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Investigation the Cytotoxic and Anti-Proliferative Effects of Crude Venom of *Euscorpius mingrelicus* (Scorpiones: Euscorpiidae) Scorpion

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ABSTRACT

Objective: Since they contain various toxins that may influence various biological events, scorpion venoms raise new hopes for cancer treatments. It is thought that the bioactive compounds contained in scorpion venom could be used in cancer treatments in near future.

Materials and Methods: In this study, different cytotoxic, apoptotic, necrotic, and anti-proliferative effects of crude venom obtained from *Euscorpius mingrelicus* (Kessler, 1874) scorpions species were tested on human breast cancer cells (MCF-7), human lung carcinoma cells (H1299), and mice fibroblast cells (L929).

Results: It was determined that the crude venom had cytotoxic and anti-proliferative effects on MCF-7 and fibroblast cells even when at low concentrations and the effect on H1299 was half of the effect on MCF-7 and fibroblast. It was observed that, as the dilution rate increased, the venom effect decreased, apoptosis and necrosis rates on H1299 decreased, and it had no effect on cell proliferation. With regards to the MCF-7 cells, apoptosis and necrosis rates were similar in H1299 cells.

Conclusion: It was found that crude venom of *E. mingrelicus* scorpion played an effective role in decreasing the proliferation of MCF-7 cells, and more comprehensive studies are needed in order to determine the toxin that is responsible for this effect.

Keywords: Scorpion Venom, Cancer, Anti-Proliferative, Apoptosis, Necrosis

INTRODUCTION

Given the death statistics of developed countries, it can be seen that, following cardiovascular diseases, cancer is the second-most lethal disease. Cancer manifests itself as an uncontrolled proliferation of cells with an invasive character and metastasis, and is hard to cure. While radiotherapy, chemotherapy, and surgical methods are widely used in cancer treatment, target-specific treatments, biological treatment methods, and hormone therapies are less frequently used. These methods are used either separately or in combination (1).

In addition to their treatment characteristics, methods with the potential to cure may also have adverse effects, i.e., negative effects on healthy cells. The medications used in chemotherapy are artificial chemicals and it is known that they have many side effects. One of the factors limiting the efficiency of anti-cancer medications in treatment is the resistance mechanism that tumor cells develop, sometimes on their own in certain cancer types and sometimes after chemotherapy in some other types of cancer (2). The objective of cancer treatment is to destroy all the cells until there is no remaining malignant cell in the body. However, at this moment,

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such a treatment is not possible, with a couple of exceptions (3). Researchers have focused on natural sources, since chemotherapeutical agents have adverse effects and they cause the development of resistance to multiple drugs. Nowadays, it is thought that *biotoxins* may be useful in cancer treatment.

Scorpion, snake, and spider venoms are mixtures that are rich in protein, polypeptide, and various biogenic amines. The venoms of these creatures have started to be used directly and indirectly in cancer treatment. Scorpion venom, in particular, comes to the forefront in cancer research because of its high protein content. Scorpions are found everywhere on earth, except for New Zealand and ocean islands, and they are more widespread in tropical and subtropical climate zones (4,5). Scorpions combine many bioactive compounds in their venoms and use them in catching their prey and protecting themselves (6). Scorpion venoms are complex mixtures containing more than 100 compounds, including mucosa, oligopeptide, nucleotide, amino acid, various ions and salts, neurotransmitter, water, low molecular weight materials, and phospholipase, hyaluronidase, lipase, alkaline phosphatase, and proteolytic enzyme (7,8).

There are numerus techniques and methods to carry out the extraction of venom from scorpions (9-12). An early method was to remove the venom glands and telsons and macerate them, followed by extracting the venom in normal saline or water. The main problem with this method was that the scorpions had to be sacrificed after the removal of the telson, and thousands of scorpions had to be sacrificed to obtain sufficient quantities of venom for research purposes (9-11). Another method was to puncture the venom gland through the chitinous covering with a sharp, hypodermic needle and suck out the venom. Again, the amount of venom that could be obtained was very limited and the process caused serious damage to the scorpion. In the widely used electro-simulation technique, a 20-25 V electric current is applied to the scorpion's telson and the venom is collected directly from the tip of the sting. The toxicity of the venom obtained with this technique is found to be higher, a higher amount of venom is obtained, and there are no permanent injures to the scorpions. Within 2-3 weeks, the same scorpions can be used for venom milking again (11,12).

