

## Assessment of cytotoxicity of isolated fractions from the venom of Iranian viper, *Macrovipera lebetina* on Brain cancer cell line, U-87MG

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### ABSTRACT

Brain cancer is the second leading cause of death after leukemia for children. In this study, brain cancer cells (U-87MG) were cultured with 10 % FBS. The *Macrovipera lebetina* venom was purified by two methods of chromatography. Anti-adhesion and cytotoxic effects were investigated by trypan blue and MTT assay. Six fractions were obtained from gel filtration chromatography. Fraction N.5 had anti-adhesion and cytotoxic effects, it was selected for ion exchange chromatography. The effect of four fractions obtained from ion exchange chromatography on the brain cells and control cell was studied and fraction N.3 had the highest level of cytotoxicity. Anti-adhesion and fatal effects on the brain and control cells were estimated to be 26 and 14 %, respectively. In this study, the active fraction had a cytotoxic effect on the brain cancer cell line.

**Keywords:** *Macrovipera Lebetina*, chromatography, MTT assay, brain cancer

## INTRODUCTION

Brain cancer is one of the types of cancer in which malignant cancer cells arise in the brain tissue [1,2]. Gliomas are invasive cancers, spreading quietly throughout the brain [2]. Because of limited efficacy that current chemotherapeutic agents offer for cancer therapy, new agents and drugs with increased the anti-cancer potential are being researched. The venom secreted by poisonous glands. There is a biological source with pharmacological effects in some animals [3,4]. Over the past years so far, venom toxins from species such as snakes, scorpions, bees and wasps have been widely studied for their potential as an origin of bioactive molecules. Apart from their therapeutic applications, studies of animal venom toxins also prepare insights into the mechanisms of toxicity and since, the design of better treatment strategies [5]. Cytotoxicity effect of animal venoms caused inhibits angiogenesis and tumour growth. Investigations show that the inhibitory effect on tumour growth from animal venoms can be used in cancer treatment studies [6]. The first studies for anti-cancer effects from the snake venoms were extracted of *Vipera lebetina turnica* and it was caused to prevent apoptosis of ovarian cancer cells [7]. Since integrin in most cancer cells is misplaced in large numbers there and Viperidae family

contains compounds that cause cell apoptosis, the venoms of the Viperidae family can be nominated for developing new anti-cancer agents [8].

## MATERIALS AND METHODS

### *Venom preparation*

The venom of Iranian viper, *Macrovipera lebetina* was from the stock of the venom and biotherapeutics molecules lab, Biotechnology Department of biotechnology research center at Pasteur Institute of Iran, which was done by the usual milking method [9,10].

### *Gel Filtration Chromatography*

In this method, the Sephacryl S-100 HR-HiPrep column (GE, USA) was used to isolate the fractions and was equilibrated with 20 mM ammonium acetate buffer. The amount of 200 mg of crude venom of Iranian viper snake in 2 ml ammonium acetate (Merck, United States) 20 mM (pH=8) was dissolved, and the solution was centrifuged to remove particle (5 min, 13000 g) then, the amount of protein in the supernatant was measured and the ready to inject into the Fast protein liquid chromatography/FPLC (ÄKTApurifier 10- GE Healthcare, United States). Fractions were manually separated at 280 nm (flow rate: 1 ml/min).

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The fractions obtained from gel filtration chromatography were dried and lyophilized by Freeze Dryer (Martin Christ, Germany).

### ***Determination of the BCA method***

The protein concentrations of fractions were measured based on BCA protein assay (Intro bio company, south Korea) on microplates with bovine serum albumin (BSA) as a standard [11,12].

### ***SDS- PAGE electrophoresis to determine molecular weight fractions***

The fractional molecular weight was evaluated by the lamelli method [13]. The vertical tanks (Bio-Rad) were used in this method. Gels were stained with Coomassie Blue Method according to the manufacturer (GE Healthcare) recommendations.

