Evaluation of in *vitro* Antioxidant Activities and Antibacterial Potentials of Two Brown Algae Extracts; *Lyengaria stellata and Padina boergesenii* Inhabiting the Persian Gulf, Iran

Pengcheng Fu[1], Maryam Akhoundian[2]*

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Abstract

In-vitro antioxidant activities of semi-purified methanolic extract *of Lyengaria stellata* and *Padina boergesenii* were evaluated using DPPH radical-scavenging activity and reducing power. Total phenolic contents (TPC) and total flavonoid contents (TFC) were also investigated. The acetone fraction of *L. stellata* contained the highest value (126.04±8.65 mg GAE/g) of TPC, and the highest concentration of TFC (94±0.13 and 82±0.09 mg GAE/g) was obtained in ethanol fractions of *Lyengaria stellata* and *Padina boergesenii,* respectively. The highest DPPH scavenging activity (88.5%) was observed in the acetone fraction of *L. stellata* (1 mg/ml). The most significant reducing power was observed in the acetone fractions of both seaweeds at 100 mg/ml concentration (1.130±0.040 and 0.839±0.010, respectively). Ethanol and acetone fractions of *L. stellata* showed the greatest antibacterial activity (29.6±0.5 and 28.0±0.7 mm, respectively) against *Staphylococcus aureus*. The minimum value of MIC was observed in acetone fractions of both seaweeds against *S. aureus* (50.7 and 50.8 µg/ml), while *Salmonella enterica* showed the highest resistance to seaweeds extracts according to the– highest MIC values (>200 mg/ml) and the smallest IZ diameters. The extract of both algae showed considerable antioxidant activity according to DPPH radical scavenging activity and reducing power assays.

Keywords: Antibacterial activity, Antioxidant Potential, Persian Gulf, Seaweeds, *Lyengaria stellate, Padina boergesenii*

Introduction

Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ethoxyquin (EQ), and propyl gallate (PG) have been used to prevent oxidative damage of aquaculture products in food industries. However, these chemical substances cause undesirable side effects such as liver damage and carcinogenesis in human consumers (Valko et al., 2007, Munir et al., 2013). Due to increased public interest in consuming healthy and natural foods, natural antioxidants are highly preferable

1- PhD, State Key Laboratory of Marine Resource Utilization in South China Sea, Hainan University, Haiko, China
2- PhD, Marine biology Department, Marine and Environmental Science Faculty, University of Mazandaran, Babolsar, Iran
*Corresponding author's email: m.akhoundian@umz.ac.ir

in the food and pharmaceutical industry as a safe replacement for synthetic compounds (Balboa et al., 2013).

Macroalgae have been considered as an appropriate choice of bioactive compounds for natural antioxidants due to their high diversity, wide distribution, and easy accessibility (Munir et al., 2013). In the past decade, many researchers have investigated and improved the antioxidant activity of several types of macroalgae (Balboa et al., 2013). It is documented in the literature that macroalgae extracts, either in the form of crude extracts or chemically processed products, have potent inhibitory effects on the destructive processes of oxidative stress in biological systems (Zengin et al., 2011, Saeed et al., 2012). Among the three phyla of macroalgae, brown algae present higher antioxidant potential than red and green algae. A detailed summary of brown algal extracts' antioxidant activity has been made, confirming the potential of brown algae as a source of novel secondary metabolites as antioxidants (Balboa et al., 2013). *Alaria esulentaobia* (Zubia et al., 2009), *Fucus vesiculosus* (Wang et al., 2012, Wang et al., 2010), *Laminaria japonica* (Wang et al., 2010); *Ascophyllum nodosum* (Audibert et al., 2010, O'sullivan et al., 2011), *Callerpa lentillifera* (Matanjun et al., 2008), *Colpemnia sinuosa* (Kelman et al., 2012), *Eklonia radiate* (Kindleysides et al., 2012), *Sargassum horneri* and *Sargassum hyxtrix* (Luo et al., 2010); *Sargassum muticum* (González-López et al., 2012) and *Undaria pinnatifida* (Plaza et al., 2008) are some of the brown algae with antioxidant properties that have been recently investigated.

Although a variety of brown macroalgae species are widely distributed in the coastal area of the Persian Gulf in the south of Iran, limited attention has been given to explore their antioxidant potential. Macroalgae in the Persian Gulf live in complex habitats and even under extreme environmental conditions such as severe sunlight, high temperature, and salinity. It is expected that macroalgal exposure to such stresses could lead to the formation of free radicals and other oxidizing agents. Surprisingly, no oxidative damages have been reported for these algae until now. This fact suggests that the algae may synthesize antioxidant metabolites to protect their DNA materials and cellular membranes, which can be used as an alternative to synthetic antioxidants in the food and pharmaceutical industries (Huang and Wang, 2004).