Scorpion venoms are toxic for humans. They cause severe reactions that may result in central nervous system damage, allergic reactions, tissue necrosis, and even death (6). Moreover, they are reported to have hemolytic, antibacterial, antifungal, insecticide, antitumor, analgesic, and (on several cells) apoptotic effects (13,14). Although scorpion venoms have very severe effects on humans, which can result in death, certain toxins found in scorpion venoms are used in central nervous system disease treatments in particular (15,16). In humans, scorpion venoms can be used in the treatment of rheumatism, pancreatic inflammation, skin cancer, breast cancer, prostate cancer, kidney tumors, male impotency, brain tumors, leukemia, epilepsy, HIV, tetanus, cardiovascular diseases, and subcutaneous nodules, and in acute and chronic convulsion therapies (17).

Scorpion venoms are classified by different channel and channel structures, binding sites, and effects. Scorpion venoms are classified as long- or short-chain peptides by molecular length, insect-, mammal-, or crustacean-specific toxins by effect groups, neurotoxins or cytotoxins by effect mechanism, and disulfide bridge peptides (DBPs) or non-disulfide bridge peptides (NDBPs) by the presence of disulfide bridge (18). DBPs are generally the ion channels showing neurotoxin activity and are bound to the membrane. They contain 3-4 disulfide bridges. These channels play important roles in arranging the cellular physiology among many mammals. After the scorpion stings, the functions of these channels deteriorate and sting causes remarkable changes (19). NDBPs are anti-carcinogenic, anti-inflammatory, antimicrobial, hemolytic, and immunomodulator (19).

The sodium channel toxins (NaTx) in scorpion venoms prefer different sodium channels in cells of mammals and insects. These toxins bind to different receptor zones and target these channels by creating pores (20). The long-chain toxins affecting the sodium channels are divided mainly into two subtypes: α-toxin and β-toxin. Scorpion α-toxins bind to Na^+ channels in mammals and suddenly inactivate the sodium channel receptor affinity. Hence, they prolong the nerve-muscle potential. In mammals, β-toxins bind to the 4th zone as sodium channel receptor and cause a sudden change in the direction of membrane potential or a more negative membrane potential (21,22). The potassium channel toxins (KTx) that scorpion venom contains function as various potassium channel blockers. The scorpion toxins targeting the potassium channels are the short-chain peptides cross-linked with 3 or 4 disulfide bridges, and it was reported that they might be used in treatment of various diseases, such as cancer, inflammation, neuropathy, and autoimmune diseases (23). Calcium channel ions play an important role in organizing functions such as regulation of gene expression, intracellular mechanism, release of neurotransmitters, and cellular stimulation Calcium channels generally spread over the body in the form of endocrine cells, smooth muscles, skeletal muscle, cardiac muscle, and neurons. Many calcium channel toxins (CaTx) that scorpion venom contains affect the functions of Ca^{++} ion channels and their activities by modulating them. Since these peptides have pharmacological and physiological properties, they have been investigated in various studies (24,25).

Scorpion venoms show their effects by influencing the movement of ions causing lethal depolarization by disintegrating the cellular membrane. The contents of the venom create pores on the cellular membrane and cause the cell content to leak outside. Moreover, some peptides contained in venom composition include hydrolases that cause the disintegration of the cellular membrane. Hydrolases distort the membrane function by causing the distribution of membrane lipids (26).

In the present study, the effects of the venom of *Euscorpius mingrelicus*, which does not have a venom that is intense enough to affect humans, was examined on two different cancer types and normal fibroblast cell lines. The effect of *E. mingrelicus* venom was examined on human breast cancer cells (MCF-7), human large cell lung carcinoma cells (H1299), and mice fibroblast cells (L929) via cytotoxic and apoptosis properties.

