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Antioxidant and Antibacterial Activity of Three Algae from Persian Gulf and Caspian Sea

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ABSTRACT The antibacterial and antioxidant activities of methanol and acetone extracts of three marine algae, including *Hypnea hamulosa*, *Gracilaria corticata* and *Enteromorpha intestinalis* were investigated. Antioxidant activities were determined by means of total antioxidant capacity, total phenolic compounds, DPPH radical scavenging activity and ferric reducing antioxidant power. Antibacterial activity was determined using a paper disc diffusion method against pathogenic bacteria, including *Listeria monocytogenes*, *Escherichia coli* and *Bacillus subtilis*. Acetone extract of *E. intestinalis* showed the highest antioxidant activity and contained the highest phenolic compounds. The highest percentage of DPPH radical scavenging activity was observed in the methanol extract of *H. hamulosa* ($p<0.05$). The highest ferric reducing antioxidant power was observed in the methanol extract of *Glacilaria* ($p<0.05$). The strongest inhibition ($p<0.05$) against *L. monocytogenes* was shown by the methanol extract of *E. intestinalis* and the highest inhibition against *B. subtilis* and *E. coli* was observed in the acetone extract ($p<0.05$). In conclusion, *E. intestinalis* extracts showed favorable antioxidant and antibacterial activity suggesting its application in food and pharmacological industries.

Key words: *Macroalgae, Solvent extraction, Antioxidant tests, Food borne pathogenic bacteria, Bacterial inhibition zone*

1 INTRODUCTION

Uncontrolled production of reactive oxygen species (ROS) including hydroxyl radical ($OH^{\cdot}$), superoxide radical ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), hydroperoxyl radical ($HO_2^{\cdot-}$), lipid peroxyl radical ($LOO^{\cdot}$), alkoxyl radical ($LO^{\cdot}$), and singlet oxygen ($^1O_2$) has been implicated in various diseases such as cancer, atherosclerosis diabetes mellitus, chronic inflammation as well as neurodegenerative diseases such as Alzheimer, Parkinson and ageing (Duan *et al.*, 2006). Furthermore, ROS can promote lipid oxidation that adversely affects the texture, color and flavor of food products, resulting in discharge of remarkable volume of food and economic loss (Min and Ahn, 2005). Synthetic antioxidants such as butylatedhydroxytoluene (BHT), butylated

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hydroxyanisole (BHA) and tert-butylhydroquinone (TBHQ) are commonly used in the food and pharmaceutical industries to retard oxidation and peroxidation processes (Amarowicz *et al*., 2000). However, due to the increasing consumer attitude for natural compounds, attention has been shifted towards natural preservatives particularly from plant sources (Cakir *et al*., 2003). In this regards, many novel antioxidants have already been isolated from various kinds of plants, herbs and spices (Ramarathnam *et al.,* 1995; Wettasinghe and Shahidi, 1999; Shon *et al.,* 2003). Natural antioxidants are not limited to terrestrial plant and some marine algae were reported to possess antioxidant activity (Duan *et al.,* 2006; Chew *et al.,* 2008; Demirel *et al.,* 2009).

