

Effects of *Haematococcus pluvialis* green alga extract on the expression of p53 and Bcl-2 genes in the MDA-MB-231 human breast cancer cell line

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ABSTRACT

Seaweeds are a natural source of a wide range of secondary metabolites with different biological activities, including anticancer activity. The present study aimed to investigate the toxicity of aqueous and organic extracts of the *Haematococcus pluvialis* green microalgae to varying concentrations on the MDA-MB-231 breast cancer cell line and to investigate the expression of genes involved in the apoptosis pathway, Bcl-2, and p53. The toxic effect of different concentrations (50, 100, 150, and 200 $\mu\text{g mL}^{-1}$) of aqueous, methanolic, hexane, and chloroform extracts of the microalgae was measured by the MTT method, and then the expressions of p53 and Bcl-2 genes were evaluated by the RT-qPCR method. The percentage of cancer cell viability was inversely related to the concentration of the extracts and the treatment time. Among the extracts, the hexane extract of microalgae at a concentration of 200 $\mu\text{g mL}^{-1}$ showed the highest cytotoxic effect (18%) compared to other extracts. Also, the expression of Bcl-2 and p53 genes in the treatment with hexane extract significantly decreased and elevated compared to the control and aqueous extract, respectively. As a result, the toxicity effect of the hexane extract of *H. pluvialis* against MDA-MB-231 breast cancer cells was efficient, so it can be a basis for further studies to introduce this green microalga as a suitable candidate for a biological compound with anticancer activity.

Keywords: Apoptosis, Breast cancer, cytotoxicity, Green microalgae, p53, Bcl-2.

Article type: Research Article.

INTRODUCTION

Over the past decades, the number of patients with breast cancer and its incidence have increased worldwide, and therefore it is considered a major health problem worldwide. Today, many approaches are used to treat breast cancer, including radiotherapy, chemotherapy, and hormone therapy (Hanahan 2022). A promising approach to treating breast cancer is to use the sensitivity of these cells to drugs that induce apoptosis. Apoptosis is the main form of programmed cell death that occurs in response to certain stimuli, as well as during the morphological differentiation process, and plays an important role in eliminating unwanted cells. Apoptosis occurs in nucleated cells through two pathways: extrinsic (death receptor-dependent pathway) or intrinsic (mitochondrial pathway). The extrinsic pathway occurs through the interaction of the tumor necrosis factor ligand (TNFL) family with the death receptor on the cell surface (Gyulkhandanyan *et al.* 2012). The intrinsic pathway of apoptosis is activated by a wide range of factors such as hypoxia, DNA damage, and reactive oxygen species and is controlled by proteins of the Bcl-2 family (van Loo *et al.* 2002). Various proteins play a role in the induction of apoptosis, including p53, which is involved in the regulation of numerous pro-apoptotic genes and is activated in the event of irreversible DNA damage, leading to the induction of the apoptosis process. In contrast, the Bcl-2 protein is an inhibitor of apoptosis and a major member of the Bcl-2 family. One of the mechanisms of action of this protein is the inhibition of the formation of channels in the outer mitochondrial membrane (Cetraro *et al.* 2022). New drugs designed to induce cell death may be more effective treatment options for breast cancer. Therefore, research into the production of drugs with greater efficacy and less toxicity is essential. Medicinal plants are important sources

of anticancer compounds, and natural compounds such as alkaloids, terpenes, flavonoids, etc. in various plants possess the highest cytotoxicity in nature. Today, finding natural compounds from marine organisms, especially algae, that have antibacterial, antiviral, and anticancer effects has been the focus of various research studies around the world. New studies are conducted in this direction every year, and valuable results are obtained (Ercolano *et al.* 2019). The *Haematococcus pluvialis* green microalga is a single-celled freshwater one distributed in many habitats worldwide. This microalga is considered the best natural source of bioactive compounds due to its valuable biological compounds such as protein, fat, carbohydrates, pigments, vitamins, minerals and antioxidant compounds, especially the pigment astaxanthin, and has wide applications in the food, cosmetic, aquaculture and pharmaceutical industries (Boussiba 2000; Li *et al.* 2020). Therefore, more comprehensive research is needed to utilize the biological potential of this microalgae and its highly valuable products. Given this, in this study, the effects of aqueous and organic extracts (methanol, chloroform, and hexane) of the *H. pluvialis* on the expression of genes involved in the Bcl-2 and p53 apoptosis pathways and the prevention of proliferation of human breast cancer cells of the MDA-MB-231 line was investigated.

