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Impacts of *Haematococcus pluvialis* extract on the expression of the SOX2 and OCT4 genes in the prostate cancer cell line, PC3

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ABSTRACT

There is a small population of cells with stem cell properties in cancer tissues that are responsible for the initiation of tumour formation and have the ability to resist apoptosis inducing agents and chemotherapy. SOX2 is a transcription factor in stem cells and OCT4 is a pluripotency factor known as cancer stem cell markers. The use of plant compounds as cancer suppressors has become increasingly important today. The purpose of this study is to investigate the expression of self-renewal genes SOX2 and OCT4 in a prostate cancer cell line (PC3) treated with different concentrations of methanolic, chloroform, aqueous and hexane extracts of the green microalga *Haematococcus pluvialis*. PC3 cells were exposed to varying concentrations of microalgae extract after cultivation. The percentage of survival was checked using the MTT method, and the change in expression change of SOX2 and OCT4 genes was studied using the RT-qPCR technique. The results of the evaluations showed that the hexane extract of microalgae caused a significant decrease in the number of living cells compared to the control and significantly decreased the expression of the SOX2 and OCT4 genes. Therefore, the hexane extract of *H. pluvialis* can be considered a factor affecting the expression pathway in self-renewal genes of stem cells.

Keywords: Prostate cancer, SOX2, OCT4, Green microalgae, MTT.
Article type: Research Article.

INTRODUCTION

Prostate cancer is the most common malignant cancer in men. This cancer is a multi-step process that begins with DNA damage followed by changes in various signals. In the early stages, it is hormone-dependent and relies on natural androgens. In the later stages, hormone-independent growth is observed. Risk factors include age, race, ethnicity, hormonal metabolism, and diet (Hosseini *et al.* 2008). According to the new theory of "the origin of cancer" not all tumour tissue cells have the power of unlimited division. Instead, but special cells called cancer stem cells (CSCs) possess ability. CSCs can create new tumours because of their self-renewal capability and their capacity to transform into various types of cancer cells. These cells can also resist apoptosis-inducing agents and chemotherapy. One of the most important characteristics of CSCs is the expression of stamens indicators. The expression of these indicators at the cellular, protein, and gene levels enhances the ability of proliferation, self-renewal, and cell migration in cancer (Frank *et al.* 2010). The requirement for unlimited and uncontrolled cell division is the activation and high expression of a group of genes known as the self-regeneration pathway genes. These genes are numerous, but the most important ones include Nucleostemin, KLF4, Nanog, OCT4 and SOX2. The SOX2, KLF4, NANOG, OCT4 and Nucleostemin genes are considered the main controlling genes of self-renewal pathway and are now used as markers for identifying CSCs (Gagliardi *et al.* 2013). By recognizing and presenting the cell theory and the similarity between the behavior of these cells and embryonic stem cells, the researchers realized that the OCT4 gene is one of the key genes and is the main factor of pluripotency and self-regeneration in these cells. It also plays a role in cancer cells. SOX2, NANOG and OCT4 play an essential role in maintaining the state of pluripotency and self-regeneration of stem cells, and recent studies show that these genes

