,B): Mass spectrum profile of $EC_{1.5(a)}$

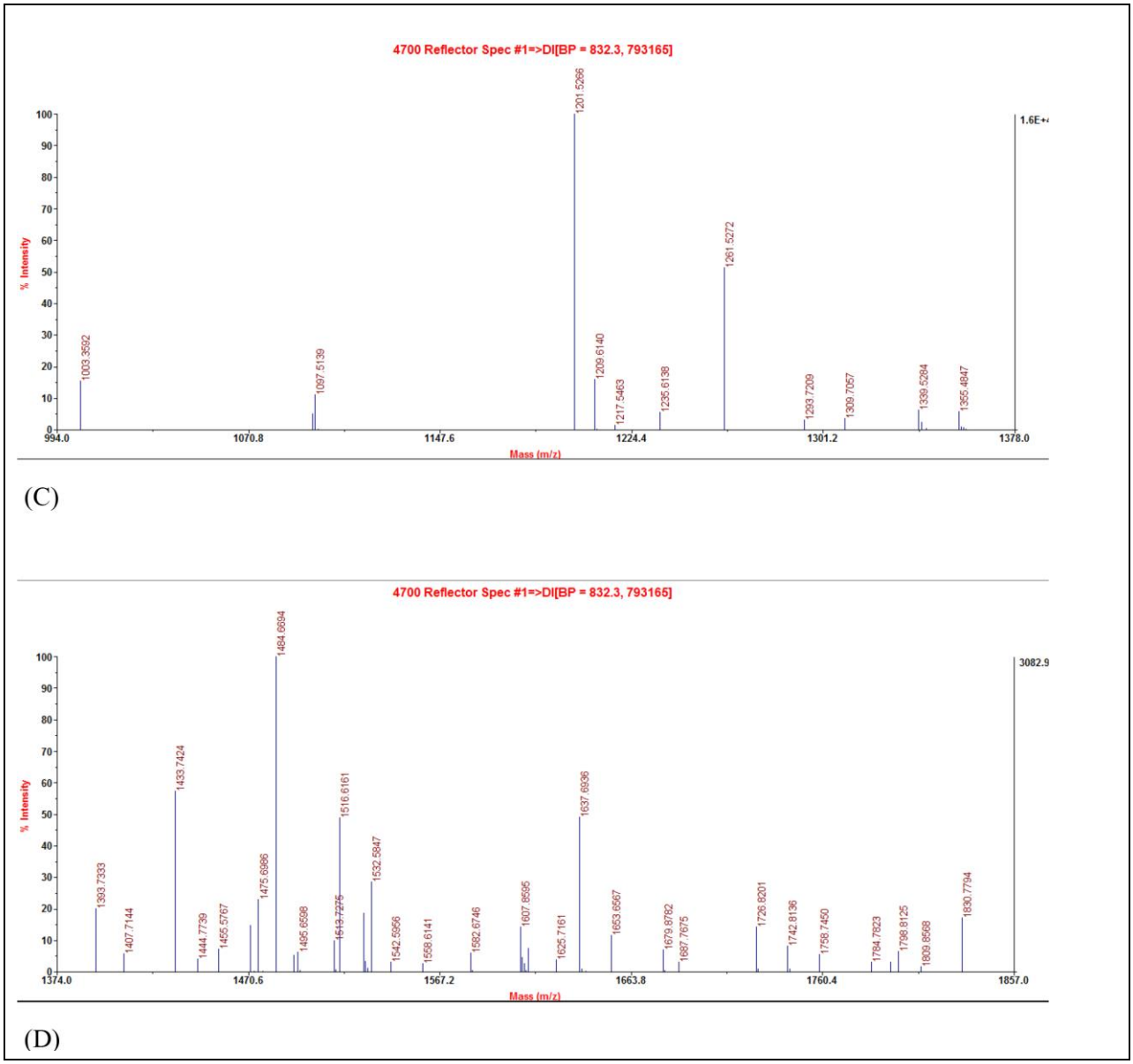
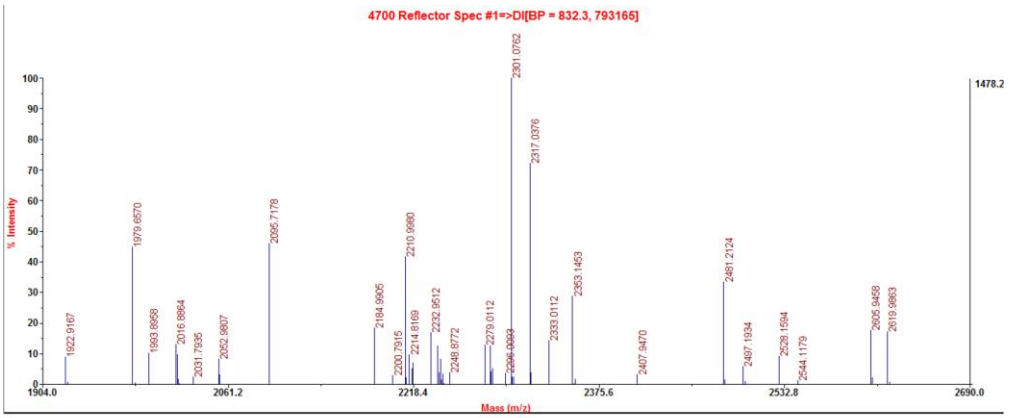