### ***Fraction N.5 isolated from the venom of Iranian viper snake using ion exchange chromatography (anion exchange)***

For higher purification of fraction N.5 from gel filtration chromatography, 1 mg of fraction N.5 was injected to an FPLC with Mono Q column, 10 cm/1.6 cm (GE, USA). The flow rate was 1 ml/min. The absorption of fractions were measured at 280/214 nm). Then, the collected fractions were desalted by dialysis Falcon tubes (Millipore, USA) with cut off: 3 kDa. Then, the fractions were

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lyophilized and the protein concentrations were determined and electrophoreses.

### ***Cell Culture***

The human embryonic kidney 293 (HEK293) cell line and the glioma cell line (U87MG) were purchased from the Pasteur Institute of Iran. The cells were cultured in DMEM medium (Gibco, Ireland), penicillin-streptomycin 1 % (v/v), 10 % (v/v) Fetal Bovine Serum (FBS) at 37 °C, 5 % CO<sub>2</sub> and humidified atmosphere. Culture medium was replaced every 3 days [14,15].

### ***Anti-adhesion effect of fractions obtained from chromatography***

The anti-adhesion effect on Brain cancer cells was examined, such as the following steps:  $2 \times 10^4$  cells were transferred to each well of the microplate and control group. Different concentrations of each fraction were added to each well (6-0.37 μg/ml). Percentage cell viability was examined by using Trypan blue staining assay.

Viability was calculated by using the formula: [16,17]

$$\% \text{ viability} = (\text{live cell count} / \text{total cell count}) \times 100$$

### ***MTT assay***

The Cytotoxic effect of candidate fraction by using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) was performed [18]. The MTT assay is based on the conversion of MTT into purple formazan crystals by living cells, which determines mitochondrial activity. The crystal formazan dissolved in DMSO and it can be measured by ELISA Reader [19].

The Brain cancer cells (U-87MG) and control cells ( $2 \times 10^4$  cells/well) were seeded into a 96-well plate for adhesion. After 24 h the cells were incubated with a medium in the absence (control cells) or presence of different concentrations of Peak N.3 (6, 3, 1.5, 0.75, 0.37 and 0.18  $\mu\text{g/ml}$ ) at 37 °C and 5 % CO<sub>2</sub>. After 24 h the cells were incubated with MTT reagent (5 mg/ml, 20  $\mu\text{L/well}$ ) for 4 h and the formazan crystals were dissolved by 100  $\mu\text{L}$  of phosphate buffered saline (PBS). After 24 h the absorption of the sample and control groups were determined at 570 nm in a plate reader (BioTek Epoch, USA).

Percentage of cytotoxicity in the negative control group was 100 and was calculated by the following formula [18,19].

Cell toxicity %

$$= \left[ 1 - \frac{(OD \text{ of treated cells} - OD \text{ blank})}{OD \text{ control} - OD \text{ blank}} \right] \times 100$$

### *Statistic Analysis*

Results based on mean  $\pm$  SD (3 replications), using 16 SPSS statistical software have been reported. To review and statistical analysis of test data, student t-test was used. ( $p \leq 0.05$ )

## **RESULTS**

Snake venom from *Macrovipera Lebetina* was submitted to chromatography on a Sephacryl S-100 HR-HiPrep column. Six peaks collected by gel filtration chromatography. The chromatographic patterns are shown in figure 1.

The gel filtration fractions and crude venom from *Macrovipera Lebetina* were analyzed by SDS-PAGE. These observations indicate that the purified fractions are composed of a polypeptide chain (figure 2).

Proliferation and adhesion assay of Gel filtration fractions was examined by trypan blue. Fraction N.5 was active on the cell line (U-87MG) and selected for ion exchange chromatography (figure 3).