play a role in the final events of tumorigenesis, i.e., invasion and metastasis (Radzisheuskaya & Silva 2014). Genes that control the self-renewal of stem cells have been identified as a new category of cancer molecular markers. Their uncontrolled expression is crucial in the process of carcinogenesis. This factor has been observed in various cancers including lung, breast, prostate, glioblastoma, colorectal, ovary and esophagus. However, the exact mechanism of how this factor functions has not been determined yet (Vaddi *et al.* 2019). Prostate tumour cells exhibit excessive expression of SOX2/OCT4, which plays a role in tumour initiation. Additionally, there is a growing body of evidence supporting the theory of CSCs in driving the progression, treatment resistance and recurrence of prostate cancer (Vaddi *et al.* 2019). Therefore, while the common cancer treatments like chemotherapy and radiation therapy effectively destroy the majority of cancer cells and reduce tumour size, but the resistance of cancer stem cells to treatment ultimately leads to recurrence, and tumour invasion. Additionally, chemotherapy drugs can have side effects such as bleeding and suppression of the immune system (Moussavou *et al.* 2014). Given the high costs of treatment, it is very crucial to identify herbal medicines with lower treatment costs as well as antioxidant activity. One plant that has garnered significant attention is alga. Seaweeds are among the most abundant natural resources (Senthilkumara *et al.* 2013). Algae are widely studied and cultivated industrially due to their valuable biological compounds including protein, fat, carbohydrates, pigments, vitamins, minerals and antioxidant compounds. These organisms have been used in aquaculture and, more recently, in the food, pharmaceutical, medical, and energy industries. Numerous bio-compounds with diverse applications such as antibiotics, antivirals, antifungals and anticancer effects have been identified and extracted from multicellular algae. Alkaloids, terpenoids and flavonoids are secondary metabolites that have anticancer effects (Rais *et al.* 2017; Sakthivel & Pandima 2019). Meanwhile, *Haematococcus pluvialis*, a single-celled freshwater microalgae, has 20-30% protein, 7-25% fat, and 30-40% carbohydrates. It also contains significant amounts of astaxanthin, the most important carotenoid pigment in nature. This information is supported by Shah *et al.* (2016) and Wu *et al.* (2021). In this study, we investigated the effects of aqueous and organic (methanolic, chloroform and hexane) extracts of the green microalgae, *H. pluvialis* on the survival rate of human prostate cancer cell line PC3 and the expression of SOX2 and OCT4 genes.

MATERIALS AND METHODS

Cultivation of microalgae

The microalga, *Haematococcus pluvialis* (Fig. 1) was purchased from Algae Bank and the standard culture medium Bold Basal (BBM Medium; Table 1) and prepared for the cultivation of microalgae under autotrophic conditions. The cultivation was conducted at the temperature of 28 ± 2 ℃ and light intensity of 3500 lux with a photoperiod of 12 hours of light and dark under aeration conditions for 20 days (Nichols 1973; Li *et al.* 2020). After the cultivation period, the biomass was removed using a centrifuge (Sigma 2-16 KL, Germany) at 6000 rpm for 5 minutes at a temperature of 25 ℃. The harvested biomass was then dried using a freeze-dryer (Germany Alpha 1-2LD plus, Christ) and stored at -20 ℃.

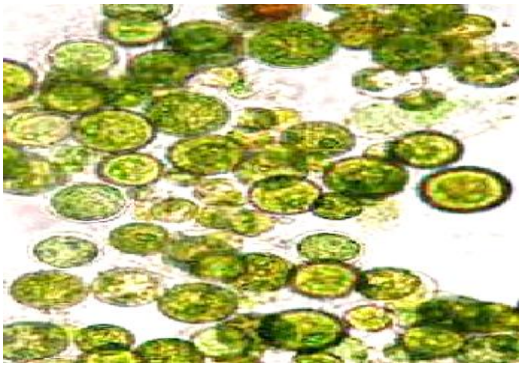

Fig. 1. *Haematococcus pluvialis* green microalgae at 400 x magnification.

Measuring the levels of photosynthetic pigments

To extract the pigments, 5 m« of 96% methanol was used for every 0.1 g of the sample. The sample was then homogenized for one minute and centrifuged at 3000 rpm for 10 minutes. The absorbance of the supernatant was measured at wavelengths of 666, 653, and 470 nm using a spectrophotometer (M501 UV/Vis CamSpec, United Kingdom). The following formulas were used to calculate the amounts of pigments (Dere *et al.* 1998): Ca for chlorophyll a, Cb for chlorophyll b, and Cc for total carotene in micrograms per gram of dry weight.

Ca (µg g^{-1} DW) = $15.65A_{666} - 7.340A_{653}$

Cb (µg g^{-1} DW) = $27.05A_{653} - 11.21A_{666}$

Cc = $1000A_{470} - 2.860Ca - 129.2Cb /245$

Table 1. BBM Standard Culture Medium (Li *et al.* 2020).

