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Research Article

Cytotoxic activities of *Padina gymnospora* and *Acanthophora spicifera* extracts against human breast cancer cell lines

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Abstract

The resistance of cancerous cells to anti-cancer drugs is one of the most common problems in medicine and therefore, finding new anti-cancer compounds with the least side effects seems to be necessary. The present study was performed to investigate the anti-cancer potential of *Padina gymnospora* and *Acanthophora spicifera*, two native algae species of the Persian Gulf, *in vitro*. In this regard, methanol, chloroform, n-hexane, and ethyl acetate extracts of both algae species were added to cultivated *MCF-7* cells at different concentrations (125, 250, 500, and 1000 μg/mL). 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was used to determine the toxicity effects of algae extracts on *MCF-7* cells. DNA isolation and agarose gel electrophoresis were also performed to assess DNA fragmentation induced by these two algae species. Based on the MTT results, the sensitivity of cultivated *MCF-7* cells to *P. gymnospora* and *A. spicifera* extracts was increased in a dose-dependent manner. The highest concentration of methanolic extract of both algae species significantly affected the *MCF-7* cells and led to the highest cell death. Moreover, the IC50 of *P. gymnospora* and *A. spicifera* methanolic extracts for the *MCF-7 cells* were equal to 557.78 and 910.61 μg/ml, respectively, which indicates *P. gymnospora* has more cytotoxic activity and anti-tumor potency. The lowest concentration of all types of algae extracts was not considerably cytotoxic to cultivated *MCF-7* cells. The DNA fragmentation of *MCF-7* cells was increased with increasing the concentration of the algal extracts. The highest amount of DNA fragmentation caused by 1000 μg/mL of *P. gymnospora* and *A. spicifera* extract; however, methanolic extract of *P. gymnospora* caused more DNA fragmentation in *MCF-7* cells than *A. spicifera*.

Keywords: Cytotoxic activity, Secondary metabolites Algae, MTT assay, *MCF-7*.

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Introduction

Nowadays, cancer is the second leading cause of death in humans. Breast cancer is one of the most common types of cancer in women. Studies in recent years show that breast cancer has been the most common type of cancer in Iranian women (about 24.41%) (Gohari *et al*., 2013). Surgery and complementary therapies such as medication, hormone therapy, chemotherapy, and radiotherapy are some of the treatments for breast cancer. Drug resistance of cancer cells is one of the major limitations of anti-cancer drugs, which may be due to the innate resistance of cancer cells to the 1000 μg/mL or may be acquired during chemotherapy. Resistance of tumor cells (whether intrinsic or acquired) leads to the selection of resistant cells from heterogeneous cells. As a result, the treatment process of resistant cells is more difficult and requires higher doses of the drug with more side effects.

Today, herbal medicines have been considered as benefit pharmaceutics because of the lack of side effects compared to chemical medicines. Marine organisms are considered an important source of bioactive metabolites and some of them have potential medicinal properties. Marine algae are one of the most important natural resources of the marine ecosystem, which contains a variety of bioactive metabolites (Ananthan *et al*., 2011). Hence, they are used in many industries such as pharmaceutical (as a fever reliever, muscle and joint pain reliever, sedative, and antibiotic), textile and food industries (Iwashima *et al*., 2005). There are numerous reports to indicate the anti-cancer, antitumor, and anti-proliferative properties of several cytotoxic compounds derived from algae such as fucoidans, laminarians, and terpenoids (Smit, 2004). Today, algae are marketed around the world as components of dietary supplements due to their anti-mutagenic, anticoagulant, and antitumor properties, as well as their high dietary fiber content (Delgado *et al*., 2013; Fedorov *et al*., 2013; Bitencourt *et al*., 2015). Many of these compounds can act directly on cancer cells or be useful in preventing cancer. Some of these seafood natural products are considered due to their availability, low toxicity, and suitability for oral use as well as having a variety of mechanisms of action (Lee *et al*., 2013).

