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Original Article

Evaluation of *in vivo* Lethality and *in vitro* Cytotoxic Effect of *Odontobuthus bidentatus* Scorpion Venom

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Abstract

The results of numerous studies have revealed that some deadly scorpion venoms are composed of various bioactive molecules that have significant cytotoxic effects on cancer cells. In this study, the in vivo lethality and cytotoxic effect of *Odontobuthus bidentatus* venom were evaluated in different cancer cell lines. Through MTT assay, the cytotoxic effects of *O. bidentatus* scorpion venom were analyzed on the MCF-7, A549, AGS, HepG2, and Ht-29 cancer cell lines and Hu02 normal cells. To this end, six venom fractions were obtained through a Sephadex G-50 column, and the cytotoxic effects of isolated fractions were evaluated on A549 lung cancer cells. The median lethal dose of *O. bidentatus* scorpion venom was determined at 0.73 mg/kg by intravenous administration of different venom doses in male BALB/c mice according to the Spearman-Karber method. The *O. bidentatus* scorpion whole venom had a significant cytotoxic effect on MCF-7, A549, and AGS cells. The treatment of A549 cells with various concentrations of fraction F1 showed that this fraction significantly induced growth inhibitory effect on the cells in a dose-dependent manner, compared to untreated cells.

Keywords: *Odontobuthus bidentatus* venom, Cytotoxic effects, LD_{50}, Cancer cells

1. Introduction

Scorpion venoms are composed of dozens of different components, such as serotonin and histamine, mucoproteins, nucleotides, protease inhibitors, histamine releasers, polypeptidyl compounds, small peptides (e.g., neurotoxins), and high molecular weight proteins (e.g., enzymes, which its composition is different from species to species) (1-3). The results of numerous investigations have exhibited that the venom components have biomedical applications and pharmacological functions (4, 5). Some of these pharmacologically active molecules are the potent natural sources for cancer treatment (6, 7).

Cancer is an important health problem and a leading cause of death globally (8). Because of the increasing incidence of severe adverse effects of the available treatment methods (chemotherapy, surgery, and radiotherapy), scientists have contributed to the development of novel venom-based drugs for cancer treatment (9, 10). In 1950, it had been reported that the venom of *Buthus martensii* Karsch (a Chinese scorpion) has antitumor properties (7). Since then, the findings of several studies have revealed anticancer activity of various scorpions' venoms, which induce their antitumor effects via different mechanisms, such as decreasing the motility of the cells and colony formation, arresting the cell cycle on G0/G1, increasing

caspase activity, blocking ion channels, suppressing proliferation and differentiation, and promoting the apoptosis (11-13).

The digger scorpion *Odontobuthus bidentatus* belong to the family of Buthidae and is found in southwest regions and Zagros Mountains of Iran and the east of Iraq (14). Its venom composition and biological activities have been poorly studied. Recently, it has been shown that the *O. bidentatus* venom induces a cytotoxic effect on hepatocarcinoma cells using mitochondria-mediated apoptosis (15). The current parallel study was conducted to investigate the cytotoxic effects of *O. bidentatus* venom on various cancerous cell lines and normal cells.

2. Materials and Methods

2.1. Chemicals

Sephadex® G-50 Medium, Thimerosal, phosphate-buffered saline components (sodium chloride, potassium chloride, disodium phosphate, Monopotassium phosphate), Bradford reagent, dimethylsulfoxide, penicillin-Streptomycin (Pen-Strep), Trypsin Solution, L-Glutamine, and 3-(4, 5 dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Aldrich (USA). Dulbecco's Modified Eagle Medium (DMEM), DMEM/F12 (1:1), Ham's F12 medium, and fetal bovine serum (FBS) were purchased from Gibco (USA).

2.2. Venom Preparation

The venom of *O. bidentatus* scorpion was obtained from Razi Vaccine and Serum Research Institute (RVSRI), Karaj, Iran, reconstituted in sterile double-distilled water at 8 mg/ml, centrifuged at 10,000 × g to remove insoluble debris. The supernatant was filtered using a nitrocellulose filter (Millipore 0.45 µm) and its protein content was measured by the Bradford method (16).