The Center for Disease Control and Prevention (CDC) estimates that each year approximately 48 million foodborne diseases occur in the United States and about 9.4 million of them have been caused by seven pathogens including *Salmonella*, norovirus, *Campylobacter*, Toxoplasma, *E. coli* O157:H7, *Listeria* and *Clostridium perfringens* (CDC, 2013). A variety of different chemical and synthetic compounds are used as antimicrobial agents to inhibit the growth of pathogenic microorganism, but they can cause a variety of negative side-effects as well (Rabiey *et al.,* 2013; Rabiey *et al.,* 2014). Compounds derived from natural sources such as marine algae can be used as safe antimicrobial agents (Dashtiannasab *et al.,* 2012). There is a great resource of algae species in the Iranian coasts of the Persian Gulf and Caspian Sea that have not been used commercially. In the form of algal bloom, they can show negative effect such as green tides (*E. intestinalis*) and biofouling mats that cause cascading effects throughout the food web. Algal mats can also deplete the available oxygen in the water and increase the production of hydrogen sulphide in the sediment, which can cause population declines in other fauna and flora (Messyasz and Rybak 2011. In certain summers, *Enteromorpha* may reach a biomass of 250 g dry wt.m^{-2}in UK (Warwick et al., 1981). So far, many marine algae have been screened for their antimicrobial potential (Salvador *et al.,* 2007; Taskin *et al.,* 2007; Chew *et al*., 2008; Vallinayagam *et al.,* 2009; Demirel *et al.,* 2009; Plaza *et al.,* 2010; Emmanuel *et al.,* 2011; Zakaria *et al.,* 2011; Ambreen *et al.,* 2012, Rosaline *et al.,* 2012; Ramalingam and Amutha, 2013), but only a few algae from the Persian Gulf and Caspian Sea have been investigated for antibacterial activity (Dashtiannasab *et al.,* 2012; Esmaeili and Khakpoor, 2012, Ghannadi *et al.,* 2013). The aim of this study was to evaluate the *in vitro* antioxidant and antibacterial activities of three marine algae collected on the Iranian Coasts of Persian Gulf and Caspian Sea.

2 MATERIALS AND METHODS

2.1 Sample collection

Two red algae, *Hypnea hamulosa* and *Gracilaria corticata*, were collected in the Bushehr coast of the Persian Gulf, Iran, and one green alga, *Enteromorpha intestinalis*, was collected in the Noor coast of the southern Caspian Sea, Mazandaran, Iran. Photos of the investigated algae species and their taxonomy are presented in Figure 1 and Table 1. The samples, collected during the spring and summer 2011, were rinsed with tap water to remove foreign materials. Then the cleaned samples were air-dried in the shade at 30°C for 5 days. Dried samples were powdered using an electric grinder and stored in polyethylene bags at -20°C until use (Zakaria *et al.,* 2011).

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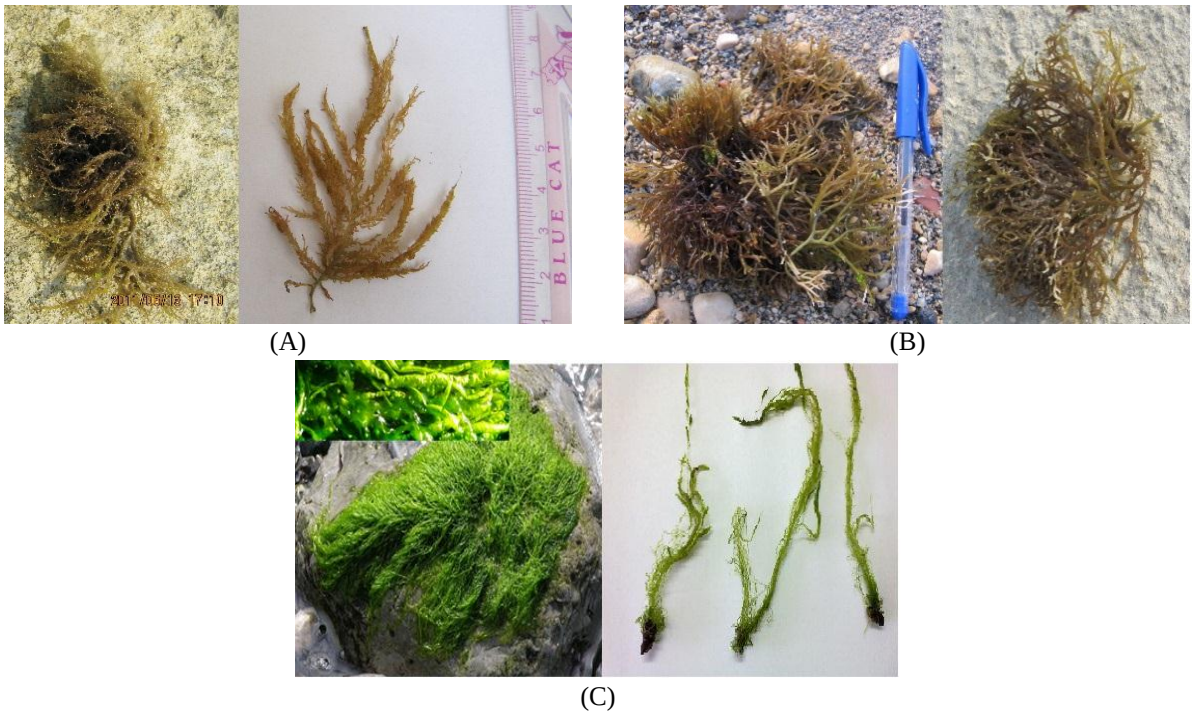