MATERIALS AND METHODS

Obtaining the Scorpions and Venoms

The *Euscorpius mingrelicus* (Scorpiones: Euscorpiidae) scorpions used in the present study were collected from nature (under stones) in Çamlıdere and Çamkoru Forests in Ankara province (33°E, 40°N, Ankara, Turkey) between 15th July and 15th September 2015. The scorpions, which were released into individual habitats that we specially prepared for them in the laboratory, were not given any food for a week in order to prevent a decrease in the amount of their venom, and venom collection was performed using electrostimulation method. Using an electrostimulator, 15-20 volts of current were applied to the tail of the scorpion through two electrodes. The crude venom was collected using the injector from the sting at the tip of telson. The crude venom was dissolved in double distilled water and centrifuged at +4°C and 15,000 g for 15 min. After the centrifuge, the supernatant containing peptide toxins was transferred to a new tube and the protein concentration was measured using the Bradford method at 405 nm wavelength (27). The protein concentration of resultant crude venom was found to be 3290 µg/µl. The crude venom was stored at -20°C until the experiment day. The venom was diluted at the ratios 1/30, 1/60, 1/120, 1/240, 1/480, 1/960, 1/1920, and 1/3840.

Cell Culture

H1299, MCF-7, and L929 cell lines were obtained from the cell collection of Kırıkkale University's Central Research Laboratory (KUBTAL). The procedures were conducted in the same laboratory in accordance with the standards. These cell lines were incubated in 25cm^3 fluxes and 15 ml medium with Dulbecco's Modified Eagle Media containing 0.1 mM L-glutamine and 10% heat-activated fetal calf serum at 37°C in an incubator with 5% CO_2.

WST-1 Cytotoxicity Test

Using a cell counting device on the cell lines that we had cultivated, we achieved cell counts of 4.5×10^5 for MCF-7, 1.7×10^5 for H1299 cells, and 3.4×10^5 for L929 cells. A WST-1 cytotoxicity test was performed using a 96-well plate by placing 5,000 cells in each well, using 2 wells for control, and leaving the rest empty. Then, DMEM containing 1% penicillin-streptomycin and 10% fetal bovine serum was added to RPMI (L-glutamine, without sodium bicarbonate) for L929, MCF-7, and H1299 cells. Cells were incubated for 24 h at 37°C in an incubator with 5% CO_2. After the 24 h incubation, 40µl of venom was diluted to 1200 µl using double distilled water. Then, it was equally distributed to the first 6 wells (100 µl). The resting portion was diluted by ½ and divided equally into the next 6 wells (100 µl). This process was continued up to the last well, and the venom doses of 1/30, 1/60, 1/120, 1/240, 1/480, 1/960, and 1/1920 µg/µl were achieved. Then, another 24 h incubation was performed. After the incubation, the cell media were removed from the wells and 100 µl media containing no phenol red were added. 10 µl WST-1 solution was added to each well, and then they were incubated for 4 hours. After the incubation, the absorbance measurement was performed at 440 nm wavelength using a spectrophotometer with a microplate scanner.

Determining the Apoptosis and Necrosis by Using Double Staining

This process was performed using a 96-well plate. MCF-7, H1299, and L929 cells were divided into 16 wells each (5,000 cells in each well). Two wells were used as control for each. DMEM containing 1% penicillin and 10% fetal bovine serum were added to RPMI for L929, MCF-7, and H1299 cells. Cells were incubated at 37°C for 24 h with an incubator having 5% CO_2. After the 24 h incubation, 40 µl of venom was diluted to 1200 µl using double distilled water. Then, it was equally distributed to the first 6 wells (100 µl). The resting portion was diluted by ½ and divided equally into the next 6 wells (100 µl). This process was continued until the last well and the venom doses of 1/30, 1/60, 1/120, 1/240, 1/480, 1/960, and 1/1920 dilutions were achieved. Then, another 24 h incubation was performed. After the incubations, the media were removed and 70 µl double staining solution was added to each well. They were incubated for 15 minutes in darkness. After the incubation, the examinations were performed using FITC and DAPI filter with a microscope with a fluorescent attachment. For each well, the total number of living cells, the total number of apoptotic cells, and the total number of necrotic cells were manually counted.

Monitoring Cell Proliferation Using Real-Time Cell Analysis System (xCELLigance RTCA)

After determining that the number of cells in flux reached an adequate number using a microscope, the medium was removed and 1 ml trypsin-EDTA was added. The flux was kept in an incubator for 3-4 min. After the incubation, flux was microscopically checked to see if the cells were detached from the surface of flux. After confirming microscopically that the cells were detached, medium was added into flux, and the cells were put into a vial and centrifuged at 2,500g for 2 min. The supernatant portion was removed after the centrifuging and the resting cell pellet at the bottom of the tube was suspended by adding 1 ml mem-α medium. Cell counting was performed. Then, 100 µl medium was added to each well of the e-plate. After the plate in the oven was placed in xCELLigence. There was a 5 min waiting period for the plate and device to reach the same temperature. After 1 min of background scanning in the device, the e-plate was removed and MCF-7 and H1299 cell lines (100 µl 3×10^3 cells) were added to each well. The e-plate was placed into the drying oven again and reading was initiated at the end of the 10min period. There was a 24 h waiting period for the cell proliferation. Moreover, venom doses of 1/30, 1/60, 1/120, and 1/240 were applied and the proliferation was monitored for 120 h.