2.3. Determination of the Median Lethal Dose

Female BALB/c mice weighing 18-20 g were used (n=4 for each group) for the determination of the median lethal dose (LD_{50}) value of *O. bidentatus* scorpion venom according to the Spearman-Karber method (17, 18). An amount of 0.2 ml of normal saline solution (control group) and different venom doses were administrated to mice through intravenous (IV) injection. Various dilutions of venom were selected with the dilution factor 1.25 to cover the entire mortality range (from 0-100%).

2.4. Cell Culture

The cell lines MCF-7 (breast cancer cell line), A549 (human lung adenocarcinoma cell line), AGS (human gastric cancer cell line), HepG2 (human liver cancer cell line), Hu02 (normal human skin fibroblast), and Ht-29 (human colorectal adenocarcinoma cell line) were purchased from Iranian Biological Resource Center, Iran. The cells A549, MCF-7, and Hep-G2 were cultured in DMEM/F12 (1:1) medium supplemented with 10% heat-inactivated FBS, Pen-Strep (1%), L-gluthamine (2 mM). Cells Ht-29 and Hu02 were cultured on DMEM complete medium and AGS cell line was cultured at Ham's F12 medium with supplements. All cells were grown at 37°C and 5% CO_2 in a humidified incubator.

2.5. Cell Viability Assay

The MTT assay was used to assess the cell viability of the cells incubated with various concentrations of the whole venom (1, 3, 5, 10, and 20 µg). The detailed instruction on reagent preparation and assay protocol for the MTT assay were explained previously (19). All cell assays were performed with three replicates and the percentage of viable cells was determined by the following equation:

$$\frac{\text{(average of absorbance of treatment cells) - (average of absorbance of medium blank)}}{\text{(average of absorbance of control cells) - (average of absorbance of medium blank)}} \times 100$$

2.6. Size-Exclusion Chromatography

In order to investigate the effects of *O. bidentatus* venom fractions on cell viability, Sephadex G-50 was used to fractionate the whole venom. Sephadex G-50 was packed in a 1 × 80 cm column according to the manufacturer's instructions. The venom of *O.*

bidentatus (25 mg/3.1 ml), equivalent to approximately 5% of total column volume, was applied to the gel. The use of 2 volumes of running buffer (0.05 M sodium phosphate, 0.15 M NaCl, pH 7.0) with the flow rate of 0.15 ml/min led to the elution of 61 sample tubes from the gel filtration column. The volume of each sample was about 0.5 ml and their protein contents were detected by monitoring their ultraviolet at 280 nm. The sample tubes under each peak were pooled, freeze-dried, weighted, and reconstituted in DMEM medium at 1 mg/ml.

2.7. Data Analysis

Values were presented as means ± standard deviation (n=3). The independent sample t-test was used to compare the difference between the experiment and control groups. Statistical significance was indicated as follows: $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$ (A *P*-value of ≤ 0.05 was statistically significant). Median lethal dose and half-maximal inhibitory concentration (IC_{50}) were estimated using the Spearman-Karber method and dose-response curve, respectively.

3. Results

3.1. Lethal Toxicity of *Odontobuthus bidentatus* Scorpion Venom

A total of 6 doses of *O. bidentatus* scorpion venom with the dilution factor 1.25 (10, 12.5, 15.5, 19, 24, and 30 µg/ml) were administered to mice with 18-20 g body weight. The lethality of venom IV injected in male BALB/c mice was estimated after 24 h. According to the Spearman-Karber method, the LD_{50} of *O. bidentatus* scorpion venom was calculated as 0.73 mg/kg.