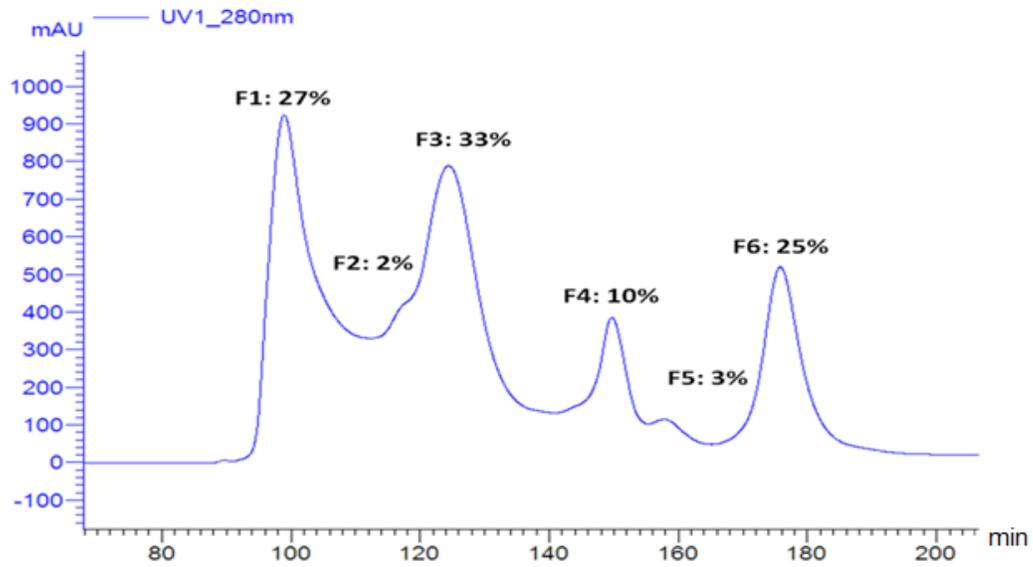


Figure 1. Chromatogram of gel filtration chromatography (S-100 column).