MATERIALS AND METHODS

Cultivation of *H. pluvialis* microalgae

Haematococcus pluvialis green microalga (Fig. 1) was purchased from Algae Bank (algaebank.ir). Standard BBM culture medium (Tripathi *et al.* 1999) was used to culture microalgae under autotrophic conditions. For better growth, it was kept for 20 days at a temperature of 28 ± 2 °C, a light intensity of 3500 lux, a photoperiod of 12 hours of darkness and 12 hours of light with aeration. The biomass was harvested by centrifuge (Sigma 2-16 KL, Germany) at 6000 rpm for 5 minutes at 25 °C and dried using a freeze-dryer (Germany Alpha 1-2LD plus, Christ) and stored in a freezer at -20 °C.

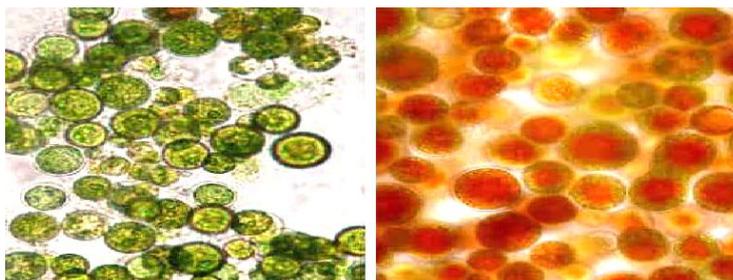


Fig. 1. Light microscope image of the *H. Pluvialis* green microalga at 400x magnification.

Measurement of photosynthetic pigment content

Five mL methanol was added to 0.01 g microalga biomass and kept in the dark at 4 °C for 24 hours. Then, it was centrifuged at 3000 rpm for 5 minutes at 4 °C. The absorbance of the supernatant solution was read at wavelengths of 470, 653 and 666 nm with a spectrophotometer (CamSpec M501 single beam UV/Visible) and the amounts of pigments were calculated according to the formula in Table 1 (Dere *et al.* 1998).

Table 1. Formula for calculating the amount of chlorophyll a, b and total carotenoids (Dere *et al.* 1998).

Chla ($\mu\text{g/g DW}$)	$= [(15.65 \times \text{OD } 666 \text{ nm}) - (7.340 \times \text{OD } 653 \text{ nm})] \times \text{Dilution factor}$
Chlb ($\mu\text{g/g DW}$)	$= [(27.05 \times \text{OD } 653 \text{ nm}) - (11.21 \times \text{OD } 666 \text{ nm})] \times \text{Dilution factor}$
T-Chl ($\mu\text{g/g DW}$)	$= \text{Chla} + \text{Chlb}$
T-Car ($\mu\text{g/g DW}$)	$= [(1000 \times \text{OD } 470) - (2.860 \text{Chla} - 129.2 \text{Chlb})] / 245 \times \text{Dilution factor}$

Measurement of phenol and flavonoid content

To measure the amount of phenol, first 1600 μL distilled water, 300 μL of 20% sodium carbonate and 100 μL of 50% Folin reagent were added to 20 μL methanolic extract of microalgae and left in a water bath at 50 °C for 30 minutes and then its absorbance was read at 720 nm with a spectrophotometer. Simultaneously with this process, a standard solution of gallic acid in the range of 0-100 mg mL^{-1} was also prepared to draw a standard graph and the measurement was made in the same way. The amount of total flavonoids was measured by aluminum chloride colorimetric method using quercetin as a standard. First, a standard solution with concentrations of 0-100 mg mL^{-1} quercetin in methanol solvent was prepared. Afterward, 0.2 mL of 2% aluminum chloride and 0.1 mL of 33% aqueous acetic acid were added to 0.2 mL microalga methanolic extract and standard solution, then mixed well.