(A) (B)

(C)

Figure 1 The investigated algae species: (A) *H. hamulosa*; (B) *G. corticata* and (C) *E. intestinalis.*

Table 1. Taxonomy of investigated algae species

Algae species	*H. hamulosa*	*G. corticata*	*E. intestinalis*
Kingdom	Plantae	Plantae	Plantae
Phylum	Rhodophyta	Rhodophyta	Chlorophyta
Class	Florideophyceae	Florideophyceae	Ulvophyceae
Order	Gigartinales	Gracilariales	Ulvales
Family	Cystocloniaceae	Gracilariaceae	Ulvaceae
Genus	*Hypnea*	*Gracilaria*	*Enteromorpha*
Species	*H. hamulosa*	*G. corticata*	*E. intestinalis*

2.2 Extract preparation

Air-dried samples were mixed with methanol and acetone (1:10) and kept at room temperature overnight. The residue was then filtered through Whatman No. 1 filter paper and dry-concentrated under reduced pressure in a rotary vacuum evaporator at 40°C (Zakaria *et al.*, 2011).

2.3 Measurement of antioxidant activity

2.3.1Measurement of total capacity

The total antioxidant capacity was measured following the method described by Prieto *et al.* (1999), by mixing 0.1 ml of algal sample (containing 100 µg) with 1 ml of reagent solution(0.6 M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate) and incubated in a boiling water bath at 95°C for 90 min. After incubation for 15 min, the absorbance was recorded at 695 nm. Ascorbic acid was used as standard.

2.3.2 Measurement of total phenolic compounds

The total phenolic content of algal extracts was estimated, following the method described by

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Taga and Miller (1984), by mixing 200 µl of the sample with 4 ml of 5% sodium carbonate solution and kept at room temperature for 1min. Then, 200 µl of 50% Folin-Ciocalteu reagent was added to the mixture and vortexed, followed by 30 min incubation at room temperature and reading the absorbance measure at 750 nm. Total phenolic content was estimated as tannic acid equivalent (TAE) per gram of the sample (Taga and Miller, 1984).

2.3.3Measurement of DPPH radical scavenging activity

Briefly, 2 ml of test sample (in methanol) (containing 40-2000 µg) was mixed with 2 ml of 0.06 mM DPPH methanolic solution and allowed to react at room temperature under dark condition, followed by 30 min incubation and reading the absorbance at 517 nm. The ability to scavenge the DPPH radical was calculated using the following equation (Yen and Chen, 1995).

Scavenging activity (%)=[1-(A_{sample}-$A_{sample\ blank}$)/$A_{control}$]×100 (1)

where the $A_{control}$ is the absorbance of DPPH solution without sample, the A_{sample} is the absorbance of DPPH solution plus test sample and the $A_{sample\ blank}$ is the absorbance of the sample without DPPH solution.

2.3.4 Measurement of ferric reducing antioxidant power

Ferric reducing antioxidant power (FRAP) was measured as described by Chew *et al*. (2008). Briefly, 2.5 ml of 0.1 M potassium phosphate buffer (pH= 6.6), 2.5 ml of 1% potassium ferricyanide and 1ml of thesample (containing 10-1000 µg) were mixed and vortexed. After 20 min of incubation at 50°C in a water bath, 2.5 ml of 10% w/v trichloroacetic acid, 2.5 ml of distillated water and 0.5 ml of 0.1% Fe Cl_3 were added to the 2.5 ml of mixture and left for 30 min at room temperature, followed by reading the absorbance at 700 nm against blank sample. The results were expressed in terms of tannic acid equivalent (TAE) per gram of sample (Chew *et al*., 2008).

