Original Article

Fractionation of Potamon Persicum Crabs hemolymph and their effects on MCf-7, MdA-231, and HUVEC

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Abstract

Introduction and aims: Breast cancer is the most common malignancy among Iranian women. The induction of apoptosis is considered a major goal in the treatment of cancer at different stages of tumor growth. The aim of this study was to fractionate the *Potamon persicum* crab hemolymph serum and assess the its cell toxicity, induction of apoptosis and anti-proliferation effects on cancer cell lines (MDA-231, MCF-7) and HUVEC. **Materials and Methods:** Crabs were transported to the laboratory in containers containing river water and anesthetized by ether and chloroform. The hemolymph was collected in a sterile tube through a cut in the forearm of the crab. Then the serum was separated from the cellular part by ion exchange chromatography. The cell toxicity and anti-proliferative effects of the total serum and fractions were investigated by LDH (Lactate dehydrogenase) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tests and their apoptotic effect was assayed by a combination of TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) and propidium iodide staining. **Results:** Four different protein fractions in terms of electrical charge were obtained from hemolymph serum by ion exchange. The fourth group (hemocyanin) contained 80% of the total protein of hemolymph serum. The fourth fraction and total serum were able to inhibit the growth and survival of MDA-231 and MCF-7 cancer cells at lower concentrations than HUVEC (noncancerous cells). They also increased the induction of apoptosis relative to the control sample, with the fourth fraction having a stronger effect than total serum. **Discussion and Conclusion:** Due to the fact that the total serum and fourth fraction of Hemolymph had low toxicity and showed anti-proliferation effect on cancer cells in lower concentrations and also caused the induction of apoptosis in these cells, so the hemolymph serum of this crab and especially the fourth fraction which has the highest amount of hemolymph protein could be a good option for more quantitative studied and continuing the study in *in-vivo* models for breast cancer treatment.

Keywords: Crab, Hemolymph, Apoptosis, Breast Cancer, LDH, MTT, Propidium iodide, TUNEL

INTRODUCTION

Cancer is caused by uncontrolled and overgrowth of the cells. This condition results from a disorder in the regulation of growth (cell cycle) in a single cell. Cancer actually is a genetic disease, and in all types of cancer there are many changes in DNA sequence or gene regulation [1]. Breast cancer is a complex disease, and the incidence and mortality of the disease has increased in recent decades. The disease consists of three distinct clinical, morphological, and molecular components. Over the past decade, research has focused on the molecular biology of the disease.

Apoptosis occurs normally during the growth and aging and, as a homeostatic mechanism, regulates cell population in tissues. Apoptosis also acts as a defense mechanism, for example in immune responses or when cells are damaged by disease or toxins [2]. Multiple mutations can cause abnormal growth in cells by interfering with apoptosis. Anticancer agents have now been shown to induce apoptosis, and disruption of the apoptosis process can reduce the sensitivity of treatment, and disruption of this process can help tumor cells survive and lead to tumor metastasis. So resistance to apoptosis helps the beginning, progress and resistance to cancer treatment [3]. Cancer is an example in which the natural mechanisms of the cell cycle are malfunctioning and it is accompanied by overgrowth of cells or reduced harvesting. In fact, suppression of apoptosis during carcinogenesis appears to play a central role in the development of some cancers. There are a variety of molecular mechanisms which

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tumor cells use them to suppress apoptosis. Tumor cells can be resistant to apoptosis by expressing anti-apoptotic proteins such as bcl-2 or by negative regulation or mutation of pre-apoptotic proteins such as bax. The expression of both bcl-2 and bax is being regulated by the tumor suppressor gene P53. Known types of human B lymphoma show overexpression of bcl-2, and this is one of the strongest evidence that disability in cell death contributes to cancer.

For many years, research has focused mainly on terrestrial organisms, due to easy access to their habitat [4]. Aquatic invertebrates, which evolve in a different environment from terrestrial animals, have unique and biologically different compounds and are a vast resource for pharmacological compounds [5]. Recently, research on aquatic life has flourished, with more than 300 new substances with different medicinal properties discovered over the past three decades, some of which have anti-cancer properties. These compounds include antimicrobial peptides present in crab homocytes such as Tachyplesins, tachystatins, crustin, as well as homocyanin macromolecules in the hemolymph of the aquatic invertebrates. The *Potamon persicum* crab, like other invertebrates, has hemolymph. In traditional medicine, it is prescribed orally for cancer patients. Hemolymph contains hemocytes, proteins, carbohydrates, and lipids [6]. The anticancer properties of homocyanin and the antimicrobial peptides of hemolymph have been demonstrated in various studies.

In another study, the anti-cancer properties of peptide fractions isolated from snow crab were investigated *in-vitro* on cancer cells. Among the fractions tested, the KCL2 isolated fraction had anticancer properties [7]. The toxicity of hemolymph antimicrobial peptides for various human normal cells, including RBCs, is much lower than the toxicity for cancer cells. These peptides bind selectively to cancer and microbial cells that have a negative charge. Cecropins, Magainins, Melittins, Tacchyplesin are examples of these anti-cancer peptides [8]. In another study the growth inhibition and apoptosis induction effects of Cecropin peptide separated from Musca domestica hemolymph on BEL-7402 human liver cancer cells were observed [9].

Until today, no study has been conducted on the anti-cancer properties of *Potamon persicum*. In this study, for the first time, the pattern of hemolymph serum proteins of this type of crab was examined by SDS-PAGE. The different protein fractions were then separated from the hemolymph serum by ion exchange chromatography with DEAE-Sepharose column. The cytotoxic effect and then the anti-proliferative effect of total serum and its fractions on breast cancer cell line (MDA-231, MCF-7) and non-cancerous, HUVEC cell line (human umbilical cord cell endothelial cell) with LDH (Lactate dehydrogenase) and MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tests were examined. Also, the apoptosis activity of total hemolumph serum and its fourth faction on these cell lines was examined by TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) test.

Materials and Methods:

The crabs were transported to the laboratory in containers containing river water and were anesthetized in the presence of ether and chloroform. The hemolymph was collected in a sterile tube through a cut in the forearm of the crab. Then the serum was separated from the cellular part by centrifugation. *Potamon persicum* crab hemolymph was first fractionated using gel filteration method with sephacryl S200 resin. Regarding that the separation range of this resin is 5-250 kDa [10] and on the other hand the molecular weight of homocyanin and some hemolymph proteins is normally higher than 350 kDa, so proper separation was not done and because of lack of resins with a higher separation range such as sephacryl S300 and S400, ion exchange chromatography was performed.