Finally, the reaction mixture was made up to 5 mL with methanol and the tubes were kept at room temperature for 30 minutes. Their optical absorption was read at a wavelength of 414 nm (Park *et al.* 2018).

Antioxidant activity measurement

DPPH (0.004% in 95% methanol) was added to 100 μL of microalgae extracts (water, methanol, hexane and chloroform) in an amount of 3.9 mL and kept in the dark at room temperature for 30 minutes. Then the absorbance of the solution was read by spectrophotometer at 517 nm and finally the DPPH free radical scavenging activity was calculated using the following equation (Burits & Bucar 2000).

$$\text{DPPHsc (\%)} = (\text{Acontrol} - \text{Asample} / \text{Acontrol}) \times 100$$

Acontrol: DPPH absorption without extract

Asample: DPPH absorption with extract

Measurement of total protein content

First, 0.1 g microalga powder was sonicated with 50 mM phosphate buffer (pH 7-7.2) for 60 seconds to break the cell wall. Then, it was centrifuged at 12000 rpm at 4 °C. An aliquot of 2900 μL Bradford reagent (Sigma Aldrich, Germany) was added to 100 μL protein-containing solution, and after 15 minutes of dark incubation at 4°C, the optical absorption at 595 nm was read by spectrophotometer. Bovine serum albumin (Sigma Aldrich, Germany) was used at concentrations of 10-50 mg mL⁻¹ to draw a standard curve and obtain a linear equation to calculate the total protein content (Bradford 1976).

Extraction from microalgae biomass

To prepare the desired extracts, 10 g dry microalga biomass was soaked in 200 mL solvents (water, methanol, hexane, and chloroform) at 25 °C for 24 hours, then passed through filter paper and the supernatant was separated. This was repeated three times. Then the solvents were evaporated by an oven (Mettler, Germany) and the extracts were dissolved in DMSO solution (Merck, Germany) and DMEM cancer cell culture medium (Bioidea) after weighing, and concentration was performed from the stock solution of the extracts (Lim *et al.* 2002).

Cancer cell line culture

Human breast cancer cell line (MDA-MB-231, NO. IBRC C10684) was purchased from the National Center for Genetic and Biological Resources of Iran (ibrc.ir/fa). These cells are a human breast adenocarcinoma epithelial cell line that is an adherent cell type (Fig. 2). For the cultivation of the desired cell, a culture medium containing DMEM (Bioidea), 10% FBS (GibCo) and 1% penicillin/streptomycin antibiotic (Bioidea) was used. The flask containing the cells was placed in an incubator (INC246 Mettler, Germany) with 5% CO₂, 37 °C and 95% humidity for cultivation.



Fig. 2. Inverted microscope image (IM-3, optika, Italy) of the MDA-MB-231 human breast cancer cell line, at 200X magnification.

Cytotoxicity assessment

At this stage, 104 cells were plated in a 96-well plate and cultured in an incubator at 37 °C with 5% CO₂ and 95% humidity. After 24 hours, the cells were treated with concentrations of 50, 100, 150, 200 $\mu\text{g mL}^{-1}$ aqueous and organic extracts of green microalga for 48 and 72 hours. An untreated group was considered as a negative control group for comparison. After the incubation times and discarding the cell supernatant, 20 μL MTT solution (5 mg mL⁻¹) was added to the cells and incubated for 3 hours. After discarding the supernatant, 100 μL DMSO was added to the cells and incubated for 15 minutes at room temperature until the formazan precipitates were completely dissolved. Optical absorption was read with a microplate reader (MPR4+ Hiperion Microplate Reader, Germany) at a wavelength of 570 nm (Li *et al.* 2012).

