→ **Original Article**

In Vitro Effects of Hemolymph Serum of *Potamon persicum* Crab (HSPPC) on MCF-7 and MDA-231 Breast Cancer Cell Lines

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Abstract
Background: Breast cancer is the most common cause of cancer-related death in women worldwide. Therefore, there is an urget need to identify and develop therapeutic strategies against this deadly disease. This study is the first to investigate the effects of Hemolymph Serum of *Potamon persicum* Crab (HSPPC) on MCF-7 and MDA-231 breast cancer cell lines.
Materials and Methods: LDH and MTT assays were performed on MCF-7 and MDA-231 breast cancer cell lines as well as human umbilical vein endothelial cells (HUVEC) to determine the cytotoxic and antiproliferative activity of the *HSPPC* at different concentrations. Further, the apoptosis inducing action of the hemolymph serum was determined by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) and cell death assay.
Results: The IC_{50} values of HSPPC for MCF-7 and MDA-231 cell lines were 960 ± 0.369 and 850 ± 1.422 µg/mL, respectively. The growth of both MCF-7 and MDA-231 cell lines were significantly ($P<0.001$) inhibited by HSPPC as compared with untreated controls at 48 hours. The results showed that HSPPC had no cytotoxic effects but significantly inhibited cell growth in a dose and time dependent manner. In addition, DNA fragmentation analysis (TUNEL) and cell death assay indicated induction of apoptosis by HSPPC in MCF-7 and MDA-231 cell lines.
Conclusion: The results suggest that HSPPC contains bioactive compound(s) with potentials for the treatment of breast cancer.
Keywords: Apoptosis, Breast cancer, Hemolymph, *Potamon persicum*

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Introduction

Breast cancer is considered the most common cause of cancer-related death in women worldwide. The incidence and death rates of breast cancer have increased in most countries worldwide in the last decades (1, 2). Five treatment methods of breast cancer are surgery, chemotherapy, radiation therapy, hormonal therapy, and targeted therapies. However, some of these methods cause undesired side effects by the non-specific targeting of both normal and cancer cells (3). Based on this, there are growing searches for the treatment of cancer using natural compounds (4). Among natural sources, marine-derived agents displayed an important role in the treatment of many forms of cancer. Cytarabine is an example of an anticancer agent originating from marine sources. Bryostatin-1, aplidine, dolastatin 10, and ET-743 are other agents originating from marine sources,

which have entered phase I and II clinical trials (4). Previous reports have shown that anticancer properties of marine sources can be mediated through the induction of apoptosis (5).

Apoptosis is a form of programmed cell death which is characterized by remarkable morphological features such as cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation. Apoptosis plays a major role as a protective method against tumorigenesis by eliminating damaged or abnormal cells (6-10).

A successful anticancer drug should kill carcinoma cells without causing excessive side effects on normal cells. This ideal situation is achievable by apoptosis induction in carcinoma cells (11). Increasing evidence demonstrates that marine-derived agents are an important source of bioactive compounds that can induce apoptosis in human carcinoma cells. Previous studies have shown that the

anticancer properties of marine sources can be mediated through the inhibition of cell growth (12, 13).

Potamon persicum crab is a genus of the family *Potamidae*. This species is widely distributed in four countries including Armenia, Iraq, Iran, and Turkey (14). In traditional medicine, hemolymph of this genus has been used for cancer treatment. Hemolymph, or hemolymph, is a fluid in the circulatory system of some arthropods (including crustaceans such as crabs and shrimp), and is analogous to the fluids and cells making up both blood and interstitial fluid (including water, proteins, fats, etc.) in vertebrates such as birds and mammals (15). Keyhole limpet hemocyanin (KLH) is a high molecular weight copper-containing protein found in the hemolymph of the sea mollusk Megathura crenulata with significant antiproliferative effects *in vitro* against esophagus, pancreas, and prostate cancers (16). KLH found in the hemolymph of the sea mollusk *Megathura crenulata* (17). This extracellular respiratory protein has many immunostimulatory properties, including the ability to enhance the host's immune response by interacting with T cells, monocytes, macrophages and polymorphonuclear lymphocytes (18). In the present study, we aimed for the first time to investigate cytotoxic, antiproliferative, and apoptotic effects of HSPPC on breast cancer cells. Furthermore, we evaluated probable side effects of the compound on human umbilical vein endothelial cells (HUVEC) as non-cancerous cells.

Materials and Methods
Collection of Animal
Crabs were collected from Sirwan River in Kurdistan, Eastern Iran, by hand picking method. The live animals were transferred to the lab in bottles containing seawater and stored in cement tanks.