Figure 6. (C,D): Mass spectrum profile of EC1.5(a)

metalloproteinase/disintegrin echistatin (Fragment), and Zinc metalloproteinase-disintegrin VMP-I, respectively. Therefore, it is expected that this is a P-II metalloproteinase that corresponds to the venom metalloproteinase (M12B), P-IIa sub-subfamily with disintegrin domains. SVMPs, according to their sizes and domains, are classified into four groups: P-I, P-II, P-III, and P-IV classes. P-II class, the medium-size SVMPs (30-65 KDa), constitute proteinase and disintegrin domains. In this group, the Arg-Gly-Asp (RGD) sequence of the many disintegrin domains was replaced by another sequence. An example of this



(E)

1/23/23, 12:25 PM Mascot Search Results: VM3_CROVE

MATRIX SCIENCE **MASCOT Search Results**

Protein View: VM3_CROVE

Zinc metalloproteinase-disintegrin-like uracoina-1 (Fragment) OS=Crotalus vegrandis OX=184545 PE=1 SV=1

Database:	SwissProt
Score:	13
Expect:	4.8e+03
Average mass (M_r):	2434
Calculated pI:	8.50
Taxonomy:	Crotalus vegrandis

Sequence similarity is available as **an NCBI BLAST search of VM3_CROVE against nr**.

Search parameters

Enzyme: Trypsin: cuts C-term side of KR unless next residue is P.

Protein sequence coverage: 100%

Matched peptides shown in ***bold red***.

1 HQKYNPFRFV ELVLVVDKAM

(F)

Figure 6. (E): Mass spectrum profile of $EC_{1.5(a)}$ and (F) PMF analysis for identification of $EC_{1.5(a)}$

group is bilitoxin-1, which is a Met-Gly-Asp (MGD) sequence instead of an RGD sequence and lacks a platelet aggregation inhibitory effect (8, 40, 41).

Former studies have shown that only two key coagulation proteins, such as factor X (FX) and prothrombin activator, could be activated by SVMPs to cause their pro-coagulant effects (12). Calcium ions are essential to activate Factor X (8, 12, 42). In the present study, $EC_{1.5(a)}$, a potent pro-coagulant agent, showed the coagulation of citrated human plasma in PCT assay without the addition of $CaCl_2$ or any other cofactors. It is, therefore, concluded that $EC_{1.5(a)}$ is thought more likely to be a prothrombin activator because calcium ions are not needed for the activity of cofactor-independent coagulation proteins. In other words, the amount of calcium ions is insufficient to trigger a coagulation cascade via cofactor-dependent coagulation factors because of the calcium-chelating activity of sodium citrate. $EC_{1.5(a)}$ is also a platelet aggregation inducer. Snake venoms that have

represented platelet aggregation effects are classified into two groups: some release reactions and induce aggregation and others inhibit platelet aggregation (12). EC_1 and $EC_{1.5(a)}$ showed aggregation inducer activity on washed human platelets without adding any agonist. Further experiments must be performed to reveal that platelet aggregation characteristics are due to the indirect generation of thrombin from prothrombin residue or due to the direct proteolytic effect on the platelet membrane receptors.