IC₅₀ measurement

To obtain the appropriate concentration of each microalgae extract for treating cells and examining the desired genes, the IC₅₀ concentration, i.e., the concentration of extracts at which half of the cells are alive and half are

dead, was used. In this way, the standard curve was drawn using the data from the MTT test, and the linear equation was obtained. Then, to obtain the concentration of the extract at which 50% of the cells are alive, Y was taken as 50, and X, which is the IC₅₀ concentration, was calculated.

Measurement of Bcl-2 and p53 gene expression with Real Time-PCR

Cancer cells were incubated in a 6-well plate at 37 °C, 5% CO₂, and 95% humidity. Then, after 80% of the cells adhered to the bottom of the plate, they were treated with IC₅₀ concentrations of aqueous and organic microalgae extracts along with fresh culture medium for 48 and 72 hours. After the end of the treatment time, RNA was extracted from the cells using the RNX Plaus kit (SinaClon, Iran), and cDNA synthesis was performed using the cDNA synthesis kit (Cinnagen, Iran). To determine the concentration and purity of the extracted RNA and synthesized cDNA, a Nanodrop device (EZDrop 1000 Blue-Ray Biotech, Taiwan) was used. Then, to check its quality, a 1% agarose gel was used. For this purpose, the optical absorption of the samples was obtained between 1.8 and 2, which are samples of appropriate purity, and the absence of smear in the gel indicates the purity and integrity of the extracted RNA and synthesized cDNA. Then, Real-time PCR was used to quantify the Bcl-2 and p53 genes. The GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) gene was used as a reference gene. In this study, the primer sequences of GAPDH, Bcl-2, and p53 genes were designed using Oligo v7.60 software and synthesized by SinaClon Company. The sequences of these primers are given in Table 2. Real-time PCR conditions, along with the time of different steps and T_m of the primers, are listed in Table 3.

Table 2. Sequences of primers for genes used in this study, manufactured by Sinaclon Iran.

Gene	forward Sequence (5'-3')	reverse Sequence (5'-3')	Gene ID	Product length (bp)
Bcl-2	CGTGATTGAAGACACCCCTC	TCTCCCGTTATCGTACCCTG	XM_017025917.2	188
p53	TCGAGATGTTCCGAGAGCTGA	AGTGGGGAACAAGAAGTGGAG	NM_001126117.2	195
GAPDH	CGTGGAAGGACTCATGACC	CAGTAGAGGCAGGGATGATG	NG_007073.2	125

Table 3. Thermal characteristics of the polymerase chain reaction.

Number of cycles	Time	Temperature (°C)
1	5 minutes	95
35	30 seconds	95
	30 seconds	Binding temperature of each primer p53: 60 Bcl-2:62 GAPDH:60
	30 seconds	72
1	5 minutes	72
-	∞	4

Statistical analysis

To reduce the error rate, each experiment was repeated 3 times, and the mean data ± standard deviation (SD) was calculated. Data analysis was performed with One-Way ANOVA and Duncan test at a significant level of $p \leq 0.05$ using SPSS Statistics 17.0 software, and graphs were drawn using Excel 2013 software.

RESULTS

Amounts of bioactive compounds of microalgae

The contents of bioactive compounds of *Haematococcus pluvialis* microalga, including chlorophyll a and b, total carotenoids, phenols, flavonoids, and total protein, are given in Table 4, and the antioxidant activities of four extracts in Table 5. According to the results, the antioxidant activity in the hexane extract was higher than in the other extracts (about 71.46 ± 1.67 %).

Investigation of MDA-MB-231 cancer cell viability

According to Fig. 3, which shows the rate (%) of MDA-MB-231 cancer cells treated with different concentrations of aqueous and organic extracts of *H. pluvialis* for 48 and 72 hours, the rate (%) of cancer cell viability was inversely related to the increased concentration of the extracts, so that the viability decreased by elevating the concentration of the extracts and the treatment time. Among the extracts, 200 µg mL⁻¹ hexane extract of microalga