More than 250 species of seaweeds have been identified in the marine resources of southern Iran (Peymani *et al*., 2014) and several studies have been performed to identify and determine the distribution map of Iranian coastal seaweeds (Rohani-Ghadikolaei and Hossaini, 2005; Rohani-Ghadikolaei *et al*., 2007). Although they are highly commercial, nutritional, and medicinal values, they have not received much attention in medicine. There are not enough reported studies relating to the anti-cancer properties of the Persian Gulf algae (Namvar *et al*., 2014). Therefore, the present study aimed to evaluate the anti-cancer potential of *Padina gymnospora* (Kützing, 1871) (Phaeophyta: Dictyotaceae) and *Acanthophora spicifera* (Bargesen, 1910) (Rhodophyta: Rhodomelaceae), the native algae species of the Persian Gulf, *in vitro*. The antioxidant effects of *P. gymnospora* were previously proved by some researchers (Murugan and Iyer, 2014). Later, more direct anti-cancer activities were seen in the genus *Padina*, which would be beneficial for healing (Al-Enazi *et al*., 2018). In this regard, the cytotoxic effects of *P. gymnospora* and

A. spicifera on the *MCF-7* cell line (breast cancer cell line) and DNA fragmentation were assessed.

Materials and methods

Preparation of algae extracts

The samples of *P. gymnospora* and *A. spicifera* were collected from the coasts of Bushehr (28°58'07"N 50°49'14"E) and Lengeh Port (26°34'24"N 54°54'43"E) in the north of the Persian Gulf, respectively (Fig. 1). After washing with clean seawater, the samples were transferred to the Marine Biology Laboratory in Khorramshahr University of Marine Science and Technology, Khorramshahr, Iran. The species were identified based on morphological characteristics (including size, color, and shape) using available taxonomic references (Sohrabipor and Rabiei, 1996; Hanyuda, 2010; Win *et al*., 2011; Amini *et al*., 2013).

Figure 1: The location of algae collection sites in Bushehr and Lengeh Port on the northern shores of the Persian Gulf, Iran.

After initial preparation, the samples were dried using the air-dry method and extracted according to Badury and Wright (2004). In the method called "maceration," first, the dried ground algae samples were added to four organic solvents (methanol, chloroform, n-hexane, and ethyl acetate) separately to prepare the primary stock (1000 µg/mL). The samples were incubated at room temperature for 14 days with continuous shaking at 250 rpm. The homogenate was centrifuged for 6 min at 3200 ×g every other day and the supernatants of each sample were added to collected extracts. The extracts were centrifuged at 3200 ×g for 15 min, and the supernatants were filtered and treated by solvent evaporator (40°C for 45 min). The resultant extract was stored at -20°C (Olivares-Molina and Fernández, 2016).

Cell culture

MCF-7, a commonly used breast cancer cell line, was obtained from the Iranian Biological Resource Center. After thawing at 37ºC in a water bath, the cells were rinsed twice with Roswell Park

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Memorial Institute (RPMI) medium. Then, 50 µL of the cells were suspended in 6 mL of RPMI 1640 medium (containing 5% FBS, 1% ITS, 100 IU/mL of penicillin, 100 µg/mL of streptomycin, 50 µL/mL of gentamicin, and 25 µg/mL of amphotericin B) and plated into 25 cm^2 tissue culture flasks and moved to CO_2 incubator at 37°C. The cell viability test and cell counting were performed using the trypan blue exclusion test and a hemocytometer (Model BL2, Marien Feld company, USA). The viability of cells was always more than 90%. After cell culture reached 80-85% confluence, subculture was conducted. For this purpose, the cells were trypsinized using 1.5 mL TEGPED solution (containing EDTA, trypsin, and pancreatin) at 37°C for 5-10 min. Trypsin was then deactivated by adding fresh media. The suspension was then centrifuged at 1500 ×g for 10min. The supernatant was removed, and the pellet was rinsed twice with fresh RPMI medium with 5% FBS, 100 IU/mL of penicillin, 100 µg/mL of streptomycin, 50 µL/mL of gentamicin, and 25 µg/mL of amphotericin B, and divided into flasks.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay

MTT assay was used to determine the toxicity effects of algae extracts on *MCF-7* cells. MTT assay was conducted according to Borenfreund and Puerner (1985). In this method, the reduction of soluble MTT tetrazolium salt (yellow) to an insoluble MTT-formazan (blue) was inhibited by succinate dehydrogenase enzyme in damaged mitochondria. Briefly, cells were detached from the 25 cm^2 flask using Dextrose solution and moved to 96-well tissue culture plates at a density of 4×10^5 cells/well and incubated at 37°C for 24 h in a CO_2 incubator. After 24 h of exposure, cells were rinsed twice with 200 µL of PBS solution and 20 µL of MTT solution (5 mg MTT/mL PBS) was then added to each well, and microplates were incubated at 37°C for 4h. Thereafter, 200 µL of dimethyl sulfoxide (*DMSO*) was added to each well and the microplate was shacked at 450 rpm for 15 min. finally, absorbance was read at 570 nm.

DNA Fragmentation

DNA isolation and agarose gel electrophoresis were performed according to the method described by Ohyama *et al*. (1998). *MCF-7* cells affected by different concentrations of *P. gymnospora* and *A. spicifera* extracts were degraded using 10 mM tris hydrochloride (pH= 7.4), 10 mM EDTA and 1% triton X-100. Low molecular weight DNA fragments were then isolated by electrophoresis in 1.5% agar gel and observed using ethidium bromide staining and ultraviolet light.

Statistical analysis

All tests were performed in triplicate and the data were presented as mean±S.E. Firstly, all data were tested by the Kolmogorov–Smirnov test for normality. One-way analysis of variance (ANOVA) was used to compare differences in cell viability between the various concentrations of algae extracts. Tukey post hoc test was used when significant differences were found. An independent t-test was used to compare the cell viability against the two algae. The half-maximal inhibitory concentration (IC50) values for 24 h were computed based on an equation presented by Reed and Muench (1938). The Statistical Package for the Social Sciences (SPSS) software (version 18, SPSS Inc., Chicago, IL, USA) was applied to determine the significant difference (p<0.05) between treatment mean values.

Results

Cytotoxicity of P. gymnospora and A. spicifera extracts

Based on the MTT assay, the sensitivity of the *MCF-7* cells to *P. gymnospora* and *A. spicifera* extracts was increased in a dose-dependent manner. Methanolic extract of both algae species (*P. gymnospora* and *A. spicifera*) with the highest concentration (1000 µg/mL) significantly affected the cultivated *MCF-7* cells and led to the highest cell death. Chloroform and ethyl acetate extracts of *P. gymnospora* and Chloroform extract of *A. spicifera* also caused high mortality of cancer cell. Although their effect was less than ethanolic extracts (p<0.05; Fig. 2B, D). N-Hexane extract of *P. gymnospora* and n-hexane and ethyl acetate extracts of *A. spicifera* showed the least cytotoxic effects. However, the lowest concentration of all extracts had not significant cytotoxic effect to cultivated *MCF-7* cells (p>0.05; Fig. 2 A, C).

The IC50 of *P. gymnospora* and *A. spicifera* methanolic extracts against the *MCF-7 cells* were equal to 557.78 and 910.61 µg/mL, respectively. It indicates that *P. gymnospora* has more cytotoxic activity and anti-tumor potency. Table 1 shows 24 h - IC50 values separately for all algae extracts.

As shown in Figures 3 and 4, the *MCF-7* cells in RPMI medium with and without the highest concentration of *P. gymnospora* and *A. spicifera* extracts. The *MCF-7* completely adhered to the culture plate 48 h after incubation. Higher concentrations of both algae extract end in cell death; however, the highest mortality rate was observed (using trypan blue exclusion test) in the cultivated *MCF-7* treated by the highest concentrations of both algae extracts. Meanwhile, the methanolic extracts of both algae species at 1000 µg/mL led to cell death more than others (p<0.05; Figs. 3 and 4).

