Original Article

Evaluation of Phenolic Content, Antioxidant Activity and Cytotoxic Effects of *Ulva lactuca and Hypnea Musiformis* Marine Algae on MDA-MB-468 Cell Line

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Abstract

Background & Objective: Breast cancer is one of the leading causes of death among women around the world. Attempts to find alternative therapies continue due to therapeutic resistance, side effects, and the high cost of conventional treatments.
Materials & Methods: Hydro-methanolic extract of *Ulva lactuca* and *Hypnea musiformis* marine algae was prepared by maceration method. The phenolic content of these extracts was compared using the standard Folin-Ciocateu method and the antioxidant capacity was evaluated by the FRAP method. The cytotoxic effects of algae were compared using MTT assay and morphological changes were evaluated by an inverted microscope.
Results: The phenolic content of *Hypnea musiformis* extract (12.46 ± 1.19 µg GAE/mg, P<0.0001), and its antioxidant activity (203.03 ± 27.87 µmol Fe^{2+}/g, P= 0.007), was significantly higher than that of the alga *Ulva lactuca*. The cytotoxic effects of *Hypnea musiformis* extract were concentration and time dependent and had severe morphological changes on the cells. Algae extract of *Hypnea musiformis* (1000 µg/mL) inhibited 84.79 ± 4.66 of % cell proliferation after 72 hours of treatment. The IC50 value of cytotoxic effect of *Hypnea musiformis* and *Ulva lactuca* on MDA-MB-468 was respectively 701.2 and >1000 µg/mL after 72h incubation.
Conclusions: *Hypnea musiformis* had higher cytotoxic effects than *Ulva lactuca* on MDA-MB-468 cells, probably due to its higher phenolic content and antioxidant capacity. Therefore, it appears that a *Hypnea musiformis* alga is a better option to continue research on drug discovery of anticancer compounds.

Keywords: Algae *(Ulva lactuca, Hypnea musiformis)*, Antioxidant, cytotoxic effects, Breast cancer

Introduction

Today, cancer is a major health issue in different societies. The disease has led to a high mortality among men and women around the world. According to the World Health Organization (WHO), the number of new cases of cancer is expected to increase by almost 70% over the next 20 years (1). Cancer is caused by the transformation or deformation of the body's normal cells. In fact, uncontrolled cell growth and metastasis to distant tissues can cause cancer. Therefore, all tissues of the body can become cancerous (2). Breast cancer is one of the most common malignancies that causes about 14,000 deaths each year. This

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cancer is a heterogeneous tumor with extensive clinical manifestations (3) that in 20% of cases lacks estrogen receptor, progesterone receptor and epidermal growth factor receptor HER2 and is called triple negative tumor. These tumors are very resistant to common therapies and do not have a good prognosis (4). Currently, the most common treatment for breast cancer is surgery, chemotherapy, radiotherapy, or hormone therapy. In addition to being costly, these methods have severe side effects such as therapeutic resistance and damage to healthy organs (5). Therefore, research to produce drugs with higher efficacy and fewer side effects, is essential.

Marine algae are one of the traditional products that have been used as medicine for a long time (6). Recently, algae have been considered for the prevention or treatment of cancer (7). Marine algae are classified as Chlorophyta (green algae), Rhodophyta (red algae) and Phaeophyta (brown algae) depending on their chemical composition (8). With the increasing tendency of the public to natural ingredients, green and red algae have attracted much attention (9). Green and red algae contain various biologically active metabolites which can benefit in cancer treatment. They are rich sources of primary and secondary metabolites such as phenolic compounds, terpenoids, phlorotannins, steroids, amino acids, alkanes and halogenated ketones (10). Ulva lactuca and Hypnea musiformis belong to the Chlorophyceae and Cystocloniaceae family and classified in green and red algae, respectively (11). A limited amount of literature has been published on anti-cancer properties of Ulva lactuca and Hypnea musiformis. The present study was conducted to evaluate the potential of Ulva lactuca and Hypnea musiformis in the production of new anticancer drugs in the future. Therefore, phenolic content, antioxidant capacity and their cytotoxic effects on MDA-MB-468 (a triple negative breast cancer cell line) was evaluated.

