

Isolation and Characterization of Crab Haemolymph Exosomes and Its Effects on Breast Cancer Cells (4T1)

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Abstract

Objective: The use of animal or plant exosomes in cancer treatment is promising because of their easy access and low cost. Freshwater crabs are used in traditional Iranian medicine to treat cancer. This study aims to determine the anti-cancer properties of exosomes removed from freshwater crabs on a breast cancer cell line (4T1) compared to bone marrow mesenchymal stem cells (BMSCs).

Materials and Methods: In this experimental study, crab haemolymph exosomes were isolated via the precipitation method and characterised by electron microscopy, dynamic light scattering (DLS), and Western blot analysis. The protein concentration and total antioxidant capacity of these exosomes were determined by bicinchoninic acid (BCA) and cupric reducing antioxidant capacity (CUPRAC). The 4T1 cells and BMSCs were treated with exosomes and we assessed the cell survival by the resazurin and MTT assays. The level of nitric oxide (NO) secretion from the 4T1 cells was determined after treatment with the exosomes.

Results: Electron microscopy, DLS and Western blot for CD63 confirmed that the isolated exosomes were <100 nm in size and expressed CD63. The total antioxidant capacity in these exosomes was 1.003 µM/ml and the protein concentration was 650 mg/ml. Resazurin and MTT assay results showed a decrease in survival of the 4T1 cells (P<0.001) after treatment with the exosomes compared to cell growth in the exosome-treated BMSCs.

Conclusion: Crab haemolymph contains protein-rich exosomes with antioxidant activities that can have anti-cancer effects on 4T1 cells. These exosomes may be proposed for breast cancer therapeutics.

Keywords: Antioxidant, Breast Neoplasms, Cell Survival, Exosome

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Introduction

Extracellular vesicles (EVs) consist of two lipid layers that are secreted into the extracellular space. This bilayer membrane prevents degradation of the content of the EVs by nucleases and proteases. EVs have a role in localized and systemic cellular communication. The transfer of the EV content between cells leads to significant changes in cellular behaviour. They also are involved in the regulation of numerous physiological and pathological methods (1). Signalling via EVs is involved in all of the stages of carcinoma development, from initiation to metastasis. EVs are categorized into three groups - exosomes, microvesicles and apoptotic bodies. Exosomes range in size from 30 nm to 100 nm. Exosome formation is a physiological process and their surface marker proteins indicate their endosomal origin. Cell-cell communication at close (micro

environment) and long distances is one of the most important roles of exosomes. Animal cells such as tumour cells, red blood cells, platelets, lymphocytes, and dendritic cells secrete exosomes (2). Exosomes have been obtained from stem cells (3) and plants (4). They are also found in biological fluids such as milk, urine and plasma (4). The normal function of exosomes is intercellular signalling and transport of proteins and RNA (5).

Cancer cell exosomes are involved in cancer development and angiogenesis (2). Exosomes, as carriers for delivery of various pharmaceutical agents or drug delivery systems, can be effective in cancer treatment (2). The most frequently used markers for characterization of exosomes are CD9, CD63 and CD81 (6). Exosomes do not have the risk of aneuploidy and transplant rejection; therefore, they can be suitable alternates for treatment of various

diseases (3). Exosomes contain a considerable amount of RNA, protein and miRNA molecules, which are responsible for the biological effects of these exosomes (5).

They are extracted from various sources, including mesenchymal stem cells (MSCs) (7), gingival MSCs (6), adipose stem cells (8), blood (9), dendritic cells and body fluids such as milk (10). Exosomes have been used in research for wound healing (6, 8), cancer treatment (11) and neurological disorders such as Parkinson's disease (12). One of the most important application for exosomes is drug delivery systems (13).

