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ORIGINAL ARTICLES

Crude venom of *Pseudocerastes persicus* snake: From the antibacterial to anticancer effects

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Abstract: *Antibiotic resistance has been reported as one of the world's most critical public health problems.*

Recent investigations have demonstrated that the venom of some species of snakes has antimicrobial and anticancer activities. The purpose of this study was to examine the antibacterial activity of Persian horned viper venom toward Escherichia coli, Bacillus subtilis, and Staphylococcus aureus bacteria and also investigate antitumor effects of concentrations of venom with higher antibacterial activity in a human hepatocellular liver carcinoma cell line (HepG2) to evaluate the potential use of its components as antimicrobial and anticancer agents. Bactericidal activity of crude venom in concentrations of 6.25-400 µg/ml was performed using MTT reduction, minimum inhibitory concentration (MIC), agar-well diffusion, and disc diffusion methods. Tetracycline (50 µg/ml) was used as a standard antibiotic. Cytotoxic effect in HepG2 cells was measured by MTT reduction assay and confirmed with neutral uptake assay following exposure of cells with different concentrations of venom (50-400 µg/ml). The apoptotic effect was investigated using the comet assay. Our findings demonstrated that venom displays higher inhibitory effects against Gram-positive bacteria as compared to Gram-negative. Furthermore, venom showed anticancer activity on the HepG2 cell line through induction of apoptosis and necrosis. The results of this study confirmed that the venom of Persian horned viper induces antibacterial and anticancer effects. These properties make the venom of this viper a potential source for isolation of effective molecule(s) having antibacterial and antitumor activity.

Keywords: *Pseudocerastes persicus, Antibacterial, Cytotoxicity, Snake venom, anti Cancer*

INTRODUCTION

In the world of pharmacology, along with the discovery and use of new antibiotics, pathogenic bacteria also have features that are gradually resistant to them. This has always been a problem for microbiologists, a constant research field for researchers to discover more effective and newer chemical antibiotics as well as antimicrobials derived from natural sources such as toxic plants and animals, including snakes (Park et al., 2012). Studies have shown that some of the active compounds in venomous animals are an important source for the separation of effective drug substances, some of which are naturally capable of destroying bacteria (Harvey and Robertson, 2004; Koh et al., 2006). Snake venom is a mixture of various biological substances, including various proteins with enzymatic and non-enzymatic properties (Chellapandi and Jebakumar,

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2008) which generally contain neurovenom, proteolytic, coagulant, phospholipase, cholinesterase, hyaluronidase, amino acid oxidase, and other enzymes (Sachidananda et al., 2007). Studies show that the effective ingredients in oral saliva or venomous snakes have long been considered by researchers and pharmacists for the treatment of some diseases, the production of various drugs, serums, and vaccines (Kuhn-Nentwig, 2003). The first reports of antibacterial activity in the venomous snakes of the family of Alpideh and Vipride in 1948 and 1968 were published (Ferreira, 1965) and Skarns also reported antimicrobial activity of some enzymes in Snake in 1970 for the first time (Aloof-Hirsch et al., 1968). Subsequently, many studies have been done about the presence of antibacterial agents in the venom of different species of snakes, which caused the isolation of some antibacterial peptides from them. Also, reports show that some of the molecules in the venoms of snakes have anti-tumor activity and prevent the growth and proliferation of cancer cells (Latifi, 2000). Chalmette's first study in 1933 examined the use of snake venom for the treatment of cancer in laboratory animals. It has introduced the results of snake venoms as an important natural source for the isolation and identification of effective molecules in the treatment of sudden illnesses like cancer in the world of pharmacology (Latifi, 2000). Pseudocerastes persicus snake is one of the most dangerous venomous snakes and a member of the Viperidae family. In addition to Iran, this Pseudocerastes persicus snake has been reported from Pakistan, Afghanistan, Oman, and Iraq, and in Iran, in the sandy, rocky and volcanic areas of Yazd, Khorasan, Kerman, Sistan and Baluchestan, Isfahan, Fars, Semnan, Central, Khuzestan, Zanjan, Tehran, Qom, and Hormozgan (Latifi, 2000). This study aimed to investigate the effect of Pseudocerastes persicus snake venom on the growth of gram-positive and negative bacteria and also the effect of probable concentrations of antibacterial effects of Pseudocerastes persicus snake venom on the growth of liver cancer cells in vitro conditions for evaluating the possibility of isolation of antibacterial and anti-cancer component.

MATERIALS AND METHODS

Venom Preparation and Protein content determination

To prepare the venom from the vein, while observing the safety considerations and placing its toxic teeth inside the glass of venom collection, and with brief pressurization to the toxic glands of the viper, the venom contained in them was injected into the glass (Lu et al., 2002). The lyophilized solution was stored and kept until it was used in experiments at -20°C. To carry out antibacterial tests, some of the venoms were pulverized in 250 µl of 50 mM sterile tris hydrochloride buffer, and a few of the venom was mixed in 150 µl water twice per sterilized distillation. The amount of protein in the homogenized toxic solution was determined using the Bradford method (Bradford 1976) at 595 nm (Bradford, 1976). To eliminate potential microbial contamination in the venom solution, the required 1% antibiotic-anti-smectic antibody (Antibiotic-Antimycotic: Invitrogen, USA) is required, and after one night at 4°C was used in experiments.