Extraction and Protein Estimation of Hemolymph
The hemolymph was collected by cutting second walking legs of the crab (male and female) with a pair of fine scissors. Four to 7 mL of hemolymph was obtained from each crab depending on the body size. The hemolymph extraction of *Potamon persicum* was performed with care to avoid any injury or damage to the animals. The hemolymph was transferred into a sterile centrifuge tube and was immediately centrifuged at 5000 rpm for 10 minutes at 4°C to separate the hemolymph plasma and hemocyte. The hemolymph serum as supernatant was decanted into new sterile tube and kept at -70°C. The amount of protein was measured by spectrometry according to the method of Bradford. Bovine serum albumin was used as a standard protein in Bradford protein assay to plot a graph of theoretical protein concentration.

Cell Culture
MCF-7 and MDA-231 cell lines (human breast cancer cell line) and HUVECs were purchased from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). The cells were grown in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal calf serum (FCS) (Sigma, Germany), 100 µg/mL penicillin, and 100 µg/mL streptomycin (United States Biochemical, USA). The cells were incubated in a humidified incubator containing 5% CO_2 at 37ºC. At 80% confluence, the cells were rinsed with phosphate buffered saline (PBS) containing 0.5% EDTA and harvested from 25 cm^2 flasks using 0.25% trypsin/EDTA solution (Sigma, Germany). Then, the cells were sub-cultured in 25 cm^2 flasks or 96-well microplates (Orange, Austria) according to experiments. The experiments were performed in triplicate.

MTT Assay
Antiproliferative effect of HSPPC was assessed on MCF-7 and MDA-231 cells as well as HUVEC using the MTT reagent (3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, Germany) according to the manufacturer's protocol. This method is based on the ability of viable cells to metabolize yellow tetrazolium salt MTT to purple formazan crystals by mitochondrial dehydrogenases. The cells (5×10^3 cells/well) were seeded in 96-well microplates and incubated for 24 hours at 37°C and 5% CO_2. The cells were treated with different concentrations of HSPPC (50, 100, 200, 400, 600, 800, 1000, and 1500 µg/mL). After 48 hours of treatment, 30 µL of MTT labeling reagent was added to each well. The microplates were then incubated at 37°C and 5% CO_2 for 4 hours. Afterwards, 150 µL of the solubilization solution (DMSO) was added to each well and shaken to dissolve formazan crystals. Finally, absorbance was read using an ELISA microplate reader (Bio Teck, Germany) at a wavelength of 570 nm. The percentage of antiproliferative activity was calculated using the following equation:

$$\text{Antiproliferative activity (\%)} = \frac{[\text{mean absorbance of treated cells ation: absorbance of negative control}]}{[\text{mean absorbance of negative control}]} \times 100$$

LDH Assay
MCF-7 and MDA-231 cells (10^4) in 96-well microplates were exposed to different concentrations of *HSPPC* (50, 100, 200, 400, 600, 800, 1000, 1500, 2000, and 3000 µg/mL) for 24 hours. The cytotoxicity of HSPPC was assessed by lactate dehydrogenase (LDH) leakage into the culture medium. The activity of LDH in the medium was detected using a commercially available kit (Roch, Germany). The assay is based on the conversion of lactate to pyruvate in the presence of LDH with parallel reduction of NAD (Nicotinamide adenine dinucleotide). To determine LDH, 100 µL/well supernatants were removed and transferred into corresponding wells of optically clear 96-well flat bottom microplates and 100 µL of reaction mixtures was then added to the wells and incubated for 25 minutes at 30°C. The formation of NADH

(Nicotinamide adenine dinucleotide hydrogen) from the above-mentioned reaction results were detected with the increase of absorbance at 490 nm. Aliquots of media and warm reagent were mixed in a 96-well microplate and the absorbances were then recorded using an ELISA plate reader (Awareness, USA). Finally, the percentage of dead cells was calculated according to the following formula:

$$\text{Cytotoxicity}(\%) = \frac{\text{OD test} - \text{OD low control}}{\text{OD high control} - \text{OD low control}} \times 100$$

Assessment of Apoptosis
TUNEL Assay for Determination of DNA Fragmentation
DNA fragmentation was detected using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) by the In Situ Cell Death Detection Kit, POD (Roche Diagnostics GmbH, Germany) as described by the manufacturer's protocol. Briefly, MCF-7 and MDA-231 cells were subcultured in 96-well microplates and incubated for 24 hours at 37°C and 5% CO_2. Afterwards, the cells were treated with HSPPC at concentrations required for 50% toxicity (CC_{50}) for 24 hours. Negative control cells were treated with the same volume of RPMI present in treated wells. After treatment, the cells were fixed with 4% (w/v) paraformaldehyde in PBS (pH 7.4) for 1 hour at room temperature and rinsed twice with PBS. The fixed cells were incubated with blocking solution (3% (v/v) H_2O_2 in methanol) for 15 minutes at room temperature and were then rinsed with PBS. Afterwards, the cells were incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 minutes on ice. Subsequently, 40 μL of reaction mixture containing TdT enzyme and nucleotide was added to the cells and incubated for 1 hour at 37°C. After washing with PBS, the wells were incubated with 50 μL converter-POD streptavidin HRP solution for 20 minutes and rinsed