Statistical Analysis
All experiments in this study were performed in triplicate. The data shown are representative results. Values were calculated as the mean and standard error. Owing to the fact that the data from vitality experiments did not meet the normality assumptions, statistical comparisons of results from control and venom application groups were made non-parametrically using the Mann-Whitney U test. Statistical package program (version 7) was utilized.

RESULTS

WST-1 Test Results
Since the scorpions examined here are small creatures, the amount of venom they provide is also very small. Hence, the dilution rates were kept high. At the dilution rate of 1/30, it was found that venom affected the vitality of MCF-7 and L929 cell lines by 50% and the effect on H1299 was found to be 27%. It was determined that the effect of venom decreased as the dilution rate increased and the cell vitality exceeds the beginning level after a specific dilution rate (Table 1).

In the study, when the viability percentages of the non-cancer cell (L929), which we used as a control, and the venom cancer cells (MCF-7 and H1299) are compared, it is seen that MCF-7 and L929 are similar, while H1299 is statistically different (Table 2).

Double Staining Results
As a result of the double staining test, it was determined that, for MCF-7 cells for which the vitality results were in corroboration with WST-1 test, 13.88±0.5% of all dead cells died of apoptosis and 12.5±1.5% died of necrosis at the dilution level of 1/30 (Table 3). Apoptosis and necrosis rates gradually decreased as the dilution rate increased (Figures 1A, B). After the dilution rate of 1/240, the decrease in necrotic deaths became sharper. While the apoptosis and necrosis rates were close to each other at the dilution rates of 1/30 and 1/60, it was determined that apoptosis rate was higher than the rate of necrosis at other doses. In H1299 cell line and at the dilution level of 1/30, apoptosis rate was found to be 10.52±0.4% and necrosis rate to be 5.26±0.5% (Table 3). Apoptosis/necrosis rates ranged between 1.41 and 4.0 at other dilution rates (Figures 1C, D).

In L929 fibroblast and at a dilution rate of 1/30, the rate of apoptosis was found to be approximately 30% and the rate of necrosis to be half of it. This ratio varied as the dilution ratio increased (Table 3).

Table 1. WST 1 test absorbance results. The cell viability (%) based on mean absorbance values at 440 nm is presented. Data are expressed as mean±standard error as calculated from 3 separate experiments.

Venom Doses	MCF-7 Vitality (%)	H1299 Vitality (%)	L929 Vitality (%)
1/30	52.69±0.2	72.96±1.0	49.12±1.0
1/60	76.27±1.5	119.45±0.7	81.33±0.1
1/120	83.12±0.8	127.33±0.8	85.79±1.1
1/240	100.59±0.7	133.34±1.1	90.36±1.4
1/480	118.80±2.0	131.81±1.4	96.30±0.5
1/960	131.36±0.5	129.54±2.1	97.82±1.0
1/1920	129.03±0.7	123.98±2.0	97.27±1.1
1/3840	122.12±0.4	124.14±0.1	100.54±0.5
Negative Control	100.59±0.1	110.45±0.5	100.54±0.1

Table 2. Paired comparisons of vitalities between L929 (control group) and each venom application (MCF and H1299) by nonparametric Mann-Whitney U test. Tests shown in bold are significant at p<0.05. (n=8).

Groups	Rank Sum L929	Rank Sum MCF	z	p-level
L929 x MCF-7	60.00	76.00	-0.84	0.40
L929 x H1299	44.00	92.00	-2.52	**0.01**
MCF-7 x H1299	53.00	83.00	-1.58	0.12

Table 3. Apoptosis and necrosis indexes. Data are expressed as mean ± standard error as calculated from 3 separate experiments.