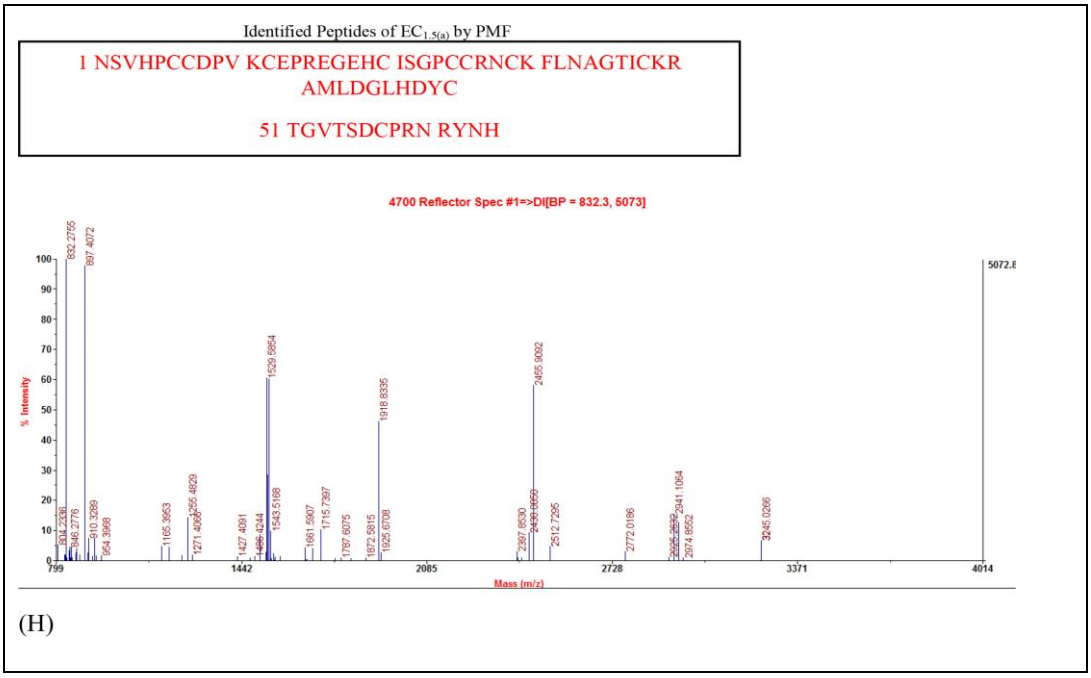
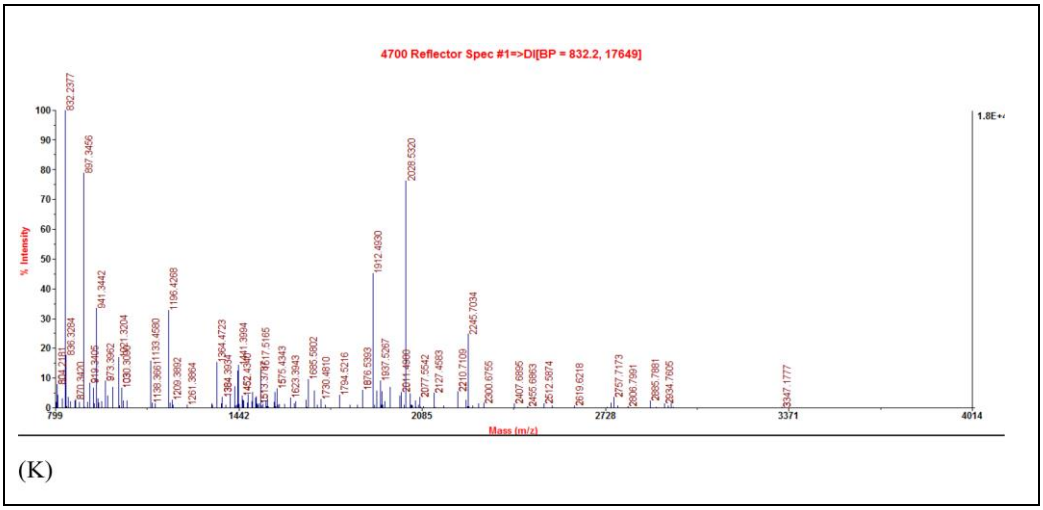