Materials & Methods

Collection and preparation of hydro-methanolic extract of algae

Marine algae samples of *Ulva lactuca* and *Hypnea musiformis* were collected from the shores of Bandar Abbas, Iran in autumn 2017. The samples were collected and identified by Dr. Kokabi at the Department of Marine Biology, Faculty of Marine Science and Technology, Hormozgan University, Iran. The identification of the algae was conducted according to checklist of the marine macro algae of Iran (12). The voucher specimens of these algae were deposited in the herbarium of Fasa Medicinal Plants Research Center (FMPRC), Fasa University of Medical Sciences, Fasa, Iran. The Voucher numbers of FMPRC-100-5 and FMPRC-100-7 were assigned to the algal samples of *Ulva lactuca* and *Hypnea musiformis*, respectively.

This experimental study was conducted in FMPRC. The collected algae were dried in a dark environment at a humidity of 10 to 15% and ground with a home grinder. Hydro-methanolic extract was obtained by maceration method. Marine algae powder (100 g) was immersed in methanol (70:30 v/v) and was kept at room temperature and in a dark environment for one week while stirring constantly. The excess solvent was evaporated at 50 °C and the concentrated extract was incubated at 50 ° C for 24 hours to dry. Dried methanol extracts (500 mg) was dissolved in 5 mL dimethyl sulfoxide (DMSO) to prepare a 100 mg/mL stock solution of the extracts. The solution was sterilized with a 0.22 µm filter, and diluted 1:100 v/v to prepare working solution (1 mg/mL) for biochemical and cell culture experiments, respectively with

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distilled water and complete DMEM (FBS 10%) (13).

Evaluation of phenolic content

To measure the phenolic content of hydro-methanolic extract of *Ulva lactuca* and *Hypnea musiformis* algae, the Folin-Ciocateu assay was used. 500 µl of Folin-Ciocateu reagent (10% v/v) was added to 100 µl of the extract (at a concentration of 1 mg/mL) and incubated in darkness for 5 minutes at room temperature. Then 400 µl of sodium carbonate (7.5% w/v) was added to the sample and the resulting solution was kept at room temperature and in the darkness for 60 minutes. Finally, the absorbance of the samples was measured by a Synergy HTX multi-mode reader at 765 nm wavelength. Gallic acid was also used as standard and the phenol content of the extracts was reported in microgram Gallic Acid Equivalent (GAE) per milligram of dry weight (µg GAE/mg) (14). Phenolic content measurement of the extracts was repeated three times.

Evaluation of antioxidant activity

The Ferric Reducing Antioxidant Power (FRAP) assay was used to evaluate and compare the antioxidant activity of *Ulva lactuca* and *Hypnea musiformis* extracts. In this method, the ability of algae extracts to reduce ferric ions (Fe^{3+}) and convert it to ferrous (Fe^{2+}) is investigated. According to previous studies, 1.5 mL of FRAP working solution (15) was poured into a test tube and 50 µl of sample or standard was added and mixed. After 10 minutes of incubation at 37 °C, colored solution absorbance was measured at 593nm wavelength. FeSO4 solution (serial dilution of 1mM concentration) was also used as standard and the antioxidant activity of the extract was reported in µmol Fe^{2+}/g (15). Antioxidant activity of the extracts was repeated three times.

MDA-MB-468 breast cancer cell line

Breast cancer cell line MDA-MB-468 was purchased from the National Cell Bank of Pasteur institute, Iran. Cells were cultured in DMEM with 10% FBS and 1% streptomycin penicillin at 37 °C in a 5% CO2 incubator (16).