The results from studies on exosomes derived from human umbilical cord stem cells, in liver tumorigenesis as well as acute and chronic liver disease models, demonstrated that these exosomes could inhibit cancer cell proliferation and treat liver damage through their antioxidant properties (14). Doxorubicin-loaded exosomes have been studied in several experiments and their impact on various breast cancer cell lines such as MCF7, MDA-MB-231 and *in vivo* cancer models were examined. The results indicated that exosomes were more active than chemotherapy alone in suppressing cancer (13, 15). Phase 1 and 2 clinical trials that investigate the effect of dendritic cell-derived exosomes on several different cancers are underway and show good results for cancer inhibition (16).

Nanoparticles are used to treat diseases, and exosomes with this characteristic can also be utilized to treat cancer (17). It takes an extensive amount of time and cost to isolate exosomes from cell cultures or blood; therefore, the use of other appropriate sources can be a major step in this field. In previous studies, we have assessed the anti-cancer effects of the shell, meat, and haemolymph serum of freshwater crabs on some cancer cell lines (18, 19). In some countries, different components of the crab were used in traditional medicine to treat diseases such as asthma and tuberculosis (20).

Worldwide, breast carcinoma is the most prevalent cancer among women. The majority of deaths associated with breast cancer are due to recurrence and metastasis (21); therefore, new and successful therapies are needed. The aim of this study is to isolate and characterize freshwater crab haemolymph exosomes, as a rich and accessible exosome source, and analyse their effects on 4T1 breast cancer cells. Chemotherapy drugs used to treat various cancers have many adverse effects and cause drug resistance. Therefore, application of a natural compound can be an appropriate alternative (22).

Materials and Methods

Crab species and location of acquisition

We used male freshwater crabs in this *in vitro*

experimental study. The freshwater crabs were prepared and identified in terms of sex and species by a zoologist (Razi University, Iran). The crabs were collected from Jajrood River in the city of Tehran (Iran) during the summer. Housing, anaesthesia, and care of the crabs was done in accordance with the standard conditions of the Animal House at Shahrekord University of Medical Sciences under the approval code of IR.SKUMS.REC.1397.136.

Exosome isolation from crab haemolymph

We injected 20 mg/kg ketamine (Sigma-Aldrich, Canada) into the leg muscles of the crabs (23). Their body surfaces were disinfected with 70% ethanol (Sigma-Aldrich, Canada). After the ethanol dried, the animals' legs were amputated and the haemolymph was collected in a sterile dish. The haemolymph was centrifuged (ROTOFIX 32A, Germany) at 3000 rpm for 10 minutes to isolate the serum. The supernatant contained serum that slowly separated from the lower phase, and was then filtered with a 0.22 µm pore size filter. The sample and reagent A from an Exocib Kit (Cib Biotech Co., Iran) were dissolved at a 5:1 ratio. The solution was vortexed for 5 minutes and subsequently incubated overnight at 4°C. After the overnight incubation, the solution was vortexed for one minute and then centrifuged at 3000 rpm and 4°C for 40 minutes. The supernatant was removed and 200 µl of reagent B was added to allow for clot formation. The samples could be kept at 4°C for a few days and at -20°C temperature for longer periods of time (24).

Characterization of the exosomes by field emission scanning electron microscopy

We used field emission scanning electron microscopy (FE-SEM, MIRA3 TESCAN, Czech Republic) to assess the morphology and size of the exosomes. The purified exosomes were fixed with 37% glutaraldehyde (Sigma-Aldrich, Canada) and dehydrated in ethanol, then placed on a dry glass slide surface and covered with a thin layer of gold. The sample was observed at multiple magnifications (25).

Dynamic light scattering measurement of the exosome

Dynamic light scattering (DLS) is one method used to analyse nanoparticles. DLS has the ability to quickly and easily measure particle sizes in a solution and does not require sample preparation. The extracted exosomes were diluted five-fold with phosphate-buffered saline (PBS, Sigma-Aldrich, Canada) and measurements were obtained by DLS (HORIBA SZ-100, Horiba Jobin Jyovin, Japan) (26).