3 times with PBS. Finally, the cells were incubated with DAB and the stained cells were analyzed with the light microscope.
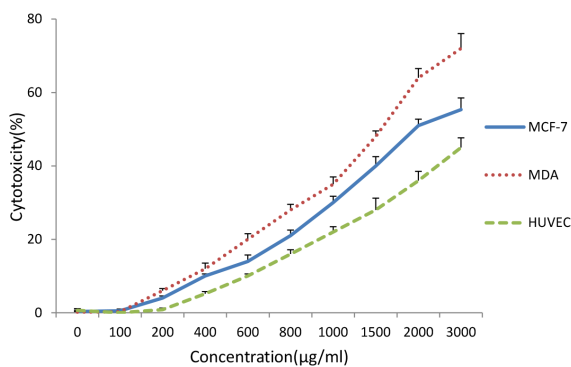
Statistical Analysis
In this study, data were represented as means ± SEM of three identical experiments made in three replicate. The statistical analysis was carried out using the analysis of variance (ANOVA), followed by post hoc test. P values ≤0.05 were considered statistically significant. All analyses were conducted using the STATISTICA (version 5).

Results
Cytotoxic and Antiproliferative Effects of HSPPC on MCF-7 and MDA-231 Cells
The cytotoxic and antiproliferative effects of HSPPC on MCF-7 and MDA-231 cells were determined by (3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and LDH assays that are shown in Figures 1 and 2. As shown in Figure 1, the cells treated with HSPPC did not show strong cytotoxic activity in comparison with the untreated control cells. The IC_{50} values indicated that the HSPPC had a more inhibitory effect on MDA-231 cells than on MCF-7 cells (Table 1). Moreover, the treated cells showed cell growth inhibition in a time and dose-dependent manner following treatment for 48 hours. At the higher concentration of this sample and the longer time of treatment, more significant cytotoxicity was achieved. The results of cytotoxicity assay showed that IC_{50} values (dose required for 50% inhibition) of HSPPC for MCF-7 and MDA-231 cells were 960 ± 0.369 and 850 ± 1.422 μg/mL for 48 hours, respectively (Table 1), indicating a higher growth inhibitory activity on cells. Both the MCF-7 and MDA-231 cell lines were significantly ($P<0.001$) inhibited by HSPPC as compared with controls at 48 hours. Further, we determined the effect of HSPPC on

The cytotoxic effects were measured using LDH assay.

Figure 1. The Cytotoxic Effects of HSPPC on MCF-7, MDA-231, and HUVEC Cells.
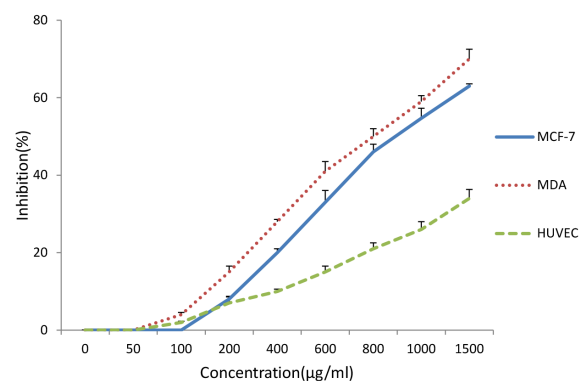

Figure 2. The Investigation of the Inhibition of MCF-7, MDA-231, and HUVEC Cells Growth Using the MTT Assay
Data are presented as mean ± SEM of three independent experiments.

Table 1. The 50% Cytotoxic Concentration (CC50) and 50% Inhibition Concentration (IC50) of HSPPC

Cell line	CC_{50}	IC_{50}
HUVEC	2470 ± 5.288	2170 ± 3.327
MCF-7	1700 ± 2.844	960 ± 0.369
MDA-231	1630 ± 1.651	850 ± 1.422

Figure 3. The Investigation of the Effect of HSPPC on the Morphology of MDA-231 Cells Using TUNEL Assay.
Control cells (A); The cells under the effect of HSPPC (B); Arrows indicate apoptotic cells; A-B: 200× magnification.

non-cancer HUVECs. Interestingly, the non-cancerous cells were not influenced by this sample (Figures 1 and 2).

Induction of Apoptosis by HSPPC in MCF-7 and MDA-231 Cells

DNA fragmentation is one of the hallmarks of apoptotic cell death. Therefore, we confirmed the presence of nucleosomal DNA fragments in treated cells by TUNEL assay in the current study. As shown in Figure 3, after the treatment of MCF-7 and MDA-231 cells with 50% cytotoxic concentration (CC50) of HSPPC at 24 hours, the apoptotic cells produced brown stained nuclei, whereas the non-apoptotic cells were found in the cells without treatment with HSPPC (negative control), which were not stained with similar observation.