during 72 hours of treatment exhibited the lowest viability rate ($18.20 \pm 0.68\%$) on cancer cells compared to the other extracts. Afterward, the aqueous, chloroform, and methanol extracts reduced cell growth, respectively. Given that the hexane and aqueous extracts of microalga showed a significant toxicity effect compared to the chloroform and methanol extracts, these two extracts were selected to investigate the expression of Bcl-2 and p53 genes. To obtain the IC_{50} inhibitory dose of the *H. pluvialis* hexane and aqueous extracts, the MTT results at 48 and 72 hours were used. The IC_{50} values for the hexane and aqueous extracts were 23.77 ± 1.01 and $170.11 \pm 0.17 \mu\text{g mL}^{-1}$ at 48 hours, respectively, while 17.66 ± 0.80 and $160.85 \pm 1.22 \mu\text{g mL}^{-1}$ at 72 hours, respectively (Fig. 4). These concentrations of the green microalga aqueous and hexane extracts were used to treat cancer cells for 48 and 72 hours to investigate the expression of genes involved in the apoptosis pathway.

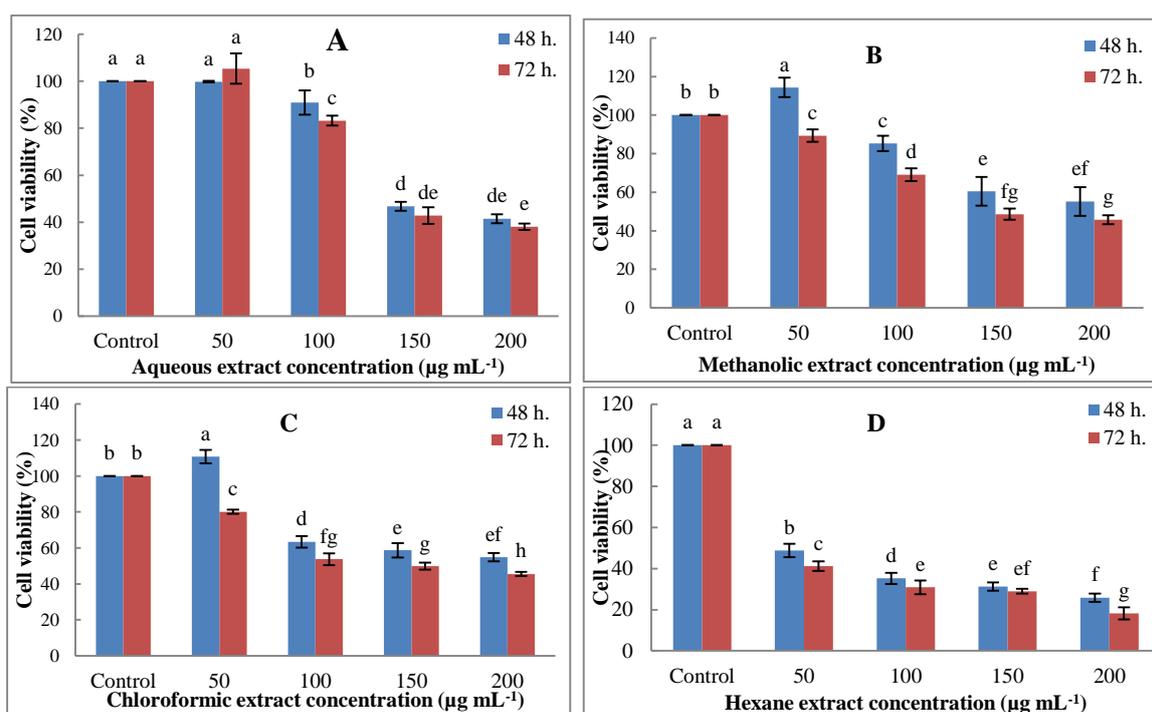


Fig. 3. Percentage of breast cancer cell viability treated with different concentrations of A) aqueous, B) methanolic, C) chloroformic, and D) hexane extracts of the *H. pluvialis* microalgae for 48 and 72 hours. Data are the mean of three replicates \pm standard deviation (SD). Different letters indicate significant differences between samples based on comparison of means using Duncan's range test at a significant level of $p < 0.05$.