Investigation of cytotoxic effects

MTT colorimetric test was used to evaluate the cytotoxic effects of *Ulva lactuca* and *Hypnea musiformis* algae. MTT assay is based on the reducing power of MTT by mitochondria of cells which shows the rate of cell proliferation and survival in treatment with various drugs. MTT assay is widely used in research on anti-cancer drugs (16). For this purpose, 150 µl of cell suspension containing 10,000 breast cancer MDA-MB-468 cells was distributed in 96-well plate wells and incubated for 24 hours. Then, according to the criteria for classifying the anti-cancer effects of drugs, cells were treated with concentrations of lower than 1000 µg/mL (250, 500, 750 and 1000 µg/mL) algae and incubated for 48 and 72 hours (17).

To evaluate the cytotoxic activity of algae extract, 20 µl of MTT solution (500 µg/mL) was added to each well (1:10 v/v) in the low light and the plates were incubated for 4 hours. Next, the supernatant was carefully removed and the formed Formazan crystals were completely dissolved by adding 200 µl of DMSO. Finally, the absorbance of the samples was measured by a Synergy HTX multi-mode reader at 540 nm wavelength. Cell viability in the treatment with algal extracts was calculated by dividing the absorbance of the treated cells by the absorbance of the control group. The results were reported as the cell viability percentage. The cytotoxic effect of *Ulva lactuca* and *Hypnea musiformis* algae extracts was determined by calculating the reduction in MDA-MB-468 cancer cell survival. Finally, IC50 value of cytotoxic effects of algal

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extract was calculated using linear regression. IC50 value indicates the minimum concentration of algal extract that can inhibit the proliferation of MCF7 and MDA-MB-231 cancer cells by 50% (18).

Investigation of cell morphological changes

After treating the cells with concentrations of 250, 500, 750 and 1000 µg/mL of *Ulva lactuca* and *Hypnea musiformis* hydro-methanolic extracts, the cell morphological changes were evaluated by inverted microscope after 48 and 72 hours of incubation.

Statistical analysis

Data were expressed as Mean ± SD. Statistical analysis of data was performed using independent t-test in GraphPad Prism 8.0.2. Significance level was also considered at $P < 0.05$ (16).

Results

Phenolic content

The phenolic content of the marine algae *Hypnea musiformis* and *Ulva lactuca* was calculated based on the gallic acid standard line equation (y = 278.59x - 48.578, R2 = 0.9902). According to Chart 1A, the phenolic content of the hydro-methanolic extract of *Hypnea musiformis* was 12.46 ± 1.19 µg GAE/mg, which was significantly ($P < 0.0001$) higher than the hydro- methanolic extract of the *Ulva lactuca* (3.08 ± 0.74 µg GAE/mg).