2.4 Evaluation of antibacterial activity

2.4.1 Microorganisms

Three different bacterial strains were used, including two gram positive bacteria, *Listeria monocytogenes* (PTCC: 1298), *Bacillus subtilis*, and one gram negative bacterium, *Escherchia coli* (PTCC: 3315). Bacterial species were provided by the Microbiology Department, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences.

2.4.2 Disc diffusion method

Mueller-Hinton agar plates were inoculated with previously prepared 24-h-old bacterial suspensions by properly spreading 0.1 ml of bacterial suspension (almost 10^6 CFU ml^{-1}) throughout the solid media in a petridish (100 mm diameter) with the help of cotton swab. Then, 250 mg ml^{-1} of different algae extracts were prepared in methanol or acetone, followed by loading the sterilized thick filter paper discs (5 mm) with 20 µl of the extracts and dried in order to evaporate organic solvent, then placed at different peripheral positions of petri dishes. Methanol and acetone served as negative controls and tetracycline (30 mg disc) served as positive control. Each treatment was replicated three times and plates were inoculated at 37°C. After 48 hours of incubation, the zones of inhibition were measured (Zakaria *et al.*, 2011).

2.5 Statistical Analysis

All data, expressed as mean±SD, were statistically analyzed by SPSS software (version 16.0, SPSS Inc., Chicago, IL, USA), using one-way analysis of variance (ANOVA). Normality was tested by Kolmogorov-Smirnov Test and homogeneity of variances checked by Levene's test. Duncan test were used for comparing the

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mean values of different treatments. P values less than or equal to 0.05 were considered statistically significant.

3 RESULTS

3.1 Antioxidant activity

Antioxidant activities of different algal extracts were analyzed by means of different in vitro tests, such as total antioxidant capacity, total phenolic compounds, % of DPPH radical scavenging activity and ferric reducing antioxidant power (Figure 2). The strongest antioxidant capacity (0.25±0.05) was observed in the acetone extract of *E. intestinalis* ($p<0.05$), while the lowest level was found in the acetone extract of *G. corticata* (Figure 2A). The methanol extracts of *G. corticata* and acetone extract of *E. intestinalis* had the highest phenolic contents with 0.14±0.00 and 0.12±0.00 mg TAE g^{-1}, respectively (Figure 2B). The DPPH radical scavenging activities of the tested extracts varied from 0.89±0.12 to 25.28±0.48 % (Figure 2C). The highest % of DPPH radical scavenging activity (25.28±0.48) was observed in the methanol extract of *H. hamulosa* ($p<0.05$). The highest ferric reducing antioxidant power was observed in the methanol extract of *G. corticata* (Figure 2D).

