RESEARCH ARTICLE

Editorial Process: Submission:10/26/2024 Acceptance:06/05/2025

The Anticancer Effect of *Haematococcus Pluvialis* Alga Extract on Breast Cancer Cell Line

Vahideh Shamskhani[1], Habib Zarredar[2], Jafar Razeghi[1], Atefeh Ansarin[2], Milad Asadi[3], Zahra Soleimani[4], Ensiyeh Seyedrezazadeh[2]*

Abstract

Objective: The aim of this study was to investigate the anticancer effects of *H. pluvialis* extract on breast cancer. **Methods:** We evaluated the cytotoxicity, suppression of migration, and apoptosis ability of *H. pluvialis* alga extract MDA-MB-468 by MTT assay, scratch test, and flow cytometry, respectively. The expression patterns of *MMP1* and *MMP9, Bax* and *Bcl2*, *Caspase3, Caspase6,* and *Caspase9* were analyzed by qRT-PCR. **Results:** Based on the findings of this study, the survival and proliferation capacity of MDA-MB-468 cells treated with the extract was reduced. Flow cytometry results showed that treatment with H. Pluvialis extracts induced apoptosis. Scratch test results showed that H. Pluvialis extract could inhibit cell migration. The expression patterns of the studied genes were changed by treatment with the extract. Expression of *Bcl2, MMP1,* and *MMP9* genes decreased, and expression of *Bax, Caspase3, Caspase8, Caspase9* genes increased. **Conclusion:** The findings of this research reveal that H. pluvialis algae extract shows a potential anti-cancer compound.

Keywords: Breast cancer- Haematococcus Pluvialis- Cell migration- Apoptosis

Asian Pac J Cancer Prev, **26 (6)**, 2071-2077

Introduction

Despite recent advancements in cancer diagnosis and treatments globally, According to statistics provided by the International Agency for Research on Cancer, breast cancer is the main concern of women and the most common type of cancer among them. Most women with breast cancer are over 45 years old, and younger women can also implicated [1, 2]. It is the leading cause of cancer among Iranian women, accounting for 27% of all malignancies in women [3]. Breast cancer is a potentially aggressive disease with highly heterogeneous molecular mechanisms that lifestyle and environmental factors including high-fat diet, obesity, shift work factors, smoking, antiperspirants, breast Implants, alcohol consumption, lack of physical exercise, hormone replacement therapy, ionizing radiation influence its progression [4]. Approximately 5 to 10% of breast cancers occur inherited with the *BRCA1* and *BRCA2* genes playing the most important role. These genes are involved in repairing damaged DNA are sometimes called tumor suppressor genes and when they have certain changes, pathogenic variants, or mutations, cancer can develop [5, 6]. Despite using the most common treatment targets including chemotherapy, surgery, radiation, and immunotherapy, natural foods such as fruit and vegetables, generally with biological and antioxidant activities improved their anticancer treatment properties. In this context, many natural foods such as citrus fruits, vegetables, macronutrients, and grains are used to prevent cancer and research and treatment strategies [7, 8]. It seems high consumption of enriched bioactive nutrient components reduces the risk of breast cancer and increases its survival rate [9]. Their mechanisms are involved in various ways consisting of down-regulation of estrogen receptor-alpha (ER-α) [10]. Microalgae contain biological substances such as unsaturated fatty acids, proteins, amino acids, pigments, antioxidants, vitamins, and minerals that are important in the diet, health, biofuels, and healthy products [11]. Among these, Haematococcus Pluvialis (*H. pluvialis*) is particular importance because of its antioxidant compound called "Astaxanthin". Astaxanthin is one of the most famous natural pigments that have very strong antioxidant activity and has a great impact on human health, including the prevention of cardiovascular disease, strengthening of immune system, prevention and treatment of various cancers, and the cell aging process prevention [12]. *H. pluvialis* is one of the important commercial algae and a unicellular freshwater microalga