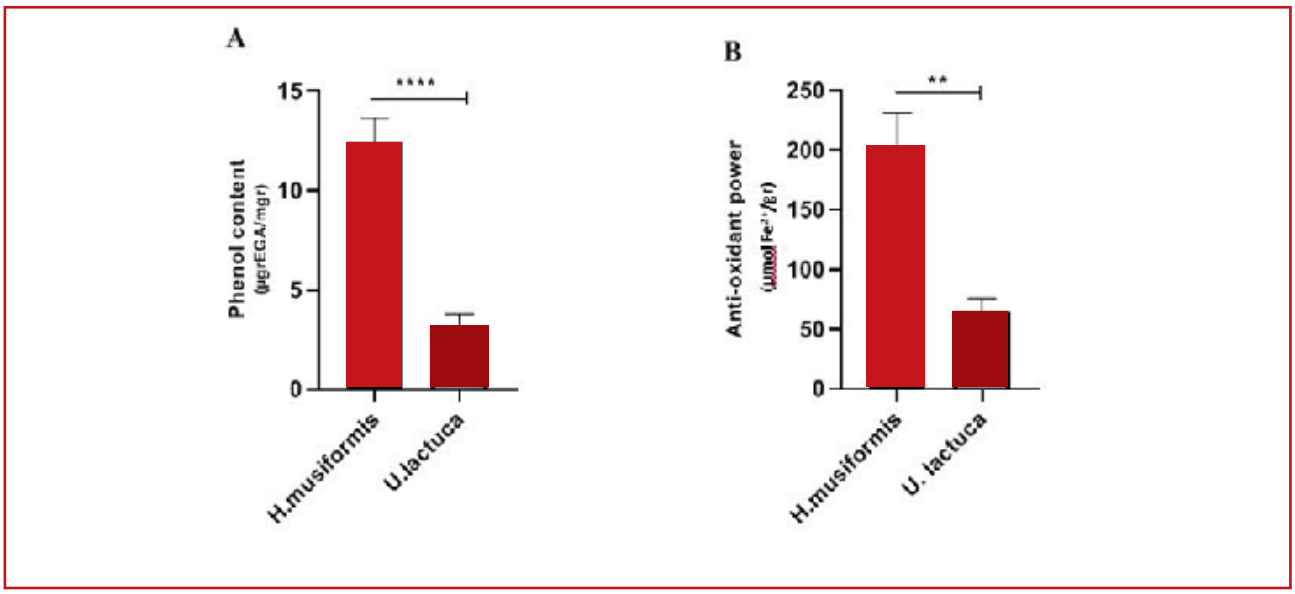

Chart 1. Comparison of phenolic content (A) and antioxidant activity (B) of hydro- methanolic extracts of Hypnea musiformis and Ulva lactuca algae

Antioxidant activity

The antioxidant activity of *Hypnea musiformis* and *Ulva lactuca* was calculated based on the $FeSO_4$ standard line equation (y = 1642.1x - 272.29, $R^2 = 0.9982$). According to Chart 1B, the antioxidant activity of *Hypnea musiformis* extract was 20.83 ± 27.87 µmol Fe^{2+}/g, which was significantly ($P = 0.007$) higher than the antioxidant activity of *Ulva lactuca* (64.89 ± 11.33 µmol Fe^{2+}/g).

Cytotoxic effects

According to the Table 1, the cytotoxic effects of *Hypnea musiformis* extract were concentration and time dependent. *Hypnea musiformis* extract (1000 µg/ml) inhibited 59.88% and 84.79% of MDA-MB-468 cell

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proliferation, respectively after 48 and 72 hours of treatment. *Ulva lactuca* algae extract had little cytotoxic effect, and treatment of the highest concentration of *Ulva lactuca* algae extract (1000 µg/mL), after 48 and 72 hours of incubation, resulted in 1.32 and 2.04% inhibition of MDA-MB cell line proliferation, respectively. According to Table 1, after 48 hours of incubation, the cytotoxic effects of *Hypnea musiformis* extract at concentrations of 500 (P= 0.003), 750 (P= 0.0001) and 1000 (P<0.0001) were significantly higher than *Ulva lactuca* extract. The IC50 value of cytotoxic effects of Hypnea musiformis after 48 and 72 hours incubation was 905 and 701.2 µg/mL respectively; while the IC50 value of cytotoxic effect of *Ulva lactuca* after 48 and 72 hours incubation was more than1000 µg/mL.

Table 1. The viability of the MDA-MB-468 breast cancer cell lines in treatment to the Hypnea musiformis extract after 48 and 72 hours of incubation

Time	CONC (µg/mL)	Hypnea musiformis			Ulva lactuca			P-value
		Viability %		IC50 (µg/mL)	Viability %		IC50 (µg/mL)	
		Mean	SD		Mean	SD		
48 hours	250	97.32	0.77	905	99.52	2.41	> 1000	0.20
	500	86.99	4.62		100.2	2.40		0.003
	750	61.18	2.71		98.72	10.28		0.0001
	1000	40.12	5.08		98.68	5.91		<0.0001
72 hours	250	92.01	3.00	701.2	98.85	3.49	> 1000	0.06
	500	75.37	3.85		100.28	2.60		<0.0001
	750	46.43	3.22		98.52	6.89		<0.0001
	1000	15.21	4.66		97.96	3.40		<0.0001