The total protein content of hemolymph serum and fractions was calculated according to the standard BSA curve.

Cell culture

After defrostion, the cells were suspended in 3 mL of RPMI 1640 culture medium containing 15% FBS and 50 µg/ mL gentamicin and 2 mM glutamine and transferred to 25 ml flasks. The flasks were placed in an incubator at 37 °C with a humidity of 95% and a CO_2 concentration of 5%. During the two to three-day period, the surface culture of the cells was slowly removed, and after two washes with PBS, 3 ml of the enriched culture medium was added to the cells again, until the number of the cells continued reached a density range of 80%.

Cell count in cell suspension by Tripan Blue staining method

To count the cells, first a drop of cell suspension is mixed with a drop of Tripan Blue (0.2% in PBS) and after one to two minutes the living cells (cells that have not been stained) and dead cells (blue cells) were counted by neubauer slide under a light microscope. In neubauer slide, there are four large squares around the central square, each containing 16 smaller squares. The number of cells in the four squares of 16 was counted and their average was calculated. This mean was multiplied by the dilution rate of 10,000 to get the number of cells per milliliter of the solution.

Determination of the toxicity effect of hemolymph serum and its protein fractions on HUVEC, MCF-7 and MDA-231 cell lines

LDH test to measure cytotoxicity

This test is a method to test the toxic effect of a substance on cells by measuring the activity of the enzyme lactate dehydrogenase in the cytoplasm of damaged cells.

The greater the destruction of the plasma membrane and the resulting cell death, the more enzymes are released into the environment and the more purple color is produced at the end of the reaction.

To study the toxicity of total hemolymph serum and fractions, concentrations of 100-2000 µg/mL of total hemolymph serum and each of the four fractions (resulting from anion-exchange chromatography) were used.

Finally, the percentage of cellular toxicity was calculated according to the following formula:

Cell toxicity percentage =

$$\frac{Mean\ absorption\ of\ test\ group - Mean\ absorption\ of\ low\ control\ group}{Mean\ absorption\ of\ high\ control\ group - Mean\ absorption\ of\ lowcontrol\ group}$$

To ensure the accuracy of the test, it should be noted that the absorption of the low control group at the confirmed concentration is not more than 0.2, and this number is approximately 1.2 for the high control group. It should be noted that to perform the LDH test, the maximum allowable concentration of FBS used in the culture medium is 2%.

Investigating the effect of hemolymph serum and its fractions on HUVEC, MCF-7 and MDA-231cell proliferation

In order to evaluate the anti-proliferative activity of hemolymph serum and its isolated fractions, the number of live and dead cells in samples under hemolymph serum treatment and control sample in logarithmic phase of cell proliferation was calculated after several steps of cell doubling. One common way to calculate the ratio of living cells is to use MTT.

The percentage of reproduction inhibition was calculated using the following formula:

Proliferation inhibition percentage = $\frac{Test\ sample\ absorbance - control\ sample\ absorbance}{control\ sample\ absorbance}$

Evaluation of apoptosis by TUNEL and peroxidase test

The effects of different concentrations of total serum as well as its fourth fractions with the same concentrations on cells compared with the control group were investigated separately with one-way analysis of variance and to determine the effective dose of each sample on cells Post hoc. Test was used. Also, to report the values of each parameter, the mean data of each group ± standard deviation was used.

P <0.05 was also considered to be the least significant difference between the treatment and control groups.

Ion exchange chromatography results of the hemolymph serum of *Potamon persicum*

After isolating the hemolymph serum proteins in the ion exchange chromatography with the DEAE-Sepharose column, four different protein fractions were obtained in terms of electric charge. The chromatograms for the fractions are shown in Fig. 1. Among the isolated fractions, the fourth, first, second and third fractions constituted 80%, 7.2%, 8.8%, and 4% of the total protein of hemolymph serum, respectively. The first fraction contained proteins that had a more positive charge and did not attach to the positively charged column and were removed before adding the salt gradient. The second protein fraction was removed at the beginning of the salt gradient, and the third fraction in the middle and the fourth fraction, which included the largest amount (80%) of the total protein of the sample and constituted of more negatively charged proteins and isoelectric points below pH 6 came out at 300 mM sodium chloride gradient.

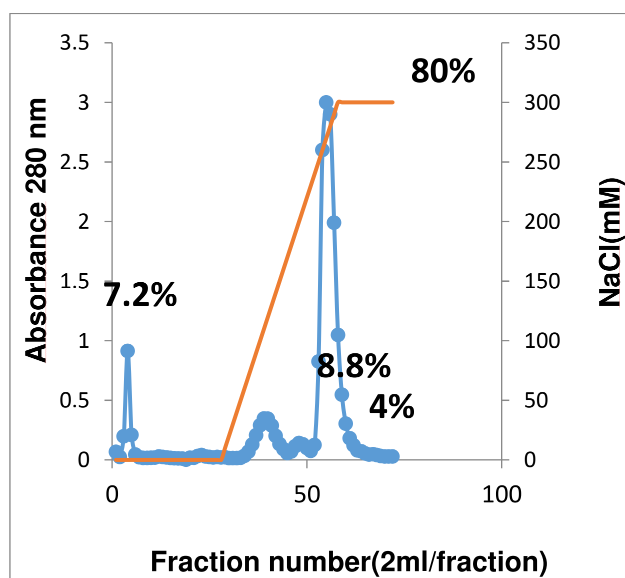

Fig. 1: Chromatogram obtained from crab hemolymph serum chromatography in the ion exchange column (DEAE-Sepharose) in a 50 Mili molar tris pH = 7.0. Fourth fraction (peak) consisted the main part of the crab hemolymph serum which was got out in a 300 mM salt gradient.

Investigating the protein pattern of ion exchange fractions in both non-regenerative and regenerative states

In Figure 2 The SDS-PAGE pattern of ion exchange fractions is shown in two modes, non- regenerative (A) and regenerative (B). the model of the fourth faction in Native mode has a heavy polymer section and a diffusion section with a weight of more than 78 kDa and a double section in the weight range of 75 kDa, But in regenerative mode (in the presence of heat and 2 ME) a large section weighing 75 kDa and a number of small protein bands with different weights. the pattern of the first and second factions is almost the same

in non-regenerative and regenerative states. in non-regenerative mode, a relatively pure band weighing 77 kDa, and regenerative mode, it includes a large band and a number of light bands, but they are different in terms of PI (isoelectric point). the third fraction has a different pattern from other fractions, in the non- regenerative state it contains a dense part weighing over 75 kDa and in the regenerative part it contains a part above 75 kDa but in broken form.