3.2. in vitro Cytotoxicity of *Odontobuthus bidentatus* Scorpion Venom

The cytotoxicity of *O. bidentatus* scorpion venom was evaluated in six types of cell lines using MTT assay, namely MCF-7, A549, AGS, Hep-H2, Ht-29, and Hu02. The treatment of the cells with various concentrations of *O. bidentatus* scorpion venom at 1, 3, 5, 10, and 20 µg/ml significantly decreased the viability of MCF-7, A549, and AGS cell lines in a dose-dependent manner. After 24 h of exposure, the cells MCF-7 and A549 displayed higher sensitivity to *O. bidentatus* scorpion venom (IC_{50}=5.74 µg/ml and IC_{50}=6.73 µg/ml, respectively) than AGS cells (IC_{50}=10.18 µg/ml). The IC_{50} for cells Hep-H2 and Ht-29 were obtained at less than 20 µg/ml (Figure 1). It was revealed that Hu02 cells, which are normal fibroblast cells, were the most resistant cells to *O. bidentatus* scorpion venom with almost 100% viability for different concentrations of venom.

3.3. Cytotoxicity Effects of Different Fractions of *Odontobuthus bidentatus* Scorpion Venom

As indicated in figure 2, a total of six main peaks were obtained indicating six venom fractions (F1, F2, F3, F4, F5, and F6). According to table 1, the aliquots related to each venom fraction were pooled, lyophilized, and dissolved in a DMEM medium at the final concentration of 1 mg/ml. A549 cells underwent treatment with 10 µg/ml of each venom fraction in parallel with 10 µg/ml of the whole venom and untreated cells in the MTT assay (Figure 3a). Fraction 1 (F1) exerted a considerable decrease in the cell viability (about 20% of untreated cells). Fraction 2 (F2) was less toxic to A549 cells with about 50% viability, compared to F1. Fractions F3, F4, F5, and F6 with a viability rate of more than 90% were not considered to be cytotoxic fractions for the cells. A549 cells were subjected to treatment with 1, 3, 5, 10, and 20 µg/ml fraction F1 and their cell viability were 90.5%, 71%, 60%, 25.5%, and 16.5% following the treatment, in comparison to untreated cells (Figure 3b).