Stock solution	Consumable material	Amount (g)	Volume (ml)
A	$NaNO_3$	10	**400**
	$MgSO_4.7H_2O$	3	
	K_2HPO_4	4	
	KH_2PO_4	6	
	$CaCl_2$	1	
	NaCl	1	
B	$ZnSO_4$	8.82	**1000**
	MoO_3	0.71	
	$Co(NO_3)_2.6H_2O$	0.49	
	$MnCl_2$	1.44	
	$CuSO_4.5H_2O$	1.57	
C	H_3BO_4	1.14	**100**
	EDTA	5	
	KOH	3.1	
D	$FeSO_4.7H_2O$	4.94	**1000**
	HCl	1 (ml)	

Determination of total phenol

The Folin-Ciocalteu (F-C) colorimetric technique was utilized to determine the total phenol concentration. In brief, 5 µL of methanolic extract of microalgae was mixed with 100 µL of 10-fold diluted F-C reagent, incubated for 5 min at room temperature, and then mixed with 100 µL of sodium carbonate (75 g L^{-1}). After an additional incubation period of 30 minutes at room temperature, the absorbance at 720 nm was measured using a spectrophotometer. To create a standard curve and establish the equation of a line, the standard solution of gallic acid in the range of 0-100 mg L^{-1} was prepared and measured in the same manner. Subsequently, the amount of total phenol in the sample was calculated in mg of gallic acid per g of dry weight using the equation of the resulting line (Mansur *et al.* 2020).

Measurement of total flavonoids

The concentration of flavonoids was measured following the method outlined by Mansur *et al.* (2020). In summary, 50 µL of a 2% (w/v) aluminium chloride-ethanol solution was combined with 50 µL of the methanol extract of microalgae. After a 1-hour incubation at room temperature, the absorbance was measured at 420 nm using a spectrophotometer. A concentration range of 0-100 mg mL^{-1} of quercetin was utilized as a standard. The total flavonoid content was determined by applying the standard curve equation and expressed in mg of quercetin per g of dry weight.

Measurement of total protein

The extraction of total protein was carried out according to the method outlined by Meijer & Wijffels (1998). Initially, 0.1 g microalgae powder was centrifuged with 5 mL of 50 mM phosphate buffer for 10 minutes at 4 °C at 5000 rpm. Subsequently, to disrupt the cells, the sample was subjected to centrifugation for 5 minutes on Sonic ice (Ultrasonic Cell, Cole-Parmer Germany Misonix Sonicator 3000) and then centrifuged at 14,000 rpm for 15

minutes at 4 °C to isolate the supernatant containing the protein. The protein content was quantified using the Bradford method with serum albumin as a protein standard (Bradford 1976).

Antioxidant activity assay

The Diphenyl Picrylhydrazyl Free Radical Inhibition Activity Test (DPPH; 1,1-diphenyl-2-picrylhydrazyl) was conducted following the method outlined by Wang *et al.* (2023). One mL DPPH at a concentration of 0.1 mol per mL in 95% methanol was combined with a 100-µL methanol extract of microalgae. This mixture was then left at room temperature in darkness for 20 minutes. The absorbance, a wavelength of 517nm, was measured using a spectrophotometer. The percentage of free radical inhibition was calculated using the formula % = [1-(AC – AS)/AC] × 100 of free radical inhibition.

Extraction from microalgae

Extraction was conducted using four solvents: methanol, chloroform, water, and hexane. To prepare each extract, 5 g microalgae powder was mixed with 50 mL solvent and placed in a shaker (TM52A, Iran) at a temperature of 25 °C for 24 hours. The extract obtained from each solvent was then filtered through filter paper and further filtered in an oven (Memmert, Germany) until completely dissolved. The dried pure extract was collected, weighed, and stored at -20 °C until the tests were conducted (Salehi *et al.* 2005).

Investigating the viability of cancer cell lines treated with MTT method

The Human Prostate Cancer Cell Line (PC-3: NCBI NO. C427) of Adenocarcinoma cancer (Fig. 2) was obtained from the Iranian Pasteur Institute Cell Bank (http://fa1.pasteur.ac.ir).