Venom	MCF-7		H1299		L929	
Dilution rates	% apoptosis	% necrosis	% apoptosis	% necrosis	% apoptosis	% necrosis
1/30	13.88±0.5	12.5±1.5	10.52±0.4	5.26±0.5	30.55±0.7	16.6±0.1
1/60	9.09±0.4	10.38±1.0	8.23±0.5	5.83±0.5	25±0.5	14.77±0.5
1/120	8.88±0.2	7.5±0.7	6.29±1.0	1.57±1.0	21.42±1.0	14.28±0.5
1/240	8.49±0.1	4.71±0.5	6.36±0.7	1.91±0.7	18.91±0.7	10.81±1.0
1/480	7.95±0.3	4.54±0.5	3.94±0.8	1.49±1.0	14.63±0.5	9.75±0.7
1/960	6.38±0.5	4.25±0.8	3.33±0.7	1.25±0.5	11.89±0.6	8.64±0.5
1/1920	6.73±0.5	2.32±0.6	3.48±0.7	1.24±0.8	11.25±0.7	7.5±0.1
1/3840	5.31±0.4	2.12±0.5	2.32±1.0	1.16±0.1	9.89±0.5	4.39±0.1

Determining Cell Proliferation Using Real-Time Cell Analysis System (xCELLigance RTCA)

During the 120-h proliferation monitoring, it was determined that the proliferation of MCF-7 and H1299 cells proportionally increased within the first 24 h. After the 24th h, when the venom was applied, proliferation stopped and even the number of living cells decreased. Then, because the effect of venom decreased, cells entered into the proliferation process again. Proliferation was found to be at the minimum level at the dilution rate of 1/30 (the highest amount of venom). It was observed that the effect of venom lasted longer (Figures 2,3). At the dilution rate of 1/30, the proliferation of MCF-7 is faster than H1299. Similar results were achieved after the 24th hour (Figure 4).

