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# Tarantula cubensis venom (Theranekron®) selectively destroys human cancer cells via activating caspase-3-mediated apoptosis

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## ABSTRACT

**Background:** *Tarantula cubensis* venom (*Theranekron®*) is used as a homeopathic medicine which has shown anti-tumor effects in veterinary medicine. The aim of this study was to assess effect of *Tarantula cubensis* venom on apoptotic cell death of human cancer cell lines. **Methods:** HEK293, MCF7 and HN5 cell lines were used. The cells were treated with different concentrations of alcoholic extract of *Tarantula cubensis* (*Theranekron®*) for different periods of time. Cell morphology was studied by light microscopic observation. Cell proliferation was evaluated by MTT assay and death rate was assessed applying trypan blue staining. Apoptosis was assessed by DNA fragmentation, cleaved caspase-3 protein western blotting and ELISA caspase-3 activity assays. **Results:** *Tarantula cubensis* venom ruined cell adhesion, reduced cell proliferation, increased cell death rates and caused DNA fragmentation in human cells. An increased caspase-3 cleavage and hyper-activation of caspase-3 was detected in the cells treated with the venom. Results also showed a significantly higher toxicity and apoptosis levels in cancer cell lines MCF7 and HN5 compared with non-cancerous HEK293 cells. **Conclusion:** We conclude that *Tarantula cubensis* venom is selectively toxic for human cancer cells via inducing caspase-3- mediated apoptosis.

**Key words:** *Tarantula cubensis*, Venom, *Theranekron*, Antitumor, Apoptosis.

## INTRODUCTION

Venom is a complex mixture of various compounds such as enzyme proteins, ions, amines, polyamines, and variety of chemicals. Most of these materials have been shown to have diverse pharmacological activities.[1] These compounds interfere with a couple of critical physiological functions of cells, result in organ injury, dysfunction or ultimately death.[2] For example, toxins which target ion channels and receptors in different cells have been isolated from spiders, marine snails, snakes, scorpions, and some other venomous animals.[3]A number of these toxins have been successfully tested for pain management, others use to treat diabetes, cancer, multiple sclerosis, and cardiovascular disease in preclinical and clinical trials.[4] The majority of spiders employ venoms that present a complex cocktail including polypeptides, polyamine neurotoxins, nucleic acids, free acids, biogenic amines, and inorganic ions. These compounds may cause a wide range of pathological effects in both vertebrates and invertebrates. Peptides are the major compound of spider venoms with a broad spectrum of bioactivities whose antimicrobial, antifungal, antiparasitic, antiarrhythmic, analgesic, cytolytic, haemolytic and antitumor activities have been described previously.[5,6]

*Tarantula cubensis*, also known the Cuban tarantula is a large arachnid belonging to the Theraphosidae family of spiders.[7] *Tarantula cubensis* utilizes venom which is chemically a mixture of wide spectrum of digestive enzymes. Injection of this venom into mammalian may cause angeioneurodema, swelling, inflammation and severe allergic reactions and even necrosis and permanent tissue death. *Theranekron®* is commercially available alcoholic extract of whole *tarantula cubensis* which is used as homeopathic therapy in veterinary medicine.[8] Many therapeutic effects of *Theranekron®* such as anti-inflammatory, antitumor, antiphlo-

gistic, demarcative, necrotizing action and wound healing have been demonstrated in clinical studies. *Theranekron®* was also reported to be effective in treatment of pododermatitis,[9] cutaneous papillomatosis,[10,11] endometritis[12,13] and foot and mouth lesions[14] in clinical veterinary. It was effective in ceasing growth of canine tumors,[15,16] reduction of inflammation and tarsal bursitis volume and also stimulating epithelialization in the cutaneous wounds.[17] *Theranekron®* also prevented retention secundinarium, improved uterine involution and treated the genital microbial diseases and oral lesions in cattle.[18,19] However, we have not observed antibacterial activity of *Theranekron®*.[20] Though antitumor effects of *Theranekron®* has been proven in clinical veterinary medicine, further *in vivo* and *in vitro* investigations are needed to address the mechanism of antitumor action of *Theranekron®*. The aim of this study was to assess the cytotoxic and anticancer bioactivity of *Tarantula cubensis* venom (*Theranekron®*).