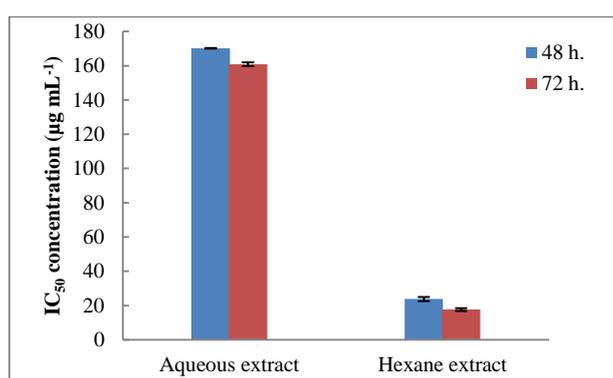


Fig. 4. IC_{50} values of aqueous and hexane extracts of the *H. pluvialis* microalgae during 48 and 72 hours of treatment of MDA-MB-231 breast cancer cells. Data are the mean of three replicates \pm standard deviation (SD).

Expression of Bcl-2 and p53 genes

According to Fig. 5, the expression of Bcl-2 and p53 genes in cells treated with the microalga aqueous and hexane extracts significantly decreased at 48 hours and increased at 72 hours ($p < 0.05$) respectively. So that, the greatest decrease in Bcl-2 gene expression was obtained by treating cells with hexane extract (0.43 ± 0.04) at 72 hours, while the least decrease in 48 hours by aqueous extract (0.85 ± 0.054). In addition, the greatest increase in p53

gene expression was obtained by hexane extract (6.26 ± 0.47) at 72 hours, while the least decrease in 48 hours by aqueous extract (1.86 ± 0.45).

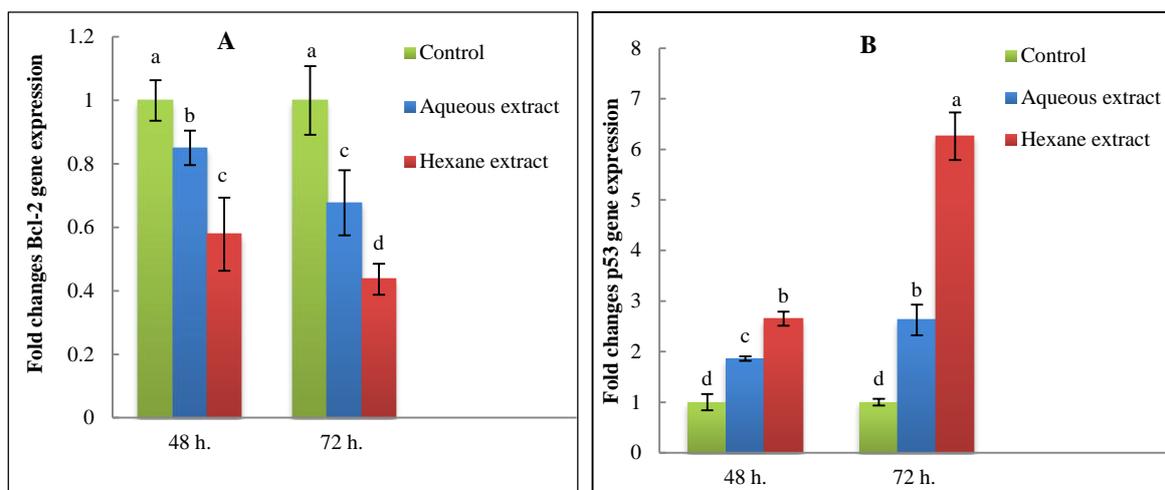


Fig. 5. A) Bcl-2 and B) p53 gene expression in breast cancer cell lines treated with aqueous and hexane extracts of the *H. pluvialis* microalgae for 48 and 72 hours. Data are the mean of three replicates \pm standard deviation (SD). Different letters indicate significant differences between samples based on comparison of means using Duncan's test at a significant level of $p < 0.05$.