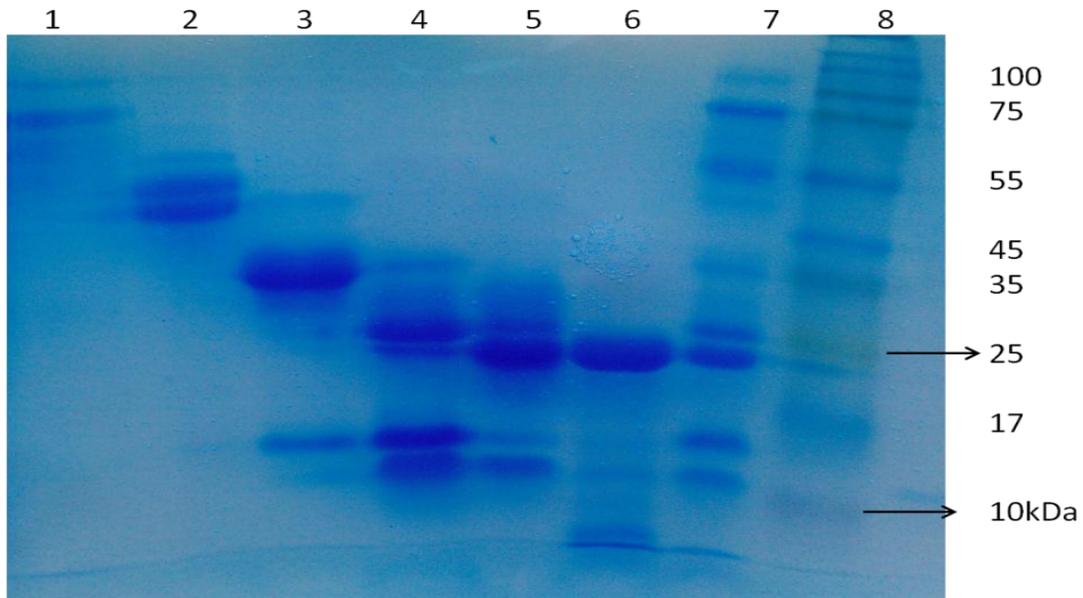
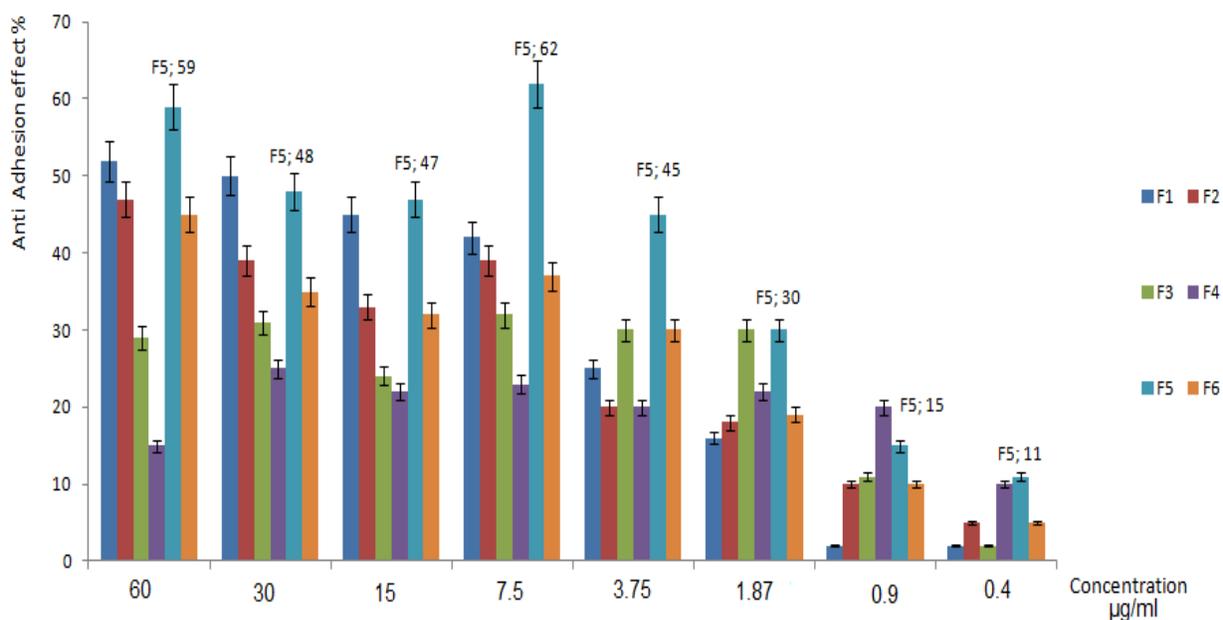


Figure 2. SDS-PAGE of gel filtration fractions & crude venom from *Macrovipera Lebetina*.



**Figure 3.** Evaluating and selecting the most powerful fraction of gel filtration chromatography (mean  $\pm$  SD of triplicates ( $p \leq 0.05$ )); Fraction N.5 was active on cell line (U-87MG) and selected for ion exchange chromatography.