## MATERIALS AND METHODS

### *The venom*

Alcoholic extract of *Tarantula cubensis* (trade name *Theranekron®*) was purchased from Richter Pharma AG (Wels, Austria).

### *Cell lines and culture*

Human embryonic kidney cell line HEK293 (#C497), human mammary gland cancer cell line MCF7 (#135) and human head and neck cancer cell line HN5 (#196) were purchased from National Cell Bank of Iran (NCBI; Tehran, Iran).The cells were grown in Dulbecco's modified eagle medium (DMEM)/F12 containing 10% fetal calf serum, 1% penicillin and 2 mmol/l L-glutamine (all from Gibco Laboratories, Grand Island, USA) with incubating at 37°C in a 5% $CO_2$ humidified incubator. After the cell culture reached 80% confluency, the cells were trypsinized with 0.25% trypsin (Sigma-Aldrich, St Louis, USA) and then harvested for next experiments.

### *Microscopic observation*

A number of $10^5$ cells were seeded as per 6-well plate and allowed to culture for 48 hours in culture conditions as described above. After the pre-culture, the cells were made to starve in serum free media for 12 h. The cells were cultured in presence of 100 μg/ml of *Theranekron®* for 24 h and then cell morphology and adhesion status were investigated as described previously.[21] Same volume of absolute ethanol was used as negative control.

### *MTT assay*

A number of $10^4$ cells were seeded as per 96-well plates and allowed to culture for 48 h in the culture conditions as described above. After the pre-culture, the cells were made to starve in serum free media for 12 h. Afterwards, the cells were treated with 20, 50, 100, 250 and 500 μg/ml of *Theranekron®* for 12, 24 and 48 h at the same condition. Same volume of absolute ethanol was used as negative control. At the end of the treatment time, 10 μl of 12 mM of MTT solution (#M5655; Sigma Aldrich, St Louis, USA) was added to the wells and then the plates were incubated at 37°C in 5% $CO_2$ incubator for 4 h. Afterwards, the solution was removed and replaced by 50 μl of dimethyl sulfoxide (DMSO) and then the plates were incubated at 37°C for 10 minutes with gently shaking until crystals melt. The absorbance of the suspension was measured at 540 nanometer wavelength using a microplate reader (BioTek, Winooski, USA). The actual absorbance value was obtained after subtracting the read absorbance value from the value of blank well.

### *Trypan blue viability assay*

A number of $10^5$ cells were seeded as per 6-well plate and allowed to culture for 48 h in culture conditions as described above. After the pre-culture, the cells were subjected to starvation in serum free media for 12 h. Then the cells were treated with 20, 50, 100, 250 and 500 μg/ml of *Theranekron®*, respectively, for 12, 24 and 48 h at the same culture condition. Same volume of absolute ethanol was used as negative control. At the end of the culture all cells collected in a micro-centrifuge tube and 50 μl were mixed with the same volume of 0.04% trypan blue solution and then 20 μl of the mixture was loaded onto a haemocytometer chamber. The viable and dead cells per square were counted under an inverted light microscope. The count of cell per ml of primary suspension was calculated as follow: cells per ml = mean of squares × 2 × $10^4$.

### *Antibodies and western blotting*

Rabbit anti human caspase-3 polyclonal IgG (#9662) which detects endogenous levels of full length caspase-3 (35 kDa) and the cleaved caspase-3 (17 and 19kDa) and rabbit anti human β-actin (13E5) monoclonal IgG (#4970) were purchased from Cell Signalling Technology (Beverly, MA, USA). HRP conjugated mouse anti rabbit polyclonal IgG (sc-2357) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Total protein was extracted by adding proper volume of lysis buffer (1% NP-40 in 20 mM EDTA, 50 mMTris-HCl, pH 7.5 plus 1 mM protease inhibitor mix) to cells and spinning down in maximum speed for 15 minutes and then protein containing supernatant was collected. A same volume of 4x lammeli buffer was added to protein sample and then incubated at 95°C for 5 min. Amount 40 μg of total protein ran in 10% polyacrylamide gel at 40 mA electric current for an hour. After electrophoresis, Proteins were transferred on nitrocellulose membrane (Bio-Rad Laboratories, Berkeley, USA) using semi-dry treansfer system at 25 V electric power for 1 h. In order to block non-specific bindings, the membrane was coated with milk proteins by incubation in 5% non-fat milk solution for an hour in 50 rpm agitation. The immune-blotting was done by incubation of the membrane in 0.2 μg/ml of primary antibody solution and then 0.1 μg/ml of secondary antibody both for 2 hours at room temperature. At the end of the blotting, the membrane was incubated in Immun-star Western C Chemiluminescent (Bio-Rad Laboratories, Berkeley, USA) substrate solution for 5 minutes then imaged using gel documentation system (Bio-Rad Laboratories, Berkeley, USA).