In this study, we aimed to determine the antioxidant activity of two endemic brown algae, *Padina boergesenii* and *Lyengaria stellata* from the coast of the Qeshm Island, Persian Gulf, Iran. *P. boergesenii* and *L. stellata* are marine species, widespread in tropical seas, extending to subtropical and warm-temperate regions, 1 m depth (Ansari et al., 2014; Edgar et al., 2010; Gharamjik and Rouhani Ghadikolaee, 2010). The results of this work may serve as information enrichment for the antioxidant properties of macroalgae in the Persian Gulf, and a way to find new sources of natural antioxidants, dietary supplements and antibiotics. These natural antioxidants from seaweeds would

be an alternative safe source for antioxidant food and medicine ingredients.

Materials and methods

Sample collection and preparation

Endemic brown macroalgae, *Padina boergesenii* (Allender and Kraft, 1983), and *Lyengaria stellata* (Børgesen, 1939) (Fig. 1), were collected from coastal and shallow water areas of the north coast of Persian Gulf, Iran (Fig. 2); between October 2018

Fig. 1. Seaweeds were collected from the Persian gulf coastal area, (a) *Padina boergesenii*, and (b) *Lyengaria stellate*

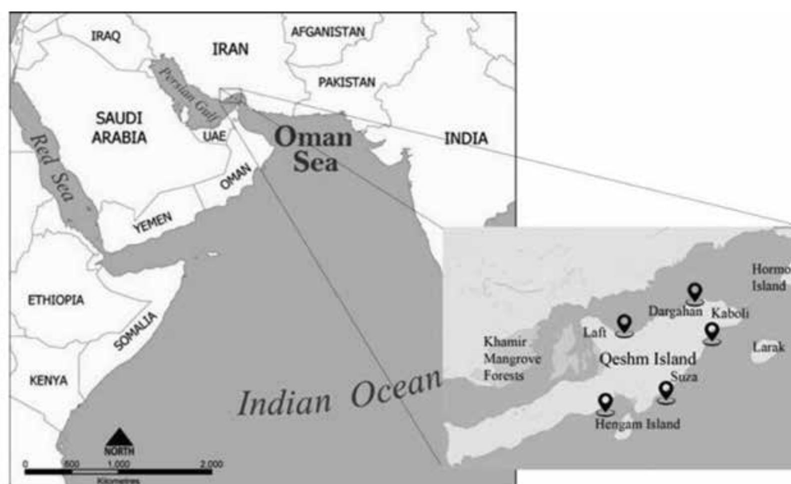


Fig. 2. Map of Persian Gulf indicating the collecting coastal area and the sampling stations in Qeshm island (Iran)

and February 2019.

Macro algae were collected in plastic jam containing seawater and transferred to the laboratory immediately, where the samples were washed thoroughly with deionized water to remove salts, sand, and epiphytes. Some samples were kept for further identification and confirmation of the species, which was carried out using available valid keys (Jones, 1986; Gharamjik and Rouhani Ghadikolaee, 2010; Richmond, 2011). The algae were dried in the shade until they reached a constant weight and then, dry samples were ground into powder and stored at -20° C.

Preparation of the crude extract

An accurately weighed 10 g sample was extracted with 50 ml of different solvents (sample: solvent=1:5). Methanol, Ethanol, and Acetone (80%) were used to compare their efficiency of extraction using three different methods of percolation, maceration (48 h), and ultrasound extraction (400W, 25 KHz, Scients-SB5200D, China) at 40° C in the water bath for 30 min (Zhou et al., 2018). Extracts were filtered and concentrated by rotary evaporation (DUAB-RE100pro, China). Each dry extract was reweighed, and the yield (extraction efficiency) was calculated and stored at -16° C (Zubia et al., 2007; Abdul Qadir et al., 2017).

Purification of the crude extract

The methanolic extract was semi purified to determine TPC and TFC as well as antioxidant potential. Semi-purified of crude extracts of *L. stellata* and *P. boergesenii* was performed using C18 cartridges by gradient elution with different organic solvents (Methanol, Ethanol, and Acetone). As a result, three semi-pure fractions were obtained, and the organic solvent of the fractions was removed using a rotary rotating evaporator at 40° C (Bergé et al., 2002; Wu et al., 2003). The fractions were stored at -20° C until analysis. To reach the favorite final concentration (1 mg/ml), 10% DMSO was used for all three fractions dilution.