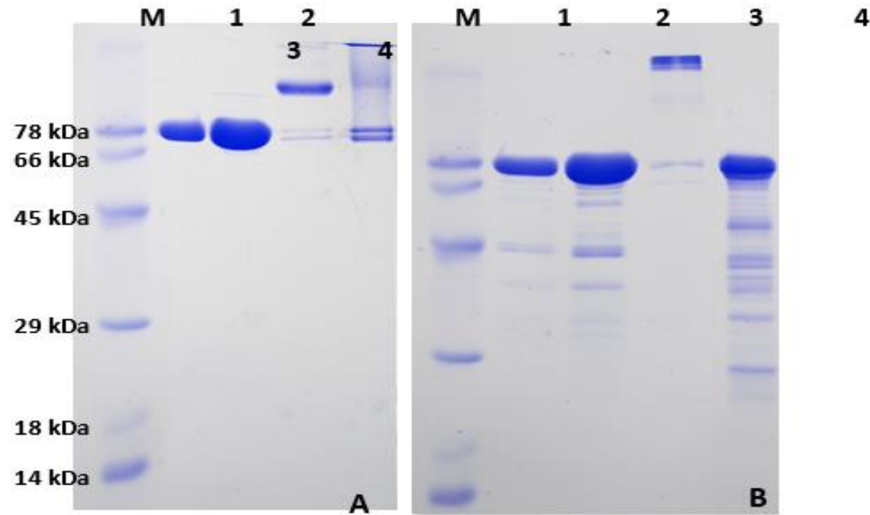

Fig. 2. The SDS-PAGE pattern of ion exchange fractions in the gel is 12.5%.

A. non-regeneration mode. B. regeneration mode. Protein values (1. 4 µg, 2. 12 µg, 3. 4 µg, 4. 5 µg per well) were given from each fraction. M. Marker, Marker proteins from top to bottom include 78, 66, 45, 29, 18 and 14 kDa (kDa).

The results of the study of the serum protein pattern of total hemolymph (high content fraction) in two-dimensional electrophoresis

The main and most valuable protein of hemolymph is hemocyanin. The isoelectric point of this macromolecule in other invertebrates is in the range of 5-6.2. Accordingly, in this study, IPG strips with a pH range of 4-7 were used to better separate proteins in the first dimension. In two-dimensional electrophoresis, proteins are first separated in the first dimension based on differences in isoelectric point. In the second dimension, which is SDS-PAGE, proteins are separated based on differences in molecular size and weight. As shown in Fig3, there is no significant difference in serum total protein (A) and fourth fractions (B). In both samples, about 13 protein spots appeared at pH intervals between 5 and 6, and their molecular weight was about 70-75 kDa. There are 6 sharp spots, a few weak spots and one diffused spot in each pattern. This pattern similarity indicates that the fourth faction includes majority (80%) of the hemolymph serum protein part. In fact, by diluting the total serum sample, the rest of the proteins disappear due to the low concentration, leaving only high content protein.

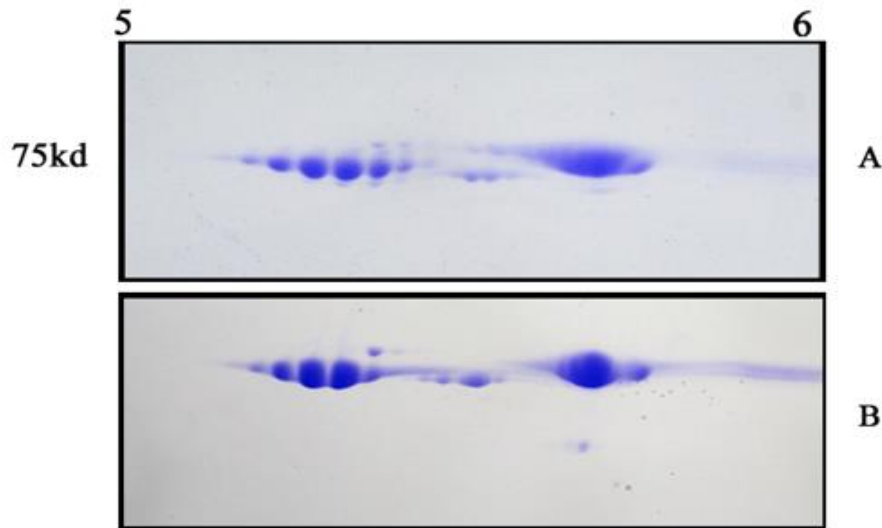

Fig. 3 Two-dimensional electrophoresis of hemolymph serum proteins in pH range: 4-7. A: Total hemolymph serum (10 µg of protein). B. The fourth (high content) fraction resulting from ion exchange (10 µg of protein).

The results of the study of cytotoxic and proliferation inhibition effects of hemolymph total serum and fractions on the studied cell lines

The MCF-7 and MDA-231 (breast cancer) and non-cancerous (HUVEC) cells were treated with different concentrations of total hemolymph serum and its isolated fractions for 24 and 48 h, and the toxicity proliferation inhibitory effects were investigated by LDH and MTT, respectively. Among the isolated factions, the fourth faction was the most effective. The IC50 of the fourth fraction on cancer and non-cancer cell lines was 800 and 1800 µg/ml, respectively. Also, the CC50 of the fourth faction on cancer and non-cancerous cell lines is 1400 and 2160 µg/ml, respectively. The results of the toxicity and the inhibitory effects percentage of different concentrations of samples on different cell lines are shown in the respective graphs. In addition, based on the results, the concentration of each sample that caused toxicity and inhibition of 50% growth of the tested cell lines compared to the controlled group. CC_{50} and IC_{50} was calculated by considering at least three repetitions of each experiment, which are listed in the tables for each fraction.

Cytotoxic effect of total hemolymph serum and its fractions on the endothelial cell line of the human umbilical cord (Huvec)

LDH testing was used to investigate the cytotoxic effect of hemolymph serum and fractions on cell lines.

As shown in Fig4, the toxicity of the fourth fraction and total serum started significantly from 600 µg to higher concentrations ($p < 0.001$) and the toxicity percentage of these two samples at 2000 µg was 36% and 39%, respectively. Also, the toxicity percentage of 1, 2, 3 fractions was started from 800 µg or higher significantly ($p < 0.001$) and is less than 15% at 2000 µg. The results suggest that hemolymph serum and its fractions are generally less toxic to noncancerous cells. However, the toxicity effects of the fourth fraction begin at concentrations above 2,000 µg.