### *Caspase-3 activity assay*

Active human caspase-3 was evaluated using commercially available enzyme-linked immunosorbent assay (ELISA) kit following manufacturer's instructions (#KHO1091; Invitrogen, Camarillo, USA). Amount of active caspase-3 was obtained by considering parallelism between optical density and known human active caspase-3 concentration according to the manufacturer's instructions.

### *Statistical analysis*

Statistical significances were examined applying two-tailed analysis of variance (ANOVA). Slope of curves was calculated by applying linear regression analysis. $p<0.05$ was considered as statistically significant. All calculation of numerical data was done using GraphPad Prism (version 5.0) software and the image basedones were analyzed using Image J javascript (NIH). All the experiments were performed as minimum triplicate (N ≥ 3).

## RESULTS

### *Cytotoxic effect of Tarantula cubensis venom on the human cells*

We treated HEK293, MCF7 and HN5 cell lines with different concentration of *Tarantula cubensis* venom and then evaluated the effects on cellular morphology, proliferation and death rates. As shown in Figure 1, a disrupted adhesion, round shaped morphology and floating in the medium were observed as in the cells cultured in the presence of 100 µg/ml of *Theranekron®* for 24 h. In contrast, in the negative control cells which were cultured in absence of *Theranekron®* or presence of ethanol, adhesion potency maintained intact and the cells were possessing morphological characteristics of healthy cells (Figure 1A). This reveals that the venom disrupted cellular adhesion which could be the causative factor behind death of most of the cells.

We also evaluated cell proliferation under effect of treatment with 20, 50, 100, 250 and 500 µg/ml of *Theranekron®* for different periods of time. Results showed a major decrease in proliferation of the all cells treated with *Theranekron®* for 12 ($p<0.001$), 24 ($p<0.0001$) and 48 ($p<0.0001$) h with increasing concentration-dependent manner in comparison with negative controls (Figure 1B). This result implicates that the venom significantly prevented cell proliferation. In order to verify whether this decline was due to cell death, we assessed