DNA fragment electrophoresis

Figure 5 shows the DNA fragment electrophoresis of the *MCF-7* cells treated with the different concentrations of methanolic extracts of *P. gymnospora* and *A. spicifera*. The DNA fragmentation of *MCF-7* cells increased with increasing the concentration of algal extracts. The highest DNA fragmentation caused by 1000 µg/mL of *P. gymnospora* and *A. spicifera* extract; however, methanolic extract of *P. gymnospora* caused more DNA fragmentation of *MCF-7* cells than *A. spicifera*. According to Fig. 5, the elongation of the base pairs up to less than

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350 kDa and more than 350 kDa was observed in the samples exposed to methanolic extract of *P. gymnospora* and *A. spicifera*, respectively. The moderate concentration (500 µg/mL) of both species of algae extract caused DNA fragmentation to the same level (to less than 500 kDa).

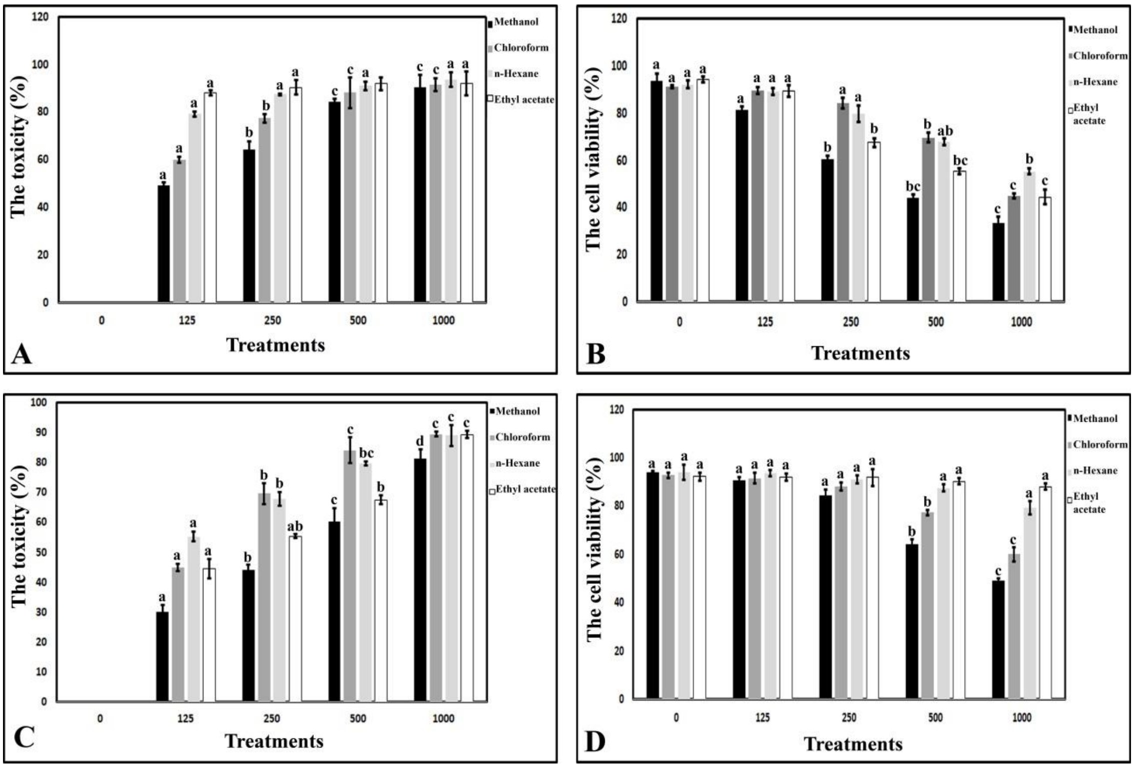

Figure 2: The *Padina gymnospora* and *Acanthophora spicifera* extract toxicity (A, C) and the cell viability (B, D) of the cultivated *MCF-7 cells* treated with different concentrations of *P. gymnospora* and *A. spicifera* (0[control], 125, 250, 500 and 1000 µg/mL) during MMT assay.

Table 1: Mean IC50 of methanol, chloroform, n-hexane and ethyl acetate extracts of *P. gymnospora* and *A. spicifera* methanolic measured on *MCF-7* cells.