*[1]Department of Plant Biology, Faculty of Natural Sciences, University of Tabriz, Tabriz, Iran. [2]Tuberculosis and Lung Diseases Research Canter, Tabriz University of Medical Sciences, Tabriz, Iran. [3]Department of Basic Oncology, Ege University, Institute of Health Sciences, Izmir, Turkey. [4]Rahat Breathe and Sleep Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. *For Correspondence: : e.s.rezazadeh@gmail.com*

belonging to the class of Chlorophyceae, order Volvocales, and family Haematococcaseae [13]. It is the richest source of astaxanthin (pink carotenoid), nowadays known as a potent antioxidant that is produced by some microalgae, plants, yeasts, and bacteria. Moreover, these algae exist in many kinds of seafood including salmon, shrimp, crab, and fish eggs [12, 14]. Astaxanthin biosynthesis usually occurs under stress conditions such as nutrient shortage, brininess, top temperature, and radiation. The cells of the green alga H.Pluvialis are green flagella in the vegetative stage. In the cystic stage, these cells, when exposed to unfavorable conditions, turn into red cysts that contain a large number of red ketocarotenoids called astaxanthin, which is immobile. When the conditions are right for growth, the cysts germinate to form new motile growth cells [15]. A temperature of 25–28 °C is the most suitable temperature for the growth of different strains of Haematococcus. Previous studies show that the extract of Haematococcus alga inhibits cell cycle progression and cancer cell proliferation and migration in different colon cancer cell lines and induces apoptosis [16]. Moreover, the Hematococcus extract toxicity of alga in lung, breast, and hepatocellular cancers is more than of Dunaliella salina extract [13]. Accordingly, astaxanthin reduces cell proliferation and migration in the breast, [17] gastric, [18]and liver cancers [19]. Different stereoisomers of astaxanthin inhibit cell growth in colon cancer by inhibiting cell line progression and enhancing apoptosis by raising anti-inflammatory effects[20]. It also reduces the incidence of esophageal cancer [21] and increases cell death in oral [22] and liver cancers [19], and it appeare to suppress inflammation in colon cancer [23]. In addition, astaxanthin inhibits the hepatoma cancer cell growth and induces cell apoptosis [24]. The purpose of this study was to evaluate the cytotoxic, apoptotic, and anti-migratory activity of ethanolic extract from *H. pluvialis* on the MDA-MB-468 cell line. Besides, the expression patterns of *Bax, Bcl-2, Caspase 3, Caspase 8, Caspase 9, MMP1,* and *MMP9* genes were evaluated as candidates in the mentioned pathways.

Materials and Methods

Preparations of H. pluvialis extract (HPE)

The dried biomasses of *H. pluvialis* were powdered with liquid nitrogen, and 35g of it is poured into 200 ml of the solvent containing 50% water and ethanol, and then were shaken at 80 rpm for 48 hours. Afterward, it was placed in an ultrasonic bath for 20 minutes, passed through a mesh and filter paper, and then centrifuged at 800 rpm for 10 minutes. To purify *H. Pluvialis* extract, the solvent phase was removed using a vacuum rotary operator, and pure algae powder was obtained. 7 mg of this powder was dissolved in 250 µl of ethanol and then 750 µl of whole culture medium was added to it to be used for the treatment of the desired cell line.

Cell Culture

MDA-MB-468 a breast cancer cell line was purchased from Pasture Institute of Iran. After de-freezing the cells, they were cultured in RPMI-1640 (GIBCO/BRL Life Technologies, USA) medium which was supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), and 1% antibiotics (100 IU/ml penicillin, 100 µg /ml streptomycin) (Sigma-Aldrich, St. Louis, MO, USA), then incubated at 37°C, with 5% CO_2, and 95% humidity. After several passages, the cells were reached the Logarithmic stage and used for different techniques.