Zeta potential measurement of the exosomes

The electrical potential in the surface layer between the slipping and the stern layers that encompasses particles and colloid drops is the zeta potential. The zeta potential is the potential difference between the static charge layer

and the rest of the continuous phase. The zeta potential is an indicator of charge accumulation in the stern layer, the degree of opposite ions attracted to the particle surface and, therefore, the degree of electrostatic stability. Hence, it is the best index for determining the surface electricity status of colloidal systems (27). In order to measure the zeta potential of exosome nanoparticles, a zeta sizer instrument (HORIBA SZ-100, Horiba Jobin Jyovin, Japan) was used. First, the column of the machine was washed twice with deionized water and a syringe was used to inject the sample into the column, which was placed in its respective compartment. Measurement of the zeta potential was carried out under the following conditions: pH=7.4, 25°C, and 2.7 V (26).

Characterization of the exosomes by Western blot analysis for CD63

Total protein was extracted from the exosomes by using RIPA buffer from a Radio immune precipitation assay kit (Sigma-Aldrich, Canada). The total protein was subjected to 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, the proteins were transferred to a nitrocellulose membrane. The membrane was incubated overnight with anti-CD63 antibody (A5271, AB Clonal). The bound antibodies were detected after incubation with horseradish peroxidase (HRP)-conjugated (Sigma-Aldrich, Canada) secondary antibody for one hour (28). Crab haemolymph was used as the control.

Total antioxidant capacity assessment of the exosome by the cupric reducing antioxidant capacity assay

The cupric reducing antioxidant capacity (CUPRAC) assay was used to measure total antioxidant capacity. Advantages of this method include the ability to measure substances such as thiol, relatively fast colour development, stable and available indicators, and no requirement for expensive or specialized instruments (29). The CUPRAC assay was used to determine the total antioxidant concentration with a kit (Kiazist, Iran). We added 975 µl PBS to the standard vial (Trolox). A 1 mM standard stock solution was prepared and subsequently diluted into six different concentrations to be used as standards. We added 30 µl of the standards and exosomes to each well of a 96-well plate followed by the addition of 150 µl of the working solution (TAC Buffer, CU+2 Solution, Chromogen). The plate was incubated at room temperature for 60 minutes and absorbance was read at 450-490 nm wavelength with an ELISA reader (Stat Fax 2100, USA) (30).

Protein concentration determination of the exosome by the bicinchoninic acid assay

The bicinchoninic acid (BCA) assay is a colorimetric assay based on the Biore reaction, which is used to determine the protein concentration of a sample. For this assay, we used a kit from Kiazist (Iran). Preparation and dilution of the standards [six different concentrations,

0-1000 µg/ml of bovine serum albumin protein (BSA)] for drawing a standard graph and preparation of the working solution were carried out according to the manufacturer's protocol. We added 10 µl of exosomes and standards to each microtube, followed by 10 µl of the working solution. The microtubes were thoroughly shaken, then incubated at 55°C for 25 minutes. After cooling the microtubes at an ambient temperature, we used a NanoDrop instrument (NanoDrop 2000, USA) to read absorbance at 56 nm. A standard curve was drawn using Excel software and standard absorbance, and the concentration of the exosome was calculated (31). Several different concentrations of the exosome protein were tested on the cells after obtaining the concentration of protein, and we evaluated three concentrations (0.1, 0.5 and 1 mg/ml).

Cell line and cell culture

We purchased the 4T1 mouse breast cancer cell line (ATCC® CRL2539) from the Pasteur Institute Cell Bank (Iran). The cells were cultured in RPMI 1640 (Sigma-Aldrich, Canada) enriched with 10% foetal bovine serum (FBS, Sigma-Aldrich, Canada) and 1% penicillin/streptomycin (Sigma-Aldrich, Canada). Cells were kept at 37°C and 5% CO₂. The BMSCs were extracted from the femur bones of 6-8-week-old adult Wistar rats in accordance with Laboratory Animal Principles and subsequently cultured in a DMEM medium (Sigma-Aldrich, Canada) that contained 10% FBS and 1% antibiotics, and incubated under standard conditions. These cells were isolated and identified as described earlier (32). The BMSCs were used to compare the effects of the exosomes on non-cancer cells.