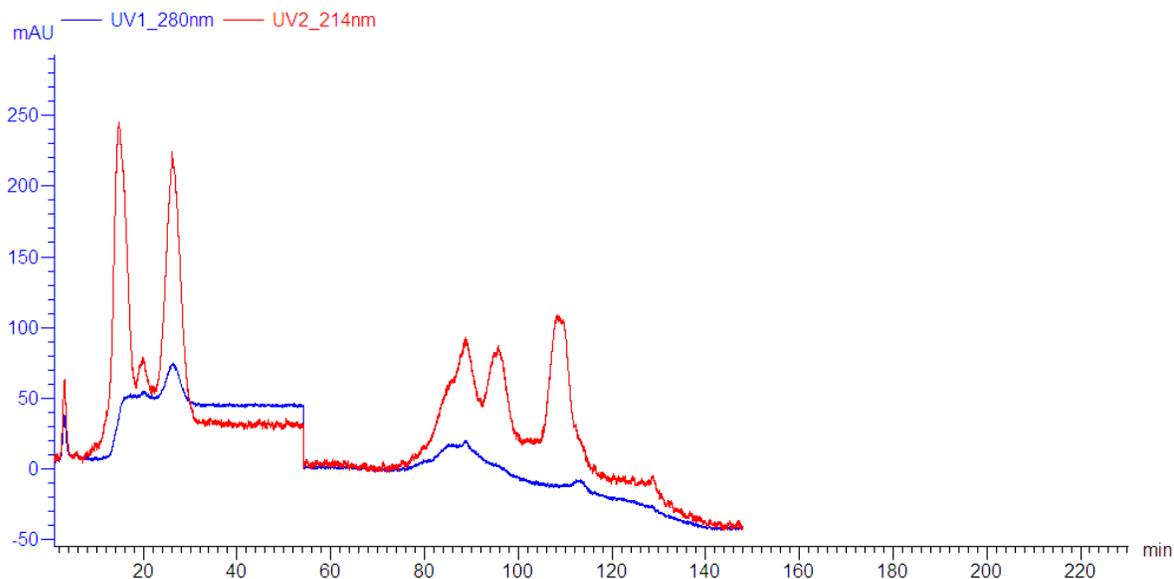
Fraction N.5 of Sephacryl S-100 column was pooled, lyophilized and dissolved in a small the volume of distilled water then dialyzed against a large volume of 25 mM Tris $\pm$  HCl, (pH=7.8), and loaded (200 mg of proteins were loaded each run) on an FPLC Mono-Q column (HR 5/5). Elution was achieved with a linear NaCl gradient. Four anionic and two cationic peaks showed at this stage and collected for anticancer activity (figure 4).

The cytotoxic activity is carried out by using the MTT assay. The effect of anionic and cationic fractions was calculated. The cytotoxic activity on U87-MG was

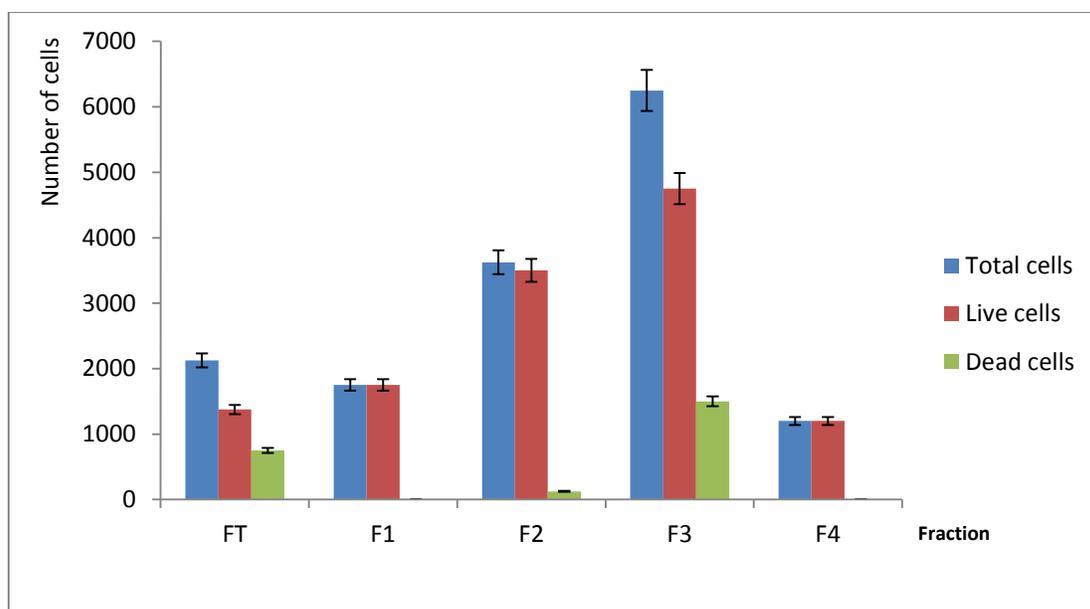
concentrated in peak 3 (anionic range), as shown in (figure 5).