Figure 6. (H): Mass spectrum profile of $EC_{5.1(b)}$

Figure 6. (I, J) PMF analysis for identification of $EC_{5.1(b)}$.,



(K)

Figure 6. (K): Mass spectrum profile of EC_4

6/5/22, 3:12 PM Mascot Search Results: VM2EA_ECHCS

MATRIX SCIENCE **MASCOT Search Results**

Protein View: VM2EA_ECHCS

Disintegrin echistatin-alpha OS=Echis carinatus sochureki OX=124223 PE=1 SV=2

Database:	SwissProt
Score:	20
Expect:	7.7e+02
Monoisotopic mass (M_r):	5877
Calculated pI:	8.20
Taxonomy:	**Echis carinatus sochureki**

Sequence similarity is available as **an NCBI BLAST search of VM2EA_ECHCS against nr**.

Search parameters

MS data file:	Venom.txt
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.

Protein sequence coverage: 67%

Matched peptides shown in ***bold red***.

1 QCESGPCCRN **CKFLK**EGTIC **KRARGDDMDD YCNGKTCDCP RNPHKGPAT**

(L)

Identified Peptides of EC_4 by PMF

1 QCESGPCCRN CKFLKEGTIC KRARGDDMDD
YCNGKTCDCP RNPHKGPAT

Figure 6. (L) PMF analysis for identification of EC_4

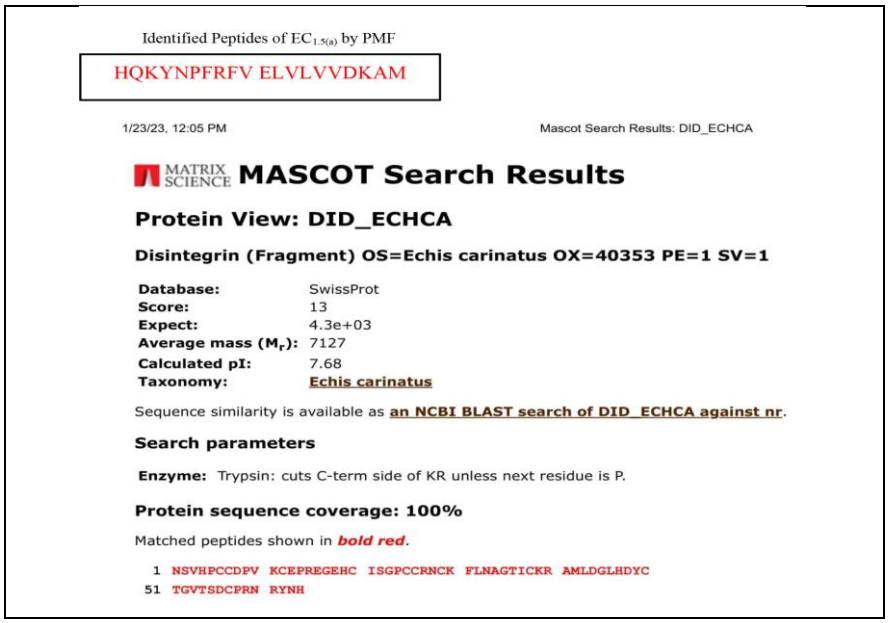


Figure 6. (G): (A, B, C, D, E): Mass spectrum profile of $EC_{1.5(a)}$ and (F, G) PMF analysis for identification of $EC_{1.5(a)}$. (H): Mass spectrum profile of $EC_{5.1(b)}$and (I, J) PMF analysis for identification of $EC_{5.1(b)}$., (K): Mass spectrum profile of EC_4 and (L) PMF analysis for identification of EC_4