Discussion

Breast cancer as a major clinical problem is the most important cause of cancer-related deaths around the world. The incidence and mortality rates of this disease have increased over the last few decades, and it mainly affects Iranian women a decade earlier than developed countries. Moreover, more than 30% of patients are under the age of 30 (19, 20).

In the present study, as a first step to provide scientific evidence for anticancer effects of *HSPPC,* the cytotoxic, antiproliferative and apoptotic activity, on MCF-7 and MDA-231 human breast cancer cells were evaluated. The results of this study, although preliminary, show that *HSPPC* inhibits the growth of MCF-7 and MDA-231 cells in vitro by apoptotic and nonapoptotic mechanisms. The activities of HSPPC may be due to the presence of a high concentration of hemocyanin. In this study, we found a differential response to HSPPC in breast cancer cells. Moreover, HSPPC did not strongly inhibit non-cancer HUVEC growth. The difference in the sensitivity to HSPPC between human breast carcinoma cells and non-cancer cells suggested HSPPC as an antiproliferative agent. There is evidence that natural compounds and many antiproliferative agents can trigger the apoptosis of cancer cells. It is evident that in apoptosis, the earliest recognized morphological changes are chromatin condensation and nuclear fragmentation (8). Progression of the condensation is accompanied by convolution of the nucleus followed by breaking up of the nucleus into discrete fragments (9). Based on this, cell death
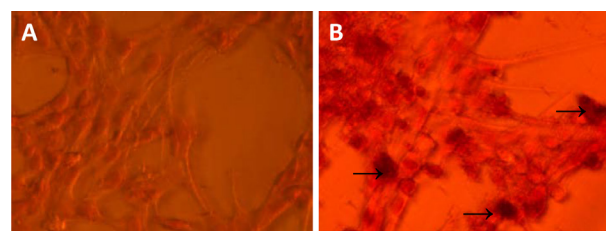
assay and DNA fragmentation assay were performed to confirm the induction of apoptotic by HSPPC. The DNA fragmentation that happened in the late stage of apoptosis was assessed using TUNEL method.

As mentioned earlier, hemolymph serum of *Potamon persicum* was investigated in our study for the first time considering that the anticancer properties of hemocyanins were reported in other invertebrates in previous studies.

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 (21).

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 (16).

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 (12).

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 (5).

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 (5, 12, 16, 21).

Given that the organism in our study is similar to other invertebrates, hemocyanin comprises 80% of the hemolymph protein, thus in order to analyze the differences between the results of our study and similar studies, further studies are needed to determine the structure and mechanisms of effective hemocyanin in our study and its differences with other invertebrates.

Considering the source diversity of the specimens used in previous (hemocyanin of the sea mollusks Megathura crenulata) and the present study (the hemolymph serum of *Potamon persicum* crab), further studies are needed. Similar to the above-mentioned results, our study also indicated a low toxicity for hemolymph serum of *Potamon persicum*. Additionally, the inhibitory mechanism of hemolymph serum of crab was apoptotic and non-apoptotic.

There are differences between the results of our study and the mentioned studies in IC50 and the apoptosis rate. The IC50 in our study is higher and apoptosis rate is lower compared to previous studies, In addition, there are differences in the cell lines used and the duration of cell treatment with the sample (72 hours in previous studies and 48 hours in our study).

According to the results of this study, it can be concluded that the hemolymph serum of crab has low toxicity and the anti-proliferative effect is more pronounced than the toxicity effect at low concentrations. However, at concentrations above 1500 μg, the inhibitory effect on cells was apoptotic, and the toxic effect of the sample was also observed. This antiproliferative effect is significantly greater on cancer cells than on non-cancer cells. Moreover, the anticancer effects of this compound are stronger on the estrogen receptor negative breast cancer cell line (MDA) than on the estrogen receptor positive breast cancer cell line (MCF-7).

Therefore, considering antiproliferative, apoptotic activities and low cytotoxic of HSPPC, it could be a good target for more quantitative and in vivo studies to find effective mechanisms of treatment of breast cancer.

However, the results of the anticancer effects of this compound in the present study are different from the conditions involved in traditional medicine. In traditional medicine, whole hemolymph (serum and cell) is orally administered to treat cancer. In the present study, only the in vitro effects of hemolymph serum of native crab were investigated; therefore, the in vivo effects of this compound should also be examined in future studies.

Conclusion
The present study demonstrated the in vitro antiproliferative and apoptotic activity of HSPPC on human breast carcinoma cells, possibly suggesting new antiproliferative agents with high potential to treat breast cancer that are not deleterious to non-cancer cells.

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