Assessment of cell viability by the MTT and resazurin assays

In order to perform these two assays, 4T1 and BMSCs (10×10³) were separately seeded in a 96-well plate and we added 200 µl of cell culture media, RPMI and DMEM, respectively, which contained 10% serum and antibiotics. The plates were incubated in an incubator at 37°C and 5% CO₂. Different concentrations of exosomes (0.1, 0.5 and 1 mg/ml) were dissolved in 200 µl serum-free medium and this solution was added to the respective wells, and the plate was incubated. The control group cells did not receive any exosomes. The cells in the control group were treated with serum-free media to ensure that the culture conditions were the same in all groups. After incubation, we prepared 5 mg/ml solutions from MTT powder in PBS (Sigma-Aldrich, Canada). We added 20 µl of the MTT solution in the dark to each well and the plates were incubated at 37°C. Subsequently, the supernatant was removed. Next, we added 100 µl of dimethyl sulphoxide (DMSO, Sigma-Aldrich, Canada) to each well in order to render the produced formazan solubilize. After 30 minutes, the optical density (OD) of each well was measured at 570-630 nm with an

ELISA reader (Stat Fax 2100, USA). For the resazurin test, the plates were incubated. The supernatant was removed after the incubation period and we added 180 μ l of the serum-free medium to each well. Next, 10 μ l of resazurin solution (Kiazist, Iran) was added to the wells that contained the cell culture medium. After incubation for three hours at 37°C, we read light absorbance at 520-570 nm using an ELISA reader. For each concentration, the cell survival percentage was calculated using its relative formula. These assays were repeated three times, at both the 48- and 72-hour incubation times (33).

Nitric oxide measurement in the 4T1 cells

Nitric oxide (NO) was measured by the Griess staining method. At 48 and 72 hours, we collected the supernatant (cell culture media) of the 4T1 cells that had been exposed to different doses of the exosomes (0.1, 0.5 and 1 mg/ml). We deproteinized 400 μ l of supernatant by adding 6 mg of zinc sulphate (Sigma-Aldrich, Canada). The vials were centrifuged at 4°C and 12 000 rpm for 12 minutes. Then, 100 μ l of the supernatant of the deproteinized samples were added to the wells of the 96-well plate and 100 μ l of vanadium chloride (Sigma-Aldrich, Canada), 50 μ l of sulphanylamide (Sigma-Aldrich, Canada) and 50 μ l of N-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD, Sigma-Aldrich, Canada) were added to each well. The plate was incubated for 30 minutes at 37°C. Next, 100 μ l standard sodium nitrate solution at concentrations of 0, 6, 12.5, 25, 50, 100 and 200 μ M, was prepared and we added vanadium chloride, sulphanylamide and NEDD to the standard wells, which was similar to the approach used for the experimental samples. The standards and the samples were read at 540 and 630 nm with an ELISA reader (Stat Fax 2100, USA) (18).

Statistical analysis

With regard to the various doses, we used one-way ANOVA test and Tukey's post-hoc test. The data were statistically analysed by GraphPad Prism software version 8 (GraphPad Software, Inc., La Jolla, CA, USA). Mean differences with a $P \leq 0.05$ were considered to be statistically significant.

Results

Assessment of the extracted exosome size and morphology

FE-SEM microscopy was used to evaluate the size and morphology of the nanoparticle exosomes. The exosomes were less than 100 nm. DLS analysis confirmed the results of the FE-SEM evaluation. The reported zeta potential for the exosome particles was -8.3 mv at 25°C, which meant that glomeration in the solution would not occur; therefore, the particles were suitable for *in vitro* and *in vivo* applications. Western blot analysis was used to determine the expression level

of an exosomal marker, CD63. The isolated exosomes expressed the CD63 protein (Fig.1).