MTT assay

MTT assay used to determine the IC_{50} dose of *H. pluvialis*. Firstly, the cell suspension was prepared and after cell count, an appropriate number of cells were dissolved in an appropriate amount of complete culture medium. The MDA-MB-468 cells were cultured for 24 hours with an initial concentration of 104 cells in 96-well plates at 200 µl of complete culture medium. The cells were treated with *H. plivialis* extract in several concentrations (0.5, 1, 2, 4, 8, 16, 25, 32 µl). After 24h incubation period, 50 µl of MTT solution was added to each well, and the plate was further incubated at 37 °C for 4 h. Then, the medium was aspirated and the wells were washed twice with phosphate-buffered saline (PBS). Later, 200 µl of DMSO was added to the wells, and the plate was located on a shaker for 30 min to dissolve the dye. Afterward, the formazan crystals were dissolved, and the absorbance was determined by an ELISA reader (Bio-Tek Instruments) at 570 nm. All the following procedures were done in a sterile area using a Laminar flow cabinet biosafety class II level. In addition, the calculations related to the inhibitor half-concentration(IC_{50}) were calculated with the GraphPad Prism 6.01 (GraphPad Software Inc., San Diego, CA), and the percent cell viability was calculated as follows:

$$\text{Cell viability (\%)} = \frac{OD\ of\ treatment}{OD\ of\ control} \times 100. \quad (1)$$

Apoptosis Assays

After culturing MDA-MB-468 cells at a density of 105 cells/well in six-well plates in 2 mL of serum complete media, the cells were treated by *H. pluvialis* extract and incubated for 24 and 48 hours with ECC at IC_{50} (75µg/mL) and higher IC_{50} (100,150µg/mL) concentrations. The cells were harvested, and adherent cells were detached by 0.25% trypsin-EDTA, then washed with 1 ml of PBS (pH 7.4) and centrifuged. The cells were suspended after adding 200 µg of binding buffer (Invitrogen CO. USA), then FITC-Annexin V reagent was added to the culture plate and incubated on ice for 20 minutes. In the next step, the cells were suspended again for the second time by a binding buffer and 10 µl of propidium iodide (PI) was added.

To evaluate cell death and diagnose early and late apoptosis from necrosis, cells were stained with Annexin V / PI according to the instructions of the Annexin / Propidium Iodide Kit (Biovision, Mountain View, CA, U.S.A.) for flow cytometry tests. Early and late apoptotic/ necrotic cells were identified using a BD LSR II cell analyzer (BD Biosciences, San Jose, CA, U.S.A.).

Wound Healing Assay

MDA-MB-468 cells (4×10^5 cells/well) were cultivated in 6-well plates, and after the cell density of each well reached more than >80%, a scratch was made on the cell layer at the plate surface by the tip of the yellow sampler. PBS (pH 7.4) was used to wash and remove cells isolated from the plate surface. In the next step, the plates were incubated for 72h at 37°C with 5% CO_2 to allow cells to migrate from the sides to the wound site. Wound area images were taken at 0, 24, 48, and 72 hours. The rate of migration and the effect of the extract on the inhibition of this phenomenon were evaluated by measuring the width of the gap area.

mRNA Extraction and cDNA Synthesis, and qRT-PCR

The isolation of total RNA was extracted from the treated and untreated cell lines by using the extraction kit (Takara Biotechnology Inc.) according to the manufacturer's instructions. The quality and quantity of RNA extracted were measured with the NanoDrop 2000c (Thermo Scientific) instrument. For cDNA synthesis, we used 2 µg of total RNA, which was used under the influence of MMLV reverse transcriptase (Takara, cat. No: RR037Q), random hexamer, and oligo dt primers according to the manufacturer's instructions, and the desired fragment was synthesized. The primers of 8 target genes and, *GAPDH* were designed by Oligo 7 software and NCBI-BLAST (Table 1). The qRT-PCR reaction was performed with a mix of 10 µl of SYBR green main mixture (Takara, RR820L) mixed with 0.5 µM of each primer, 1 µl of cDNA template and 8 µl of nuclease-free water. Initial stage of enzyme activation at 95°C for 1 minute with 45 cycles at 95°C for 10 seconds, 59 to 63°C for 30 seconds, and 72°C for 20 seconds for different primers Followed. qRT-PCR was performed by the StepOne qRT-PCR system. Finally, the relative expression quantification levels of target genes using the cycle threshold (Ct) values were calculated with the $2^{-\Delta\Delta Ct}$ method [25].