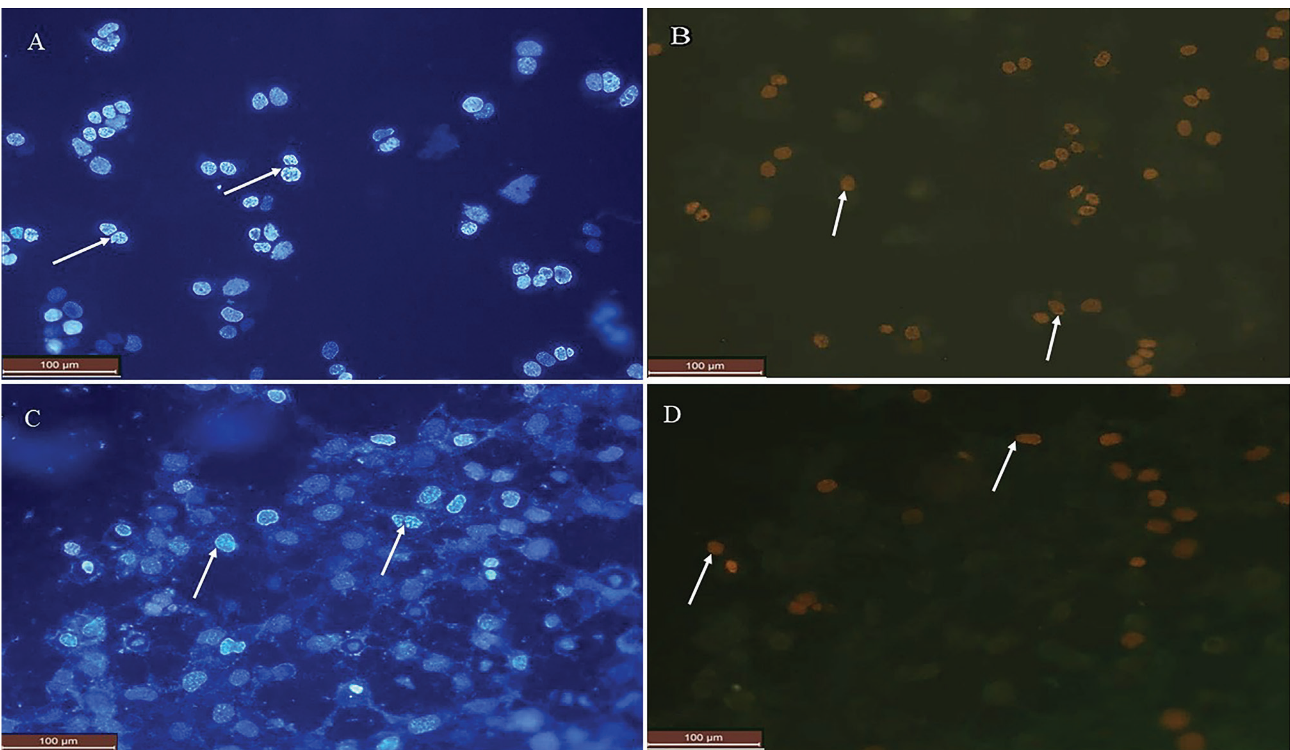

Figure 1. Inverted microscope image of MCF-7 cells at the dilution rate of 1/30; apoptosis (A) and necrosis (B), H1299 cells at the dilution rate of 1/30; apoptosis (C) and necrosis (D). Apoptotic and necrotic cells are shown with arrows.

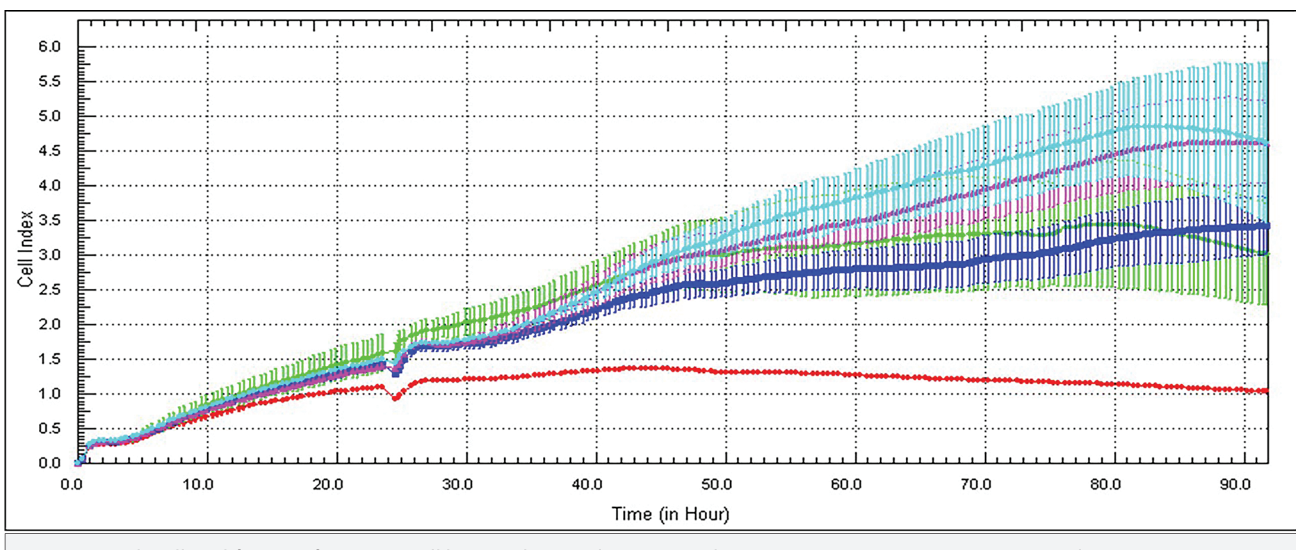

Figure 2. 24-h cell proliferation for MCF-7 cell line (Red 1/30, Blue 1/60, Pink 1/120, Turquoise 1/240, Green control).