Table 4. Content of bioactive compounds of *H. pluvialis* microalgae. Data are the mean of three replicates \pm standard deviation (SD).

Algae species	Chlorophyll a Content ($\mu\text{g g}^{-1}$ DW)	Chlorophyll b Content ($\mu\text{g g}^{-1}$ DW)	Carotenoid Content ($\mu\text{g g}^{-1}$ DW)	Phenolic Content (mg GA g^{-1} DW)	Flavonoide Content (mg QE g^{-1} DW)	Protein Content (mg mL^{-1})
<i>H. pluvialis</i>	3.69 ± 0.15	2.81 ± 0.53	5.37 ± 0.36	66.13 ± 0.43	13.28 ± 0.35	28.20 ± 0.16

Table 5. Antioxidant activity of aqueous and organic extracts of *H. pluvialis* microalgae. Data are the mean of three replicates \pm standard deviation (SD).

Algae extract	DPPHsc (%)
Aqueous extract	65.82 ± 2.90
Methanolic extract	59.65 ± 0.37
Hexane extract	71.46 ± 1.67
Chloroform extract	60.82 ± 0.92

DISCUSSION

The breast cancer cell line MDA-MB 231, with its specific molecular pattern, is known as a triple-negative cell line with cancer stem cell properties, which has high invasiveness. It grows adherently to the bottom of the flask, and is considered the most common cause of cancer-related death among women (Alateyah *et al.* 2022). Research into the production of drugs with greater efficacy and less toxicity is essential. Nowadays, finding natural compounds from marine organisms, especially algae, has attracted attention all over the world. Therefore, more comprehensive research is needed to utilize the biological potential of algae and their highly valuable products. Given this, in this study, the effects of *H. pluvialis* aqueous and organic extracts (methanol, chloroform, and hexane) on the expression of genes involved in the Bcl-2 and p53 apoptosis pathway was investigated. In addition, the inhibition of proliferation in the human breast cancer cells of the MDA-MB-231 line was examined to find compounds of natural origin with less toxicity and side effects as stimulants of the apoptosis signalling pathway. In this study, given the bioactive compound amounts including chlorophyll pigment, carotenoids, phenols, and flavonoids (Tables 4 and 5), which possess antioxidant properties, the highest antioxidant activity of the microalga