The bacteria and cell line used

Gram-positive bacteria Bacillus subtilis (nonpathogenic) (Bacillus subtilis subsp. spizizenii, ATCC 6633), gram-negative bacteria of Escherichia coli (ATCC 25922 Escherichia coli), and gram-positive strain of Staphylococcus aureus (pathogenic)(ATCC 25923) from the collection center of Persian Type Culture Collection (PTCC) and liver cancer cells (HepG2) were developed and used from the cell bank of Pasteur Institute of Tehran.

Antibacterial properties of crude venom

Culture media and antibiotics: For a culture of bacteria used from liquid and solid Mueller Hinton (MH) and for comparing the toxic effect of venom with a standard antibiotic used from tetracycline (Sigma, USA) at a concentration of 50 µg/ml.

MTT assay

MTT test is a colorimetric method based on the regeneration and breakdown of tetrazolium yellow crystals by the enzyme succinate dehydrogenase present in the cytoplasm and eventually the formation of insoluble blue crystals. These crystals are dissolved by adding DMSO (Wang et al., 2010). Test microorganisms were grown in MH broth for 5 h (exponential phase) and adjusted to 0.5 McFarland turbidity standard (A600 = 1.0), corresponding to 1.5×106 cfu/ml (Yalcın et al., 2014), 5µl was added in sterile plate 96-wells. Also, the final volume of each well was measured using a liquid culture medium (MH Broth) to 100µl using serial dilution. The bacteria were contacted at concentrations of 6.25, 12.5, 25, 50, 100, 200, and 400µg/ml of crude venom. In this experiment, tetracycline (50µg/ml) as a positive control, a culture medium containing bacteria as negative control, and a non-bacterial culture medium was used as blank. The plate was incubated for 23 hours at 37°C. Then, 5µl MTT (concentration 5µg/ml) was added to all wells and placed in dark conditions at 37°C for one hour. Then, 100µl of DMSO was added to each well. After 2 hours of incubation in dark conditions, the optical absorption of wells at 595 nm was measured using a plate reader (Biotek, USA). The above experiment was performed 3 times and at each time 3 wells (3 repetitions) were considered for each concentration. The

percentage of bacterial survival after contact with different concentrations of the venom was calculated using the following formula (Yalcın et al., 2014).

Bacterial vital percent:

$$\text{Viable Bacterial} = (a/b) \times 100$$

a = Optical Absorption Sample - Blank Optical Absorption

b = Negative optical absorption - Blank optical absorption

Minimum Inhibitory Concentration Test (MIC)

The steps of performing the minimum inhibitory test on plate 96-well were similar to the MTT assay but after placing the bacterium in a culture medium containing different concentrations of venom and incubating them for 24 hours at 37°C without adding any marker. Also, the absorbance of wells at 605 nm was measured using a plate reader (Biotek, USA). This experiment was repeated 3 times and at each concentration 3 wells (3 repetitions) were considered for each concentration. The inhibitory percentages of the effect of venom and standard antibiotics on the tested bacteria were calculated using the following formula (Shebl et al., 2012).

$$\text{Percentage of inhibition of bacteria} = [1-(a/b)] \times 100$$

a = Optical Absorption Sample - Blank Optical Absorption

b = Optical Absorption Control - Blank Optical Absorption

Disc diffusion assay

This experiment was carried out according to Bauer et al (Bauer et al., 1966).

The bacteria were first cultured in a culture medium Mueller–Hinton broth and incubated for 24 to 18 h at 37°C. A solid culture medium with a thickness of 4 mm was prepared in glass plates. 200µl of the 0.5 MacFarland suspension was placed on a plate surface and a cotton seedling was cultured using a sterile loop. Using sterile pin disks (prepared from antibacterial medicine) containing 40µl of venom in the concentration of 3.125, 6.25, 12.5, 25, 50, 100, 200, and 400 µg/ml on the surface plate was placed. In this experiment, 50µg of tetracycline antibody was used as a positive control for 50 µM tetracycline, and 50 mM Tris hydrochloride was used as a negative control. Plates were incubated for 24-18 hours at 37°C, and then an inhibition zone was determined and its size was determined. This experiment was performed 3 times for each bacterial strain.

Well diffusion assay

This method is similar to the disk diffusion method, except that in this test, the venom solution was added to the well instead of being placed on the disk (Torres et al., 2010). In summary, after the cultivation of grass, using the end of the pasteurized pipette, the wells were transferred to the medium and cut into pieces of gel, and 20µl of liquid agar medium was used to block the bottom of the well and prevent leakage and solution leakage. The venom was poured into the well under the medium. After pouring 40µl of Crude venom in concentrations of 3.125, 6.25, 12.5, 25, 50, 100, 200, and 400 µg/ml, the plate was incubated for 24 to 18 hours in an incubator at 37°C. After the mentioned time, the presence of a halo around the wells was investigated and its diameter was determined. This experiment was performed 3 times for each bacterial strain in two replicates.