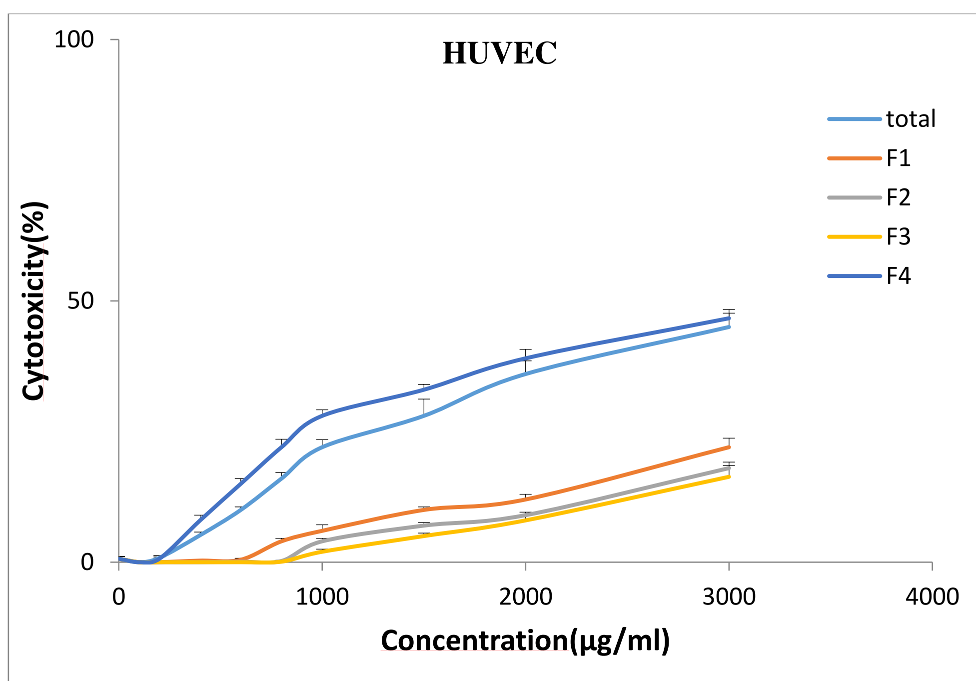

Figure 4. The toxicity effect of different concentrations of total hemolymph serum and its fractions on Huvec cell line. F (fraction), Total (total serum).

The effect of total hemolymph serum and its fractions on the proliferation of endothelial cells of the human umbilical cord (Huvec)

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method, was used to investigate the effect of hemolymph serum and fractions on cell proliferation.

As shown in Fig5, the inhibitory effects of total serum and the fourth fraction started from 400 µg and above concentrations, significantly ($p < 0.001$) and at 1500 µg the inhibition rate was 33% and 42%, respectively. The inhibitory effects of other fractions started significantly ($p < 0.001$) at the concentrations of 1000 µg and above and were less than 17% at concentrations of 1500 µg. Total serum and its fourth fraction showed relatively weak inhibitory effects on the proliferation of noncancerous cells.

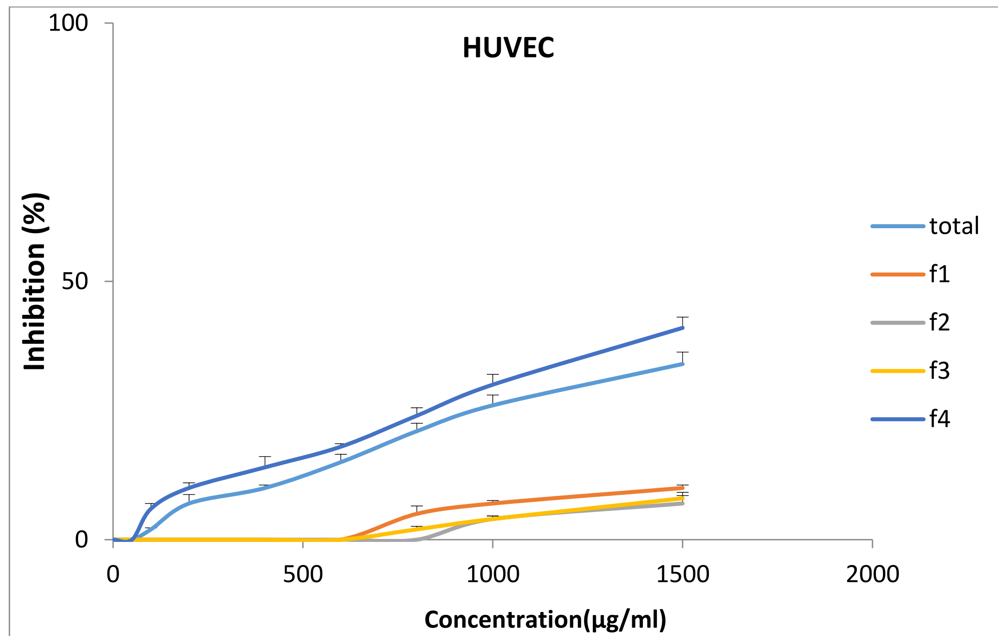

Fig. 5 - The effect of different concentrations of total hemolymph serum and its fractions on the proliferation of Huvec cell line. F (fraction) Total (total serum).

The toxicity effect of total hemolymph serum and its fractions on the MCF-7 cancer cell line

As shown in Fig. 6, the toxicity of total serum and the fourth fraction started at a concentration of 400 µg and above ($p < 0.05$) and reached 55% and 66% at 2000 µg, respectively. The toxicity of other fractions started at a concentration of 800 µg or more ($p < 0.05$) and was less than 18% at the concentration of 2000 µg.