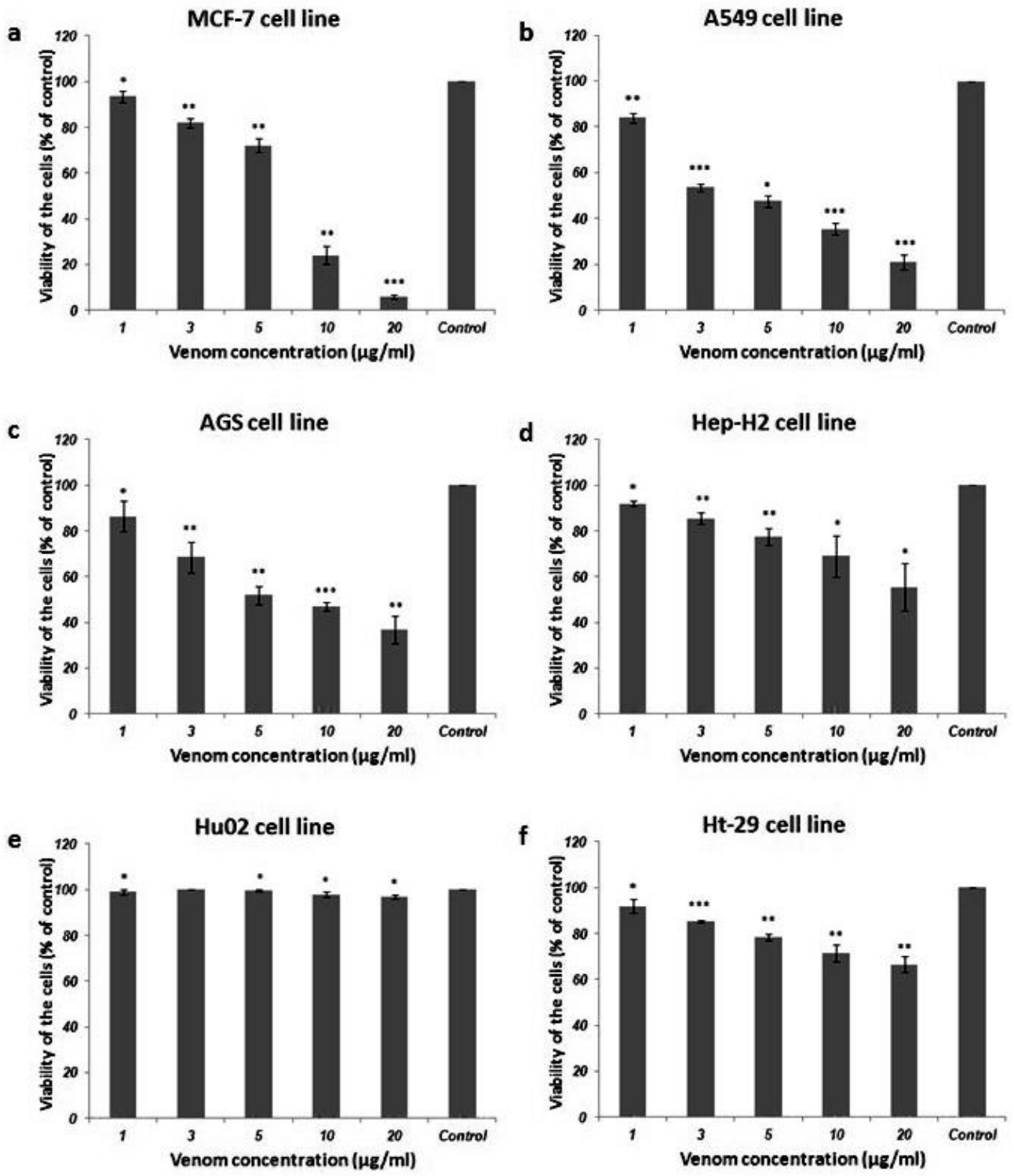

Figure 1. Cytotoxicity effect of *Odontobuthus bidentatus* scorpion venom on a) MCF-7 cells, b) A549 cells, c) AGS cells, d) Hep-H2 cells, e) Hu02 cells, and f) Ht-29 cells

All data were presented as mean ± S.D (n=3). **P*<0.05; ***P*<0.01; ****P*<0.001

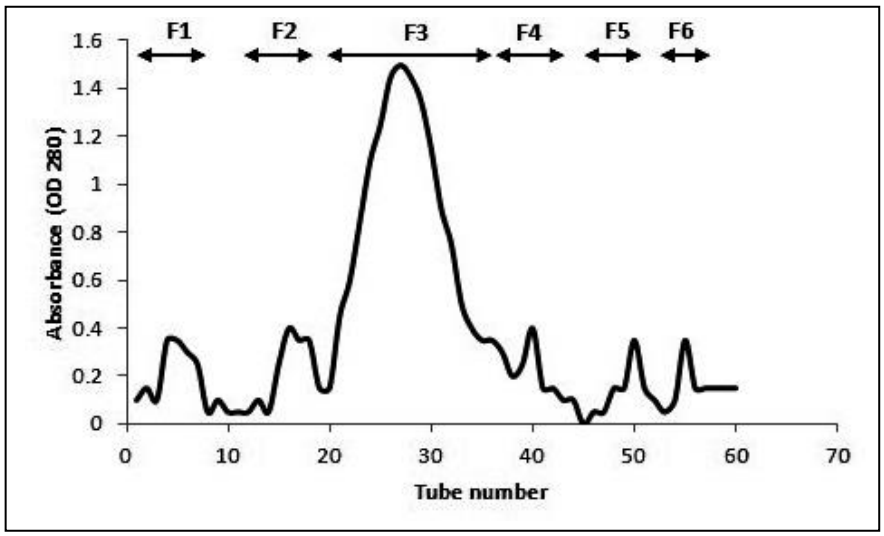

Figure 2. Gel filtration chromatography of *Odontobuthus bidentatus* scorpion venom on Sephadex G-50 Six fractions related to main peaks were numbered F1 to F6.

