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An in vitro study on cytotoxic effects of *Androctonus crassicauda* Scorpion Venom on K562 Cell Line.

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ABSTRACT

The objective of the present study was to measure the ability of *Androctonus crassicauda* scorpion venom on inhibition of cell proliferation in K562 cell line derived from human Chronic Myeloid Leukemia. After calculating protein concentration of venom by Bradford method, K562 cells were treated with venom of this scorpion and after 24h incubation, MTT assay was performed and the obtained results were used for calculating IC50 value. In this study, the estimated value of IC50 was approximately equal to 111.46 µg/ml.
Keywords: *Androctonus crassicauda*, Scorpion venom, K562 Cell Line, MTT, IC50

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INTRODUCTION

Nowadays, cancer is considered as one of the major problems in the world. The incidence of cancer and its associated mortality rates are increasing as the World Health Organization predicts that 21. 4 million people will be diagnosed with cancer annually worldwide by 2030. [1]

In 2014, International Agency for Research on Cancer recognized more than 100 chemical, physical and biological carcinogens. Development of effective anti-cancer treatment strategies has had a slow pace. The ancients believed that there was no useful treatment for cancer and intervention to treat cancer might be more harmful than no treatment. However, surgery is a preliminary therapy for cancer and there have been major advances in cancer surgery since early 20th century. Hormone therapy was another treatment that was discovered in 19th century and provided an important modern method against breast cancer. [2, 3]

After discovery of X-ray by Roentgen, radiotherapy was immediately used for diagnosis and treatment and was recognized as an essential therapy for cure or relief of cancer patients. [2, 4]

The use of chemotherapy in cancer treatment began in early 20th century. First, Paul Ehrlich used chemicals to treat this disease [5] and finally, this method overtook other methods of cancer treatment, including surgery and radiotherapy. Other therapeutic methods against cancer are immunotherapy and targeted therapy. [2]

Despite the use of several therapeutic approaches against cancer, the efficacy of these treatments is not favorable and they are associated with significant side effects such as nausea, vomiting, loss of appetite, weight and hair loss. Therefore, there is great demand for effective treatments with minimal side effects against cancer. [6]

Accordingly, several natural substances produced by animals, plants and bacteria have been employed for development of new treatments for diseases such as thrombosis, AIDS as well as cancer. [7]

In fact, Clardy and Walsh in 2004 showed that 23% of new drugs approved by FDA were natural-product-derived molecules. [8] Venom-producing animals are usually known for their mortal effects. Their venom is a complex of toxins with different physiological activities that lead to mild symptoms, including dermatitis and allergic reactions, or severe symptoms such as hemorrhage and respiratory arrest. In addition to these negative effects, the venom is a rich source of pharmacologically active substances that can be used as a subject for research of new molecules for diagnosis and treatment of some diseases. Anti-cancer therapy is a main reason for using animal's venom, which induces an anticancer role by affecting cell proliferation, permeabilization of cell membrane, angiogenesis and finally cell death. Venomous arthropods like scorpions, bees, wasps, spiders, ants and caterpillars are among the animals that generate active biomolecules. [7]

Scorpion is one of the oldest creatures that has lived on earth more than 400 million years and is widely found all over the world. More than 1500 species of scorpion have been characterized to date. [9]

The use of scorpion venom has been prevalent in traditional medicine for thousands of years, especially in Asia and Africa. Scorpion venoms contain a mixture of peptides, enzymes, mucoproteins, free amino acids, nucleotides, lipids and amines. [10]

The venom has extensive pharmacological activities including antibacterial, antifungal, antimalarial, antiviral and anticancer activities and its antitumor activity has been observed both in cell lines and animal models. [11]

In a study by Gina D'Suze et al in 2010, two new peptides called neopladine 1 and neopladine 2 were isolated from *Tityus discrepans* scorpion venom, both of which induced apoptotic effect on human breast carcinoma cells but had an inconsiderable effect on non-malignant monkey kidney cells. [12]

In another research in 2010, a new protein called Bengalin was extracted by Gupta from Indian black scorpion venom and exhibited antiproliferative and apoptotic effects against U937 and K562 human leukemic

cells. Bengalin could inhibit the growth of U937 and K562 cell lines with no significant effect on normal cells at IC50 values of 3.7 µg/ml and 4.1 µg/ml, respectively. [13]