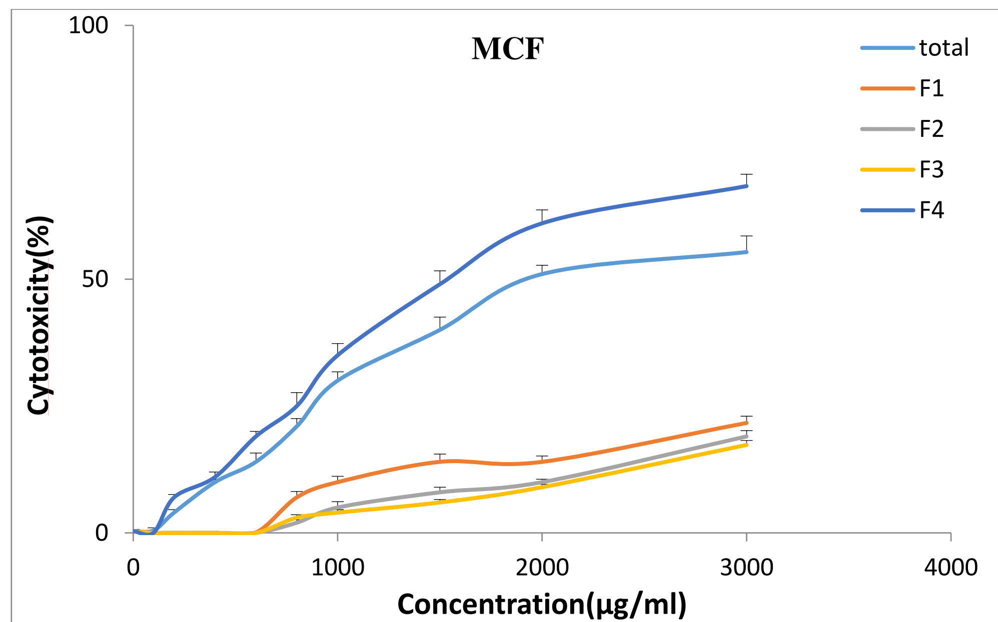

Fig. 6. The toxicity effects of different concentrations of total hemolymph serum and its fractions on MCF-7 cell line. F (fraction) Total (total serum).

The toxicity effect of total hemolymph serum and its fractions on the MDA-231 cancer cell line

As shown in Fig. 6, total serum toxicity, such as it was on the MCF-7 class, started from a concentration of 400 µg significantly ($p < 0.001$) but for the fourth fraction it started from a concentration of 200 µg ($p < 0.001$). At a concentration of 2000 µg, the toxicity of total serum and the fourth fraction reached 61% and 72%, respectively. The toxicity of the rest of the fractions started at a concentration of 800 µg or more ($p < 0.001$) and was less than 25% at the concentration of 2000.

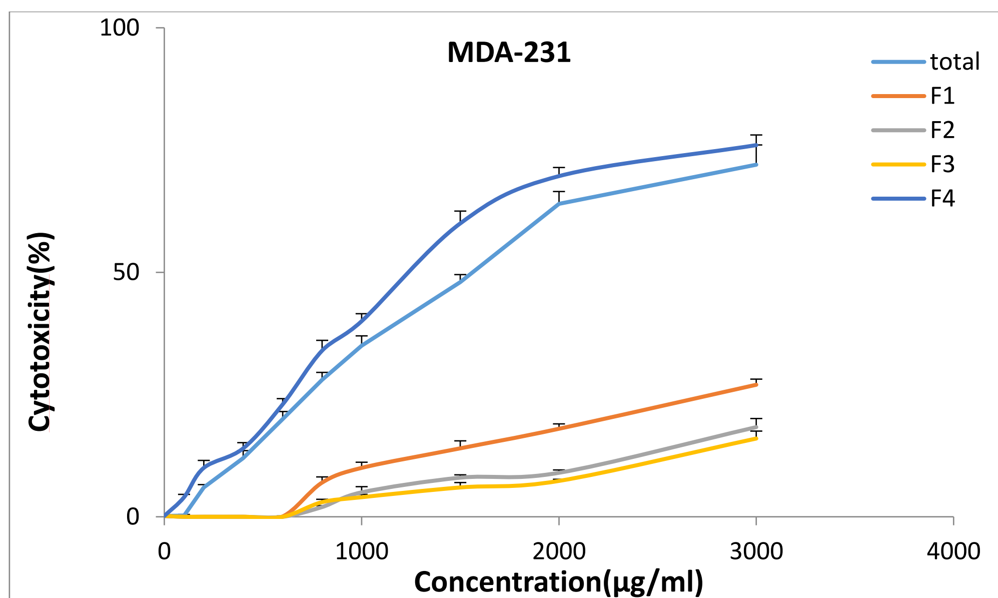

Fig. 7. The toxicity effect of different concentrations of total hemolymph serum and its fractions on MDA-231 cells. F (fraction) Total (total serum).

The effect of total hemolymph serum and its fractions on the proliferation of MCF-7 cancer cell line

As shown in Fig. 8 the inhibitory effects of total serum ($p < 0.05$) and the fourth fraction significantly ($p < 0.001$) started at 200 µg and at 1500 µg reached 63% and 74%, respectively. The inhibitory effect of 1,2,3 fractions started significantly ($p < 0.001$) from a concentration of 800 µg and above and at a concentration of 1500 µg was less than 18%, and with increasing concentration of these fractions, their inhibitory effect did not increased significantly.

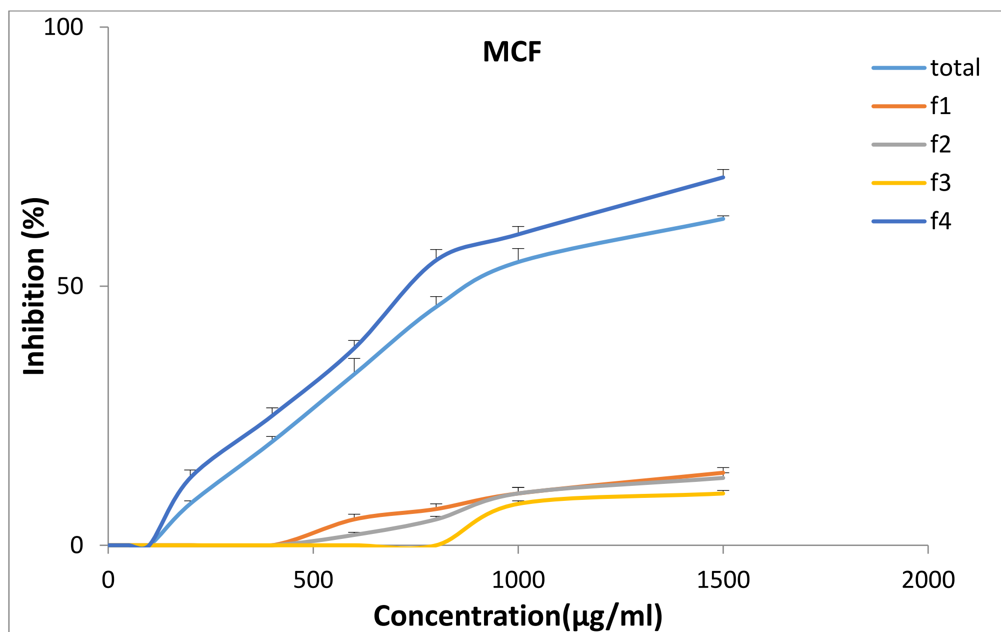

Fig. 8. The effect of different concentrations of total hemolymph serum and its fractions on MCF-7 cell line proliferation. F (fraction), Total (total serum).