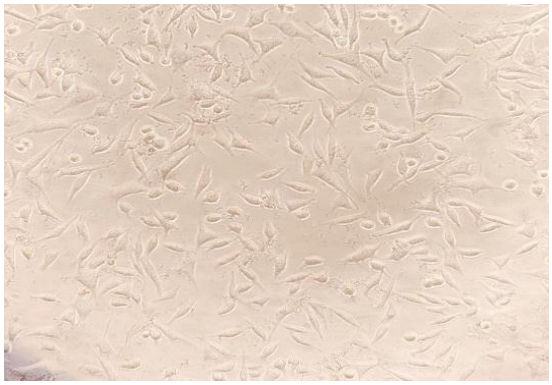

Fig. 2. The human prostate cancer cell line (PC3) at 100 x magnification.

The cells of this cell line were cultured in DMEM culture medium containing 10% FBS, 2 mM L-glutamine, penicillin and streptomycin at a rate of 100 mL $Unit^{-1}$ at 37 °C, 5% CO_2 and 95% humidity in an incubator (INC246 Memmert, Germany) in the Cell Culture Room, Biochemistry Research Laboratory, Faculty of Basic Sciences, University of Guilan, Iran. To perform the MTT test, the third passage cells were transferred to a 96-well plate and cultured for 24 hours. Then the cells were treated with concentrations of 50, 100, 150 and 200 µg mL^{-1} of all four microalgae extracts and a control (cancer cells without treatment with microalgae extract) for 48 and 72 hours. After this period, the supernatant solution was discarded and 20 µL MTT solution (5 mg mL^{-1} PBS) was added to each well in the dark and incubated for 3 hours. After this time passed and the supernatant was discarded, 100 µL dimethyl sulfoxide (DMSO) was added to each well and placed on a shaker for 15 minutes to dissolve the resulting insoluble formazan crystals. Then the optical absorption of formazan was measured at 570 nm using an ELISA reader (MPR4+ Hiperion Microplate Reader, Germany). The IC_{50} concentration was used to determine a non-toxic concentration of each microalgal extract for the treatment of cancer cells and to measure the expression changes of the desired genes (Li *et al.* 2012).

Investigating the expression of SOX2 and OCT4 genes using Real Time-PCR technique

Cancer cells adhered to the bottom of a 6-well plate were treated with the IC_{50} concentration of each microalgal extract for 48 and 72 hours. RNA extraction was performed using RNX PLUS (SinaClon, Iran). The concentration and purity of RNA samples were determined using a nanodrop device (EZDrop 1000 Blue-Ray Biotech, Taiwan). cDNA synthesis was carried out using a cDNA synthesis kit (Cinnagen, Iran). Real Time q-PCR machine (Lightcycler 96 instrument, Roche, Germany) with SYBR Green was used to analyze the expression changes of

SOX2 and OCT4 genes. The program for the device was as follows: 10 minutes of initial denaturation at 95 °C, followed by 40 consecutive cycles including 30 seconds at 95 °C, 30 seconds at the binding temperature of SOX2 and OCT4 primers, and 45 seconds at 72 °C. GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) gene was used as the reference gene for RT-PCR reaction. The sequence of primers used in this study is provided in Table 2 (Moradi *et al.* 2023).

Table 2. Sequence of primers for the genes used in this study, which are created by Metabion Iran.

Gene	forward Sequence (5′–3′)	reverse Sequence (5′–3′)	Gene ID	Product length (bp)
SOX2	GGAAAACCAAGACGCTCATG	CCGTTCATGTGCGCGTAAC	NM_003106.4	154
OCT4	CCAAGGTGGTATCTCGTGAG	GGCAACCGAAATGACAAGTC	NM_112957.3	219
GAPDH	CGTGGAAGGACTCATGACC	CAGTAGAGGCAGGGATGATG	NG_007073.2	125

Statistical analysis

The data was described using tables and graphs and the results were displayed as an average with standard deviation. To compare quantitative variables among three groups, One-Way ANOVA and Duncan's test were utilized at a significant level of $p < 0.05$ with SPSS software (Statistics 17.0). Excel 2010 software was used to create graphs.

RESULTS

Amounts of microalgae biological compounds

Table 3 displays the quantities of various biological compounds found in microalga, *Haematococcus pluvialis*, such as protein, phenol, flavonoid, carotenoid, chlorophyll a and b, as well as the level of antioxidant activity.

Table 3. Amounts of biological compounds present in *H. pluvialis* microalgae. The results represent the average of three repetitions with standard deviation (SD).

