

## Cytotoxicity, anti-adhesive and anti-angiogenic effects of Caspian Cobra snake (*Naja oxiana*) venom on human endothelial cells

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### ABSTRACT

Snake bite was known as one of the most important public health concerns world wide. Respiratory and cardiac problems and destruction of endothelium system occurs as a result of snake bite. Some proteins and peptides in snake venom have potential for drug discovery studies. According to the important role of cell adhesion and angiogenesis in cancer development, identification of new therapeutics that targets this process seems indispensable. Evaluation of cytotoxicity, anti-adhesive and anti-angiogenic effects of Caspian Cobra snake (*Naja oxiana*) venom on human endothelial cell was the main aim of current study. Methods: Crude Cobra snake venom was fractionated by fast protein liquid chromatography (FPLC) using S-200 column. Purity of crude venom and each fraction was monitored by SDS-PAGE. Cytotoxicity of Caspian Cobra snake (*Naja oxiana*) crude venom and fractions on human endothelial cells was evaluated by MTT assay Adhesion and tube formation

assay was performed to evaluate anti-adhesive and anti-angiogenic activities of snake venom. Results of FPLC revealed eight individual fractions. Cobra crude venom and fractions showed dose-dependent cytotoxic effect on human endothelial cell. Fraction 6 (IC<sub>50</sub>=5.7 µg/ml) and fraction 7 (IC<sub>50</sub>=5 µg/ml) showed higher cytotoxic effects on human endothelial cells. Therefore, further assays carried out with fraction 6 (F6) and 7 (F7) and results showed that both of fractions inhibited *in vitro* adhesion and tube formation of human endothelial cells. Results evaluated cytotoxicity, anti-adhesive and anti-angiogenic effects of Caspian Cobra snake (*Naja oxiana*) venom on human endothelial cell and represents promising tool for drug discovery and development.

**Keywords:** Cytotoxicity, adhesion, angiogenesis, snake venom.

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## INTRODUCTION

Snake venom contains various proteins, enzymes and peptides that have physiological and pharmacological effect to mammalian [1]. Unique properties of component, makes snake venom as potential for drug development. Captopril is the first successful example of venom derivations [2]. Many therapeutics from snake venom have been developed for treatment of different disease like hypertension, thrombosis, and cancer [2, 3]. Many other venom derivative molecules are to characterize for drug discovery [4]. In fact venom component have been considered in many biopharmaceutical companies because they can directly use as therapeutic agent and serve as potent leads [5]. Venoms of *Crotalidae* and *Viperidae* family affect human endothelium cells and causes hydrolysis of vessel and bleeding. Snake venom proteins including:

metalloproteinase, disintegrins, phospholipases and C-type lectins [6, 7]. Metalloproteinases (MPs) are most abundant toxin in the venom of Viperidae family. They play important role in hemorrhage through disturbing interactions of endothelial cells and basement membrane. There are several reports describing inhibitory, anti-adhesive and apoptotic effects of snake venom metalloproteinase on human HUVEC endothelial cells. Disintegrins are non-enzymatic proteins in snake venom and play crucial role in inhibition of platelet aggregation and angiogenesis of cancer cells.

Phospholipases are proteins with enzymatic activity and have been shown anti-tumoral and anti-angiogenesis activities [9]. Cytotoxic and pathophysiological effect of different snake venoms on human endothelial cells has been determined in various studies [10]. However further studies are need to evaluate the

cytotoxicity of all venomous snake on endothelial cells. Here, for the first time we evaluated the cytotoxic, anti-adhesive and anti-angiogenic effect of Iranian Cobra crude venom and fractions on human endothelial cell

## **MATERIALS AND METHODS**

Lyophilized Caspian Cobra crude venom prepared in Pasteur Institute of Iran, Tehran, Iran. BCA protein concentration kit was from Pierce Rochford, USA. DMEM medium, FBS (fetal bovine serum), trypsin/EDTA and pen/strep (penicillin/ streptomycin) were bought from Invitrogen. Ham's F12 medium was purchased from Lonza. MTT powder (3, 4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was bought from Sigma, USA. DMSO (dimethylsulfoxide) was purchased from Sinaclon, Bioscience. Human umbilical vein endothelial cells (HUVEC) isolated from human umbilical cord according to our previous study [9]. HUVEC cells culture in DMEM- Ham's F12 medium (1:1) supplemented by 10 % FBS and 1% pen/strep at 37 °C in 5 % CO<sub>2</sub> incubator.