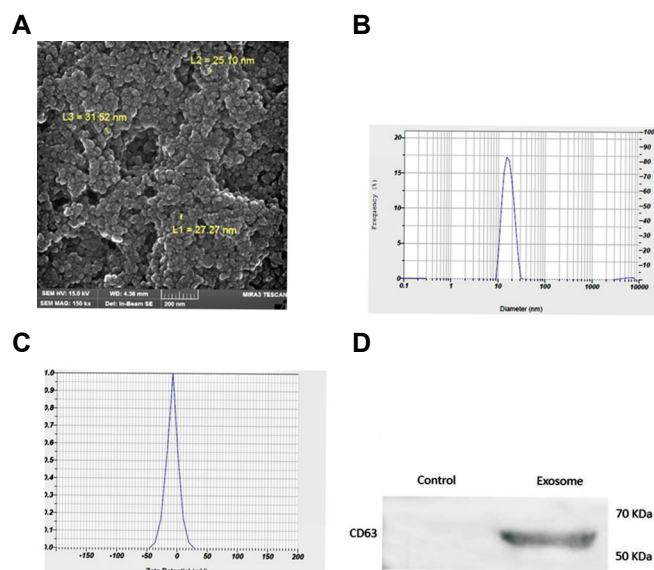


Fig.1: Assessment of the crab haemolymph exosomes was done by several methods. **A.** Field emission scanning electron microscopy (FE-SEM) and **B.** Dynamic light scattering (DLS) showed that the exosomes had a spherical structure and were less than 100 nm in size. **C.** Zeta potential of the exosomes was -8.3 mv. **D.** Western blot analysis showed that the exosomes expressed CD63.

Cupric reducing antioxidant capacity and bicinchoninic acid assays

We created a standard graph for total antioxidant capacity and protein concentration. According to the calculated formula, the exosomes had a total oxidant capacity of 1.003 μ M/ml and protein concentration of 650 mg/ml. The purpose of this test was to determine protein concentration in the exosomes in order to prepare different doses of the exosomes and analyse their effect on BMSCs and the 4T1 cancer cell line. We prepared three concentrations (0.1, 0.5 and 1 mg/ml) of the exosomes to assess their effects on the cells at 48 and 72 hours (Fig.2).

MTT and resazurin assays

We used the MTT and resazurin assays to assess the viability of the 4T1 cancer cells and the BMSCs that were treated with 0.1, 0.5 and 1 mg/ml of crab haemolymph exosomes. In both methods, the percentage of cell viability in the 4T1 cancer cell line decreased in a dose- and time-dependent manner. This difference between all of the experimental groups and the control group was significant ($P \leq 0.001$). The inhibitory concentration (IC_{50}) was obtained with both tests at the 1 mg/ml dose during 48 and 72 hours. However, in the BMSCs, cell growth continued to increase over time in the BMSCs, which was verified by the MTT and resazurin assays. Therefore, crab haemolymph exosomes decreased the breast cancer cell viability but did not affect the BMSCs (Fig.3).