The molecular weight of active fraction (N.3 Anionic Peak) determined by SDS-PAGE. This observation indicates that the purified fraction is composed of a single polypeptide chain (figure 6).

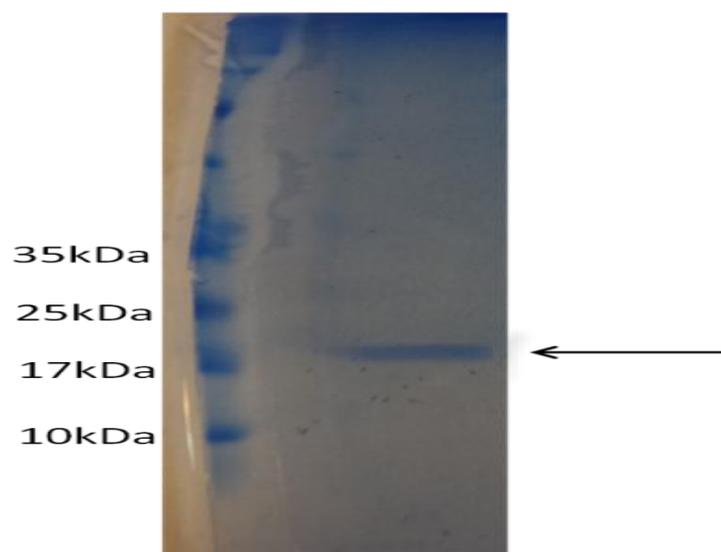
Results of MTT assay from an active fraction (peak 3) showed a significant effect on the U87-MG and HEK-293 cell lines (figure 7).



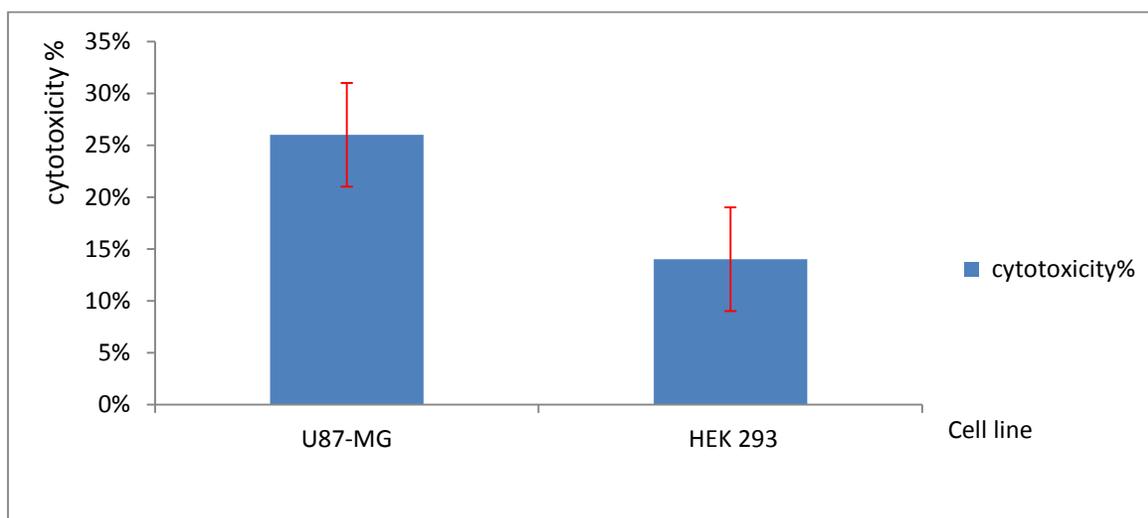
**Figure 4.** Chromatogram of ion exchange (Mono Q Column); four anionic and two cationic peaks showed at this stage and collected for anticancer activity.



**Figure 5.** Evaluating and selecting the most powerful anti-adhesion fraction of ion exchange chromatography fractions (mean  $\pm$  SD of triplicates ( $p \leq 0.05$ )).