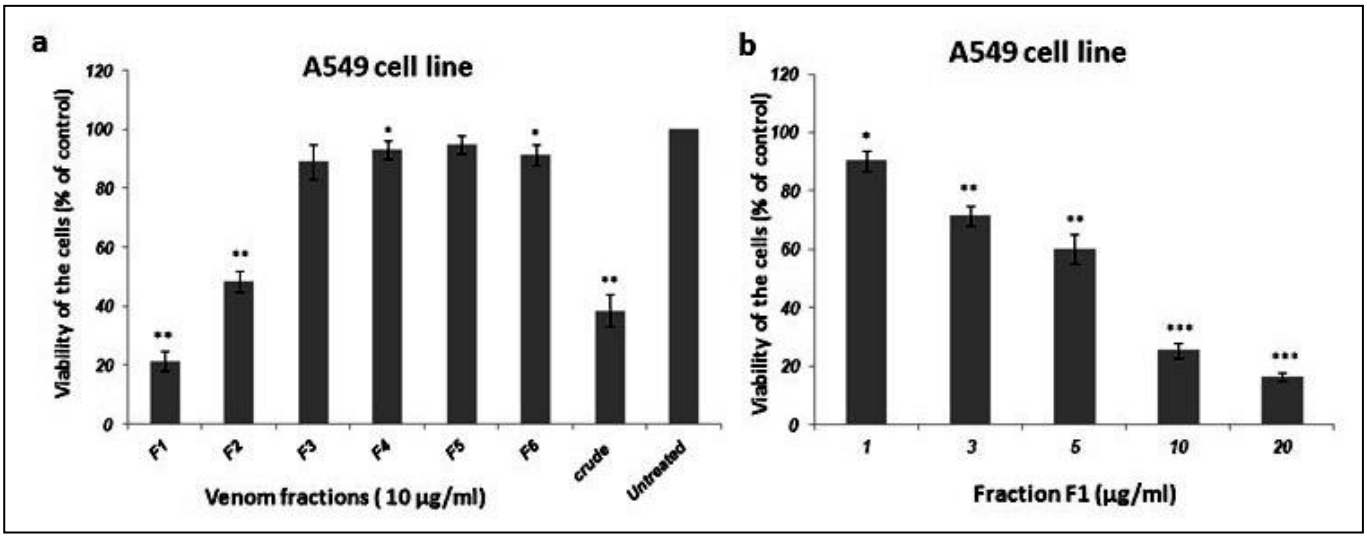

Figure 3. Cytotoxicity effect of *Odontobuthus bidentatus* scorpion venom fractions on A549 cells; a) the viability assay for the cells treated with 10 µg/ml of six venom fractions and b) the viability assay of the cells for different concentrations of venom fraction 1 (F1) All data were presented as mean ± S.D (n=3). **P*<0.05; ***P*<0.01; ****P*<0.001

Table 1. Protein content and the relative yield of the protein in each peak obtained from *Odontobuthus bidentatus* scorpion venom fractionation using Sephadex G-50

Peak	F1	F2	F3	F4	F5	F6
Sample tubes	2-8	12-19	20-37	38-45	48-51	54-56
Amount of protein in each venom fraction (mg)	1.2	1.8	9.2	2.2	1.7	0.7
Relative yield of the protein in each fraction obtained from whole venom (%w/w)	4.8	7.2	36.8	8.8	6.8	2.8

4. Discussion

Odontobuthus bidentatus scorpion from the Buthidae family is a digger scorpion that is a habitant of stone areas in the southwest and Zagros regions of Iran (14). The LD_{50} of *O. bidentatus* venom for mice was determined to be 0.73 mg/kg through the IV route. In comparison to the LD_{50} of medically important scorpions of Iran (used for antivenom preparation) from Buthidae family species, its LD_{50} is higher than those of *Odontobuthus doriae* venom (LD_{50}=0.19 mg/kg) (20, 21) and *Androctonus crassicauda* venom (LD_{50}=0.4 mg/kg) (22) and lower than those of *Hottentotta saulcyi* venom (LD_{50}=1.01 mg/kg) (20), *Mesobuthus eupeus* venom (LD_{50}=1.45 mg/kg), and *Hottentotta schach* venom (LD_{50}=3.36-4.2 mg/kg). Since scorpions with an LD_{50} of less than 1.5 mg/kg are considered to be dangerous for humans (23, 24), *O. bidentatus* venom with LD_{50}=0.73 mg/kg is a medical threat to human health. However, its danger has been ignored because of its distribution in mountainous areas, which are not high-risk areas for humans.