Statistical Methods

Data analysis was presented as the mean ± SD. Differences between the two groups were evaluated by Student's t-test and one-way ANOVA, respectively. p values <0.05 were considered significant. Data were analyzed using Graph Pad Prism software version 6.01

Results

Anti-proliferative activity of H. pluvialis extract on breast cancer cell

The anti-prolative effects of HPE were assessed using MTT methods. The IC_{50} values were experimented using twofold dose ranges consisting of 2, 4, 8, 16, 32, and 64 µg. The results were shown the IC_{50} of the concentrations of 8, 16 ($p < 0.05$), 32, and 64 µg ($p< 0.001$) significantly decreased the viability of breast cancer cells compared to control ones (Figure 1).

Table 1. The Primer Sequences

Gene		
Bax	Forward	5'- GACTCCCCCGAGAGGTCTT-3'
	Reverse	5'- ACAGGGCCTTGAGCACCAGTT-3'
Bcl-2	Forward	5'- GACTCCCCCGAGAGGTCTT-3'
	Reverse	5'- TGCCGGTTCAGGTACTCAGTC-3'
Caspase- 3	Forward	5'- ATGGTTTGAGCCTGAGCAGA-3'
	Reverse	5'- GGCAGCATCATCCACACATAC-3'
Caspase- 8	Forward	5'- ACCTTGTGTCTGAGCTGGTCT-3'
	Reverse	5'- GCCCACTGGTATTCCTCAGGC-3'

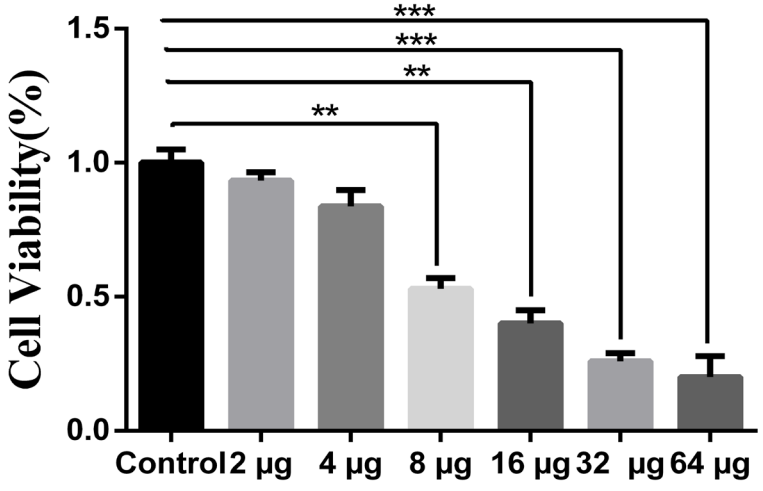

Figure 1. Effects of IC_{50} Concentration of *H. pluvialis* Extract on MDA-MB-468 Cell Viability: *H. pluvialis* extract decreased the cell survival to 22.71% compared to the control cells (** $p < 0.05$) (***$p < 0.001$).