Evaluation of anti-cancer properties of crude venom

Cell culture

Liver cancer cell line (HepG2) was cultured using DMEM-F12 (Gibco, USA) containing 10% Fetal Bovine Serum (Gibco, USA). These cells were cultured in flasks of 25-50 ml cubic meters and stored at 37°C and 5% carbon dioxide. Replacing every 2-3 days of the culture medium and after providing the cell population required for each test, using a Trypsin-EDTA prepared from Sigma-Aldrich, the USA from a flask and after counting using a Neobar lam were placed.

MTT reduction assay

This experiment was carried out according to the method performed by Zargan et al. (Zargan et al., 2011). To perform this test, 3×104 cells/well were plated in 96 wells containing 100µl of non-serum culture media. After overnight incubation at 5% CO_2, moisture content of about 80% and temperature of 37°C were removed outside the culture medium and the cancerous cell was exposed to a new culture medium (lacking serum) containing different concentrations of venom (50, 100, 200 and 400µg/ml). After 24 hours, 5µl of MTT solution (concentration 5mg/ml) was added to each well. After the formation of crystalline (formalin) in living cells, the culture medium of each well was removed and after washing in the well with PBS, 100µl of DMSO was added. After 4 to 3 hours of incubation in dark conditions and dissolving purple crystals in DMSO, the optical absorption of wells at 570 nm was measured using a plate reader (Biotek, USA). In this essay, the culture media was used as a blank and control medium containing the cells as control. This experiment was repeated 3 times, and 3 wells (3 repetitions) were considered for each concentration. The percent survival of the cell after contact with different concentrations of the venom was calculated using the following formula (Shebl et al., 2012).

$$\% \text{ Viable cell} = (a/b) \times 100$$

a = the absorbance of treated cells - the absorbance of the blank

b = the absorbance of the control - the absorbance of the blank

Cytotoxicity study by Neutral Red Uptake Assay

The steps of performing a neutral color red color test (Waheed et al., 2013) were similar to the MTT assay, but after incubating the cell with a toxic solution for 24 hours, a microplate of neutral red solution (5 mg/ml) instead of MTT was added to each well. After binding of neutral red matter to the lysosome surface of the living cell and the formation of red crystals in the living cytoplasm, the culture medium containing the neutral red of each well washed and after washing twice with each PBS, 100 µl of fixative buffer (37% formaldehyde, Calcium chloride (10%) was added. After one minute, the buffer was removed and 100µl of solvent buffer (0.5% acetic acid) was added. The plate was measured on a shaker for twenty minutes under microwave conditions and incubated at lab temperature and absorbed by wells at a wavelength of 540 nm using a plate reader (Biotek, USA). This experiment was repeated 3 times, and 3 wells (3 repetitions) were considered for each concentration at each concentration. The mortality rate due to different concentrations of venom on cell growth was calculated using the following formula.

Percentage of cell death = [1-(a/b)] ×100

a = the absorbance of treated cells - the absorbance of the blank

b = the absorbance of the control - the absorbance of the blank

Investigation of induction of apoptosis by venom using comet assay test

This test is one of the best methods for checking the damaged DNA in the cell (Sajad et al., 2013). To conduct this experiment, at first, in each well of the plate, 24 sterile wells containing 300µl of serum medium, cultured 12×104 cells. The plate was incubated overnight under conditions of 5% CO2, humidity 80%, and temperature 37 ° C. In this assay, the culture media was used as a blank and control medium containing the cells as control. After that, the initial culture medium of each well, evacuation, and cells was exposed to 300µl of a new culture medium (without serum) containing different concentrations of venom (50, 100, 200, and 400µg/ml).

After incubating the cell for 24 hours at 37 ° C, 80% moisture content, and 5% CO2, cells of each well were isolated using trypsin. Microtubes containing cells were evacuated for 5 minutes at 4 ° C with 1500 rpm of the centrifuge and aqueous solution. 400µl of PBS (pH = 7.4) was added to each of the tubes to wash the cells, and after centrifugation, the supernatant was evacuated. Then, 200µl of PBS was added to the tubes, and cells were separated from each other by the separate sampler and insulin syringe. Also, the slides needed for testing were coated with agarose with normal melting point (NMA% 1), test cell suspension and control with low melting point agarose (LMA% 1), one to two on the slides a flat layer was placed. To lyse the cell and nucleus membrane, all slides are placed in the refrigerator in a refrigerator buffer (NaCl 2.5M, EDTA 100mM, Tris 10mM, NaOH 0.2M, Triton X-100% 1 pH = 10) for 16-18 hours. After removing the buffer, the slides were washed for 20 minutes and 2 times with electrophoresis buffer (3M NaOH, EDTA 1mM, and pH>13), and then to open the DNA for 40 minutes in a cold electrophoresis buffer temperatures were maintained at 4°C. The slides were then extracted from the solution and placed in a buffer containing an electrophoresis tank and electrophoresed at 45°C for 45 minutes at a voltage of 25 and 300 mA. In the next step, to undo the playing environment, the slides were placed in a neutralizing buffer (Tris% 4 M, pH = 7.5) for 10 minutes. To staining cells, 100 µl of ethidium bromide solution (concentration 20 µg/ml) was added to each slide, and after 10 minutes incubated at the laboratory for 10 minutes, they were distilled twice with water. DNA of each slide cell was studied by fluorescence microscopy. For each sample, different slide positions and at least an image of hundreds of cells' DNA were prepared and the results were analyzed statistically.