Algae extract	IC50 (µg/mL) for 24 h			
	Methanol	**Chloroform**	**N-hexane**	**Ethyl acetate**
Padina gymnospora	557.78	912.44	1078.86	715.9
Acanthophora spicifera	910.61	1032.4	1192.5	1308.6

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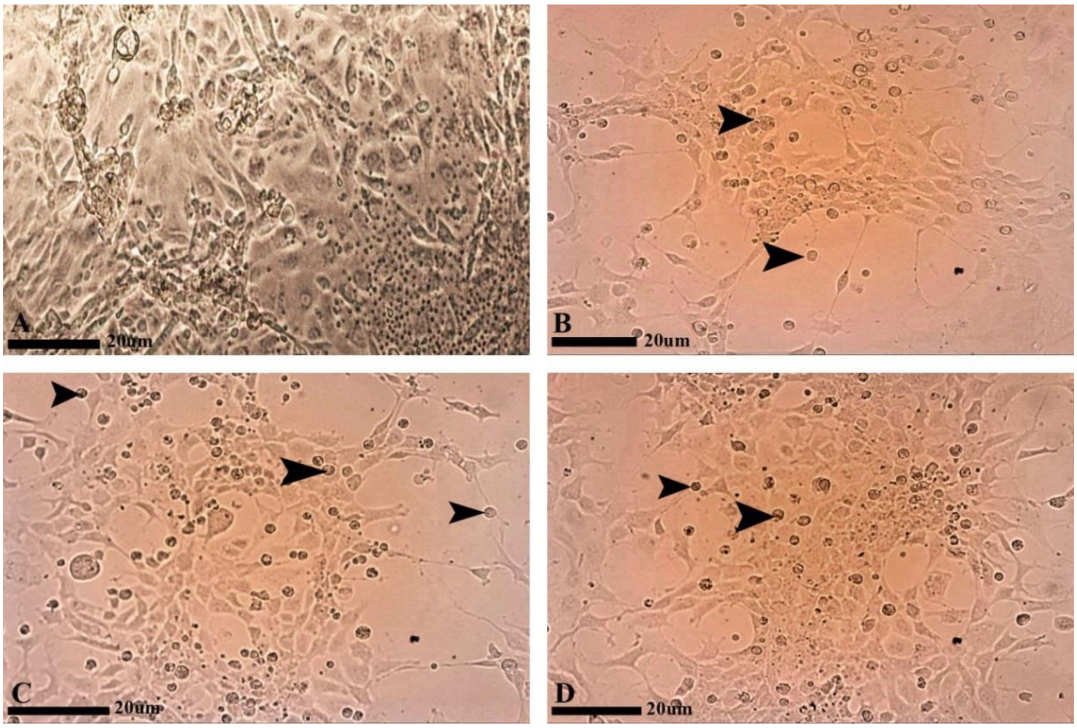

Figure 3: Photomicrograph of *MCF-7 cells* cultivated in RPMI medium without (A) and with 1000µg/ml of methanolic (B), chloroform and ethyl acetate (C) and n-hexan (D) extracts of *Padina gymnospora* extract; dead cells (black arrowheads); (×7250).