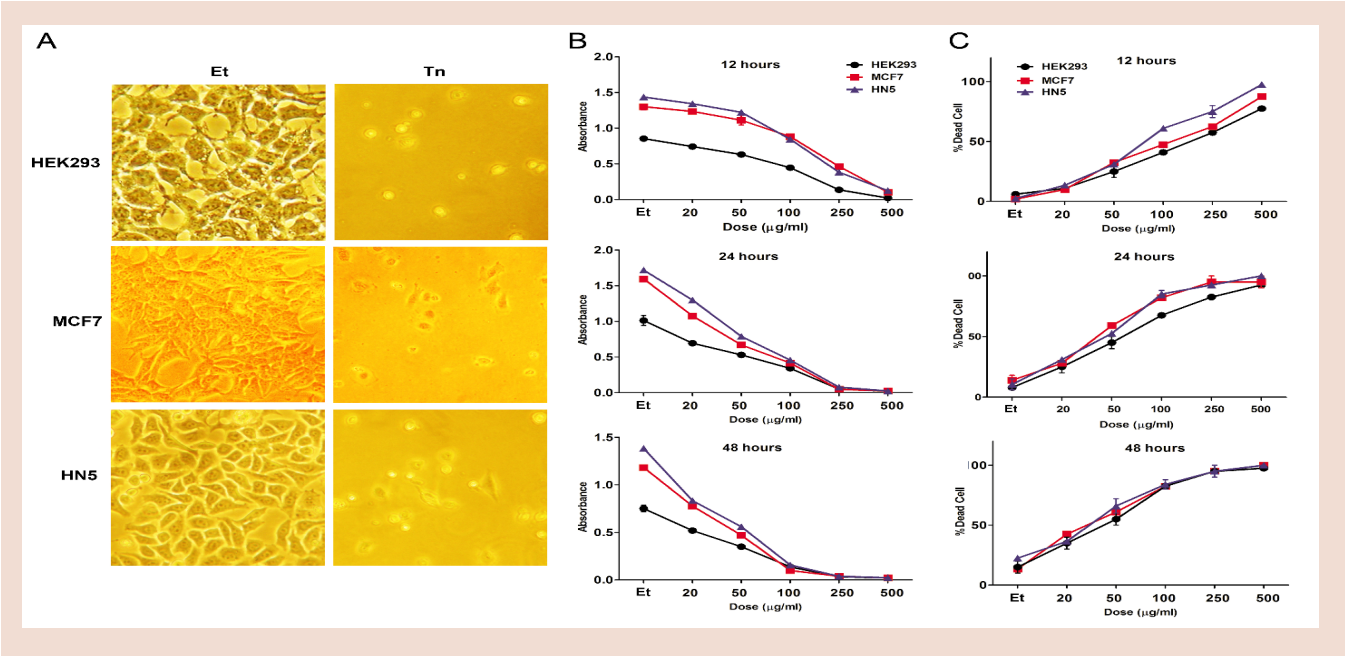


**Figure 1: Cytotoxic effect of Tarantula cubensis venom on the human cells. (A) Morphological change of the cells. HEK293, MCF7 and HN5 cell lines showed aberrant adhesion morphology after treatment with 100 µg/ml of Theranekron® (Tn) for 24 h. (B) MTT cell proliferation results. The results of indicated significant decrease in proliferation of the cells under treatment with 20, 50, 100, 250 and 500 µg/ml of Theranekron® for 12, 24 and 24 h. (C) Percentages of dead cells. The cells were treated with the various concentrations of Theranekron® for the various periods as described above and then were stained with trypan blue dye. There was found significant increase in death rate of the cells under effect of Theranekron®. Absolut ethanol (Et) was used a negative control. P-values are not indicated on this figure.**

dead cells after treatment with same concentration of the *Theranekron*® for the time as described above. Results indicated a dramatic increased death rate of the cells treated with different concentrations of the *Theranekron*® for 12 ($p$<0.0001), 24 ($p$<0.0001) and 48 ($p$<0.0001) h with an increasing concentration-dependent manner compared with negative controls (Figure 1C). Taking together, these results indicated high level cytotoxicity of *Theranekron*® on the human cancer cells.

## *Tarantula cubensis venom is more toxic for cancer cells*

In order to examine sensitivity of the cells against *Theranekron*® we compared the levels of proliferation and death between the cells treated with 50 µg/ml of *Theranekron*® for 0, 6, 12, 24 and 48 h. For this we calculated change slope valuesof proliferation and death curves under effect of single concentration and time-dependent manner. MTT assay results showed that proliferation was reduced with different coefficients between the cells. The mean negative slope of proliferation curve for HEK293, MCF7 and HN5 cell lines was as respectively -0.041 (standard deviation; SD=0.0001), -0.062 (SD=8.505e-005) and -0.070 (SD=2.517e-005). There was found a significant difference between the proliferation slope values of HEK293 and MCF7 ($p$<0.0001), HEK293 and HN5 ($p$<0.0001) and even MCF7 and HN5 ($p$<0.0001; Figure 2A). A similar assessment on the rate of cell death identically showed that there were different coefficients in positive slopes of the cells. The mean slope of cell death curve for HEK293, MCF7 and HN5 cell lines was as respectively1.196 (SD=0.089), 2.056 (SD=0.085) and 2.601 (SD=0.044). There was also very significant difference between HEK293 and MCF7 ($p$=0.0003), HEK293 and HN5 ($p$<0.0001) as well as MCF7 and HN5 ($p$=0.0006) slope values (Figure 2B). Taking together, these results indicate that *Theranekron*® shows significantly more toxicity on the cancerous cell lines MCF7 and HN5 in comparison with non-cancerous cell line HEK293.