Statistical analysis

The results of each test were reported as Mean±SD and the data were analyzed using software (GraphPad InStat). Different concentrations of venom were compared to the control group and compared to each other by one-way ANOVA and Tukey tests. $P<0.05$ was considered significant in all tests. Draw diagrams in the Microsoft Excel software (2013 version).

RESULTS

Evaluation of the Anti-Bacterial Properties of Crude venom

Test results MTT assay

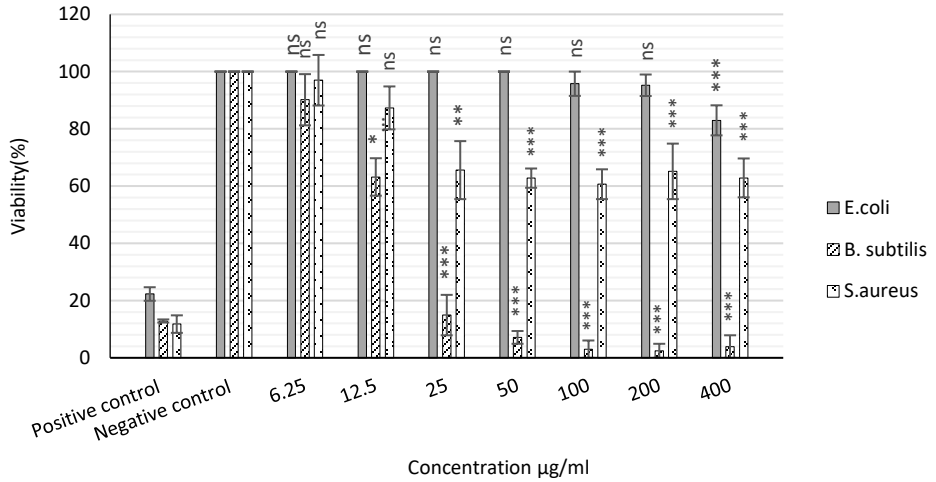
The results showed that venom only at 400µg/ml concentration significantly inhibited the growth of Escherichia coli bacteria compared to the negative control (normal culture medium). The critical percentage of this bacterium in concentrations of 6.25-50 µg/ml was 100 and in 100, 200, 400 µg/ml concentrations respectively were 95.79, 96.29, and 83 (Figure 1).

In the case of the Bacillus subtilis strain, Crude venom in the concentration of 12.5 to 400µg/ml produced a significant

inhibitory effect on bacterial growth compared to the negative control.

The percentage of vital bacteria in different concentrations of venom 6.25, 12.5, 25, 50, 100, 200 and 400 µg/ml respectively were 89.62, 62. 49, 13.73, 6.56, 2.14, 1.63 and 4.6.

Figure 1: Antibacterial effect of different concentrations of Pseudocerastes persicus snake crude venom on Escherichia coli, Bacillus subtilis, and Staphylococcus aureus using MTT assay. The effect of concentrations was evaluated in comparison with the negative control group.



(: p<0. 05), (**: p<0. 01), (***: p<0.001), (ns: non-significant).*

Venom hurt the growth of this bacterium at a concentration of 6.25µg/ml, but this effect was not significant in comparison with negative control. In the range of 25-400, the inhibitory effect of the venom was greater than the positive control (tetracycline). Statistical analysis showed that the effect of venom in a concentration of 50-400 µg/ml was significant for this bacterium but it was not significant compared to each other. According to the results, IC50 of the venom for Bacillus subtilis was determined to be 15.32µg/ml. The results also showed that the vital percentage of Staphylococcus aureus bacteria in the concentration of 6.25, 12.5, 25, 50, 100, 200, and 400µg/ml of crude venom were 95.11, 85.29, 72.48, 62.23, 59.98, 63.31, and 63.96. Crude venom at a concentration of 25-400 µg/ml produced a significant inhibitory effect on the bacteria compared with the negative control. Venom in the concentration of 12.5µg/ml hurt the growth of this bacterium, but this effect was not significant compared to negative control. Statistical analysis showed that the effect of venom in concentrations of 25-400 micrograms per ml was significantly less than the negative control, but compared with each other, it is not significant (Figure 1).