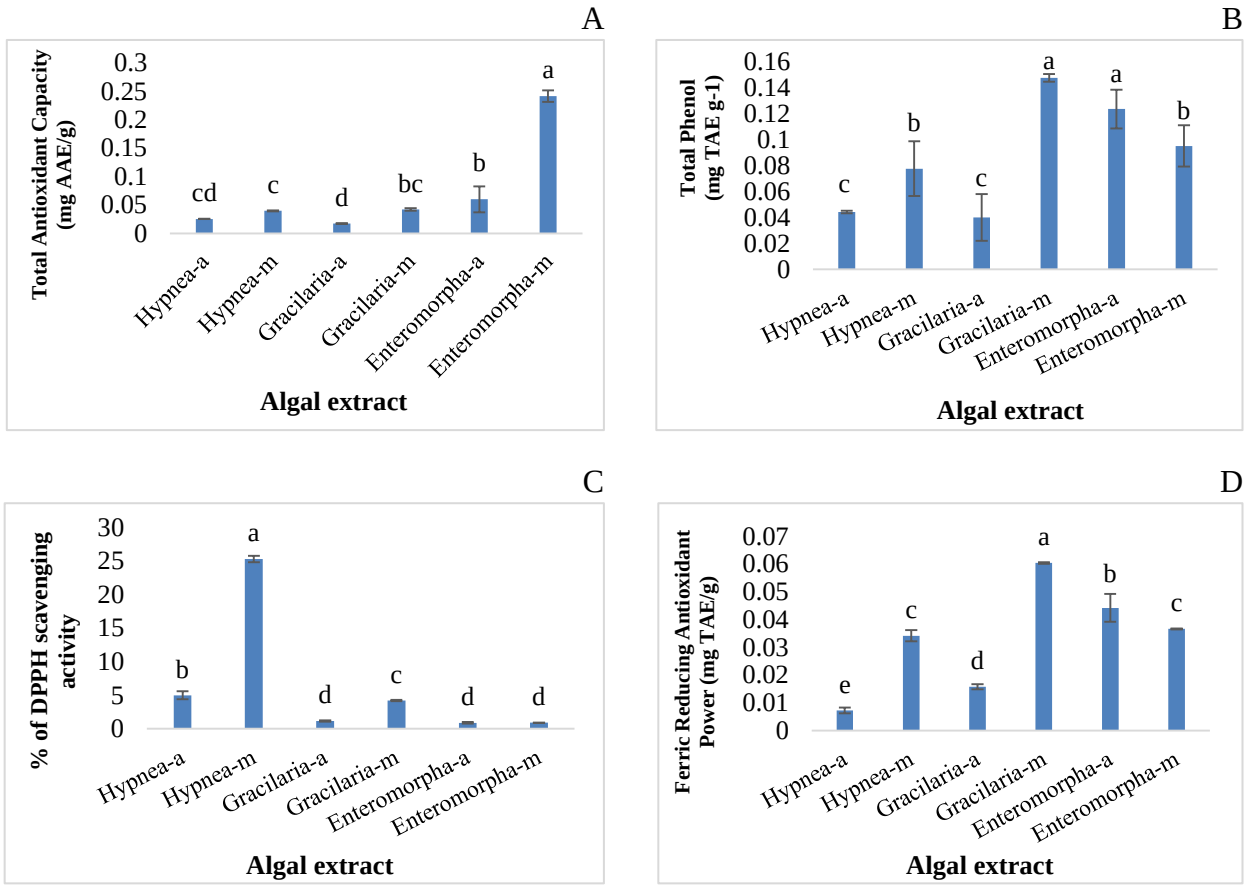

Figure 2A:Total Antioxidant Capacity (mg AAE g^{-1}); B: Total Phenol Content (mg TAE g^{-1}); C: % of DPPH Radical Scavenging Activity; D: Ferric Reducing Antioxidant Power (mg TAE g^{-1})

a: acetone extract; m: methanol extract

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3.2 Antibacterial activity

Antibacterial activities of the acetone and methanol extracts of the algae were determined against *L. monocytogenes*, *E. coli* and *B. subtilis* (Table 2). The methanol and acetone extracts of the algae showed various activities against tested bacteria, among which the acetone extract of *E. intestinalis* demonstrated the highest inhibition ($p<0.05$) against *E. coli* and *B. subtilis* with halo diameters of 15.04±0.01 mm and 18.96±0.99 mm, respectively. Furthermore, the strongest activity against *L. monocytogenes* was observed in the methanol extract of *E. intestinalis* (Zone of inhibition of 19.15±0.46 mm) ($p<0.05$).The results of tetracycline as positive control and organic solvents as negative controls against pathogenic bacteria are presented in Table 3.