Morphological changes of MDA-MB-468 cells

Morphological changes of MDA-MB-468 as a triple negative breast cancer cell line in treatment with hydro- methanolic extract of *Hypnea musiformis* were concentration and time dependent. And as shown in Figure 1, the morphological changes were exacerbated with increasing incubation time and *Hypnea musiformis* concentration. These morphological changes included increased cell shrinkage, granulation, cell membranes rupture and remnants of cell death. However, the algae extract of *Ulva lactuca* did not show a clear change in the morphology of MDA-MB-468 cells.

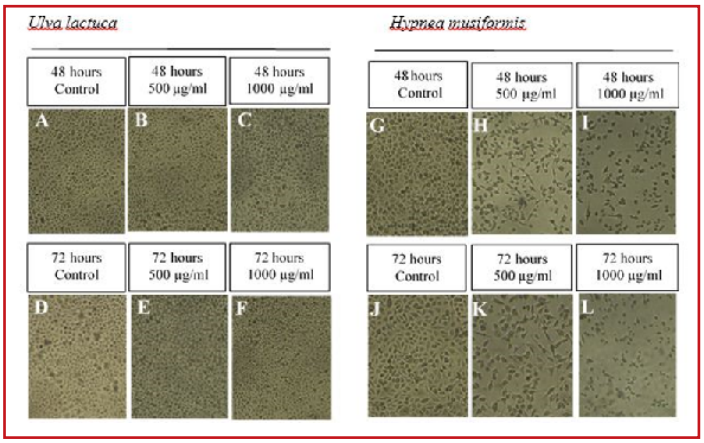

Figure 1. Morphological changes of MDA-MB-468 triple negative cell line in treatment with *Ulva lactuca* and *Hypnea musiformis* algae extracts after incubation of 48 hours (A-F) and 72 hours (G-L)
For brevity, the images of cell lines treated with the concentration of 250 and 750 µg/mL of the extracts were not shown

Discussion

Breast cancer occurs due to the uncontrolled growth of mutant epithelial cells in breast tissue. Normal cells with exposure to free radicals and oxidative stress, undergo physiological changes. These physiological changes include production of growth signals, inhibition of inhibitory signals, and angiogenesis, that induce a variety of breast cancers in the host (19). Improving oxidative stress and strengthening the antioxidant system is one of the effective strategies in the prevention and treatment of cancer.

In the present study, it was shown that the alga *Hypnea musiformis* had higher antioxidant activity, compared to *Ulva lactuca.* Therefore, *Hypnea musiformis* has a higher ability to inhibit the proliferation of triple negative breast cancer cells. The higher antioxidant effects of *Hypnea musiformis* were probably due to its higher phenolic content. In fact, there was a direct association between phenolic compounds and cytotoxic effects, consistent with previous studies (20).

The cytotoxic effects of *Hypnea musiformis* were concentration and time dependent. After 48 and 72 hours of incubation, the cytotoxic effects on 1000 µg/mL concentration of the alga compared to 250 µg/mL increased by 57.2% and 76.8%, respectively. *Ulva lactuca* had less cytotoxic effects. These cytotoxic effects of *Hypnea musiformis* algae extract are probably due to higher phenolic content and antioxidant capacity. The results of morphological changes on MDA-MB-468 cells also confirm the cytotoxic effects of *Hypnea musiformis* and *Ulva lactuca.* The rate of cell shrinkage, granulation, cell membranes rupture and remnants of MDA-MB-468 cell death in treatment with *Hypnea musiformis* increased sharply, contrary to *Ulva lactuca.* These morphological changes were also concentration and time dependent, along with cytotoxic effects. The most morphological changes were observed in treatment of MDA-MB-468 cells with a *Hypnea musiformis* extract concentration of 1000 µg/mL, after 72 hours of incubation.