Algae species	Chlorophyll a (µg g^{-1} DW)	Chlorophyll b (µg g^{-1} DW)	Carotenoid (µg g^{-1} DW)	Phenolic (mg GA g^{-1} DW)	Flavonoid (mg QE g^{-1} DW)	DPPHs (%)	Protein (mg mL^{-1})
H. pluvialis	3.94 ± 0.14	2.62 ± 0.37	4.22 ± 0.18	46.48 ± 1.67	13.89 ± 0.61	62.27 ± 0.83	23.13 ± 1.50

Investigating the toxicity of microalgae extracts on cancer cells

The percentage of cancer cell viability decreased significantly ($p < 0.05$) in all concentrations treated for 48 and 72 hours compared to the control. The decrease in cell viability is dependent on the dose of microalga extract and treatment time. By an elevation in the extract concentration and treatment time, the death rate of the cells also increased, demonstrating the toxicity of the extract on cell lines. According to the results, the concentration of 200 µg mL^{-1} of the microalgae hexane and methanol extracts in 72 hours showed survival rates (%) of 30.41 ± 3.6 and 43.79 ± 3.1% respectively, compared to the control sample and other extracts. This concentration had the highest toxicity on prostate cancer cells (Fig. 3). The IC_{50} values of these extracts for prostate cancer cells were 177.71 ± 1.3 and 211.37 ± 2.5 µg mL^{-1} in 48 hours and 47 ± 3.7 in 72 hours with IC_{50} values of 147.1 and 169.38 ± 1.8 µg mL^{-1} respectively (Fig. 4).

Investigating the expression of the SOX2 and OCT4 genes

It was found that the expression of both self-renewal genes, OCT4 and SOX2 in the treated samples decreased compared to the control sample. The passage of time also influenced gene expression, with a significant decrease ($p < 0.05$) observed over time. After 72 hours, the expression change was most notable, with the highest decrease in expression seen in the OCT4 gene after treatment with the microalga hexane extract (0.63 ± 0.02) and in the SOX2 gene after treatment with methanol extract (0.64 ± 0.06). The expression of target genes treated with methanolic and hexane extracts did not show any significant difference ($p < 0.05$) compared to each other (Fig. 5).