### **Venom preparation and fractionating**

Lyophilized Venom of Iranian Cobra snake was dissolved in distilled water, homogenized and centrifuged at 1000mm for 10 min. pure venom in the supernatant was collected and kept at -70 °C. Pure venom was fractionated using FPLC

with S-200 column (GE Healthcare, Bio-Sciences). Briefly, Cobra venom was dissolved in FPLC buffer containing 20 mM ammonium acetate, pH=6.8 (50 mg of venom /500 µl of buffer). Each fraction was collected with flow rate of 1 mg/ml and fractions lyophilized in freeze drier (Christ, 2 alpha , Germany) at -55 °C and kept at -20 °C. Protein concentration in each fraction was evaluated using BCA protein concentration Kit (Pierce Rochford, USA). Crude venom and each fraction were analyzed by Coomassie brilliant blue staining 15% SDS-PAGE under reduction condition.

### **Cytotoxicity of Cobra venom on human endothelial cell**

HUVECs (10<sup>4</sup> cells) were isolated in this study from umbilical cord according to the Baudin B et.al protocol [10] and were cultured in 200 µl of DMEM Ham's F12 medium supplemented by FBS 10 % and 1 % pen/strep and incubated at 37 °C for 24 h. HUVEC cells were treated with fresh medium containing various concentrations (up to 200 µg/ml) of each fraction as well as crude venom and incubated for 24 h at 37 °C. About 20 µl of MTT (5mg/ml of PBS 1X) was added to each well and incubated for 4 h. Then MTT solution was removed and wells incubated with 100 µl of DMSO for 15 min at RT. Absorbance of formazan crystal was measured at 570 nm using

ELISA plate reader (Epoch, Bio-Tek, USA). IC50 referred as concentration which show 50% inhibitory effect on cell proliferation according to the control

#### **Adhesion assay**

About  $2 \times 10^4$  HUVEC cells with DMEM Ham's F12 medium supplemented by 10% FBS were cultured in 96-well plate with different concentration of fraction 6 and 7 (up to 200  $\mu\text{g/ml}$ ) and incubated for 1 h at 37 °C, 5% CO<sub>2</sub>. Then, the cells in the supernatant were collected, centrifuged at 900mm for 1 min, counted and defined as non-adhesive cells. The adhesive cells also were trypsinized, counted and defined as adhesive cells. The assay was performed in triplicate.

#### **Angiogenesis assay**

Geltrex matrix (Gibco, Invitrogen) was thawed at 4 °C and 50  $\mu\text{l}$  of it transferred to 96-well plate. Subsequently, the plate was incubated at 37 °C for 1 h to solidify Geltrex. About  $10^4$  HUVEC cells resuspended in 100  $\mu\text{l}$  of DMEM-Ham's F12 medium and transferred into 96-well plate containing 50 $\mu\text{l}$  solid Geltrex matrix. Various concentrations of fraction 6 and 7 (up to 200  $\mu\text{g/ml}$ ) added to the wells and then the plate incubated for 6 h at 37 °C. Formation of tube-like structures and HUVEC cells conditions

monitored by invert microscope (INV100-FL, BEL- Italy).