## *Tarantula cubensis venom induces apoptosis in cancer cells*

In order to investigate whether *Theranekron*® causes apoptosis in the cells, we did DNA fragmentation assay and assessed activation of caspase-3 by western blotting and ELISA-based active caspase-3 assays in the cells treated with 100 µg/ml of *Theranekron*® for 24 h. As result, we detected a considerable DNA fragmentation in the cells treated with 100 µg/ml of *Theranekron*® but not in negative controls (data not shown). Generally, DNA laddering is caused by caspase-mediated DNase activity with subsequent cleavage of DNA into multiple fragments that is a key event indicating apoptosis. Blotting of full length caspase-3 (35 kDa) and the cleaved caspase-3 (17 and 19 kDa) showed significant increased level of cleaved caspase-3 in HEK293 ($p$=0.0028), MCF7 ($p$=0.0002) and HN5 ($p$=0.0008) cells which were treated with 100 µg/ml of

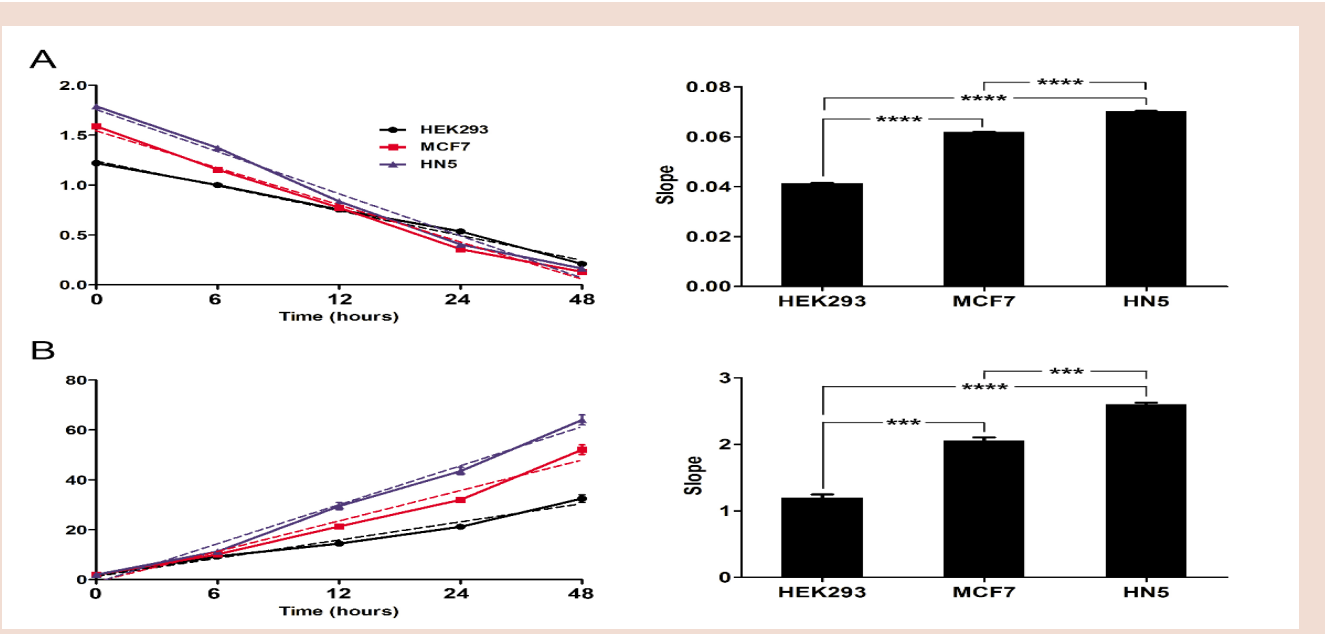


**Figure 2: Tarantula cubensis venom is more toxic for cancer cells. (A) Proliferation curves and related slope values. HEK293, MCF7 and HN5 cells were separately seeded and allowed to be cultured in the presence of 50 µg/ml of Theranekron® for 0, 6, 24 and 48 h. Then, the cell proliferation rates were assessed at the end of each period using MTT assay. Regression analysis was performed to evaluate related slope of each curve. Results showed very significant difference between the negative slopes. The order of proliferation subsidence was found as HN5 > MCF7 > HEK293. (B) Cell death curves and related slope values. The cells were cultured as described above and then percentage of dead cells was evaluated by trypan blue staining. Positive slope for each curve was assessed by applying regression analysis. Results showed very significant difference between the positive slopes. The order of cell death increase was found as HN5 > MCF7 > HEK293. Slope lines are indicated as dotted. *** p < 0.001, **** p < 0.0001.**