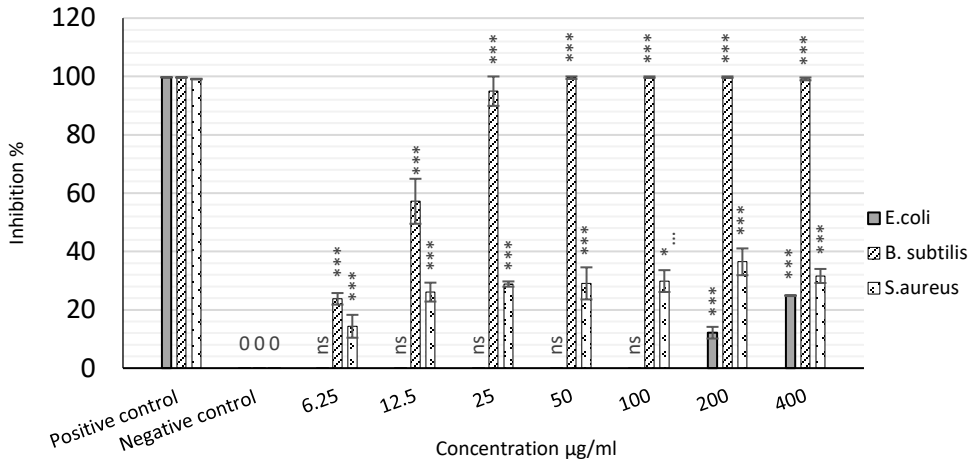
Minimum Inhibitory Concentration Test results

Pseudocerastes persicus snake venom at concentrations of 6.25-100 did not inhibit the growth of Escherichia coli, but induced inhibitory effects at concentrations of 200 and 400 µg/ml, respectively were 12.15 % and 24.99 % which, compared to the intolerance of tetracycline, and the difference in their effect is significant (Figure 2).

The inhibitory effect of venom on Bacillus subtilis bacteria in the concentrations of 6.25, 12.5, 25, 50, 100, 200, and 400 µg/ml was 23.22, 58.92, 92.80, 99.48, 99.65, 99.71, and 99.3, respectively. According to the results, the IC50 of the venom for Bacillus subtilis was determined by 10.81µg/ml. The statistical analysis showed that Crude venom at a concentration of 6.25-400 µg/ml had a significant inhibitory effect on Bacillus subtilis bacteria growth compared to the negative control. The inhibitory effect of venom was 25-400 µg/ml in comparison to the negative control, but compared with each other and in comparison with standard antibiotics was not significant (Figure 2).

Crude venom has an inhibitory effect on the growth of Staphylococcus aureus, and the inhibitory effect induced in it was 6.25, 12.5, 25, 50, 100, 200, and 400 were 13.95, 25.28, 29.13, 27.30, 30.52, 35.91, and 31.69 µg/ml, respectively. Statistical analysis showed that the toxic venom has a significant inhibitory effect on the growth of this bacterium at concentrations of 6.25-400 µg/ml, compared to the negative control. The losses caused by the effect of venom in different concentrations ranged from 25-400 µg/ml compared to the negative control but were not significant in comparison with each other (Figure 2).

Figure 2: Antibacterial effect of various concentrations of Pseudocerastes persicus snake crude venom on Escherichia coli, Bacillus subtilis, and Staphylococcus aureus using MIC assay. The effect of concentrations was evaluated in comparison with the negative control group.

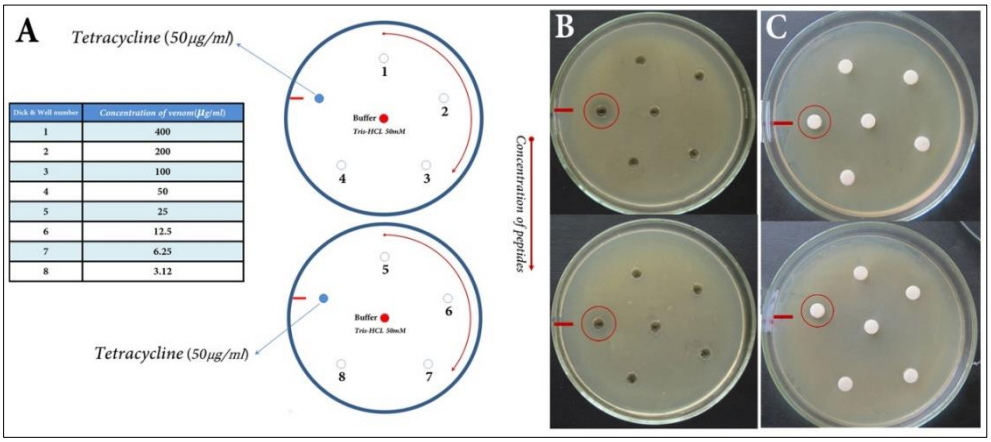


(: p<0. 05), (**: p<0. 01), (***: p<0.001), (ns: non-significant)*

Results of diffusion tests on disk and wells

The results of the effect of various concentrations of venom in the diffusion test on the disk and wells indicate that the results of these two tests, especially at low concentrations of venom, are not repeatable. However, according to the results of this study, it was found that the venom in these concentrations did not inhibit the growth of Escherichia coli (Figure 3).

Figure 3: Antibacterial effect of various concentrations of Pseudocerastes persicus snake crude venom on Escherichia coli, Bacillus subtilis, and Staphylococcus aureus using MIC assay. The effect of concentrations was evaluated in comparison with the negative control group.