was obtained in the hexane extract ($71.46 \pm 1.67\%$). Vahdat *et al.* (2017), in a study on the fresh weight of *H. pluvialis* reported the amount of chlorophyll a to be 4.73 ± 0.21 , chlorophyll b 1.63 ± 0.54 , and total carotene $7.19 \pm 0.76 \mu\text{g g}^{-1}$. Naderi *et al.* (2015) reported in their study that the amount of protein in *H. pluvialis* in BBM medium was $32.40 \pm 0.04\%$. In addition, in the study by Mourya *et al.* (2023), the amount of chlorophyll a was reported to be 3.46, chlorophyll b 0.67, and total carotene $0.83 \mu\text{g L}^{-1}$. Moreover, in the present study, by increasing in the extract concentration and treatment time, the cancer cell viability rate (%) significantly decreased ($p < 0.05$). The cancer cell viability rates (%) under treatment with different concentrations of microalgae extract exhibited the following trend: methanol > chloroform > aqueous > hexane. So that methanol and hexane extracts showed the least and the most toxic effect, respectively. Among the four solvents used, water has the highest polarity, methanol and chloroform are solvents with medium polarity, and hexane is non-polar. Therefore, hexane solvent has a greater ability to extract non-polar compounds than the other solvents, which in turn plays a role in cytotoxicity. Namvar *et al.* (2014) reported that methanolic extracts of three algae species, *Sargassum ilicifolium*, *Ulva faciata*, and *Gracilaria corticata* displayed inhibitory activity against the breast cancer cell line MCF7. In another study, these researchers reported that methanolic extract of *Sargassum muticum* exhibit a toxic effect against breast cancer cell lines MDA-MB 231 and MCF-7. In addition, the antiproliferative activity of the alga extract is positively correlated with the polyphenol content (Namvar *et al.* 2013; Namvar *et al.* 2014). In another study, Dellai *et al.* (2013) reported that hydroalcoholic extract of *Laurencia obtusa* displays antiproliferative activity on the HCT15, A549, and MCF7 human cancer cell lines. They also stated that this extract contains phenolic and flavonoid compounds, and its inhibitory effect increases by the elevated dose (Dellai *et al.* 2013). Hemasudha *et al.* (2019) reported that the methanol extract of *Gracilaria edulis*, which has high antioxidant activity due to its phenolic and flavonoid compounds, showed a significant toxic effect on breast cancer (MDA-MB231). Taheri *et al.* (2018) also reported that by elevating the concentration of methanol, chloroform, ethyl acetate, and hexane extracts of *Sargassum glaucescens*, the survival rate of breast (MCF-7) and colorectal (HT-29) cancer cells decreased. Cancer cells inhibit this process by using various mechanisms, including increasing anti-apoptotic and decreasing pro-apoptotic proteins, thus achieving high survival rate, which in turn causes cancer proliferation and progression. For this reason, apoptosis has attracted much attention in anticancer research (Chandler *et al.* 1994). The Bcl-2 protein family plays an important role in mitochondrial-dependent apoptosis, and members of this family are divided into two main groups: apoptosis inhibitors and apoptosis promoters (Dakubo 2010). On the other hand, p53 is an important protein in inducing apoptosis in the damaged or cancerous cells, which causes the death of mutated or cancerous cells by inducing apoptosis (El-Deiry *et al.* 1993). In contrast, anti-apoptotic members prevent the process from starting. In cases of increased apoptosis, it is expected that the expression of the p53 gene will increase, while that of the Bcl-2 gene will drop (Fulda & Debatin, 2006). In the present study, the expression level of the Bcl-2 in cells treated with aqueous extracts of microalga decreased, while that of p53 genes with hexane extract increased. Palozza *et al.* (2009) reported that the extract of *H. pluvialis* displayed dose- and time-dependent inhibitory effects by stopping cell cycle progression and promoting apoptosis in HCT-116 colon cancer cells. Thus, the expression of p53, p21, and p27 increased, while the expression of cyclin D1 and phosphorylation of AKT were simultaneously decreased (Palozza *et al.*, 2009). Kawsji & Fatemi Moghadam (2019) stated that *Spirulina* extract reduced the expression of the Bcl-2 gene in the glioblastoma U87 cancer cell line. In most studies, the existence of different properties about bioactive compounds present in algae is known, each of which is capable of cytotoxic and apoptotic activities.

CONCLUSION

In this study, the hexane extract of the *Haematococcus pluvialis* exhibited a more toxic effect on the MDA-MB-231 human breast cancer cell line than the other extracts and was able to affect the expression of the p53 and Bcl-2 apoptosis pathway genes, in a way that increased the expression of the p53 gene, while decreased that of the Bcl-2 gene. As a result, it can be a basis for conducting further studies to further investigate the effects of this alga in cancer treatment and identifying anticancer compounds. The findings of the present study can be used for further research to identify, isolate, and characterize specific phytochemical compounds of algae.

Author contributions. The idea, data collection, experiments and calculations were carried out by Pouya Ghaleb. Other authors contributed to the literature review, analysis and interpretation of data, as well as drafting, editing, critical revision, and approval of the final version.

Funding. This research was extracted from Pouya Ghaleb's M.Sc. project in the Biochemistry Research Laboratory of the Faculty of Basic Sciences, at University of Guilan, Iran.

Conflict of interest. The authors declare no conflict of interests.

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Bibliographic information of this paper for citing:

Ghaleb, P, Moradi, F, Aghamaali, M 2025, Effects of *Haematococcus pluvialis* green alga extract on the expression of p53 and Bcl-2 genes in the MDA-MB-231 human breast cancer cell line. *Caspian Journal of Environmental Sciences*, 23: 1303-1311.