*Theranekron*® (Figure 3A). ELISA-based assessment of active caspase-3 revealed that the mean concentration of active caspase-3 in ethanol treated HEK293, MCF7 and HN5 cell lines were as respectively 3.2 (SD: 2.83), 1.53 (SD:1.86) and 1.85 (1.76) ng/mg of total protein. While active caspase-3 concentration for the cells treated with 100 µg/ml of *Theranekron*® were as respectively 29.33, (SD: 4.16; $p$=0.0008), 31.40 (SD: 6.7; $p$=0.0004) and 31. 33 (SD: 4.3; $p$=0.0004) ng/mg of total protein. In addition, the mean positive fold change of active caspase-3concentration due to treatment with *Theranekron*® in HEK293, MCF7 and HN5 cells were found as respectively 9.17 (SD: 1.47), 20.48 (SD: 3.1) and 17.09 (SD: 2.45). These results mean that activation of caspasse-3 in MCF7 ($p$=0.0045) and HN5 ($p$=0.0087) was significantly higher than HEK293 (Figure 3B). Taking together, our results equivocally show that *Theranekron*® induces apoptosis in human cells through activating caspase-3 protein, obviously more in cancerous cells compared with non-cancerous cells.

## DISCUSSION

Using venom from venomous animals like snake and bee in order to treat diseases is a precedent method of homeopathic biotherapies which is traced back thousands of years. In middle of recent century, many researchers of alternative medicine and pharmacologists realized the importance of spider venom as a potent homeopathic medicine. The increasing use of homeopathy in veterinary medicine without side-effects demonstrates that many veterinary surgeons apply this treatment as a natural stimulator of self-healing mechanisms in animals.[22] There is also demonstrated that spider venom is a rich source of substances with pharmacologic and therapeutic potential.[5,6] In particular, *Tarantula cubensis* venom has been pharmacologically characterized as useful homeopathic medicine in the treatment of wide range of conditions such as animal wounds and tumors. Koch and Stein (1980) reported the first evidence of effectiveness of *Tarantula cubensis* venom on mammalian tumors. They showed that the venoms topped growth of canine tumor and decreased the tumor size after a week treatment.[15] Based on this report, Gultekin and Vural (2007) investigated the effects of *Tarantula cubensis* venom during pre and postoperative period on canine mammary tumors. They found a significant regression in benign tumors after three times injections a week at one week intervals with a dosage of 3 mg per 10 kg of body weight. They suggested that *Tarantula cubensis* venom may be used as an effective anti tumor homeopathic medicine.[16]

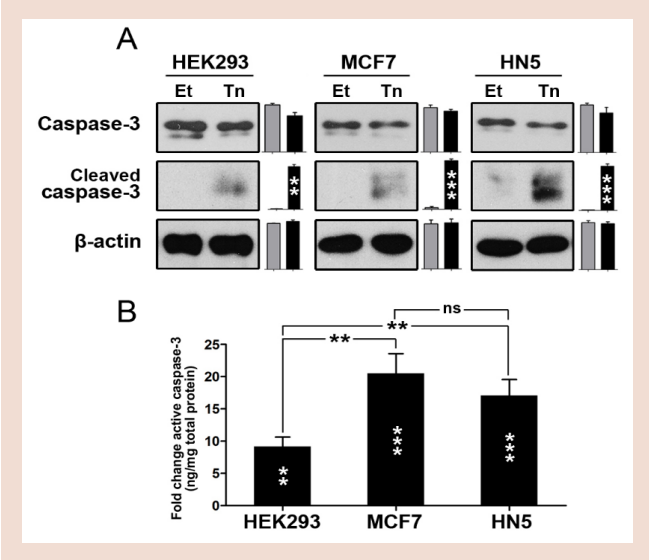


**Figure 3: Tarantula cubensis venom induces apoptosis in cancer cells. MCF7, HN5 and HEK293 cells were treated with 100 µg/ml of Theranekron® (Tn) or same volume of ethanol (Et) for 24 h. (A) Western blot protein level of full length (35 kDa) and cleaved (17 and 19 kDa) caspase-3 in the cells. Increased level of cleaved caspase-3 in Theranekron® treated cells. (B) Fold change of ELISA active caspase-3 in the cells in the result of treatment with Theranekron®. MCF7 and HN5 cells showed higher level of increased active caspase-3in comparison with HEK293 cells. ** $p<0.01$, *** $p<0.001$, ns = Not Significant.**