Li Qinjing et al in 2015 focused on the effect of *Heterometrus liangi* scorpion venom on proliferation of human esophageal cancer cells. Cell growth inhibition was assessed by MTT test within 24h and 48h and IC50 values were estimated 50 µg/ml for 24h and 34.5 µg/ml for 48h. [14]

A new study in 2016 was performed by Mohamed L. Salem et al. They evaluated the anti- tumor efficacy of *Androctonus amoreuxi* scorpion venom. In vitro MCF-7 cell line was treated with venom and its IC50 was calculated. [15]

Androctonus crassicauda is one of the most dangerous scorpions medically and the second dangerous scorpion in Iran, which is widely found all over the world. The venom of this scorpion includes neurotoxins with a high affinity for sodium channels in nerve and muscle cells. *A.crassicauda* causes severe pain, hyperemia, edema, disturbance of autonomic central nervous system and muscle function, cardiac involvement, myocarditis, convulsions and death. [16-18]

While epidemiological and clinical characterizations of *A. crassicauda* are well documented by many studies, few studies have been conducted on therapeutic effect of the venom against diseases. Therefore, in vitro cytotoxic effect of *A. crassicauda* venom on K562 cell line was evaluated in this study.

MATERIALS AND METHODS

Scorpion collection and venom preparation

More than one thousand scorpions were collected from different parts of Khuzestan Province using UV light technique overnight and brought to the animal breeding center of Ahvaz Jundishapur University of Medical Sciences. In the laboratory, *Androctonus crassicauda* species were detected under stereomicroscope. Then, crude venom of this scorpion was obtained by electrical stimulation of telson. The extracted venom was centrifuged at 14000 RPM for 15 min at 4˚C. The supernatant was separated from mucus and immediately freeze dried. This lyophilized venom was stored under refrigeration conditions and was dissolved in distilled water on the day of experiment.

Evaluation of protein concentration

Bradford assay was employed to measure the protein concentration. First, serial dilutions were prepared from the standard protein (BSA) and then the absorbance was read at 595 nm by spectrophotometer and finally the protein concentration of *A. crassicauda* venom was calculated.

Cell culture

K562 cell line was purchased from Pasteur Institute, Tehran, Iran. This cell line is derived from patients with Chronic Myelogenous Leukemia (CML) and is composed of undifferentiated granulocytes that are rounded and suspended in culture medium. The cells were cultured in RPMI-1640 (Biosera, UK) with 10% heat inactivated fetal bovine serum (Gibco, USA), 100 units/ml penicillin and 100µg/ml streptomycin (Gibco, USA) and then were incubated in a humidified incubator with a temperature of 37˚C and 5% CO2.

Cell growth inhibition

Measurement of cell viability and metabolic activity is the basis of a large number of in vitro assays, including MTT. MTT is a colorimetric assay that measures the cell proliferation rate or cytostatic activity. 5×10^4 cells/well of K562 cells were seeded in a 96-well tissue culture plate. The cells were treated with increasing concentrations of *A. crassicauda* venom from 40 to 160 µg/ml for 24h and 3 [4-dimethylthiazol-2-yl] -2-5-diphenyl tetrazolium bromide (MTT) (Sigma, USA) assay was performed. After incubation time, 20 µL MTT reagent as well as 180 µL RPMI-1640 medium was added to both untreated control cells (with culture medium but without scorpion venom) and treated cells and they were again incubated for 4h. Eventually, insoluble formazan crystals were dissolved in DMSO (Sigma, USA) and a purple color was produced. The absorbance was

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read at 570 nm in a micro-plate reader. The percentage of inhibition for each concentration was calculated using the following formula:

$$\% \text{ Viability} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

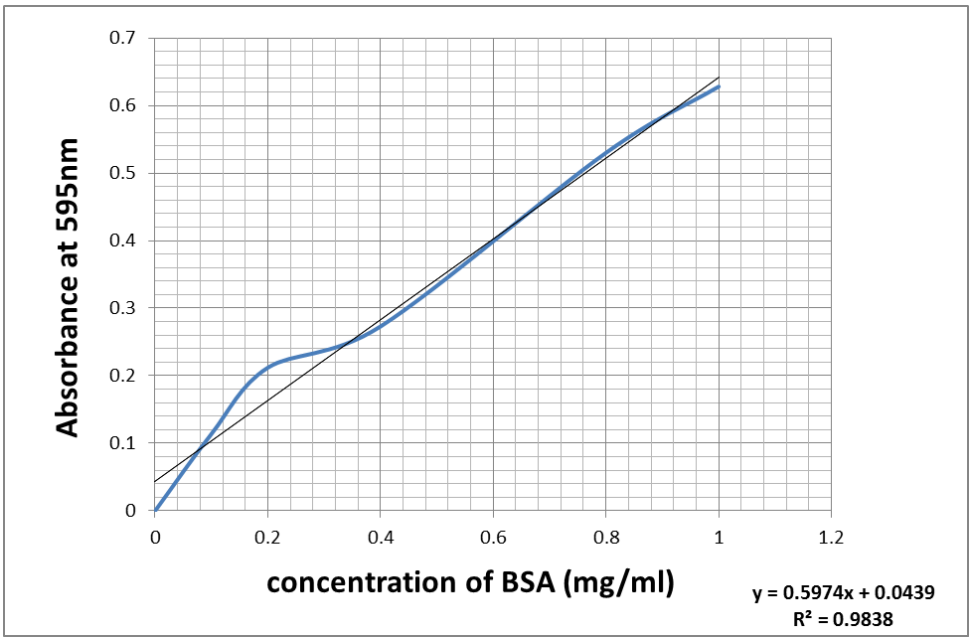
% Cytotoxicity = 100 - % Viability

Results obtained from this formula were used in Microsoft Excel to calculate the IC50 value of *A.crassicauda* venom. Also, ANOVA test was performed to determine the significance level.

RESULTS

Protein concentration of *A. crassicauda* venom

The protein concentration in *A. crassicauda* venom was determined by Bradford assay. OD for venom sample was 0.420 and after insertion in relevant equation, the numerical value of protein concentration of venom was calculated 0.63 mg/ml (Graph 1).



Graph 1: In this graph, the absorbance of various concentrations of BSA at 595nm is shown that was used to calculate the amount of protein in *A. crassicauda* venom.

Cell growth assay and MTT assay

In this study, the growth of K562 cells was inhibited at 40-160 µg/ml concentration of *A. crassicauda* venom. The results obtained from this research proved that the viability of K562 cells was decreased after treatment with venom. In other words, growth inhibition of K562 cells was enhanced with increasing venom concentration, which showed a dose dependent trend. The results of MTT assay were used to determine IC50 value, i.e. the venom concentration that caused 50% reduction of K562 cells. The calculated IC50 value after 24h incubation of K562 cell lines with *A. crassicauda* venom was 111.46 µg/ml. Furthermore, ANOVA test results confirmed significant increase of dose dependent growth inhibition (Graph 2).

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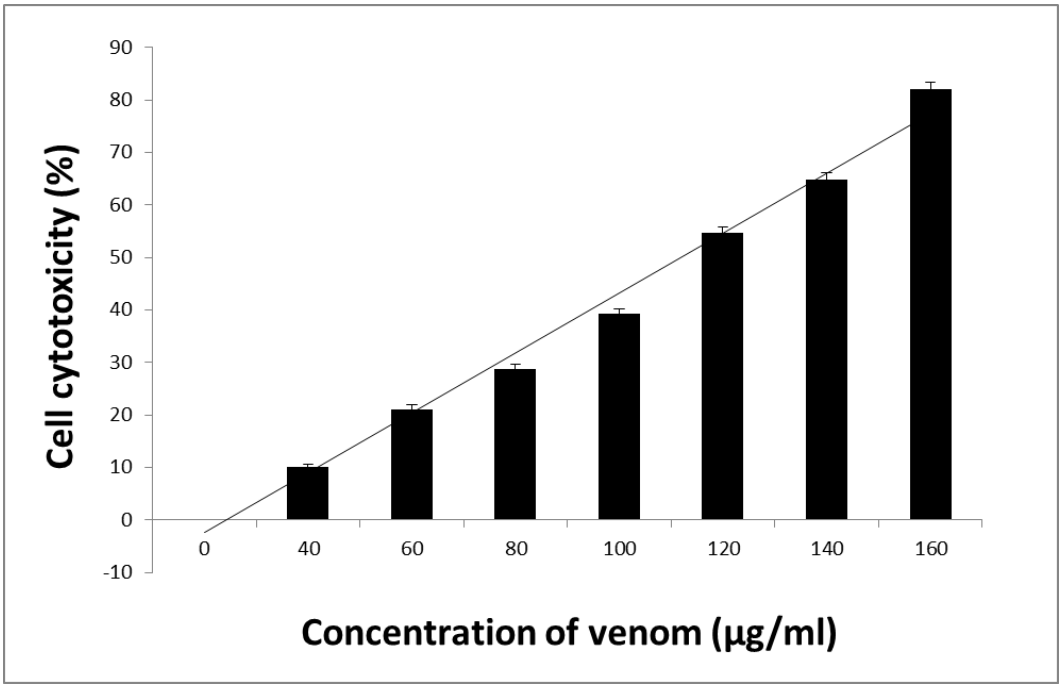