Another feature confirmed for these metalloproteinases is hemorrhagic activity. Systemic and local hemorrhage is a common pathological complication of *Crotalidae* and *Viperidae* snakebites that can be attributed to metalloproteinases (26). $EC_{1.5(a)}$ elicits a hemorrhagic response, and compared to EC venom (1 mg/mL), $EC_{1.5(a)}$ (47.8 µg/mL) shows hemorrhagic activity with less intensity. The results of defibrinogenating activity demonstrated an increase in the coagulation time of the blood taken from the injected mice heart. This event occurs due to the employment of specific plasma proteins (e.g., coagulation cascade factors), the fall of platelet, and the activation of the fibrinolytic system by pro-coagulant toxins (e.g., $EC_{1.5(a)}$).

One of the most important events that occur following a snakebite by EC is a considerable reduction of fibrinogen in addition to an increase in fibrinogen degradation products (26). Clinically, these events lead to declining concentrations of fibrinogen and other blood factors resulting in a potentially lethal pathological syndrome, known as Disseminated Intravascular Coagulation and Venom-Induced Consumption Coagulopathy (26). In victims of snakebites, this causes intravascular coagulation, leading to cardiovascular collapse. In human victims, the employment of the majority of available essential clotting factors to the formation of millions of microthrombies following the dilution of the venom into a much larger blood volume can be lethal. Coagulopathy also contributes to internal hemorrhages, such as cerebrovascular accidents (23, 43-47). Ecarin is the best candidate for group A prothrombin activators. This Ca^{2+}-independent pro-coagulant protein was isolated and purified from the venom of the EC (Kenya carpet viper or saw-scaled viper) by Morita et al. (1987). It is a P-III metalloprotease with 426 amino acids and shares 64% similarity to the heavy chain of RVV-X (Russell's viper venom factor X activator) from *Daboia russelli* (43).

Ecarin has no inhibitory effect on platelet aggregation because of the replacement of RGD sequence with MDC domains in the disintegrin-like domain. Previous studies have also reported that Ecarin is a platelet inducer. It has even been reported

to induce platelet aggregation despite the presence of disintegrin domains (48-50). A few SVMPs have been reported to induce platelet aggregation. Alborhagin and crotarhagin, which are SVMPs from *Trimeresurus albolabris* and *Crotalus horridus horridus* snake venom, respectively, induce platelet aggregation through a mechanism involving Glycoprotein VI (12, 26). EoVMP3 is another SVMP platelet aggregation inducer from the venom of *Echis* species, the first fraction being Ecarin (50). EoVMP3 is SVMP class P-III metalloendopeptidases consisting of metalloproteinase, disintegrin-like, and cysteine-rich domains (50). Ecarin does not independently induce aggregating washed platelets [32]. In contrast, EoVMP3 and $EC_{1.5(a)}$ can aggregate platelets independently of plasma (50). $EC_{1.5(a)}$ has pro-coagulant and aggregation activities on washed human platelets, which may show the replacement of the platelet inhibitor sequence in a disintegrin-like domain similar to Ecarin. The full characterization of the mechanism of $EC_{1.5(a)}$ activity and its interaction with platelets requires further studies.

Halystase is a serine protease isolated from *Agkistrodon halys blomhoffii* snake venom. Although it has an RGD sequence, it did not inhibit the platelet aggregation induced by adenosine diphosphate (ADP) and collagen (51). Ecarin clotting time is a laboratory test used to assay anticoagulation during treatment with hirudin (22, 34). Chudzinski-Tavassi et al. (2005) introduced insularinase A, which belongs to the class P-I fibrin(ogen)olytic metalloproteases and is functionally a member of group A prothrombin activators (52). Insularinase A, similar to Ecarin and in contrast to $EC_{1.5(a)}$, did not show hemorrhagic activity. It has a pro-coagulant effect independent of any cofactors like $EC_{1.5(a)}$ (Silva et al., 2003; Nishida et al., 1992) (52, 53).