Disk & Well number	Concentration of venom(µg/ml)
1	400
2	200
3	100
4	50
5	25
6	12.5
7	6.25
8	3.12

The results of these experiments on Bacillus subtilis bacteria showed that the venom had an inhibitory effect on bacterial growth at a concentration of 50-400 µg/ml, but did not have an inhibitory effect on the growth of the bacteria in the concentration of 6.5- 25 µg/ml (Figure 4).

Also, venom only prevented the growth of Staphylococcus aureus at concentrations of 200 and 400 µg/ml and did not inhibit the effects of lower concentrations (Figure 5).

Evaluation of anti-cancer properties of Crude venom

Results of cytotoxicity testing by MTT method

Venom at concentrations of 50, 100, 200, and 400 µg/ml causes high mortality in liver cancer cells, respectively, in which the vital percent of the cell at the above concentrations were 14.12, 10.97, 10.43, and 9.96% has dropped. The cells losses caused by the different concentrations of venom are not significant (Figure 6).

Cytotoxicity test results with neutral red colorimetric method

The mortality rate of liver cancer cells in the concentration of 50, 100, 200, and 400 µg/ml of crude venom was 60.13, 72.58, 81.61, and 77.48 %, respectively. Venom has a significant inhibitory effect at concentrations of 100-400 µg/ml in comparison with the control (containing cell and culture media), but the mortality rate is compared between all concentrations is not significant (Figure 7).

Figure 4: Antimicrobial effect of crude venom on diffusion and disk diffusion methods against growth Bacillus subtilis bacteria. A) map and concentrations used; b) well distribution; c) disk diffusion test.

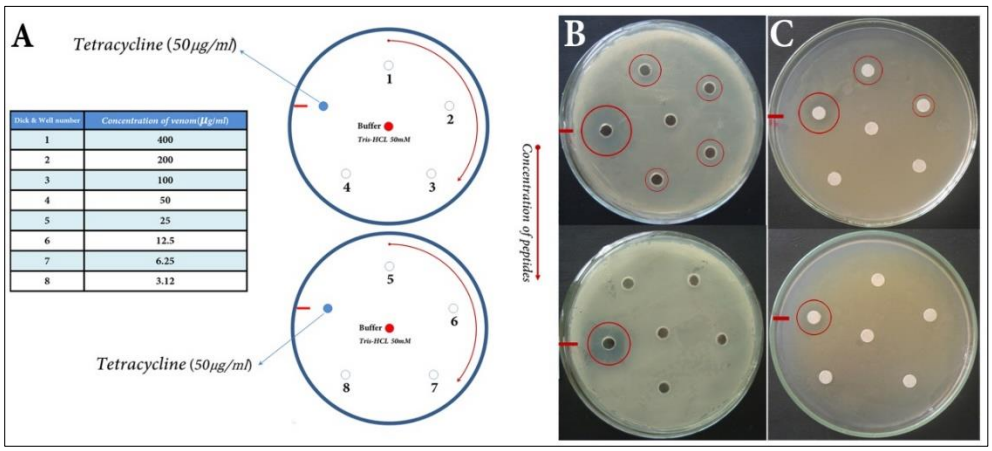


Figure 5: Antibacterial effect of crude venom on diffusion and disk diffusion methods against growth Staphylococcus aureus bacteria. A) map and concentrations used; b) well distribution; c) disk diffusion test.

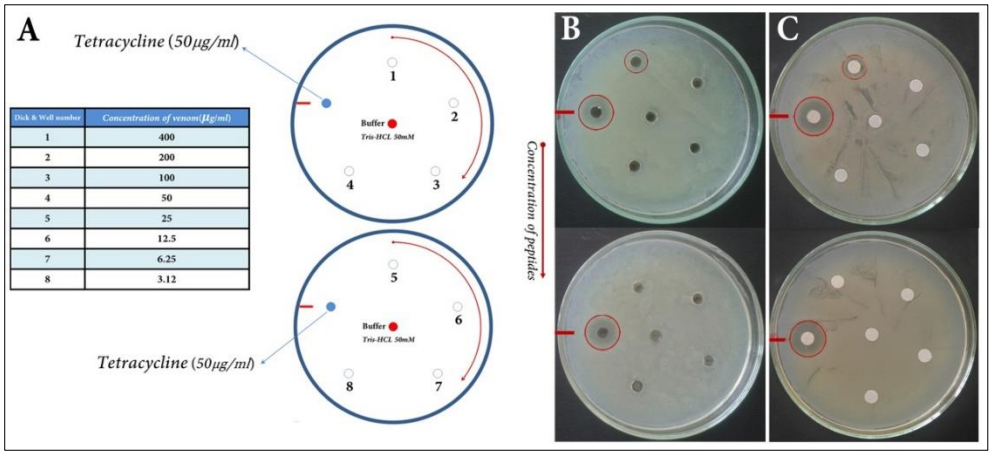


Figure 6: Percentage of vital liver cancer cells after 24-hour contact with different concentrations of the crude venom according to the MTT assay method. The effect of concentrations was evaluated in comparison with the control group. (***: p<0.001).