The effect of total hemolymph serum and its fractions on the proliferation of MDA-231 cancer cell line

As shown in Fig.9, the inhibitory effects of total serum and the fourth fraction ($p < 0.001$) started significantly at a concentration of 200 µg and at a concentration of 1500 µg the inhibition rate reached to 72% and 84%, respectively. The inhibitory effects of other fractions on this cell line are not different from those of MCF-7 cell line and are less than 18%.

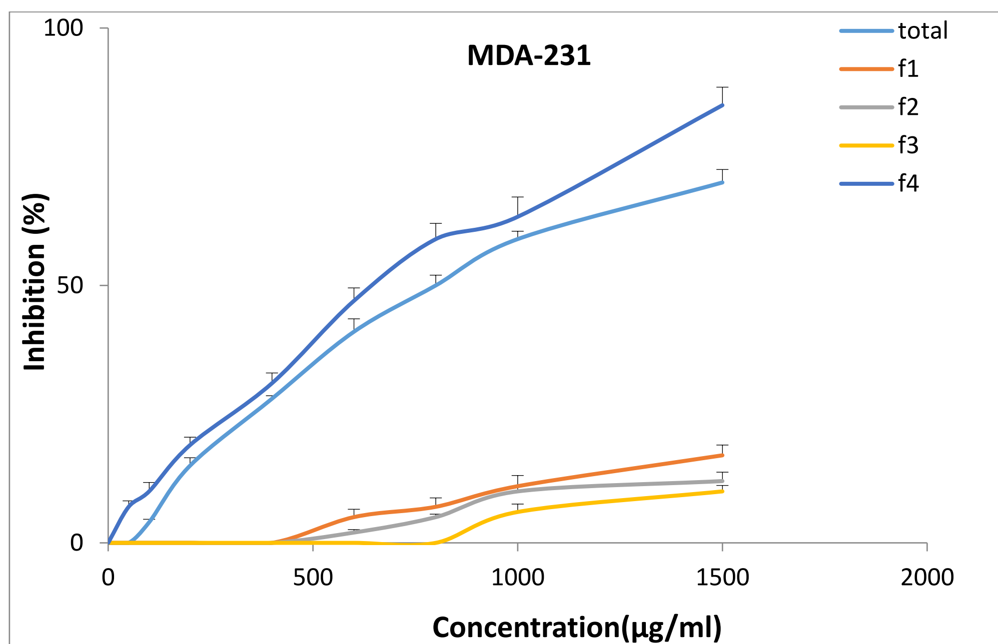

Fig. 9: The effect of different concentrations of total hemolymph serum and its fractions on MDA cell line proliferation. F (fraction) total (total serum).

Overall, the toxicity of total hemolymph serum and its fractions on cancer and non-cancerous cells was low, as well as the inhibitory effect of total serum and the fourth fraction was at lower concentrations than their toxic concentration. On the other hand, the toxic effect and especially the inhibitory effect on cancer cells was significantly higher than on non-cancer cells. Although the 1, 2, 3 factions had lower toxicity compared to the total serum and the fourth fraction, but their inhibitory effects were very weak compared to the total serum and the fourth fraction. Their inhibitory effect did not increase significantly with increasing the concentration. Therefore, the 50% toxicity and inhibition on cell lines were very high, so it was calculated only for the total serum and the fourth fraction.

Table 1-CC_{50} and IC_{50} of the total serum and the fourth fraction on the Huvec cell line

Sample	CC_{50}(µg/ml)	**IC_{50}(µg/ml)**
Total serum	2470	**2170**
Fourth fraction	2160	**1800**

Table 2-CC_{50} and IC_{50} of the total hemolymph serum on the MCF-7 and MDA-231 cell lines

Sample	CC_{50}(µg/ml)	**IC_{50}(µg/ml)**
MCF-7	1700	**960**
MDA-231	1630	**850**

Table-3 CC50 and IC50 of the fourth fraction on the MCF-7 and MDA-231 cell lines

Sample	CC_{50}(µg/ml)	**IC_{50}(µg/ml)**
MCF-7	1500	**890**
MDA-231	1370	**760**

Results of the determination of apoptosis by TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) test

The cells were treated by the CC_{50}-equivalent (effective) concentrations of total and fourth fraction for 24 hours.

Total serum

At a concentration of 2000 µg, it caused 5% apoptosis in HUVEC cells.

At a concentration of 1800 µg, it caused apoptosis by 18% in MCF-7 cells and 25% in MDA cells.

Fourth faction

At a concentration of 2000 µg, it caused apoptosis by 8% in HUVEC cells.

At a concentration of 1800 µg, it caused apoptosis by 22% in MCF-7 cells and by 35% in MDA cells.