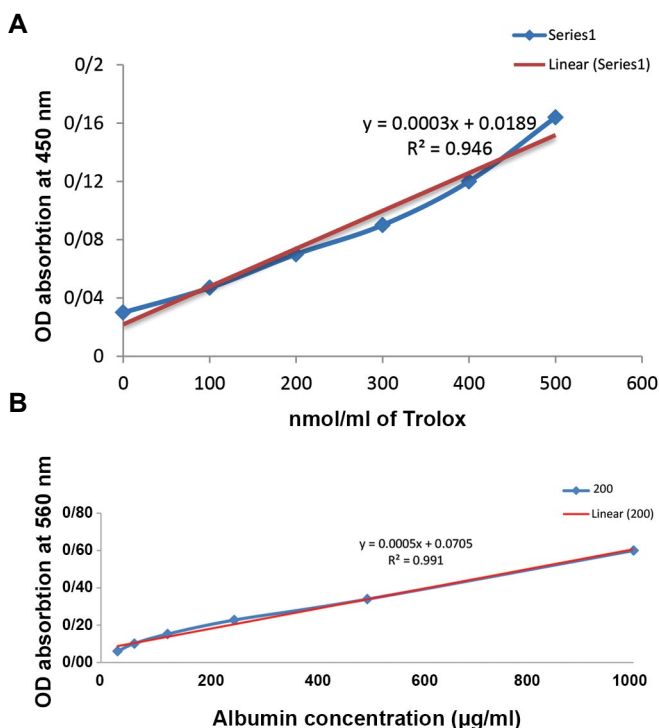


Fig.2: Draw a standard chart. The standard curve for calculating **A.** Total antioxidant capacity and **B.** Protein concentration of the crab haemolymph exosomes. OD; Optical density.

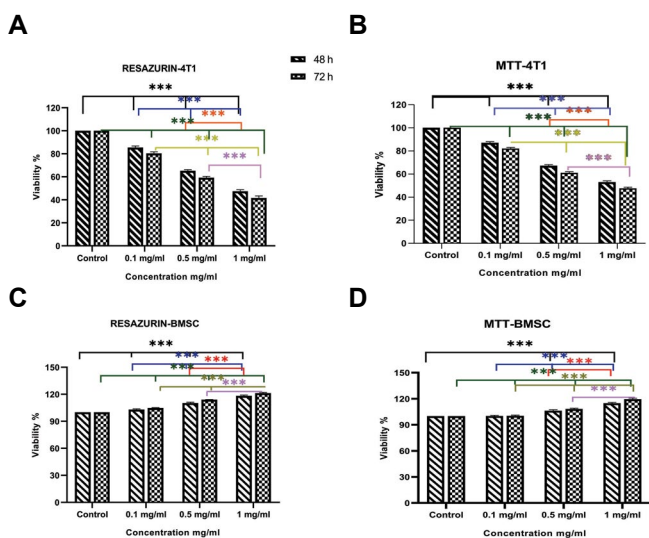


Fig.3: Viability assessment. Viability assessed by the 4T1 **A.** Resazurin and **B.** MTT assays after treatment with different concentrations of the exosomes. Bone marrow mesenchymal stem cell (BMSC) viability assessed by the **C.** Resazurin and **D.** MTT assays after treatment with different concentrations of the exosomes. Cell viability in the 4T1 cancer cell line decreased in a dose- and time-dependent manner according to both assays. In the BMSCs, there was an increase in cell growth, which was dose- and time-dependent manner in both assays. Comparing with the control group was reported to be significant. Comparing different groups and with comparison to the control group was reported to be significant. ***, P≤0.001. The data are shown as mean ± SD.

Nitric oxide assay

The level of NO increases in cancer tissues. Therefore, we examined the amount of NO secretion in the 4T1

cancer cell line that was treated with different doses of the crab haemolymph exosomes. There was a dose- and time-dependent reduction in NO secretion (Fig.4).