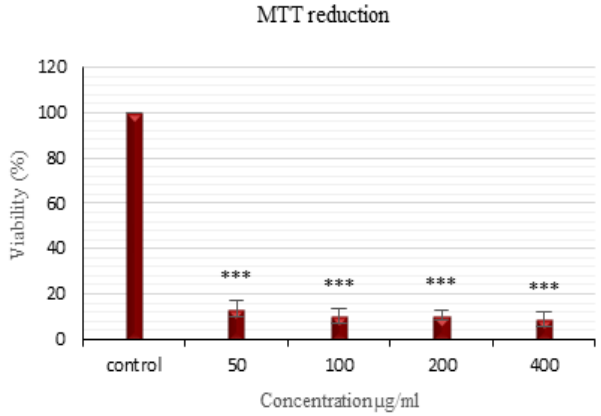
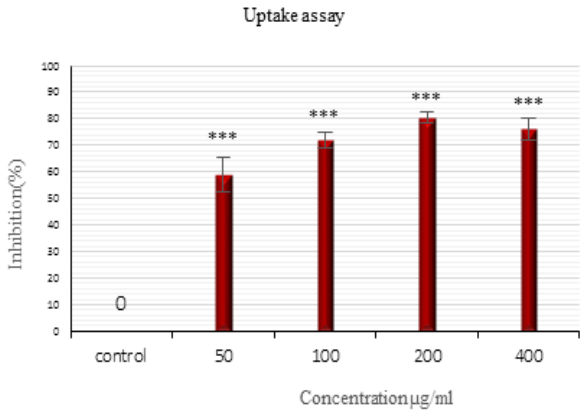


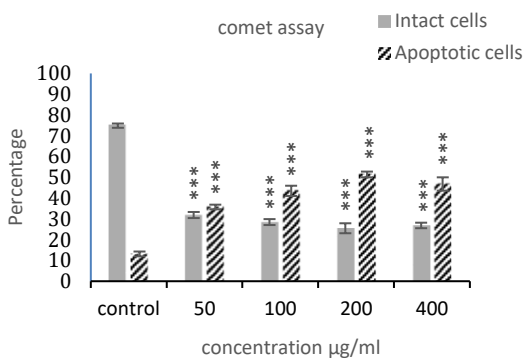
Figure 7: Percentage of mortality of liver cancer cells after exposure to different concentrations of crude venom Pseudocerastes persicus snake based on Neutral Red Color. The mortality due to the effect of each concentration is compared with that of the control group. (***: p<0.001)

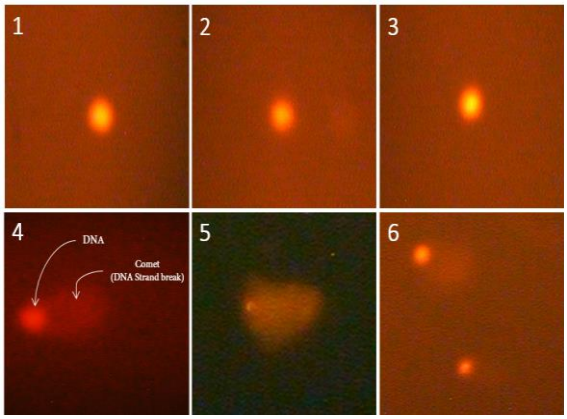


Investigation of venom apoptosis induction using comet assay test

The results of this study showed that Pseudocerastes persicus snake venom induces apoptosis and causes mortality in liver cancer cells. Venom at concentrations of 50, 100, 200, and 400 were 36.6, 43.28, 51.5, and 47.13%, respectively, induced apoptosis in the cell. The morphological study of venomous cells also showed that the venom of this aphid, in addition to apoptosis, induces necrosis in liver cancer cells, and this effect has an upward trend of more than 200 µg/ml in comparison with lower concentrations. These results indicate that mortality in cancer cells is due to the common effects of apoptosis and venous necrosis (Figure 8).

Figure 8: Rate of apoptosis induced by Pseudocerastes persicus snake crude venom in liver cancer cells based on the alkaline test. A: The morphological changes in DNA from the concentration of each concentration compared to the control group. B: Morphological changes in DNA of proximal cells with Pseudocerastes persicus snake crude venom compared to control cells [1-3]: DNA image of the normal cell(×40), [4-6]: DNA image of apoptosis cell(×40).





DISCUSSION AND CONCLUSIONS

According to the World Health Organization, 40% of the 50 million annual human mortality is due to bacterial contamination and microbial diseases (Ivnitski et al., 1999). Also, bacterial infections caused by resistant strains and problems due to the lack of treatment of people with them have become an important health problem. This has led researchers to investigate more in living organisms, including various venomous snakes, to discover more effective and new antibiotics. According to studies conducted in recent years, venomous snakes are a source of unknown molecules of active substances (25) that can be considered as an appropriate candidate for the production of antimicrobial and anticancer drugs. In this study, for the first time, the antibacterial and cytotoxic effects of Pseudocerastes persicus snake were studied in vitro conditions to evaluate the possibility of isolating molecules with antibacterial and toxic effects for cancer cells. MTT reduction, MIC assay, Disc diffusion assay, and Well diffusion