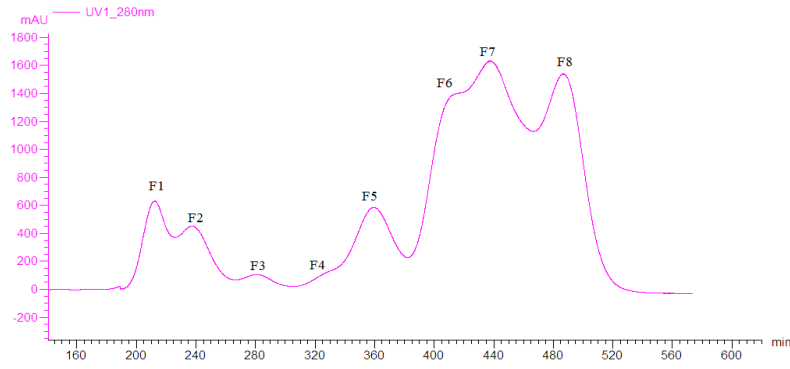
## **RESULTS**

#### **Fractionating**

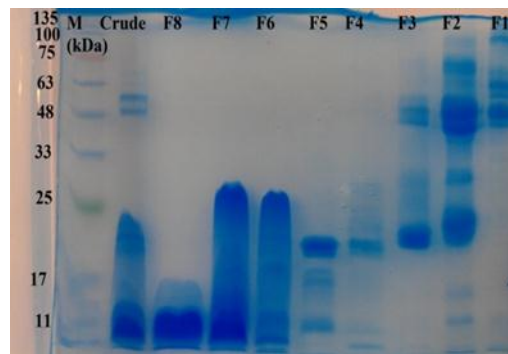
Eight individual fractions of Cobra snake crude venom were detected in fast protein liquid chromatography (FPLC) diagram (Fig. 1). Isolation of fractions in FPLC was performed by S-200 column. Purity of isolated fractions was evaluated by SDS-PAGE analysis and results are shown in Fig. 2. According to SDS-PAGE results, Cobra crude venom successfully fractionated with S-200 column and the venom contains protein and peptides with various molecular weights.

#### **MTT assay**

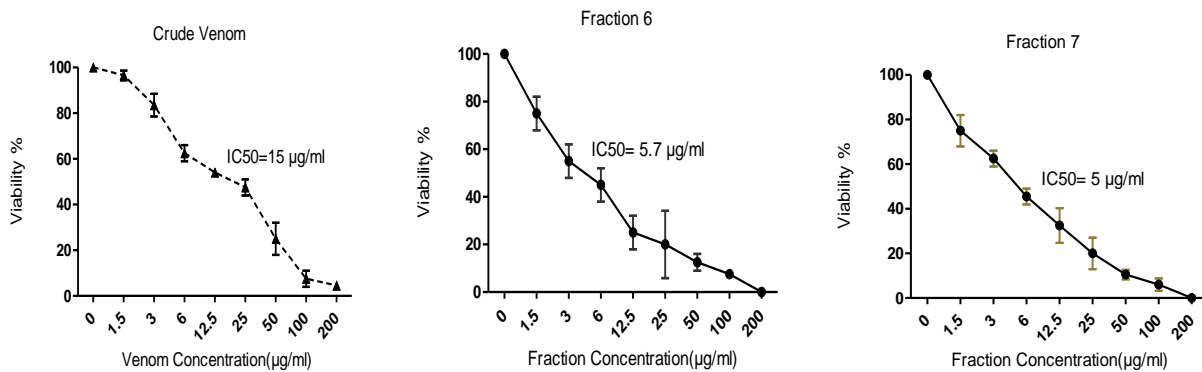
MTT assay revealed the cytotoxicity effect of Cobra venom on HUVEC cells. Treatment of HUVECs with various concentrations of Cobra crude venom and fractions resulted in morphological change of cells. Cobra crude venom and all fractions showed dose-dependent cytotoxicity activity on HUVEC cells. According to IC50 results, the highest inhibitory effect on HUVEC proliferation was determined for fraction 6 (IC50=5.7  $\mu\text{g/ml}$ ), and 7 (IC50=5  $\mu\text{g/ml}$ ). Therefore, fraction 6 and 7 were chosen for further functional analysis. IC50 of crude venom was 15  $\mu\text{g/ml}$  (Fig. 3).



**Figure 1.** FPLC results. Eight fractions isolated by fast protein liquid chromatography and S-200 column.



**Figure 2.** SDS-PAGE 15% results. Cobra crude venom and fractions evaluated by coomassie brilliant blue SDS-PAGE analysis. M; Protein marker, F: fraction.



**Figure 3.** MTT assay results. Cytotoxic activity of Cobra snake crude venom, fraction 6 and 7 was evaluated on HUVEC cells through MTT assay. Graphs represents triplicate of each assay±SD.

### Adhesion assay

It has been demonstrated that in snake venom there are potential anticancer agents with anti-adhesion activity. In this study, we used fraction 6 and 7 (according to their high cytotoxicity effect) to evaluate their effect on adhesion of HUVEC cells to the wells. Our results revealed that both fraction 6 and 7 had dose-dependent effect on HUVEC adhesion. However, this effect was different among fractions 6 and 7 (Fig. 4). As shown with increasing the concentration of fraction 6 and 7 more cells observed in supernatant, indicates in higher concentration the adhesion of cells on plate were not happened. In low concentration of fraction 6 and 7 most of the cells were adherent and low number of cells detected in supernatant (non-adhesive cells).