**Figure 6.** SDS-PAGE of fraction N.3 (anionic peak).



**Figure 7.** Compare of cytotoxicity % by MTT assay on U87-MG & HEK-293 cell lines (mean ± SD of triplicates ( $p \leq 0.05$ )) ; Anti-adhesion and cytotoxicity effect on brain cancer cell lines and control cells  $26 \pm 0.8$  and  $14\% \pm 1$  , respectively in total were calculated.

## DISCUSSION

Cancer is an abnormal division of the cells of the body and this fragmentation extends

beyond genetic damage to DNA. This genetic damage affects the normal functioning of the cell, including apoptosis and DNA repair, and ultimately leads to the

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production of a tumour and also metastasizes to other parts of the body [1]. According to the NCI and the American Cancer Society, 23,000 people with brain cancer die each year, of which 13,000 die out of this 23,000 people [1,3].

Integrins are cell-surface proteins that contribute to the cellular attachment to the intracellular matrix. Integrin also acts as receptors that can send messages to cells and regulate cell membranes, cell migration, as well as cell cycle and inflammatory responses. Blocking of integrins usually inhibits cell migration and angiogenesis of the tumour [20].

Snake venom is a complex mixture of proteins and peptides that have broad biochemical and medicinal functions. Disintegrins are polypeptides that obtained from the venom of the family of snakes. Disintegrins blocks the selective action of the receptors [21,22]. The results of this study were based on *in vitro* tests. The results examined the effects of Iranian viper venom on U-87 MG cell line and normal cells in order to achieve an effective fraction of disintegrin (anti-adhesion) and cellular toxicity.

Maryam Kakanj and her colleagues in Shahid Beheshti University in 2015 evaluated the cytotoxicity of *Vipera Lebetina* crude venom by exposing the

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HUVECs. In this study by MTT assay revealed that the venom had a toxic effect (44.4 %) on the cells (HUVECs) at a concentration of 20 µg/µl.

However, in our study, using the MTT assay, the active fractions obtained from FPLC and ion exchange chromatography on the control cells and U87-MG cell line had significant toxicity. Also, in the study on HUVECs, morphological changes in cells were observed under a microscope, while we carried out did not see their changes [23].

Hye Lim Lee and his colleagues in Korea in 2015 found that venom of *Vipera lebetina turnica* could contain PRDX6. PRDX6 is a component of peroxidase and it's also known to stimulate the growth of lung cancer cells. PRDX6 has a site for bonding to a gene promoter (AP-1). This research was carried out on crude venom [24].

Also, in 2014, Pushpa Saranya Kollipara et al. investigated the anti-cancer effect of snake venom activated Natural killer cells (NK-92MI) in lung cancer cell lines. The effects of NK cells showed a significant inhibitory effect on cell growth and also prevented DNA binding and release of cytokines, which also prevented to Proliferation of cells and apoptosis, however, due to the lack of normal cell

controls, Cannot be as a perfect result. It is not possible to obtain a comprehensive result from its study for the purpose of performing the pharmacological studies [25]. In another study by John Michael Conlon and his colleagues in 2014, it was shown that toxicity of the Eastern green mamba venom on A549 cell line in Non-Small Cell Lung Cancer (NSCLC) has a significant effect on inducing apoptosis and inhibiting the growth of cancer cells, but it was not used by the normal control cell [26].

But the basis of our study was based on the fact that given the toxic effect from snake venom of *Macrovipera lebetina* on cancer cells [27].

Therefore, based on our research results, this hypothesis has proven that anti-cancer fractions derived from snake venom of *Macrovipera lebetina* have a lower toxicity effect on normal cells than cancer cells.

### CONCLUSION

The venom of *Macrovipera lebetina* may have anti-adhesion and anti-proliferative effects on brain cancer cells in low value. We have shown that the active fraction of *Macrovipera lebetina* venom is a possible anti-cancer agent with clinical potential.

### ACKNOWLEDGMENT

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