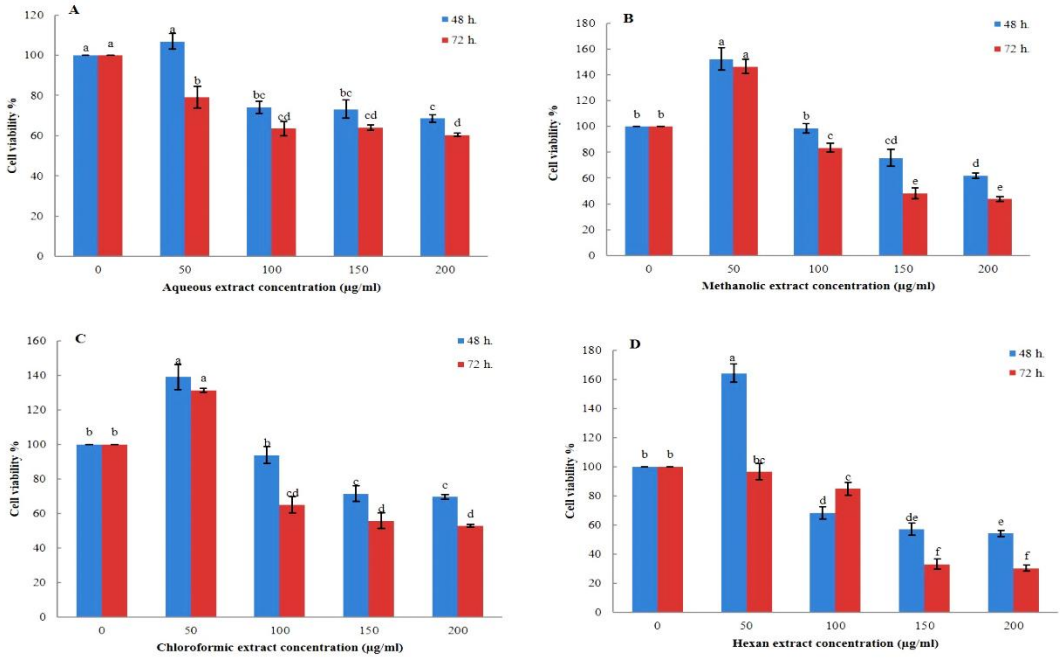

Fig. 3. shows the percentage of PC3 prostate cancer cell survival after 48 and 72 hours of exposure to A) aqueous extract, B) methanolic extract, C) chloroform extract and D) hexane extract of *H. pluvialis* microalgae at various concentrations compared to the control. The results are the average of three repetitions ± standard deviation (SD).

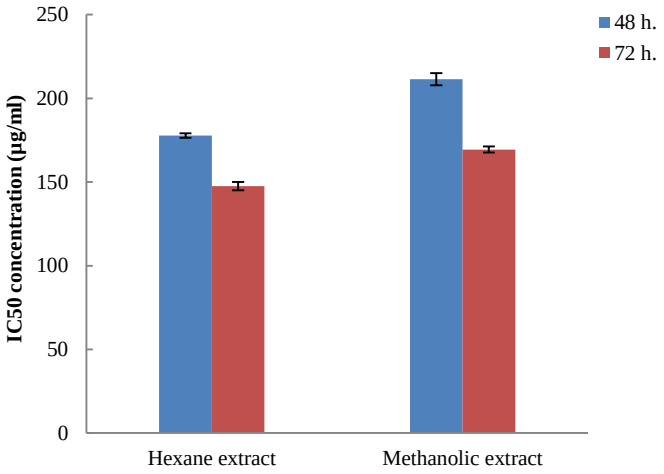

Fig. 4. shows the IC_{50} values of hexane and methanol extracts of *H. pluvialis* microalgae for PC3 prostate cancer cells at 48 and 72 hours. The results represent the average of three repetitions ± standard deviation (SD).

DISCUSSION

The diversity and multifunctional properties of materials extracted from algae are attributed to the abundant bioactive metabolites found in them. Bioactive metabolites identified in seaweeds include phenols, carotenoids, oxygen heterocycles, nitrogen hetero cycles, kanic acids, guanidinium derivatives, phosphazine derivatives, amino acids and amines, sterols, poly sulphated saccharides and prostaglandins (Kazłowska *et al.* 2013). According to reports, the most effective free radical inhibiting activity of algae is attributed to their phenolic compounds. Additionally, a significant relationship between DPPH free radical inhibitory activity and total phenol content has been reported (Namvar *et al.* 2013). Polyphenols belong to a heterogeneous group of compounds found in plant sources; they are strong antioxidant agents or act as scavengers of free radicals (Kampa *et al.* 2000). According to the results of this research, microalga, *H. pluvialis*, has protein equivalent to 23.13 ± 1.50 µg mL^{-1}, phenol equivalent to 46.48 ± 1.67 mg GA/g DW and flavonoid 13.89 ± 0.61 mg QE/g DW, as well as a significant percentage of antioxidant activity at about 0.83 ± 62.27%. The MTT test results indicate that the microalgae hexane and methanol extracts at 200 µg mL^{-1} over 72 hours exhibited the most lethal effect on PC3 cells compared

to the control and other extracts. In many studies, a several of solvents are used for extraction in algae research. However, it still remains unclear which type of solvent is most effective for extraction (Yi *et al.* 2001). In the present study, it appears that the hexane and methanol solvents contain more effective compounds for cancer cell toxicity compared to other solvents.