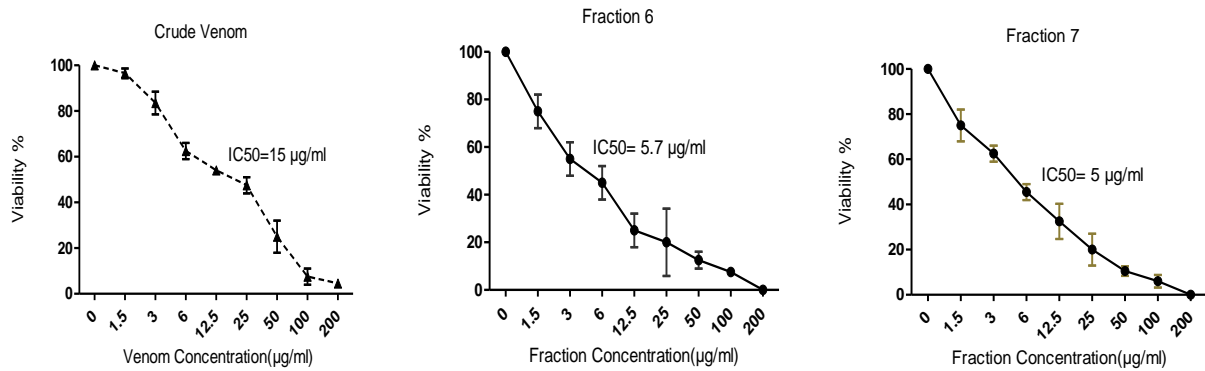
### Angiogenesis assay

It has been demonstrated that HUVEC cells forms tube-like structures using existing growth factors of Geltrex matrix. Thus, tube formation assay was performed to evaluate anti-angiogenic activity of fraction 6 and fraction 7 of Cobra snake venom. According to the achieved results, fraction 6 and 7 significantly inhibited tube formation of HUVEC cells on Geltrex matrix. However, HUVEC cells formed complete tube-like structures on Geltrex matrix in absence of fraction 6 or 7 (Fig. 5).

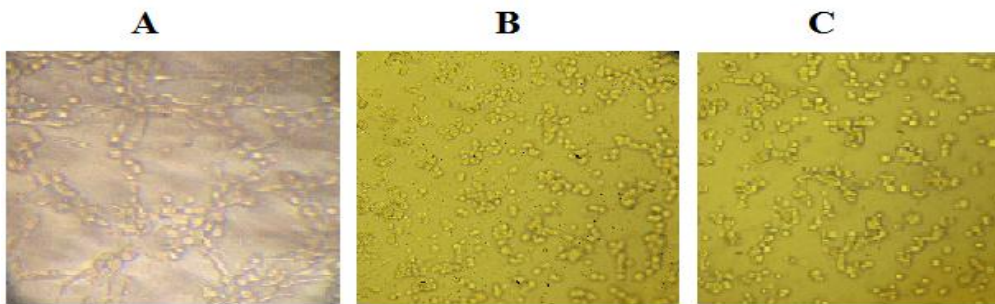
### DISCUSSION

Snake venom contains mixture of various components like enzyme, protein and peptides with different molecular weight and biological activity against mammalian cells [8]. However snake venom contains several molecules with neurotoxicity, cytotoxicity, cardiotoxicity and other effects, but it has been demonstrated that they can be used for treatment of many diseases like cancer [11]. Here, for the first time cytotoxicity, anti-adhesion and anti-angiogenicity of Iranian Cobra snake venom on HUVEC cells were evaluated. Using S-200 column, eight fractions were observed by FPLC. The highest cytotoxicity effect of snake venom on HUVEC was related to fraction 6 and 7. The cytotoxicity effect of crude venom and fractions was dose-dependent and in higher concentrations, the highest cytotoxicity on HUVEC cells was observed. Our cytotoxicity results are in consistent with the study of Kakanj *et. al* which showed cytotoxicity of *Vipera lebetina* crude venom on HUVEC cells [8]. In Kakanj study, *Vipera lebetina* crude venom showed cytotoxicity to HUVECs in dose dependent manner. They also showed that exposure of cells with *Vipera lebetina* crude venom resulted in morphological change which our study confirmed their results. The MTT assay demonstrated as standard test for *in vitro* cytotoxicity [12, 13]. In many studies, were

evaluated that snake venom contains phospholipases A2 which effect on endothelial components like metalloproteinase, and cells and causes hemorrhage [14, 15].