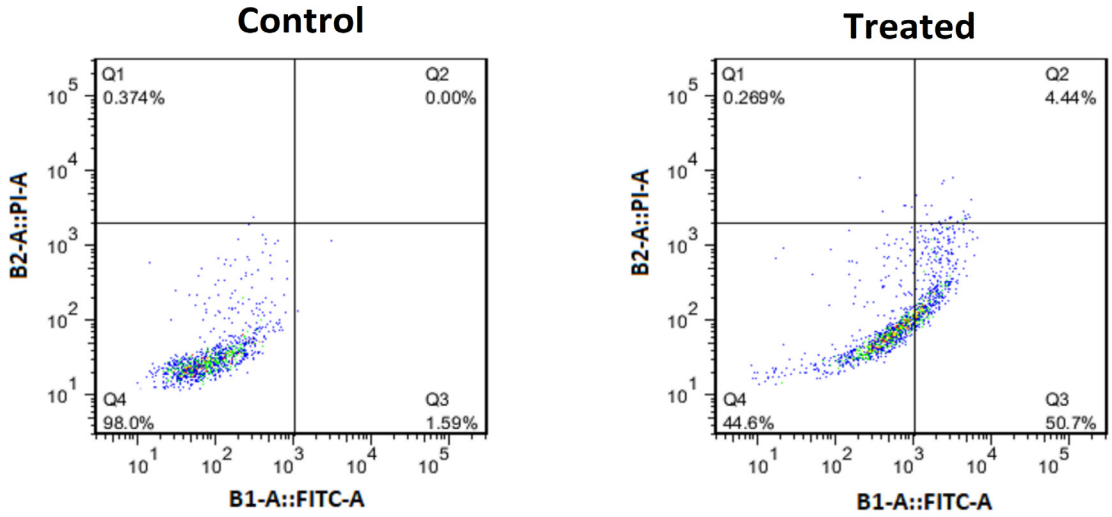

Figure 2. Flow Cytometry Analysis Indicating the Effect of HPE (*H. pluvialis* extract) Treatment on the Apoptosis Rate of MDA-MB-468 cells. LL: live cells, LR: early apoptosis, UR: late apoptosis, UL: necrotic cells.

Table 2. Flow Cytometry Analysis of MDA-MB-468 Cells Treated with *H. pluvialis* Extract

	LL (%)	LR(%)	UL(%)
Control	98	1.59	0.037
Cases	44.6	50.7	0.27

The effects of H. pluvialis on apoptosis induction

In this study, dual staining of cells with Annexin V / PI was used to quantify the percentage of apoptotic cells. The four areas Q1 to Q4 recorded after analyzing the results by the device software are: Q1 represents (Annexin V- / PI +) in the necrotic cells in the upper left quadrant (UL), Q2 represents late apoptotic cells (Annexin V + / PI +) in the upper right quadrant (UR), Q3 represents primary apoptotic cells (Annexin V + / PI-) in the lower right quadrant (LR), Q4 live cells (Annexin V- / PI-) is shown in the lower left (LL) (Figure 2) (Table 2).

H. pluvialis extracts prohibits the migration of breast cancer cell

To evaluate the effect of HPE on cell migration of MDA-MB-468, the migration ability of extract-treated cells to fill the wound site was evaluated and compared with the control sample at different time intervals. The results of wound healing are shown in Figure 3. Although the tumor invasion is remarkable in control cells, the *H. pluvialis* extract inhibited cell migration to the gap area in three-time evaluations.

The Effects of H. pluvialis extract treatments on the expression of BAX, Bcl-2, MMP-1, MMP-9, Caspase3, Casapese-8, and Caspase 9

The effects of the *H. pluvialis* extract treatments on the expression of *Bcl-2, Caspase 9, Bax, Caspase 3, Caspase 8, MMP-1*, and *MMP-9* in the MDA-MB-468 cell line were evaluated by qRT-PCR. In the *H. pluvialis* extracts-treated group, 24 h after treatment, the expression level of *Bcl-2* (p =0.002) and MMP-9 (p=0.0413) decreased. Also, after treatment by *H. pluvialis* extracts, the level of *Bax* (p =0.004), *Caspase 3* (p =0.021), and *Caspase 9* (p =0.006) significantly increased (Figure 4). Also, the expression levels of the *Cas-8* (p=0.087) and *MMP-1* (p =0.14) genes have not significantly changed.