Another example of pro-coagulant agent is berythractivase, which was previously described as a novel prothrombin activator enzyme, was isolated from *Bothrops erythromelas* (*jararaca-da-seca*) snake venom (Chudzinski-Tavassi et al., 2003). Similar to $EC_{1.5(a)}$, it contains metalloproteinase, disintegrin-like, and cysteine-rich domains. In contrast to $EC_{1.5(a)}$, berythractivase showed no hemorrhagic activity. According to the results, although the basic structure of berythractivase is related to snake-venom hemorrhagic metalloproteinases and is functionally identical to group A prothrombin activators, it is a prothrombin activator without hemorrhagic response. Additionally, the role of DCD in its disintegrin-like domain of berythractivase in platelet function has not yet been explored (44).

Basparin A has been noted as a pro-coagulant metalloproteinase, from the venom of the Crotaline snake (*Bothrops asper*), inhibiting platelet aggregation as well as inducing defibrination and thrombosis (Gutiérrez et al., 2003). Apart from its clotting activity, basparin A can obstruct collagen-dependent platelet aggregation *in vitro*. Basparin A is a single-chain P-III metalloproteinase structured like $EC_{1.5(a)}$, with the metalloproteinase, disintegrin-like, and high-cysteine domains. In contrast to $EC_{1.5(a)}$, Basparin A lacks hemorrhagic effects; it has pro-coagulant activity, independent from additional cofactors; therefore, similar to $EC_{1.5(a)}$. It shows a higher clotting ability in human plasma similar to $EC_{1.5(a)}$ (54).

Babaie et al. in 2013 introduced one pro-coagulant fraction from Iranian saw-scaled viper (EC) with a molecular weight of about 65 kDa and potent pro-coagulant activity on PPP without the requirement of any cofactors similar to $EC_{1.5(a)}$ (29). Velmurugan reported a novel P-III class pro-coagulant SVMP from Indian EC venom named EC-PIII and displayed a pro-coagulant effect under *in vitro* conditions similar to $EC_{1.5(a)}$. It is devoid of hemorrhagic, unlike $EC_{1.5(a)}$ (24). RVV-X (Russell's viper venom factor X activator) is a pro-coagulant agent purified by Williams and Esnouf in 2003 from *Daboia russelli*. It consists of a metalloproteinase, a disintegrin, and a cysteine-rich domain similar to $EC_{1.5(a)}$. The structure of factor X activator RVV-X is very similar to that of carinactivase-1 from EC *leucogaster*. Unlike $EC_{1.5(a)}$, it requires Ca^{2+} ions at millimolar concentrations for

optimal activity (55).

Another example of a pro-coagulant is carinactivase-1 (Ca^{2+}-dependent) isolated from EC *leucogaster* venom. They consist of metalloproteinase and C-type lectin-like domains similar to $EC_{1.5(a)}$; however, unlike $EC_{1.5(a)}$, they require Ca^{2+} ions for activity (23, 39). According to the results of PMF analysis (Table 2 SD), the protein sequence coverage of $EC_{5.1(b)}$ is 100%, 100%, 79%, and 73% homologous in sequence to basic phospholipase A_2 Smb-N6 (Fragment), basic phospholipase A_2 Cll-N6 (Fragment), phospholipase A_2 homolog ECS_00014, and basic phospholipase A_2 homolog ecarpholin S, respectively. $EC_{5.1(b)}$ exhibits potent anti-coagulant activity compared to the control. It also displays platelet aggregation inhibitor properties.

Table2. List of identified proteins by MALDI-TOF/TOF of the $EC_{5.1(b)}$of EC Venom