Images of apoptosis are shown in Figures 10 and 11

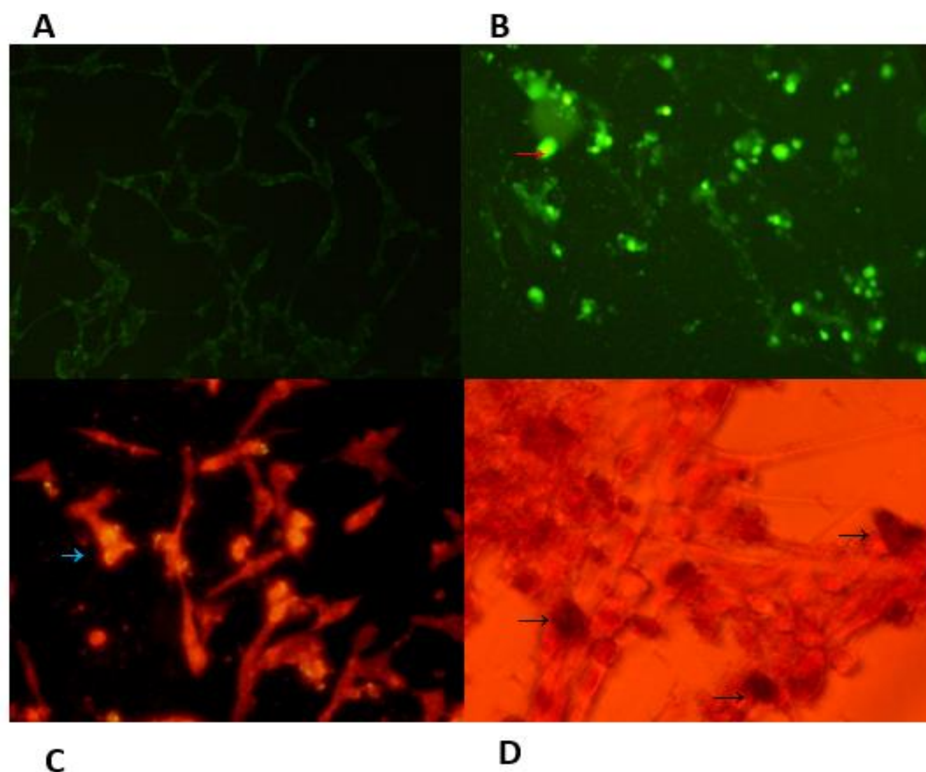

Fig. 10. Effect of hemolymph serum (fourth fraction) on apoptosis in MDA cells. Control group cells (without hemolymph effect) in TUNEL (A) staining. Apoptotic cells (exposed to hemolymph) with bright green nuclei in TUNEL (B) staining and bright yellow nuclei with the addition of PI (C) dye and apoptotic cells with gray nuclei in the enzymatic method of peroxidase-di-aminobenzidine (D). (Arrow refers to apoptotic cells) (magnification × 200).

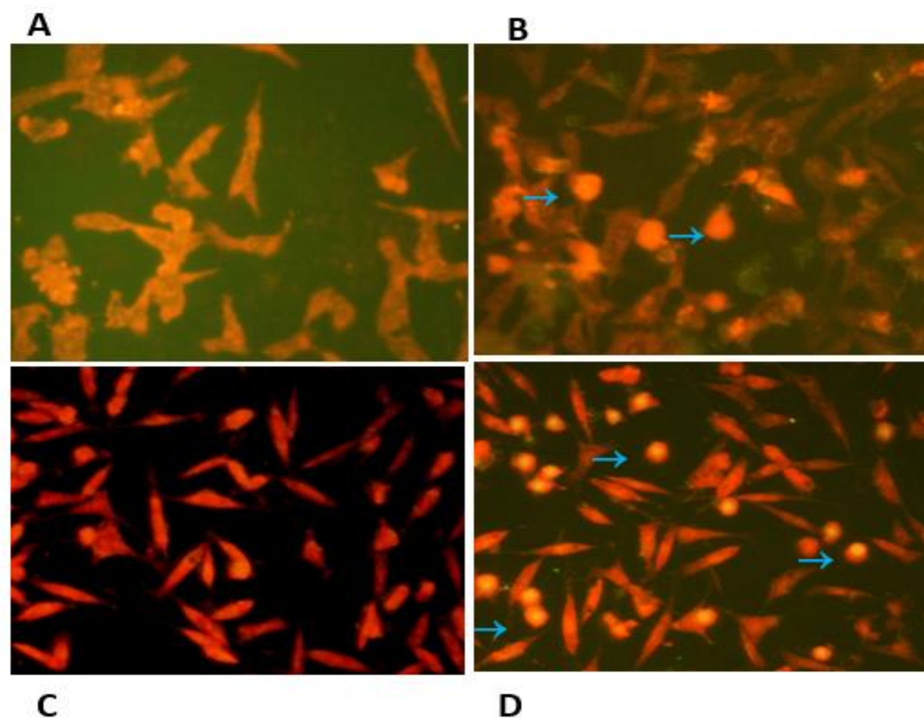

Fig. 11. Effect of hemolymph serum (fourth fraction) on apoptosis in HUVEC and MCF-7 cells in combined TUNEL and PI staining. HUVEC cells control group (A). Treated with hemolymph (B). MCF-7 cells control group (C) Treated with hemolymph (D). As can be seen, the incidence of apoptosis in MCF-7 cells is higher than in HUVEC cells. (Arrow refers to apoptotic cells). (Magnification: × 200).

Discussion and Conclusion

Breast cancer, as a major clinical problem, is the leading cause of death from cancer in women around the world. The mortality by this disease has been increased in recent decades. Also, the age of this disease in Iran is one decade earlier than in developed countries, with more than 30 percent of patients under the age of 30 [11, 12]. Despite the availability of surgical techniques, radiotherapy and chemotherapy drugs in the treatment of this disease, the treatment of this cancer still remains a major problem due to its heterogenecity and drug resistance as well as side effects of treatment [13].

Due to the anti-tumor properties of other invertebrate hemocyanins as well as the oral use of *Potamon persicum* crab in traditional medicine to treat cancer, in this study for the first time the anti-cancer properties of hemolymph serum of this crab as separate fractions were examined in-vitro on two cancer cell lines of breast cancer, MDA-231, MCF-7, as well as non-cancerous cell line (HUVEC).

In examination of the electrophoretic pattern of hemolymph crab serum proteins in regenerative polyacrylamide gel with SDS-PAGE method, in the cathode range (negative pole) a band with a molecular weight above 78 kDa, in the middle area a high content band with a weight of 75 kDa and below it, other bands with different weights, the lightest of which was 30 kDa, appeared. There was no difference in the electrophoretic pattern of hemolymph serum proteins of large and small crabs and crabs from spring and autumn seasons. It is noteworthy that the majority of serum protein in this type of crab is a high content protein (fourth fraction). Therefore, we performed ion exchange chromatography to separate the protein components. Since the isoelectric point of hemocyanin is pI 5-6.2, this protein binds to the DEAE column at pH 7 and can be separated from other proteins.

In a study by Maridass et al. in a SSD-PAGE examination of Parathelphusa hydrodromous serum proteins in a 7% in a regenerative poly acrylamide gel, three protein regions: the area close to the cathode, including 6 slow-moving bands, the middle zone (diffuse bands), the anode zone (4 dense bands) were reported. In this study, the total protein concentration of hemolymph serum was reported to be 60 mg / ml by Biore method [14]. The protein concentration of this study is almost the same as our study, but its electrophoresis pattern is different due to different electrophoresis conditions, such as differences in gel percentage (12.5% in our study and 7% in this study) and the differences in sex and species of the living.

Rouchu discovered the effect of buffering conditions on estimating the molecular weight of hemocyanin. In fact, by comparing the tris buffer with the phosphate buffer, it was concluded that the electrophoretic pattern is different in the two buffered conditions [15-17]. All of the above studies have identified hemocyanin as the main protein in hemolymph, which includes 80-90%. In our study, 80% of the total protein in hemolymph serum belonged to the four fractions obtained during the ion exchange chromatography. Also, the results of the two-dimensional electrophoresis pattern of total serum and the fourth faction showed that the main and valuable protein of crab hemolymph studied by us was the fourth faction and considering that the fourth faction was greenish blue and the rest of the factions were transparent, it could be concluded that hemocyanin was isolated in the fourth fraction.