**Figure 4.** Adhesion assay results. (A) Adhesion assay results for fraction 6. As shown with increasing fraction concentration more cells were in supernatant (F6 S) (non-adhesive cells) and low number of cells were observed in pellet (F6 P) (adhesive cells). (B) Adhesion assay results for fraction 7. According to graph in lower fraction 7 concentrations more cells were in pellet (F7 P) and low number of cells was in supernatant (F7 S).



**Figure 5.** Angiogenesis assay. A) Control well (absence of snake venom fractions), B) Fraction 6, C) Fraction 7. As it can be seen HUVEC cells formed tube-like structures in absence of venom fractions. Fraction 6 and 7 completely inhibited tube formation of HUVEC cells.

According to the cytotoxicity results, fraction 6 and 7 were chosen for adhesion and tube formation assay. Our results showed that fraction 6 and 7 of Cobra snake venom inhibited *in vitro* adhesion of HUVEC cells. Anti-adhesive effect of fraction 6 and 7 was dose

dependent and in higher concentration of fractions, more cells detected as non-adhesive. Our results were in agreement with some related studies which evaluated anti-proliferative and anti-adhesive activity of snake venom on cancer cells [16, 17]. It has been discussed that such

effect on cancer cells was related to C-type lectin proteins [17, 18]. Ebrahimi *et. al* evaluated anti-cancer effect of Cobra snake venom on HepG2, MCF7 and DU145 cell lines. The observed anti-cancer effect in Ebrahimi *et. al* study, was related to induction of apoptosis in cancer cell lines [19]. Recently, anti-angiogenic effect of Cysteine-rich secretory protein (CRISP) which isolated from snake venom of *Echis carinatus sochureki*, was evaluated on endothelial cells [20]. It has been determined that all venoms don't show cytotoxic effect on endothelial cell even in long time exposure [21]. However we showed that Iranian Cobra venom significantly decreased *in vitro* proliferation, adhesion and tube formation of human endothelial cells in dose dependent manner.

### CONCLUSION

In this study, by using FPLC and S-200 column we fractionated Cobra crude venom. Eight fractions were eluted and evaluated by SDS-PAGE analysis. Cytotoxic, anti-adhesive as well as anti-angiogenic effect of crude venom and fractions was determined by MTT, adhesion and tube formation assay. Results indicated the significant cytotoxic, anti-adhesive and anti-angiogenic effects of Cobra crude venom and fractions (fraction 6 and 7) on human endothelial cells. These results indicate the

potential of Cobra venom fractions (fraction 6 and 7) as novel therapeutic of cancer.

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### REFERENCES

- [1]. Reeks TA, Fry BG, Alewood PF. Privileged frameworks from snake venom. *Cell Mol Life Sci*, 2015; 72(10): 1939-58.
- [2]. Koh CY, Kini RM. From snake venom toxins to therapeutics—cardiovascular examples. *Toxicon*, 2012; 59(4): 497-506.
- [3]. Fatani AJ. Snake Venoms and Scorpion Venom Research in the Middle East: A Review. *Clinical Toxinology in Asia Pacific and Africa*, 2015; 327-55.
- [4]. Rodrigues VM, Lopes DS, Castanheira LE, Gimenes SN, Naves de Souza DL, Ache DC, Borges IP, Yoneyama KA, Rodrigues RS. Bothrops pauloensis Snake Venom Toxins: The Search for New Therapeutic Models. *Curr Top Med Chem*, 2015; 15(7): 670-84.
- [5]. Zhan C, Li C, Wei X, Lu W, Lu W. Toxins and derivatives in molecular pharmaceuticals: drug delivery and targeted therapy. *Adv Drug Deliv Rev*, 2015; 90: 101-18.
- [6]. Marcinkiewicz C. Applications of snake venom components to modulate integrin