Graph 2: Cytotoxic effect of different concentrations of *A. crassicauda* venom on K562 cell line after 24h exposure (MTT). Results are shown as mean±SD, P<0.001 compared to the control cells, with using one-way ANOVA test.

DISCUSSION

In this study, the effect of *A. crassicauda* scorpion venom on cytotoxicity induction in K562 cell line was examined. The protein concentration of this venom and its IC50 value was calculated 0.63 mg/ml and 111.46 µg/ml, respectively.

The results obtained from this research may have numerical differences with other studies in this field. The source of these differences is due to the variation in composition and quality of venom, which is due to different geographic locations, stimulation time and the voltage used to extract and collect venom. Venom composition can be a reflection of its performance and in fact, the diversity and complexity of the venom can lead to large variation in cytotoxic property of venom. Also, the cell line type, incubation time and experimental conditions may be involved. [19-21]

In Alexis Díaz-García study in 2013, the effect of venom isolated from Cuban scorpion *Rhopalurus junceus* was investigated against a panel of human cancer cell lines (A549, Hep-2, Hela, K562, U937, etc.) and MTT results were checked after 72h incubation. The observations showed that *R.junceus* venom had no cytotoxic effect on K562 cells, whereas in the present study, it was demonstrated that *A. crassicauda* venom causes significant reduction in the viability of this cell line in comparison to control cells only after 24h incubation. [22]

Gupta et al in 2006 examined the anti-proliferative effect of Indian black scorpion venom *(Heterometrus bengalensis* Koch) on K562 and U937 cell lines and obtained the IC50 value of 88.3 µg/ml for K562 after 48h incubation, which was close to IC50 of the present study. In addition, *H. bengalensis* venom induced apoptosis in this cell line. [23]

Another study was performed in 2010 by the same researcher. This time, the effect of a component of Indian black scorpion venom called Bengalin was tested and IC50 value for K562 cell line was reduced to 4.1 µg/ml. In fact, it showed better performance than the total venom of this scorpion. [13]

In the research of Jamil Zargan et al in 2011, the effect of *A. crassicauda* venom was evaluated on SH-SY5Y (human neuroblastoma) and MCF-7 (breast cancer) non- leukemic cell lines. After MTT assay, they observed that the cytotoxic effect of venom was not significant in low doses while in higher doses, the growth

of these two cell lines was significantly inhibited in comparison to control cells. IC50 value for SH-SY5Y and MCF-7 was 207.7 µg/ml and 269 µg/ml, respectively. [24]

Acra 3 peptide is a component purified from A.crassicauda crude venom. This isolated peptide was determined to evaluate the cytotoxicity of mouse brain tumor cell line (BC3H1). Results showed that this extracted component had very strong effect on the mentioned cell line and a dose dependent decrease was observed in viability after 48h incubation since the IC50 value was 5 µg/ml. [25]

Our study and similar studies confirmed the cytotoxic nature of A.crassicauda venom on some cancer cell lines. According to the above-mentioned studies, the peptides isolated from venom had better effect on cell lines as IC50 value of them was lower than the whole venom. The venom from the studied scorpion was neurotoxic and it seemed to be more effective upon cell lines of nervous origin like SH-SY5Y and BC3H1. However, the results obtained from our study showed that A.crassicauda had a dose dependent cytotoxic effect on K562 cell line, which was not a cell line of nervous origin. Anyway, ability of scorpion venom to inhibit the growth of cancer cells in the above studies confirmed our results in inhibition of K562 cell line.

CONCLUSION

The results of this study proved that *A.crassicauda* venom induces growth inhibition in K562 cell line. According to other studies as well as the current study, which demonstrated the cytotoxic effect of natural toxins on cancer cells, apoptotic effects induced by *A.crassicauda* in K562 cell line will be dealt with in our future research, which is hoped to be a step in the improvement of cancer treatments using natural products.

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