There was a contradiction in the SDS-PAGE pattern of the four fractions obtained from ion exchange. Factions 1 and 2 were similar in size to the fourth fraction, despite differences in caming out of the column. The reason for this can be explained as follows: In Fredrick's study of hemolymph proteins in marine crustaceans, including crabs, a protein called cryptocyanin was mentioned, which is similar in size, sequence, and structure to hemocyanin (it was actually hexamer and each of its subunits were 78 kDa), but its isoelectric point was 6.5-8 and was different from that of hemocyanin. It also lacked copper, which was why it was also called apo hemocyanin. It is even believed that it is produced from the doubling of the the homocyanin gene and its concentration increases during metamorphosis. Its role in metamorphosis and structure of living skeletons has been mentioned [6]. Also, the reason for the high concentration of protein in small crabs (in metamorphosis) compared to large crabs in our study is the high concentration of cryptocyanine in small crabs.

After isolating the major hemolymph serum fraction (hemocyanin) in this study, the cellular toxicity, anti-proliferation and apoptosis effects of total serum of crab were compared with hemocyanin and other fractionation on two cell lines of breast cancer (MDA-231, MCF) and the HUVEC cell line as a non-cancerous cell line.

In a study of the anti-cancer properties of *Potamo persicum* crab serum and its fractions on breast cancer cell lines and human umbilical vein endothelial cells (HUVEC), we concluded that hemolymph serum and its fractions have poor toxicity (average percentage of toxicity of the effective faction (Fourth), using the LDH method at the concentration of 2000 µg / ml is 70% on cancer and 39% on healthy cell lines.

In a study by Riggs et al, the effects of KLH on PANC-1(pancreatic) cancer cell line were investigated and an IC50 of 400 µg/mL was reported within 72 hours. In this study,it was claimed that KLH inhibits cancer cell proliferation through apoptotic and non-apoptotic mechanisms [18]. In addition, in another study by Riggs et al., the anticancer effects of KLH on breast cancer lines, MCF-7 (estrogen receptor positive) and ZR75-1 (estrogen receptor negative), were investigated, and it was revealed that the effect of this protein was stronger on ZR75-1 cells than on MCF-7. The IC50 values of 600 and 250 µg/mL were reported for ZR75-1 and MCF-7 cells for 72 hours, respectively [19]. In a study by McFadden et al, the effects of KLH on esophageal, BIC-1 and SEG-1 cancer cells were evaluated and it was reported

that KLH inhibited 50% proliferation of SEG-1 cells and 70% proliferation of BIC-1 cells at 300 μg/mL for 72 hours. It induced apoptosis in SEG-1 cells but no change was observed in BIC-1 cells [20]. In another study by Somasundar et al, the anti-cancer effects of KLH on melanoma cancer cells HTB68 and HTB72 were evaluated and it was reported that KLH inhibited 50% proliferation of HTB68 cells and inhibited 30% proliferation of HTB72 cells at a concentration of 300 μg/mL during 72 hours of treatment. The rate of apoptosis in HTB68 cells is twice as high as that of HTB72 cells [21]. In the above-mentioned studies, the difference in the effect of KLH on cells was due to the presence and absence of the expression of cell surface receptors (including estrogen receptor expression on MCF-7 cells and lack of expression on ZR75-1 surface). The mutations in the p53 gene were positive or negative. SEG-1 cells were apoptotic due to the absence of mutation in p53 gene and BIC-1 cells were mutated in the p53 gene and did not have apoptosis. Moreover, lack of toxic effects of KLH on the cell lines has been noted in all of the above-mentioned studies. [18-21]

In examining the apoptosis induction and anti-proliferation effects of isolated fractions, the fourth faction was more effective and was 10% higher than the total serum sample. This indicates the presence of impurities and the inhibitory effect of other fractions in the total sample. The inhibitory effects of the fourth fraction on the cancer cell lines were dose-dependent, so that the toxic effect of the sample at concentrations above 1500 μg / ml could also be due to the toxic effect of the sample. The 3, 2, and 1 factions were less toxic than the total serum and the fourth fraction, but the inhibitory effect of these fractions was almost the same and much less than the inhibitory effect of the fourth fraction and the total serum. Total hemoplymph serum, and especially the fourth fraction in much lower concentrations than HUVEC inhibited the proliferation of the cancer cells (MDA-231, MCF-7). The mean concentrations of IC_{50} (inhibition of 50% proliferation) of the fourth and total serum fraction using MTT method on cancer cell lines were 800 μg/ml and 900 μg/ml, respectively. This number reached 2000 μg/ml in HUVEC cells (the concentration of 800 μg/ml of the fourth fraction was the most effective concentration, as it has the toxicity less than 35% and inhibited 55% of cancer cells and 20% of noncancerous cells). In the study of the effect of apoptosis induction, the percentage of apoptosis in cancer cells was higher than in HUVEC cells, so that the incidence of apoptosis caused by the fourth faction in cancer cells averaged 30% and in noncancerous cells 7%. Also, the anti-cancer effects of total hemolymph serum and its fourth faction on the MDA-231 cells (negative receptor cell) were about 10% stronger than on the MCF-7 cell (positive receptor cell).

According to the results of this study, *Potamon persicum* crab serum has poor toxicity and in low concentrations the anti-proliferative effect is greater than the toxicity effect, although in concentrations higher than 1500 μg the inhibitory effect on cells in addition to apoptotic effect could be due to the toxic effect of the sample. This anti-proliferative effect on cancer cells is significantly higher than non-cancerous cells. Also, the anticancer effects of this compound on the negative receptor cell (MDA-231) are stronger than the positive receptor cell (MCF-7). Due to the fact that common drugs in the treatment of breast cancer are of the receptor-dependent type and in addition to side effects, the resistance against these drugs has been increased, so in this regard, hemolymph serum of this crab, and especially its fourth faction, if the studies are completed, could be a good option for the treatment of breast cancer, but it should be noted that the results of the anti-cancer effects of this compound in this study differs from that in traditional medicine because in traditional medicine, total hemolymph (both serum and cell fractions) is given orally to treat cancer and undergoes secondary changes. On the other hand, this study examined only the effects of crab hemolymph serum as native compound and by invitro methods. Therefore, it is necessary to study the effects of this compound using invivo methods conditions in the future studies.

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