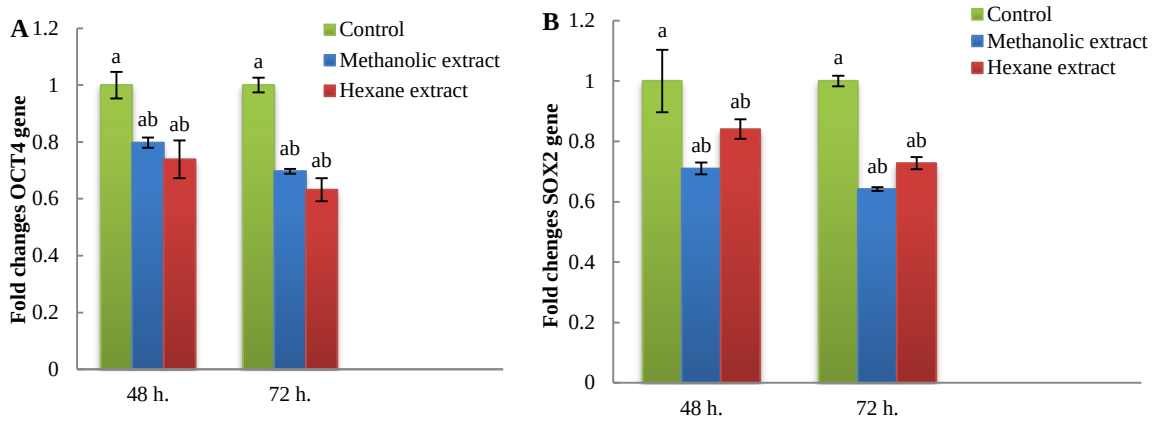

Fig. 5. Changes in gene expression of OCT4 (A) and SOX2 (B) in the prostate cancer cell line PC3 after treatment with methanolic and hexane extracts of *H. pluvialis* microalgae for 48 and 72 hours. The results represent the average of three repetitions ± standard deviation (SD).

This could be due to the chemical structure and polarity of the components in the algae and the solvent. Kampa *et al.* (2000) conducted a study on the impact of various antioxidant polyphenols (catechin, epicatechin, quercetin, and resveratrol) on the growth of three prostate cancer cell lines (LNCaP, PC3, and DU145). They found that the inhibition of cell growth by polyphenols is dependent on both the dosage and duration of exposure. Flavonoids are particularly effective at inhibiting cells. Their data demonstrate the direct inhibitory effect of low concentrations of antioxidant phenols on the proliferation of human prostate cancer cell lines. Narasimhan *et al.* (2013) investigated the bioactive properties of three seaweed samples, *Enteromorpha antenna*, *E. linza* and *Gracilaria corticata* on Hep-G2, MCF7 cancer cell lines and normal VERO cell lines. They found that the methanol extract of these algae has a higher total phenol content and a higher percentage of inhibitory activity. SOX2 and OCT4 are the most important genes that control the process of self-renewal and differentiation of embryonic stem cells in prostate cancers. Their expression frequency increases with the increase in the degree of malignancy (Zhang *et al.* 2020). Sotomayor *et al.* (2009) demonstrated the expression of OCT4 gene in prostate cancer. Their studies also revealed a correlation between the level of OCT4 gene expression and the degree of tumour malignancy. Specifically, the expression of the OCT4 gene and the degree of malignancy increased in prostate tumour samples. In the current research, it was discovered that the microalgae methanolic and hexane extracts possess the potential to reduce the expression of the OCT4 and SOX2 genes. This effect was found to intensify significantly over time. The hexane extract showed the greatest drop in OCT4 gene expression. In addition, the methanol extract exhibited the highest decrease in SOX2 gene expression within 72 hours of treatment. Zamani *et al.* (2017), investigated the effect of *Spirulina* algal extract on prostate cancer cells and changes in OCT4 gene expression. They found that the number of live cells decreased significantly compared to the control group. It was also true for the expression of the OCT4 gene. This decrease was significant with the elevation in concentration and time.

CONCLUSION

The discovery of natural products and new metabolites extracted from microorganisms, animals and plants with high effectiveness against tumour cells without any toxicity on normal cells, are significant advancement in scientific research. Given the focus on using herbal medicines in cancer treatment, this study revealed that the hexane and methanol extracts of *Haematococcus pluvialis* had an impact on PC3 prostate cancer cell survival and the expression of self-renewing genes OCT4 and SOX2. This led to a decrease in cell proliferation and the expression of self-renewing genes, preventing the development of cancerous tissue. The findings of this study can be employed for further research to identify, isolate and characterize compounds responsible for cytotoxic activities.

Author contributions. The idea, data collection, experiments and calculations were carried out by Mahsa Khatami. 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.

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

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