- activities in cell–matrix interactions. *Int J Biochem Cell Biol*, 2013; 45(9): 1974-86.
- [7]. Ogawa T, Chijiwa T, Oda-Ueda N, Ohno M. Molecular diversity and accelerated evolution of C-type lectin-like proteins from snake venom. *Toxicon*, 2005; 45: 1-14.
- [8]. Kakanj M, Ghazi-Khansari M, Zare Mirakabadi A, Daraei B, Vatanpour H. Cytotoxic Effect of Iranian *Vipera lebetina* Snake Venom on HUVEC Cells. *Iran J Pharm Res*, 2015; 14: 109-14.
- [9]. Kazemi-Lomedasht F, Behdani M, Pooshang Bagheri K, Habibi Anbouhi M, Abolhassani M, Khanahmad H, Shahbazzadeh D, rzahoseini H. Expression and Purification of Functional Human Vascular Endothelial Growth Factor-A121; the Most Important Angiogenesis Factor. *Adv Pharm Bull*, 2014; 4(4): 323-28
- [10]. Baudin B, Bruneel A, Bosselut N, Vaubourdolle M. A protocol for isolation and culture of human umbilical vein endothelial cells. *Nat. Protoc*, 2007; 2(3): 481-85.
- [11]. Vyas VK, Brahmabhatt K, Bhatt H, Parmar U. Therapeutic potential of snake venom in cancer therapy: current perspectives. *Asian Pac J Trop Biomed*, 2013; 3(2): 156-62.
- [12]. Berridge MV, Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnol Annu Rev*, 2005; 11: 127-52.
- [13]. Rismanchian M, Khodaeian N, Bahramian L, Fathi M, Sadeghi-Aliabadi H. In-vitro comparison of cytotoxicity of two bioactive glasses in micropowder and nanopowder forms. *Iran J Pharm Res*, 2013; 12(3): 437-43.
- [14]. Bhavya J, Francois NN, More VS, More SS. Scorpion Toxin Polypeptides as Therapeutic Agents: An Overview. *Protein Pept Lett*, 2016; 23(9): 848-59.
- [15]. Boukhalfa-Abib H, Laraba-Djebari F. CcMP-II, a new hemorrhagic metalloproteinase from *Cerastes cerastes* snake venom: Purification, biochemical characterization and amino acid sequence analysis. *Comp Biochem Physiol C: Toxicol. Pharmacol*, 2015; 167: 65-73.
- [16]. Jebali J, Fakhfekh E, Morgen M, Srairi-Abid N, Majdoub H, Gargouri A, El Ayeb M, Luis J, Marrakchi N, Sarray S. Lebecin. a new C-type lectin like protein from *Macrovipera lebetina* venom with anti-tumor activity against the breast cancer cell line MDA-MB231. *Toxicon*, 2014; 86: 16-27.
- [17]. Sarray S, Berthet V, Calvete JJ, Secchi J, Marvaldi J, El-Ayeb M, Marrakchi N, Luis J. Lebectin, a novel C-type lectin from *Macrovipera lebetina* venom, inhibits integrin mediated adhesion, migration and invasion of human tumor cells. *Lab Invest*, 2004; 84(5): 573–81.

- [18]. Sarray S, Delamarre E, Marvaldi J, El Ayeb M, Marrakchi N, Luis J. Lebectin and lebecetin, two C-type lectins from snake venom inhibit alpha5 beta1 and alphaV-containing integrins. *Matrix Biol*, 2007; 26(4): 306–13.
- [19]. Ebrahim K, Vatanpour H, Zare A, Shirazi FH, Nakhjavani M. Anticancer Activity a of Caspian Cobra (*Naja naja oxiana*) snake Venom in Human Cancer Cell Lines Via Induction of Apoptosis. *Iran J Pharm Res*, 2016; 15: 101-12.
- [20]. Lecht S, Chiaverelli RA, Gerstenhaber J, Calvete JJ, Lazarovici P, Casewell NR, Harrison R, Lelkes PI, Marcinkiewicz C. Anti-angiogenic activities of snake venom CRISP isolated from *Echis carinatus sochureki*. *Biochim Biophys Acta*, 2015; 1850(6): 1169-79.
- [21]. Borkow G, Lomonte B, Gutiérrez JM, Ovidia M. Effect of various Viperidae and Crotalidae snake venoms on endothelial cells in vitro. *Toxicon*, 1994; 32(12): 1689-95.