test were used to study the antibacterial effects of crude venom. The results obtained from MTT reduction and MIC tests showed that Pseudocerastes persicus snake venom has an inhibitory effect on Escherichia coli bacteria as a representative of gram-negative bacteria only at the highest concentration (400 µg/ml). However, bacteria Bacillus subtilis and Staphylococcus aureus have been prevented from bacterial growth at concentrations of 12.5-400 and 25-400 µg/ml, respectively. The reason for the difference in the results of MTT and MIC is due to the difference in the calculation of the inhibitory effect of the venom in two methods, in the MIC method, the opacity is due to the presence of bacteria, which includes dead and living bacteria, is measured at a wavelength of 605 nm. While in the MTT method, the optical absorption of formazone particles formed in the cytoplasm of the living bacteria is determined after dissolution in DMSO at 570 nm. MTT testing seems to provide more accurate results than the MIC method. Also, the results of the study showed the antibacterial effects of crude venom using two methods of diffusion in wells and discs. In this study, snake venom has no inhibitory effect on gram-negative bacteria but has an antibacterial effect on two-gram positive bacteria. The results showed that these two methods qualitatively confirm the effects of MTT and MIC, but did not quantitatively produce repeatable results. One of the reasons for this issue, it seems, is that the proteins and toxic peptides are deposited at the bottom of the well, and the walls and around the wells act as a filter and penetrate the entire contents of the venom into the well prevents solid culture. In the case of a disk, it is also possible that the disk itself acts as a filter and prevents the outflow and full penetration of the components of the venom into the medium. The rapid absorption of water in the upper surface of the medium using a paper disk and the lack of complete absorption of toxic compounds that allow heterogeneous release in the medium is another disadvantage of this method. In this study, it was determined that Pseudocerastes persicus snake crude venom is more effective than gram-positive bacteria. This selective antibacterial activity may be due to several factors such as the difference between bacterial species such as density and lipopolysaccharide structure present in the wall of gram-negative bacteria or fat composition in the cytoplasmic membrane and electrostatic potential across the membrane in gram-positive bacteria Be (San et al., 2010). The results of studies by Husniye et al. (2012), Shebell et al. (2012), Stabley et al. (2004), Steels et al. (1991), and similar studies show that venomous affect gram-positive bacteria (Stiles et al., 1991; Lu et al., 2002; Yalcın et al., 2014; de Lima et al., 2005; Stábeli et al., 2004). Also, the results of the antibacterial effect of Jafri's venom (Jami et al., 2011) showed that snake venom has a better effect on Staphylococcus aureus bacteria than Escherichia coli bacteria. The results obtained in this study are consistent with previously reported results regarding the antibacterial effects of snake venom. On the other hand, the inhibitory effect of Pseudocerastes persicus snake crude venom in the highest concentrations studied in this study on E. coli is an effective component of this bacterium at very low concentrations in venom. The results of studies by Chalapandi et al. (2008), Bostley et al. (2008), Starker and Trierworth in 1986, and similar studies have reported the inhibitory effects of venomous organisms on gram-negative bacteria (Chellapandi and Jebakumar, 2008; Stiles et al., 1991; Shebl et al., 2012; Bustillo et al., 2008). Snakes venom are mainly composed of proteins and peptides that have diverse biological activities. Some of these molecules have been shown to have inhibitory effects on cancer cell growth in clinical trials and may find their way toward developing anticancer drugs in the future (Jain and Kumar, 2012). In this research, the effects of toxicity of venomous snake, which has antimicrobial properties induced by the use of MTT assay, Neutral red uptake assay, and Single cell gel electrophoresis (Comet assay) on liver cancer cells (HepG2 cell line) was taken into consideration in vitro conditions. Reports on the presence of cytotoxic effects in the venom snake have led to the use of HepG2 cells in this study (Latifi, 2000). The results of the MTT assay and uptake assay showed that crude venom at concentrations of 50, 100, 200, and 400 µg/ml has cytotoxic effects and causes the death of liver cancer cells. These results are consistent with findings from Bozemans et al. (2005), Yang et al. (2006), Depeca et al. (2012), Sang et al. (2012), to induce cytotoxic properties by venoms of a snake (Jain and Kumar, 2012; Bosmans et al., 2005; Cancer Control Office, 2005; Song et al., 2012; Yang et al., 2006). As well as the results of Shebel, et al suggesting that mortality in the cell would be increased by increasing the concentration of venom (Shebl et al., 2012). To investigate the possibility of inducing cell death through crude venom through apoptosis in HepG2 cells, this study was conducted using Comet assay. The results showed that crude venom at concentrations of 50, 100, 200, and 400 µg/ml caused apoptosis in cancer cells the liver causes it to die. It was also found that with an increase in concentrations of 50 to 200 µg/ml of crude venom, apoptosis has a tendency to increase, which is consistent with previous reports (Sajad et al., 2013). Generally, the results of this study showed that the crude venom of snakes contains molecules that can have anti-bacterial and anti-cancer activity. These results suggest that the venom of a snake is a suitable candidate for the isolation, purification, and introduction of valuable drug molecules to combat antibiotic-resistant bacteria as well as

anti-cancer agents.

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