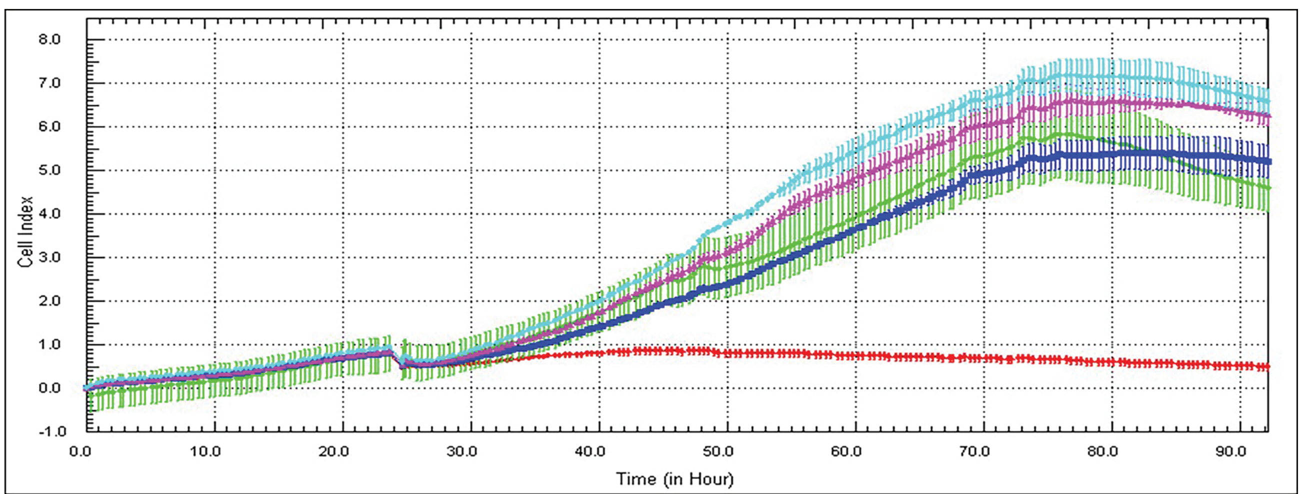

Figure 3. 24-h cell proliferation for H1299 cell line (Red 1/30, Blue 1/60, Pink 1/120, Turquoise 1/240, Green control).

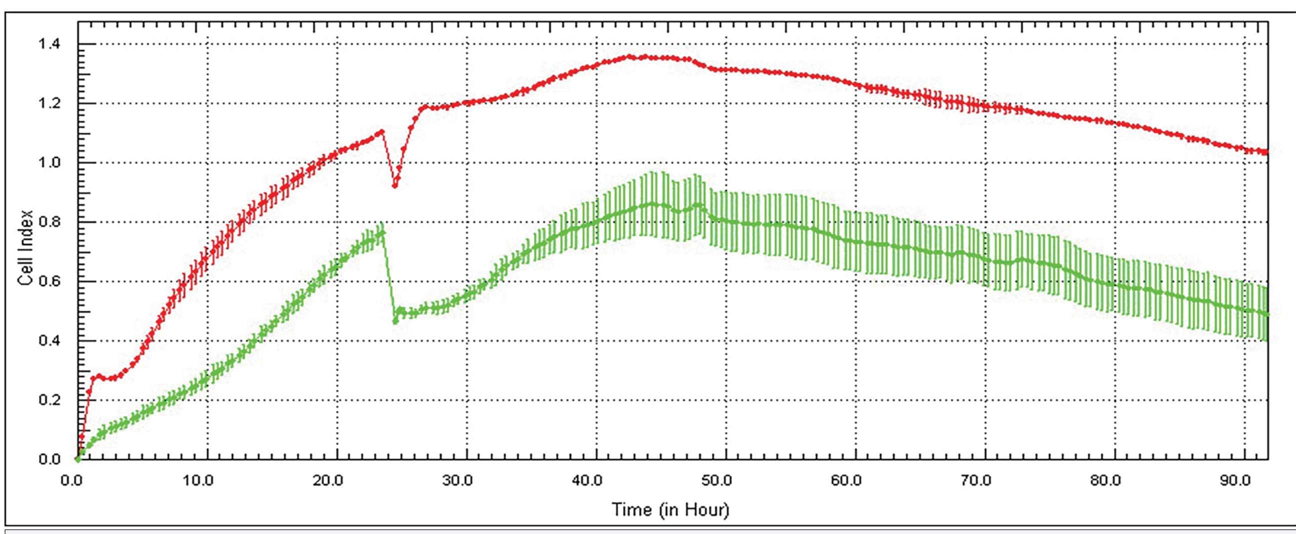

Figure 4. Comparison of proliferation curves of MCF-7 and H1299 cells at the dose of 1/30 (Red MCF-7, Green H1299).

DISCUSSION

Radiotherapy, chemotherapy, and surgical interventions are used in cancer treatment. Although they have curative potential, their negative effects on healthy cells limit the treatment potential. Because of their low side effects, biotoxins have drawn attention as a new hope for cancer treatment in recent years. Cytolytic and cytotoxic activities of peptides that venom contains show their effects by strengthening the immune system of the body. It is thought that biotoxins could replace the anti-neoplastic in the near future (1).