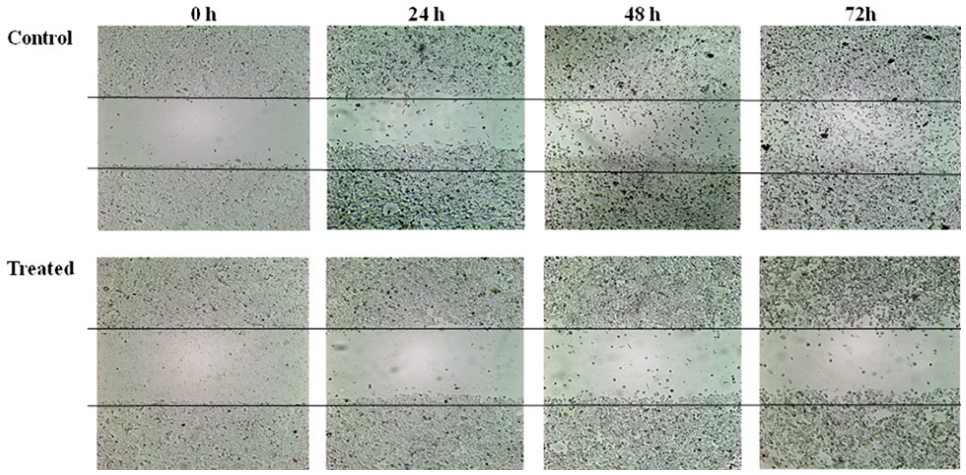

Figure 3. The Images from Scratch Healing Rates of the HPE (*H. pluvialis* extract) treatment effects on tumor cell migration in comparison to normal cell.