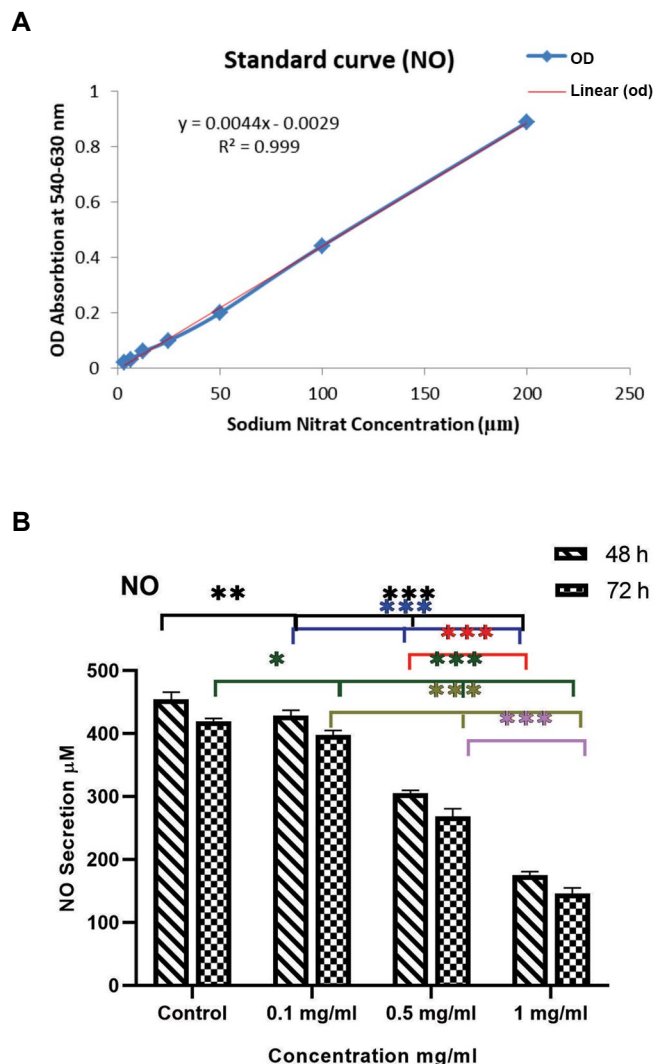


Fig.4: Draw a standard chart. **A.** Standard curve of nitric oxide (NO) secretion from 4T1 cells treated with exosomes. **B.** NO secretion of 4T1 cells decreased in a dose- and time-dependent manner. NO; Nitric oxide, OD; Optical density, *; P≤0.02, **; P≤0.003, and ***; P≤0.001. The data are shown as mean ± SD.

Discussion

In this study, FE-SEM, DLS and Western blot analysis for CD63 confirmed that the exosomes were <100 nm in size and they expressed CD63. The exosomes had a total antioxidant capacity of 1.003 µM/ml and protein concentration of 650 mg/ml. The resazurin and MTT assays showed a decrease in cell survival in the 4T1 cancer cells after treatment with the exosomes. Although exosomes are excreted in cell culture and body fluids, the amount of exosomes obtained from these sources is low. Consequently, the production of exosomes in large quantities for clinical practice is difficult. However, blood provides a better source for exosomes. The amount of exosomes extracted from the blood is higher than the previous sources. Blood reticulocyte-derived exosomes

prevent immune activity and can be an appropriate and safe source of exosomes (9). Our team was the first to extract exosomes from crab haemolymph because the occurrence of invertebrate cancer is very rare and, if the tumour forms, it is benign (34). In our previous study, crab shell extract inhibitory effects on the MCF7 and LNCap cancer cell lines were attributed to chitosan and antioxidants (18, 19). Because of the anti-cancer effects of various crab compounds, cancer rarely occurs in this species (34). In this study, exosomes from freshwater crab haemolymph were extracted and we evaluated their effect on the proliferative abilities of a breast cancer cell line (4T1) and BMSCs from Wistar rats. Exosomes from stem cells have been extracted for use in multiple studies and characterized by electronic microscopy, DLS, and Western blot analysis of CD9, CD81 and CD63 (6). In our study, crab haemolymph exosomes had a zeta potential of -8.3 mV at 25°C. Repulsion and attraction between exosome nanoparticles is measured by the physical properties of the zeta potential. The zeta potential measurement showed that the exosomes were stable with no agglomeration; hence, they were suitable for cell treatments. The results of different studies have shown that the zeta potential range of exosomes is between -2.8 mV to -29.12 mV (35), which agreed with our analysis. The 4T1 cancer cells and BMSCs ability to proliferate were examined via both the MTT and resazurin assays. The results of both assays showed that growth of the 4T1 cells was inhibited at the 0.1, 0.5 and 1 mg/ml doses of exosomes after 48 and 72 hours of treatment. The IC_{50} was obtained with both tests with the 1 mg/ml concentration of exosomes at 48 and 72 hours. The exosomes did not inhibit the growth of BMSCs. Human umbilical cord (MSC)-derived exosomes have been shown to prevent tumour development in the liver (14). In a study, the anti-cancer effects of exosomes isolated from stem cells of mouse bone marrow were investigated on breast cancer *in vivo*. miR16 in stem cell-derived exosomes suppresses tumour by inhibiting VEGF(3). This result was similar to the conclusions of the present report.