According to Baharum et al. (17), the cytotoxic power of plant extracts and traditional medicinal products is divided into four categories according to IC50 value. In this categories, plants with an IC50 value of 0-20, 20-100, 100-1000 and >1000 µg/mL are classified respectively as very active, relatively active, weakly active and inactive cytotoxic drugs. *Hypnea musiformis* extract consider as a weakly active compounds; due to its IC50 value of cytotoxic effects, which was 905 and 701.2 µg/mL respectively after 48 and 72 hours of incubation. Therefore, it appears that the total extracts of *Hypnea musiformis* and *Ulva lactuca* have a low effect on inhibiting the cancer cells proliferation.

Consistent with the present study, Erfani et al. reported that the Persian Gulf native algae extract of *Hypnea flagelliformis* has weak cytotoxic effects on breast cancer MDA-MB-231, MCF-7 and T-47D cell lines (21). In the study of Mashjoor et al., the IC50 value of the methanolic extract of *Ulva flexuosa on MCF7* cell line was 107.48 µg/mL and was included in the category of weak active compounds (22). Mosaddegh et al. also indicated that the IC50 value of different algae species of the *Hypneaceae* and *Ulvaceae families*, such as *Hypnea boergeseni, Hypnea charoides, Hypnea valentiae, Ulva fasciata* and *Ulva rigida on MCF7* cell line was more than 100 µg/mL and was considered as a weak active compound (23). Namvar et al. indicated that *Ulva fasciata* had a relatively weak effect on MDA-MB-231 negative triple cells, and it was classified as a weak active compound

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with an IC50 value of 104 µg/mL (24). In the study of Alghazeer et al., the IC50 value of *Hypnea musiformis* cytotoxic effects on Caco-2 cell line was more than 200 µg/mL and was included in the category of weak active compounds (25).

Unlike the present study, some studies have shown higher cytotoxic and antioxidant effects. For example, it was shown that the ethyl acetate extract of Ulva flexuosa had higher cytotoxic effects than methanolic extract. The IC50 value of *Ulva flexuosa* ethyl acetate extract on MCF7 cell line was 60.04 µg/mL and classified as a relatively active compound (22). Arsianti et al. indicated that the hexane extract of *Ulva lactuca* with IC50 value of 45.1 ± 1.7 µg/mL on MCF7 cell line was included in the category of relatively active anti-cancer compounds (26). The potent antioxidant and cytotoxic effects of Ulva lactuca and *Hypnea musiformis* native to Libya were shown in the study of Alghazeer et al. (25). It was shown that the phenolic content of *Ulva lactuca* and *Hypnea musiformis* was 440.5 ± 39.13 and 264.44 ± 24.18 µg GAE/ mg, respectively. The *Ulva lactuca* extract also showed a high cytotoxic effect. In fact, *Ulva lactuca* extract (50 µg/ mL) inhibited the cell viability of Caco-2 cell line by 55%. This higher antioxidant activity and cytotoxic effects of the extract may have been due to differences in extraction methods and geographical conditions.

To the best of our knowledge, the present study evaluated the cytotoxic effect of *Ulva lactuca* and *Hypnea musiformis* for the first time on MDA-MB-468 cell line. The major limitation of the present study was lack information of cytotoxic effects of the algae extracts on normal cells.

Conclusion

Hypnea musiformis red algae had higher phenolic content, antioxidant activity and cytotoxic effects than *Ulva lactuca*. Therefore, it appears that the *Hypnea musiformis* extract is a better option to continue research on drug discovery of anticancer compounds. For a comprehensive conclusion, it is suggested that the total extracts of *Hypnea musiformis* should be prepared in different solvents such as n-hexane, chloroform, ethyl acetate, n-butanol and water. Their cytotoxic and antioxidant effects should be reviewed. If effective results are observed, additional studies such as effects on normal cells of the body, induction of apoptosis and disruption of the growth cycle of cancer cells should be performed.

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Conflict of interest

There is no conflict of interest.

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