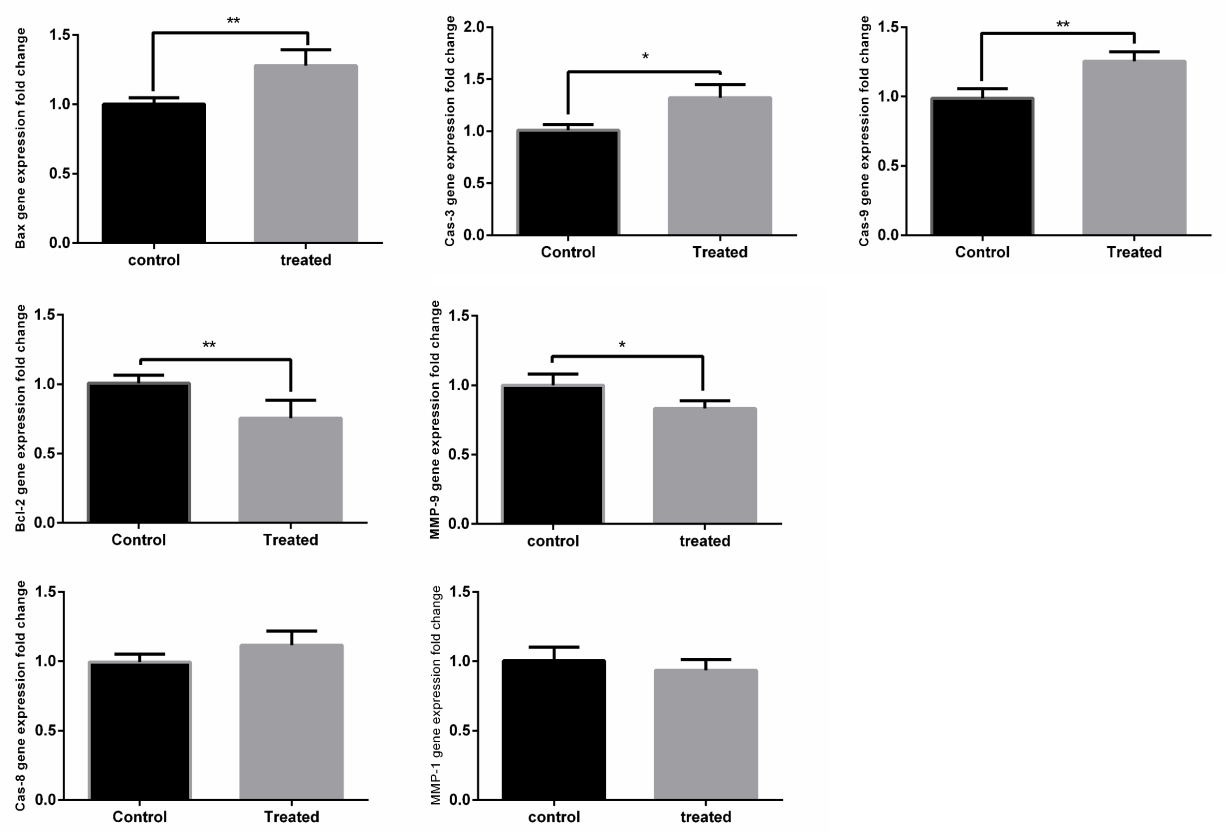

Figure 4. Effects of HPE (*H.pluvialis* extract) Treatment on the Gene Expression of *BAX, Bcl-2, MMP-1, MMP-9, Caspase 3(Cas-3), Caspase 8 (Cas-8),* and *Caspase (Cas-9)*. The data was presented as mean ± SD. *P < 0.05

Discussion

In summary, our findings exhibit that *H. pluvialis* extract significantly influences the proliferation, migration, and apoptosis pathways in the MDA-MB-468 cell line. It inhibited cell growth in a dose- and time-dependent manner, by arresting cell cycle progression and promoting apoptosis. *H. pluvialis* extract showed significant inhibitory effects on migration and survival of MDA-MB-468 cell line. The cytotoxicity effect of 8 to 64 µg/ml *H. pluvialis* extract on cancer cells was significantly different from other concentrations and control samples. In addition, the treated cells with *H. Pluvialis* extract, cell migration was significantly reduced, which is quite evident in the results of the wound healing test. Moreover, Flow cytometry data confirm the increased incidence of cell apoptosis in *H. Pluvialis* extract-treated cells compared to the control sample.

Activating or deactivating the expression of genes associated with the apoptotic pathway plays an important role in the control and treatment of many malignancies, especially cancer. In this study, the expression of *caspase-9* as a positive indicator of intrinsic apoptosis was significantly increased after treatment with *H. Pluvialis* extract. However, the increase in *caspase-8* expression as a mediator of induction of external apoptosis was not significant in the treated sample compared to the control sample. But the most important member of the caspase family, *caspase 3*, which is involved in both mitochondrial and external pathways, after treatment with *H. Pluvialis* extract, its expression has increased significantly. Key genes in the regulation of apoptosis include *Bcl-2* and *Bax.* Accordingly, the expression of the *Bcl-2* gene as a negative regulator of apoptosis and *Bax* as a positive regulator in the treatment and control groups were examined. It was observed that in the treated sample, the expression of both genes was significantly regulated. In addition, *MMP-1* and *MMP-9* as key members of the matrix metalloproteinase family which play a considerable role in cell migration and metastasis were significantly reduced in the extract-treated sample compared to the control. Elevated *MMP-1* expression has been reported in bladder cancer and revealed to be associated with a poor prognosis in these patients [26] as well as in patients with prostate cancer [27], and gastric cancer [28]. The *MMP-1* level is also markedly upregulated in breast cancer and stromal cells and is associated with breast cancer progression and poor prognosis [29].