Table 2 Antimicrobial activity of algal extracts against pathogenic bacteria (mm)

Algae	Extract	*L. monocytogenes*	*E. coli*	*B. subtilis*
G. corticata	Acetone	9.10 ± 0.52^{d}	9.66 ± 1.2^{b}	8.85 ± 0.99^{c}
	Methanol	10.17 ± 0.71^{cd}	na	na
H. hamulosa	Acetone	10.75 ± 0.36^{c}	8.50 ± 1.00^{b}	7.78 ± 0.66^{c}
	Methanol	na	8.45 ± 0.11^{b}	na
E. intestinalis	Acetone	12.95 ± 0.20^{b}	15.04 ± 0.01^{a}	18.96 ± 0.99^{a}
	Methanol	19.15 ± 0.46^{a}	9.28 ± 0.79^{b}	10.91 ± 0.99^{b}

a, b, c, indicate significant difference in each column ($p<0.05$)
na: No activity

Table 3 Antimicrobial activity of tetracycline as positive control and organic solvents as negative controls against pathogenic bacteria (mm)

	L. monocytogenes	*E. coli*	*B. subtilis*
Tetracycline (positive control)	14.94±1.48	26.75±0.02	9.59±0.73
Acetone (negative control)	na	na	na
Methanol (negative control)	na	na	na

na: No activity

4 DISCUSSION

The methanol and acetone extracts of three algal species, including *H. hamulosa*, *G. corticata* and *E. intestinalis* were investigated for antioxidant and antibacterial activities. The methanol extracts of *G. corticata* and the acetone extract of *E. intestinalis* showed the highest phenolic contents, followed by the methanol extract of *E. intestinalis* (Figure 2B).Great variations in the phenolic contents even among different species within the same genus of algae might be related to extrinsic and intrinsic factors. For instance, Ganesan *et al*. (2011) reported that the increased period of storage and drying can decompose the phenol content of *Enteromorpha*. In our study, the samples were shade dried, but it seems that freeze-drying can result in better results that should be considered in future studies. A positive correlation has been reported between the total antioxidant capacity and the total phenolic content (Wangensteen *et al.*, 2004; Duan *et al.*, 2006). Among the algal extracts in this study, the methanol extract of *E. intestinalis* possessed the strongest antioxidant activity, followed by the acetone extract of *E. intestinalis* and the methanol extract of *G. corticata* (Figure 2A). These three extracts also had the highest phenolic contents which can justify our results.

DPPH method is widely used to measure the ability of antioxidant compounds to act as free

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radical scavengers. It is based on the reduction of the stable, purple-colored radical DPPH into the yellow-colored DPPH (Shon *et al.,* 2003). Among the algal extracts, the methanol extract of *H. hamulosa* revealed the strongest DPPH radical scavenging activity (Figure 2C).A positive correlation between the total phenolic compounds and DPPH radical scavenging activity has been reported (Siriwardhana *et al.,* 2003). The higher DPPH radical scavenging activity of the methanol extract of *H. hamulosa* in this study might have resulted from non-phenolic compounds, such as carotenoids, polyunsaturated fatty acids, and polysaccharides.

FRAP assay is often used to measure the ability of an antioxidant to donate an electron. In this assay, the extract causes the reduction of Fe^{3+}/ferricyanide to the ferrous (Fe^{2+}) form and therefore, the Fe^{2+} complex can be monitored by measuring absorbance at 700 nm (Vallinayagam *et al.,* 2009). In this study, the ferric reducing antioxidant power of the algal extracts ranged from 0.007 to 0.06 mg TAE g^{-1}, the highest value of which belonged to the methanol extract of *Gracilaria* ($p<0.05$)(Figure 2D).