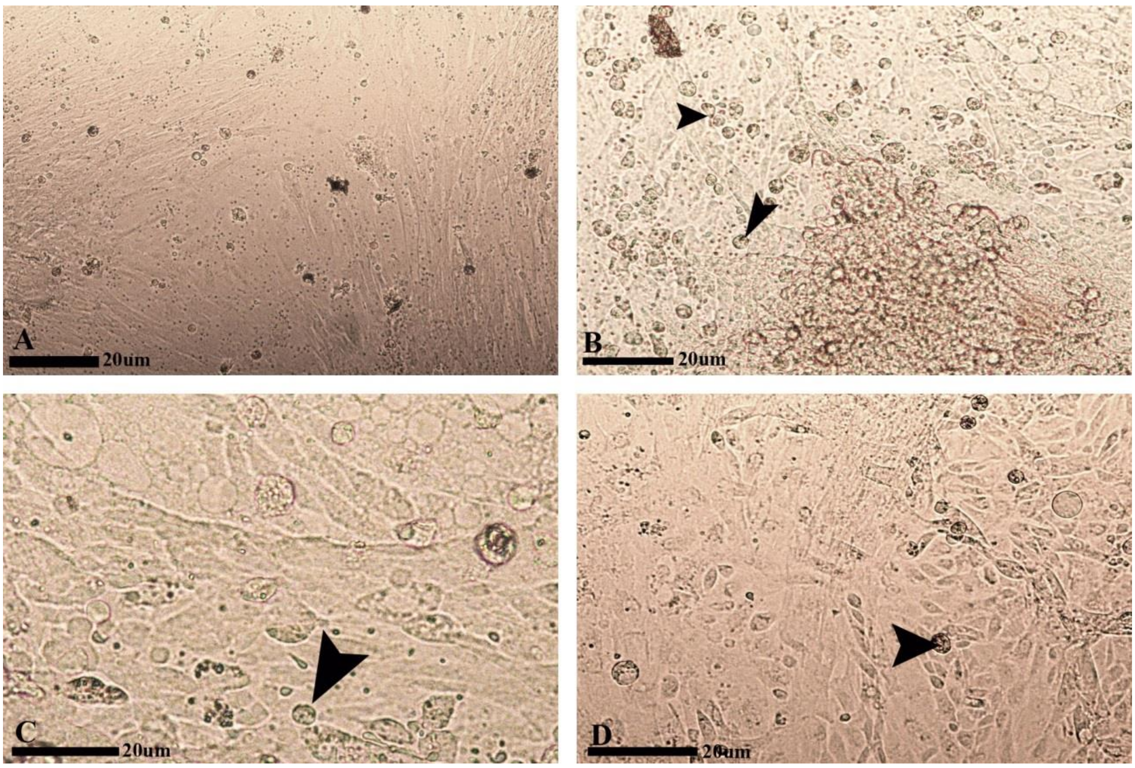

Figure 4: Photomicrograph of *MCF-7 cells* cultivated in RPMI medium without (A) and with 1000 µg/mL of methanolic (B), chloroform (C), n- hexan and ethyl acetate (D) extracts of *Acanthophora spicifera* extract; dead cells (black arrowheads); (×7250).

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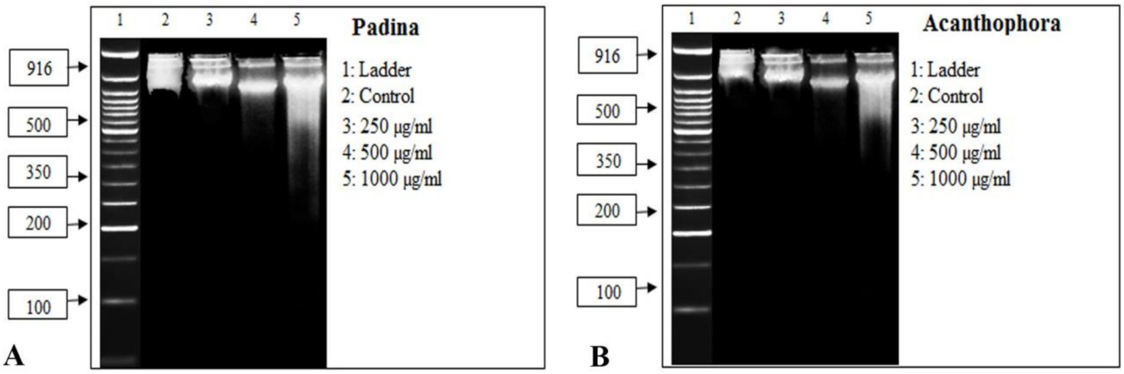

Figure 5: The DNA fragment electrophoresis of cultivated *MCF-7* cells treated with the different concentrations of methanolic extracts of *Padina gymnospora* and *Acanthophora spicifera*.