NO.	protein	Calculated PI	Protein sequence coverage	Matched peptide	species	Subfamily
1	Basic phospholipase A2 Smb-N6 (Fragment)	10.18	100%	1 NLLQFNKMIK IMTKKNAFPF YTS	Sistrurus miliarius barbouri	Belongs to the phospholipase A2 family. Group II .subfamily
2	Basic phospholipase A2 Cll-N6 (Fragment)	10.18	100%	1 NLLQFNKMIK IMTKKNAFPF YTS	Crotalus lepidus lepidus	Belongs to the phospholipase A2 family. Group II .subfamily
3	Phospholipase A2 homolog ECS_00014	8.35	79%	1 SIVELGKMII QETGKSPFPS YTSYGCFCGG GERGPPLDAT DRCCLAHSCC 51 YDTLPDCSPK TDRYKYKREN GEIICENSTS CKKRICECDK AMAVCLRKNL 101 NTYNKKYTYY PNFWCKGDIE KC	Echis carinatus sochureki	SIMILARITY: Belongs to the phospholipase A2 family. Group II subfamily. S49 sub-subfamily.
4	Basic phospholipase A2 homolog ecarpholin S	8.35	73%	1 SVVELGKMII QETGKSPFPS YTSYGCFCGG GERGPPLDAT DRCCLAHSCC 51 YDTLPDCSPK TDRYKYKREN GEIICENSTS CKKRICECDK AVAVCLRKNL 101 NTYNKKYTYY PNFWCKGDIE KC	Echis carinatus	SIMILARITY: Belongs to the phospholipase A2 family. Group II subfamily. S49 sub-subfamily.

Table 3. List of identified proteins by MALDI-TOF/TOF of the $EC_{4(a)}$of EC Venom

NO.	protein	Calculated PI	Protein sequence coverage	Matched peptide	species	
1	Disintegrin echistatin-alpha	8.20	67%	1 QCESGPCCRN CKFLKEGTIC KRARGDDMDD YCNGKTCDCP RNPHKGPAT	Echis carinatus sochureki	"Significance of RGD loop and C-terminal domain of echistatin for RT recognition of alphaIIb beta3 and alpha(v) beta3 integrins and expression RT of ligand-induced binding site."; RL Blood MISCELLANEOUS: The disintegrin belongs to the short disintegrin subfamily.
2	Disintegrin echistatin-	6.86	38%	1 DCASGPCCRD CKFLKEGTIC KRARGDNMDD YCNGKTCDCP	Echis pyramidum	MISCELLANEOUS: The disintegrin

	beta			RNPHKGEHDP	leakeyi	belongs to the short disintegrin subfamily.
3	Disintegrin echistatin-gamma	4.70	34%	1 DCASGPCCRD CKFLEEGTIC NMARGDDMDD YCNGKTCDCP RNPHKWPAP	Echis pyramidum leakeyi	MISCELLANEOUS: The disintegrin belongs to the short disintegrin subfamily.

The circulatory system is one of the vital physiological systems of the body attacked by the anti-coagulant PLA_2 from snake venom (26). Crude venom mixture consists of several isoforms of PLA_2, which are classified as acidic, basic, or neutral PLA_2 enzymes according to their overall net charge. The mechanism of anti-coagulant action of snake venom PLA_2 enzymes is to destroy or make unavailable pro-coagulant phospholipids that are necessary for starting the coagulation cascade. Some of the PLA_2s can bind to other blood coagulation factors, such as factor Xa, factor Va, prothrombin, and thrombin, or inhibit the formation of prothrombinase complex (complex of factor Xa, factor Va, phospholipids, and Ca^{2+}) and thus can inhibit the initiation of blood clotting pathway (56).

In comparison to the anti-coagulant introduced by other researchers, we can mention Cc1-PLA_2 and Cc2-PLA_2, two PLA_2s purified from *Cerastes cerastes* venom. The anti-coagulant effect is due to the interaction of them with factor FXa through a noncatalytic PL-independent mechanism leading to no released thrombin. These agents also have anti-platelet activity similar to $EC_{5.1(b)}$ (57). NnPLA2-I, acidic PLA_2 purified from Indian cobra (*Naja naja*) venom, has both anti-coagulant and anti-platelet activities similar to $EC_{5.1(b)}$ (58). Daboxin P, a major PLA_2 enzyme from the Indian *Daboia russelii* venom, targets both factor X and factor Xa for its anti-coagulant activity (59). The results of a study by Babaie showed that F_2C and F_2D fractions from IEC venom could delay the prothrombin time, and thus can be considered anti-coagulant factors. They suggested that the anti-coagulant activity of these fractions could be caused by proteolytic enzymes (27).