We also screened the antibacterial activity of the acetone and methanol extracts of three algae against different pathogenic bacteria (Table 2). The strongest inhibition against *L. monocytogenes* was shown by the methanol extract of *E. intestinalis*, while the highest inhibition against *B. subtilis* and *E. coli* were observed in the acetone extract of this alga. In other words, in the three tested algae, *E. intestinalis*showed higher antibacterial activity. A great variety in antibacterial activities of different algal species has been reported. For example, Ambreen *et al.* (2012) reported higher antibacterial activity in the brown algae than the green and red algae, but Vallinayagam *et al*. (2009) found higher degrees of antibacterial activity in the extracts from the red algae. The difference between the results of the present study and those of other studies may be due to the algal species, organic solvents used, geographic area, seasons of the year, temperature of the water and the antibacterial assay methods (Dashtiannasab *et al.,* 2012). In our study, the methanol extracts showed higher activity against *L. monocytogenes*, but the acetone extracts showed higher activity against *B. subtilis* and *E. coli*. While Emmanuel *et al.* (2011) and Rosaline *et al*. (2012) found acetone as the best solvent for extracting the antimicrobial agents from marine algae, Ravi Kumar *et al.* (2009) reported that methanol was the best solvent. The antibacterial activity of the algal extracts may be due to the presence of amino acids, terpenoids, phlorotannins, steroids, phenolic compounds, halogenated ketones and alkanes, cyclic polysulphides, fatty acids and acrylic acid as well as lipophilic, lipid soluble phenol components and pigments such as β-carotene (Taskin *et al*., 2007; Bhagavathy *et al*., 2011). Depending on the specific compounds extracted by each solvent, it seems that different extracts even from the same alga can show various activities against the specific bacterium. So, it can be said that different bacteria can show various degrees of sensitivity when treated with these extracts. Generally, gram positive bacteria such as *L. monocytogenes* and *B. subtilis* are more sensitive to algal extracts than gram negative bacteria such as *E. coli* (Sreenivasa-Rao and Parekh, 1981; Pesando and Caram, 1984). In conclusion, among algal spices tested in this study, methanol extract of *E. intestinalis* showed the best antimicrobial and antioxidant activities, which makes it interesting for use as natural preservatives in the food and pharmaceutical industries. However, further studies are needed to identify the compounds causing the activity and to evaluate their effects in real food systems such as fish oil and fish mince.

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فعالیت ضداکسیدانی و ضدباکتریایی سه گونه از جلبک‌های خلیج فارس و دریای خزر

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چکیدهفعالیت ضد اکسیداسیونی و ضد باکتریایی عصاره‌های متانولی و استونی دو گونه جلبک قرمز (*Hypnea hamulosa* و *Gracilari acorticata*) و یک گونه جلبک سبز (*Enteromorpha intestinalis*) بررسی شد. فعالیت ضد اکسیداسیونی به‌وسیله اندازه‌گیری ظرفیت آنتی اکسیدانی کل، محتوی فنول کل، فعالیت جذب رادیکالDPPH، قدرت کاهندگی آهن تعیین گردید. فعالیت ضد باکتریایی به‌وسیله روش انتشار دیسک بر علیه باکتری‌های بیماری‌زا شامل *Listeria monocytogenes*، *Escherichia coli* و *Bacillus subtilis* تعیین گردید. عصاره استونی جلبک *E. intestinalis* بیش‌ترین فعالیت ضد اکسیداسیونی و بیش‌ترین سطح فنول را نشان داد ($p<۰/۰۵$). بیش‌ترین فعالیت جذب رادیکال‌های DPPH برای عصاره متانولی جلبک *H. Hamulosa* مشاهده شد ($p<۰/۰۵$). عصاره متانولی جلبک *G. corticata* دارای بیش‌ترین قدرت کاهندگی آهن بود ($p<۰/۰۵$). بیش‌ترین فعالیت بازدارندگی بر علیه باکتری *L. monocytogenes* به‌سیله عصاره متانولی جلبک *E. intestinalis* نشان داده شد ($p<۰/۰۵$) و بیش‌ترین فعالیت بازدارندگی بر علیه باکتری‌های *B. subtilis* و *E. coli* نیز توسط عصاره استونی این جلبک مشاهده شد ($p<۰/۰۵$). در نتیجه‌گیری کلی، عصاره‌های جلبک *E. intestinalis* فعالیت‌های ضد اکسیداسیونی و ضد باکتریایی قابل توجهی را نشان دادند که کاربرد این جلبک را در صنایع غذایی و دارویی پیشنهاد می‌کند.

کلمات کلیدی: جلبک، حلال‌های مختلف، آزمایش‌های ضداکسیدانی، باکتری های بیماری زای غذازاد، بازدارندگی رشد باکتری

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