Nevertheless, the life-threatening venoms sometimes turn into life-saving therapeutics. There are numerous preclinical investigations on the anticancer effect of scorpion whole venom and some purified proteins and peptides (7, 25, 26). There are various promising medication candidates of scorpion venom that have anticancer activities, such as the anticancer effects of the whole venom of *Buthus martensii* Karsch on human glioma, lymphoma, breast cancer, and hepatoma cells (7, 27); the whole venom of *Androctonus amoreuxi* on human breast cancer cells (28); the whole venom of *Androctonus crassicauda* on human breast cancer, ileocecal adenocarcinoma, and colorectal carcinoma cells (29); Bengalin protein of *Heterometrus bengalensis* Koch scorpion on human leukemic cells (13, 30). Recently, it has been observed that the *O. bidentatus* scorpion whole venom induces apoptosis effects via the mitochondria pathway on liver hepatocellular cells (HepG2 cells) (15). In this study, using the MTT assay, it was shown that *O. bidentatus* scorpion whole venom had a significant cytotoxic effect on MCF-7 (breast cancer cell line), A549 (human lung adenocarcinoma cell line), and AGS (human gastric cancer cell line) cells in a dose-dependent manner and moderate toxicity toward HepG2 (human liver cancer cell line) and Ht-29 (human colorectal adenocarcinoma cell line) cells. The treatment of Hu02 (normal human skin fibroblast) cells even with a high concentration of *O. bidentatus* scorpion whole venom failed to induce considerable changes in the viability of the cells. These results suggested that *O. bidentatus* scorpion whole venom had an anticancer activity on MCF-7, A549, and AGS cancer cells.

In the next step, the whole venom was fractionated using gel filtration chromatography and the potential cytotoxicity of the six venom fractions was evaluated on A549 cells using MTT assay. The fraction F1, which is the first fraction containing the content of the tubes of numbers 2 to 8, with a concentration of 10 µg/ml, induced up to 78.5% cell growth inhibition, in comparison to untreated A549 cells. The cell viability in the A549 cells treated with this fraction was about 21.5% of untreated cells. However, the survival rate of the cells after treatment with 10 µg/ml whole venom was about 38.5% of control. The low percentage of viable cells receiving treatment with Fraction 1 was due to the presence of a higher amount of proteins with anticancer activities in this fraction. Fraction F2, containing the content of the tubes of numbers 12 to 19, also inhibited the growth of A549 cells up to 50%. Fractions F3, F4, F5, and F6 did not significantly alter the cell viability, comparing to untreated cells.

The results of studies conducted on the dose-response curve showed that the fraction F1 induced higher inhibitory effects on the A549 cells in a dose-dependent manner. Accordingly, the treatment of the A549 cells with 20 µg/ml of venom fraction F1 led to the cell death of up to 83.5% of the untreated cells. These findings have indicated that the cytotoxic properties of *O. bidentatus* scorpion whole venom are related to fraction F1. Since in gel filtration chromatography, the large protein is eluted first from the column, it is suggested that fraction F1 is a high molecular weight

protein rather than a small peptide. However, this was a preliminary study to find out the anticancer properties of *O. bidentatus* scorpion venom, and further studies are needed to evaluate the nature of the proteins in fraction F1 and deep analysis to investigate the potential anticancer properties of the proteins in this fraction.

Authors' Contribution

Z. S. N. analyzed and interpreted the biochemical data regarding the cytotoxicity effects of the venom and prepared venom fractions from gel filtration chromatography. M. S. performed the in vitro cytotoxicity assays. H. R. G. performed the LD50 test for scorpion venom. All the authors read and approved the final manuscript.

Ethics

All studies were performed in compliance with the rules of the animal ethics of the Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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