The CUPRAC test showed the antioxidant capacity of the exosomes. Many antioxidants in the diet, through reducing oxidative stress, may act alone or in conjunction with chemotherapy to prevent cancer and avoid metastasis. Antioxidants like resveratrol (36) vitamin C, vitamin E, and α -tocopherol increased the effects of chemotherapy drugs like 5-fluorouracil. Vitamin C antioxidant activity increased the therapeutic effects of doxorubicin, cisplatin and paclitaxel for breast cancer treatment (37). Exosomes, such as human umbilical cord MSC-derived exosomes, may have antioxidant properties that could be effective as treatments for cancers and other diseases (14). The amount of antioxidant in these crab haemolymph exosomes according to the CUPRAC method was 1.003 μ M/ml and the amount of antioxidant was reported in another study for example Green Tea and Chokeberry extract 1.24 mM/ml and 189.6 μ M/ml respectively (30, 38). Less than other studies, the amount of antioxidants in the exosome was reported but its effect on tumour control

was significant. It is likely that the antioxidant properties of the exosomes used in this study could inhibit cancer cell growth. The time- and dose-dependent secretion of NO decreased in cancer cells treated with exosomes. NO is considered to be a free radical that causes an increase in oxidative stress (39); its secretion increases in breast (19) and prostate cancer cells (18). Treatment with antioxidant compounds reduced levels of NO in both cancers. In our study, there was a decrease in NO levels (cancer cell) after treatment with the crab haemolymph exosomes. Given that exosomes have antioxidant effects, this may be one reason for the decrease in NO levels. The 4T1 cancer cell line is derived from breast cancer and is equivalent to stage 4 human breast cancer. This cell line produces triple negative breast cancer, which constitutes approximately 15% of breast cancers in women (40). Not many chemotherapy drugs exist for triple negative breast cancer in comparison to endocrine receptor positive (oestrogen and progesterone positive) and triple positive [positive for oestrogen, progesterone, and human epidermal growth factor 2 (Her2)] receptors. Therefore, inhibiting the growth of these cancer cells with crab haemolymph exosomes can be considered a novel treatment for triple negative breast cancer. BMSCs were also exposed to exosomes in this study and this comparison helped us to evaluate the effect of exosomes on non-cancer as well as cancer cells. This study showed that cell viability in the BMSCs did not decrease after treatment with crab exosomes.

Conclusion

Freshwater crab haemolymph is rich in exosomes that are less than 100 nm in size. These exosomes have antioxidant properties and can inhibit mouse breast cancer cell (4T1) proliferation. These exosomes may be used to carry pharmaceutical agents such as anti-cancer drugs. More extensive studies on haemolymph exosomes of freshwater crab are suggested.

Acknowledgments

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Authors' Contributions

L.R., M.A.; Performed the experiments, collected and interpreted the data, drafted and edited the manuscript. E.Sh., M.S.; Participated in data analysis, evaluations and editing the manuscript. A.A.; Conceived the study idea, participated in the study design, and supervised the project. All authors read and approved the final manuscript.

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