It was reported that ion channels and potassium channels associated with voltage-related the human ether-à-go-go-related gene decelerated the DNA synthesis and cellular proliferation in tumor cells (28). Çalışkan et al. (29) purified Ac8, Ac9, and Ac10 peptides from *Androctonus crassicauda* species using High performance liquid chromatographic analysis and determined that these peptides had a cytotoxic effect on BC3H1 (mouse brain tumor cell) cells. It was stated that these peptides would have an antitumor effect in humans. A peptide consisting of 36 amino acids was isolated from the *Leiurus quinquestriatus* species. Blocking the chlorine channels, this peptide is named chlorotoxin (ClTx). It was found to have an apoptotic and necrotic effect on the human embryonic renal cells and mouse myoblast cells (30). Chlorotoxin is specifically sensitive to glioma cells. It was found to prevent proliferation in human glioma cells and have a therapeutic effect on brain cancer (31-33). Chlorotoxin is a noninvasive scanning tool for the early diagnosis of colon, esophageal, cervical, lung, and skin cancers. Binding with ferrous oxide nanoparticles, chlorotoxin forms a polyethylene glycol binder. This structure successfully binds to ligands targeted via the medications. These ligands preferably accumulate on tumor cells and increase the cytotoxicity in tumor cells. This multifunctional nanoparticle system is believed to be potentially useful in cancer diagnosis and treatment (34). *Buthus martensii* Karsch antitumor-analgesic peptide isolated from *Buthus martensi* Karcsh species is a sodium channel neurotoxin. This peptide was reported to stimulate apoptosis by influencing the ion channels in malignant glioma cells (35). It was observed that the crude venom of *E. mingrelicus*, which we used in the present study, decreased the proliferation of MCF-7 cancer cell line. However, further studies are needed to elucidate this mechanism of action.

The venom called bengaline and isolated from *Heterometrus bengalensis* was reported to inhibit cellular proliferation by stimulating the apoptosis in human leukemia cells (36,37). The polypeptide extract from scorpion venom (PESV) is a peptide consisting of 50-60 amino acids isolated from *Buthus martensi Karsch* scorpions and it was determined to have anti-proliferative, cytotoxic, and (for human belly vein cells) apoptotic effects. It was revealed that polypeptide extract from scorpion venom was found to be effective against the androgen-dependent prostate cancer cell line. Hormone-resistance prostate cancer (HRPC) remains a problem (38). Breast cancer is a cancer that might originate from any point in breast tissue. The death that is most frequently seen among breast cancer patients is due to the metastatic spread of cancer cells invading the angiogenic blood vessels growing within the tumor. A hyaluronidase (BmHYA1) was purified from the venom of *Buthus martensii*. The human breast cancer line (MDA-MB-231) contains many hyaluronidases. Hyaluronidase constitutes a new class for anti-cancer treatment without therapeutic and toxic side effects (39,40). It was reported that protease isolated from *Mesobuthus gibbosus* scorpion venom yielded a significant decrease in human lung adenocarcinoma cell lines. These peptides show very strong gelotinotylic and cytotoxic effects against human lung adenocarcinoma (41). TRAIL peptide (TNF-related apoptosis-inducing ligand) obtained from scorpion venom is used for inducing apoptosis in melanoma cells. While normal cells are not affected, these peptides target only the cancer cells. In melanoma cancer cells, TRAIL initiates proapoptotic Bcl-2 and caspase. Resulting in the release of mitochondrial factors such as TRAIL, SMAC (secondary mitochondrial caspase activator), and AIF (apoptosis-inducing factor), it causes depolarization and permeabilization of the external membrane of mitochondria. These factors inhibit the proliferation of cancer cells and induce apoptotic death (42). In literature, the scorpion venoms that have been examined are those that are strong enough to intoxicate humans. In this study, the crude venom of *E. mingrelicus*, a scorpion species that does not have enough venom to intoxicate humans, was tested on two different cancer types and normal fibroblast cell lines. This venom reduced proliferation of mcf-7 cancer cell line. This showed that there are agents that can affect cancer cells in the venom of scorpions, which do not cause intoxication in humans.

CONCLUSION

In the present study, it was observed that the scorpion venom used reduced the proliferation of the MCF-7 cancer cell line. Since the amount of venom was limited, dilution rates were set to be high. By carrying out further studies determining the protein content of venom and increasing the venom concentration, it is thought that this scorpion venom can be used as a biotherapy agent in the treatment of human breast cancer.

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