Discussion

The discovery of natural products and new secondary metabolites from microorganisms, animals, and plants with high efficacy against tumor cells without any toxicity to natural cells is a breakthrough in scientific research (Lim *et al*., 2002). Various compounds produced by seaweeds facilitate their survival in highly competitive environments. The seaweed biodiversity has made them unique in terms of chemical composition and mineral contents. Many primary and secondary metabolites of seaweeds could be considered bioactive compounds in the pharmaceutical industry. Several studies reported the significant advantages of marine algae on human health due to their various contents (Real *et al*., 2004). It has been reported that the extract of some marine algae species can inhibit the growth and proliferation of human cancer cells (Senthilkumara *et al*., 2013; Moussavou *et al*., 2014). Dellai *et al*. (2013) stated that the hydroalcoholic extract of Mediterranean red algae, *Laurencia obtusa*, had significant anti-proliferative effects on three cancer cell lines including MCF7, HCT15 and A549. Senthilkumara (2013) reported that the methanolic extracts of marine algae species, including *Ulva lactuca*, *Enteromorpha intestinalis*, *P. gymnospora*, *Sargassum wightii*, *A. spicifera* and *Laurencia papillosa*, possess a high content of primary and secondary metabolites, the presence of which play an important role in the biological activity. According to Dellai *et al*. (2013) the phenol and flavonoid contents of algal extracts have inhibitory effects. Wang *et al*. (2006) studied the effect of aqueous extract of 12 algae species on cancer cells (MCF7 and HT-29). They reported that the extract of *P. arborescens* and *Hydroclathrus clathratus* inhibited the growth and proliferation of cancer cells. Ahmadzadeh (2009) showed the cold water soluble extract of the native Persian Gulf brown alga, *Sargassum oligocystom*, inhibited the growth and proliferation of K562 and BLL cell lines at 610-650 µg/ml. The antioxidant and anticancer effects of the *P. gymnospora* could be caused by some active polyphenols such as fucosterol and 1-heptacosanol (Murugan and Iyer, 2014).

Evaluation of the proliferation and survival of healthy and cancerous cells is important to determine the effectiveness of natural anti-cancer drugs. In the present study, the effect of various extracts of both selected algae species was

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evaluated on the viability of human breast cancer cell line (MCF7 cells) by MTT assay. According to the results, the amount of treated cell viability was decreased with the increase in extracts concentration. The cytotoxic potential of selected algae extracts (especially methanol extracts) on cancer cells indicated the probable presence of antitumor metabolites. The cytotoxic effect of 1000 μg/mL methanolic extract of both algal species was significantly higher than others. Taheri *et al*. (2018a) reported that the viability percentage of colorectal cancer cells (HT-29) was decreased by increasing the concentration of *Sargassum glaucescens* extract. They stated that the highest concentration of methanolic extract of *S. glaucescens* (1000 μg/mL) resulted in the most cytotoxic effects and the least viability. It has been confirmed that the solvents with lower polarity have better results than the solvents with higher polarity, because higher-polarity solvents often extract more compounds and other algal bioactive components. Therefore, in higher-polarity solvents, the ratio of bioactive substances with anti-cancer properties to the total extracted solution decreases compared to the extracts with lower polarity (Dellai *et al*., 2013; Taheri *et al*., 2018a).

Activation of deoxyribonuclease due to caspase activation is one of the apoptosis characteristics. Deoxyribonuclease is a cellular endonuclease that cleaves the DNA strand in areas not covered by histones. When these fragments are electrophoresed on an agarose gel, they are separated and broken, indicating apoptosis (Youle and Strasser, 2008). In the present study, fragmentation of genomic DNA of MCF7 cells treated with methanolic algae extract, especially at the highest concentration, was observed by electrophoresis of DNA fragments on the agarose gel. Taheri *et al*. (2018b) reported genomic DNA fragmentation as an indicator of the apoptotic effects of *Gracilaria arcuata* extract on colorectal cancer cell lines. Accordingly, the resulting bands indicate the ability of the algae extract in DNA fragmentation.

According to the present study, the methanolic extract of both algae species (*P. gymnospora* and *A. spicifera*) at 1000 μg/mL had a significant cytotoxic effect on MCF7 cells. Future research will focus on identifying, isolating, and characterizing the effective compounds of these algae.

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