Inhibitory effects of astaxanthin extracted from various sources or synthetic astaxanthin on cancer cell migration and invasion have been reported in different types of human cancers, but for the first time based on our knowledge, it seems the effect of *H. pluvialis* extracts on the MDA-MB-468 cell line of breast cancer has been studied. This study evaluated the cytotoxic, apoptotic, and anti-migratory activity of extract from *H. pluvialis* on the MDA-MB-468 cell line.

The growth-inhibitory effect of the CO_2 extract rich in astaxanthin from *H. Pluvialis* was previously studied by Palozza et al. against HCT116 colon cancer cells [16]. *H.*

pluvialis algae extract inhibits cell growth in a dose- and time-dependent manner by stopping the cell cycle at the G0/G1 phase, and increasing expression levels of *P53*, *P21, CIP, WAF-1, P27* and reducing cyclin D1 expression and AKT phosphorylation[30]. In addition, *H. pluvialis* algae extract by decreasing *BCL2* expression and increasing Bax expression strongly promotes apoptosis [16]. The cytotoxic activity of different fractions of *H. pluvialis* was investigated on 4 cell lines HePG2, MCF7, HCT116, and A549 by El-Baz et al.; They found that among the various carotenoids contents of the algal extract, Astaxanthin, have the greatest effect on the growth inhibition of HePG2, MCF7, HCT116, and A549 cells [13]. The effects of astaxanthin on proliferation, migration, and gene expression on breast cancer cells including estrogen receptor (ER)+ MCF7 and MDA-MB-231 cells were assessed [17]. It significantly inhibits cell migrations, reduces the number of MDA-MB-231 cells, and induces apoptosis compared to the normal breast epithelial cells (MCF10A), therefore altered gene expression ratios [17]. Moreover, in human gastric cancer cell lines, Astaxanthin through inhibition of ERK phosphorylation and increased *p27(kip-1)* expression, disturbs cell cycle progression and proliferation [23]

The survival of cancer cells under the influence of Astaxanthin is controlled in a dose-dependent manner. For example, the survival of KATO-III and SNU-1 cancer cell lines was suppressed by Astaxanthin in a dose-dependent manner with a significant reduction in proliferation. Astaxanthin increased the number of cells in the G0/G1 phase [20] but redused the proportion of the S phase in both cells [18]. Different stereoisomers of astaxanthin inhibit cell growth in colon cancer by inhibiting cell line progression and enhancing apoptosis by raising anti-inflammatory effects by inducing G2/M cell cycle arrest [20]. Flow cytometry analysis indicated that astaxanthin induced G2/M cell cycle arrest and cellular apoptosis in cancer cells. The cell cycle arrest caused by astaxanthin was associated with increases in the expression levels of *p21Cip1/Waf1, p27,* and *p53*, as well as decreases in the levels of *CDK4* and *CDK6* [20].

In summary, *H. pluvialis* intervention significantly and dose-dependently (32 and 64 µg) decreased the viability of breast cancer cells. Its intervention promotes cell apoptosis and significantly inhibits cell migration. In this way, the related biomarkers displayed significant upregulated expression levels of *Caspase 3* and *Caspase 9* and downregulated levels of Bcl-2 and Bax. Therefore, *H.pluvialis* extract may be an effective anticancer that developed cytotoxic properties.

In conclusion, in the current research, we found that *H. pluvialis* extraction significantly and dose-dependently promotes apoptosis and decreases the viability of breast cancer cells.

Author Contribution Statement

All authors contributed equally in this study.

Acknowledgements

The authors are thankful from patients and their families for their contribution in the study. This study was financially supported by a grant from Department of Tuberculosis and Lung Diseases Research Center, University Tabriz University of Medical Sciences, Tabriz, Iran.

Funding

This study was supported by a grant from research deputy of Department of Tuberculosis and Lung Diseases Research Center, University Tabriz University of Medical Sciences, Tabriz, Iran.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request

Ethical approval

All procedures performed in were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Conflict of Interest Statement

The authors declare no potential conflict of interest.

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