We here hypothesized that *Tarantula cubensis* venom may has a cytotoxic effect on tumor cells. Thus, we examined our hypothesis by investigating effect of *Tarantula cubensis* venomon human cancerous and non-cancerous cell's apoptosis. Our results indicated that *Tarantula cubensis* venom disrupted cell adhesion, decreased proliferation and increased death rate of human cells particularly cancerous cell lines MCF7 and HN5. The results also showed that the venom significantly induced caspase-3 activation and caused DNA fragmentation in the cells. Interestingly, caspase-3 activation was higher in MCF7 and HN5 cells compared with HEK293 cells. Caspase-3 and other members of caspase protein family are important death-inducer proteins that play prevail role in inducing apoptosis and necrosis.[23] Equivocally, our results indicate that the venom causes apoptotic cell death through activation of caspase-3 protein however we do not exclude the possible involvement of other pro-apoptotic proteins.

It can be concluded that the venom caused dramatic cell death in MCF7 and HN5 cell lines due to hyper-activation of apoptosis with higher rate in comparison with HEK293 cells. Despite the lower aggression of HEK293 as a non-cancerous cell line, also higher invasion of MCF7 and HN5 cells as two different cell types derived from different solid tumors and their higher tolerance to environmental stressors, we observed a higher level susceptibility of MCF7 and HN5 against the venom in comparison with HEK293 cells. This finding suggests that *Tarantula cubensis* venom might efficiently target malignant cells via hyper-activation of apoptosis in the cells. The vulnerability of the malignant cells could because of different cellular biochemistry of the cells and their unique molecular patho-physiology. Due to the rapid metabolism and hyper-activity of synthesis machinery, cancer cell is more vulnerable against some critical conditions such as starvation and growth inhibitors. Many previous studies described cytotoxic effect of spider *Macrothele revani* venom on various human cancer cells.[24–26] The *in vitro* and *in vivo* assessments showed that *Macrothele revani* venom induced apoptosis and necrosis, caused an inhibition of cell

growth and conferred a systemic effect of tumor regression by arresting cell cycle progression though regulating C-myc, protein 21 (p21), caspase-3 and caspase-8.[24–26] C-myc is an important transcriptional factor which targets a bunch of genes involved in cell cycle progression, cellular metabolism and proliferation whose hyperactivity has been demonstrated in many cancers. While p21 is a tumor suppressor protein that charged to inhibit cell growth and proliferation.[23] Despite possible differences in composition of venom of *Macrothele revani* and *Tarantula cubensis*, the cytototoxic functions and mechanisms of their venom might be similar due to the phylogenetical proximity. Besides, polypeptides as major components of spider venoms that can promote death modulators by attacking cellular ion channels, disrupting the ions balance and eventually demolition of cell membrane. Therefore, we suggest that venom of *Tarantula cubensis* may contain a set of enzymatic polypeptide with toxic potentials, although components of the venom and mechanism of action on the cell have not yet investigated.

## CONCLUSION

We conclude that venom of *Tarantula cubensis* which is commercially available under the name *Theranekron*® induces apoptotic death via activating caspase-3 in cancer cell lines. According to the finding we suggest that *Tarantula cubensis* venom has a remarkable anticancer activity and it may be exploited as an antitumor cell death-inducer biotherapeutic in cancer therapy. Based on this conclusion, we suggest future studies to biochemically analysis of *Tarantula cubensis* venom, describing its functional compounds and to address the mechanism of effect on cancer cells.

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## CONFLICT OF INTEREST

The authors hereby certify that all work contained in this article is original. The author declare no conflicts of interest exists.

## AUTHOR CONTRIBUTIONS

A.G. and N.A. conceived and designed the experiments; A.G. performed all the experiments; A.G. analyzed the data. A.G. and N. A. contributed reagents/materials/analysis tools. B.N. provided the cell lines and wrote the manuscript.

## ABBREVIATION USED

ANOVA: analysis of variance ; DMSO: dimethyl sulfoxide ; ELISA: Enzyme-linked immunosorbent assay ; Et: Ethanol ; p21: protein 21 ; Tn: Theranekron

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