Mirakabadi introduced EC_{217} as an anti-coagulant fraction from IEC venom and showed that the venom of EC contains at least one anti-coagulant factor (60). Wilkinson et al. reported that PLA_2s were the most likely candidates responsible for anti-coagulant effects stimulated by *Naja nigricollis* venom (3). Damotharan et al. designed to purify a novel anti-clotting PLA_2 component from the sea snake venom of *Enhydrina schistose*. They suggested that this fraction could be a candidate for the development of novel compounds for pharmacological applications in the near future (61). $EC_{4(a)}$ inhibits both pathways of platelet aggregation induced by collagen and arachidonic acid. The results of the PMF analysis showed that this fraction was Echistatin-like. According to the results of PMF analysis (Table 3 SD), the protein sequence coverage of $EC_{4(a)}$ is 67%, 38%, and 34% homologous in sequence to Disintegrin Echistatin-alpha, Disintegrin Echistatin-beta, and Disintegrin Echistatin-gamma, respectively.

Echistatin, a platelet aggregation inhibitor, was purified from the venom of the EC (saw-scaled viper). It inhibits platelet aggregation by binding to the GP IIb/IIIa receptors throughout the RGD sequence. It inhibits platelet aggregation stimulated by ADP, thrombin, epinephrine, collagen, or platelet-activating factor (30). Gowda et al. introduce an acidic PLA_2 platelet aggregation inhibitor from Indian saw-scaled viper (EC) venom termed EC-me-PLA_2. It inhibits human platelet aggregation when stimulated by ADP, collagen, and epinephrine (62).

Vatanpour et al. investigated platelet aggregation inhibitory and anti-coagulant properties of venoms of *Cerastes persicus feldi* and EC. They isolated two fractions from *Cerastes persicus feldi* with anti-platelet aggregation activity on ADP-induced platelet aggregation (28). Fig. SD8 displays the alignment of

MALDI-TOF/MS-derived $EC_{1.5(a)}$, $EC_{5.1(b)}$, and $EC_{4(a)}$ peptide sequences of IEC venom with the homologous proteins from the *Viperidae* snake venom protein families in protein databases. Alignment analysis according to the results of Mascot indicated that $EC_{1.5(a)}$ demonstrated high homology with Disintegrin metalloproteinase/disintegrin from EC *sochureki*, Metalloproteinase 2 from *Crotalus adamanteus*, and Zinc metalloproteinase-disintegrin-like from *Crotalus vegrandis*. Moreover, $EC_{5.1(b)}$ showed high homology with Phospholipase A_2; Short=svPLA_2 from *Crotalus atrox*, and $EC_{4(a)}$ revealed high homology with Disintegrin multisquamatin from *Echis multisquamatus* and crystal structure of Echistatin, from EC venom.

In conclusion, EC venom contains components that exhibit both pro- and anti-coagulant properties. To the best of our knowledge, this was the first study to introduce three fractions of EC and their functions on the hemostatic system by MALDITOF MS. $EC_{1.5(a)}$, metalloproteinase/disintegrin region with disintegrin-like domains, is platelet aggregation inducer on human washed platelets that possesses strong pro-coagulant effect without the addition of calcium and any other co-factors. Similar to other metalloproteinases, it displays hemorrhagic activity. This factor decreases the thrombin and fibrinogen time compared to the control. Non-hemorrhagic $EC_{5.1(b)}$, with structural similarity to PLA_2, is a strong anti-coagulant that inhibits the aggregation of human-washed platelets induced by collagen and arachidonic acid, and non-hemorrhagic $EC_{4(a)}$ is an Echistatin-like with platelet aggregation inhibitor.

Authors' Contribution

All the authors contributed significantly to this research. N. NN wrote the original draft, prepared figures and tables and was involved in all tests. NM, MA, HV are supervisors. MAB, GP assisted in the experimental research and in the preparation of the paper. MN assisted in writing the paper and Statistical analysis. All authors have read and approved the final manuscript.

Ethics

Not applicable.

Conflict of Interest

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

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