Assessment of antioxidant activity

DPPH radical scavenging activity

DPPH (1, 1 Diphenyl 2- Picryl Hydrazyl) radical scavenging activity was determined using the earlier reported method with slight modification (Wu et al., 2003). Briefly, different concentrations (1, 0.5, 0.25, 0.1, 0.05 mg/ml) of sample solutions (1.5 ml) were mixed with an equal volume of 0.1 mM DPPH (in 95% methanol). The mixture was stirred vigorously for 30 min at room temperature. The absorbance was measured by spectrophotometer at 517 nm. DPPH is a stable free radical in powder form with red color, which turns yellow when scavenged. The lower the absorbance, the higher the DPPH scavenging activity. The scavenging effect is expressed as below:

[(blank absorbance–sample absorbance)/ blank absorbance] ×100%

The DPPH test was performed in triplicate, and ascorbic acid (10 mg/ml) was used as a reference.

Reducing power assay

The reducing power activity of algal extracts was determined by Fe^{3+} reduction, using protocols described by Lee and Kim (Lee and Kim, 2015), with slight modifications. 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% $K_3Fe(CN)_6$ were mixed with algae extracts. The resulting mixture was incubated for 20 min at 50° C. After adding 2.5 ml of 10% trichloroacetic acid, the mixture was centrifuged at 3000 rpm for 10 min. Then, 2.5 ml of supernatant was diluted with an equal volume of deionized water and 0.5 ml of 0.1% $FeCl_3$. The absorbance was measured at 700 nm by spectrophotometer (Philes-T6, China).

Determination of total phenolic contents (TPC)

To determine the total phenolic contents of algal crude extracts, the Folin-Ciocalteu

reagent was used as reported earlier (Zubia et al., 2007). Briefly, 0.5 mL of Folin-Ciocalteu reagent and 2.9 ml distilled water were added to 0.1 ml algae extract in test tubes. After 10 min at room temperature, 1.5 ml of 20% Na_2CO_3 was added into the test tubes and kept in the dark place at room temperature for one hour. Absorbance was measured at 725 nm. The total content of phenolic compounds was expressed in GAE mg/g dry weight based on a standard curve of gallic acid. Calibration curve was y=0.0584x+0.0077 (R 2 = 0.9926). Every sample was used in triplicate.

Total flavonoid content (TFC)

The total flavonoid content of the crude extract was determined by the aluminum chloride colorimetric method (Chang et al., 2002). Briefly, 500 µl of samples (1 mg/ml) was mixed with 1.5 ml methanol, and 3 ml distilled water, made up of 0.3 ml 10% AlCl3 solution, 0.3 ml potassium acetate (1 M). After 20 min incubation at room temperature, the mixture was allowed to stand for 15 min, and then, the absorbance of samples was measured at 415 nm using a spectrophotometer (PHILES-T6, China). The total flavonoid content was expressed as milligrams of quercetin equivalents (mg QE/g).

In vitro evaluation of antimicrobial activity

Microbial strains and growth conditions

Antimicrobial activity of semi-purified algae extracts was tested in both Gram-positive (*Staphylococcus aureus* (ATCC 29213), *Bacillus circulance* (ATCC 4516)) and Gram-negative bacteria (Escherichia coli (ATCC 25922), *Salmonella enterica* (ATCC 9150) obtained from the Microbial culture collection, Pasteur Institute, Tehran, Iran. Bacterial strains were cultured overnight at 37 °C in Mueller–Hinton agar (MHA).

Disk diffusion method

For the determination of the antimicrobial activity, the disk diffusion method was used as described by Kozekidou et al. (2008) with some modifications. Briefly, a suspension of the tested bacteria (10^8 CFU/ml) was spread on Sterile BHI Agar media plates. Sterile 6 mm filter paper discs were impregnated with 15 µl of extracts and placed on the inoculated plates and incubated at 37° C for 24 h. Negative controls were prepared using 15 µl of 30% DMSO.

Ampicillin (10 µg/disc) was used as positive growth control. Antimicrobial activity was determined by measuring the inhibition zone diameter around the discs using a metric scale and evaluation of the sensitivity of bacteria to extracts was interpreted by the inhibition zone diameter (IZD), according to the criteria mentioned by Sreepian et al. (2019); when IZD of the extract≤ 6 mm (No activity), 6 mm≤ diameter≤ 12 mm (weak activity), 12 mm≤ diameter≤ 20 mm (moderate activity) and≥20 mm (strong activity); and all the tests were conducted in triplicate.

Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) was estimated by using the both micro dilution method (Akremi et al., 2017, Salah et al., 2019).

All tests were carried out in Mueller Hinton broth (MHB). Bacterial strains cultures were incubated at 37° C for 24 h in MHA, and the tested strains were suspended in MHB to reach a final density of 5.01 CFU/ml. All the extract's fractions were dissolved in 10% DMSO and geometric dilutions ranging from 12.5 to 500 µg/ml of each sample, were prepared in a 96-well micro-titer plate, including one growth control (MHB+10% DMSO). Plates were incubated at 37° C for 24 h. The MIC was determined as the lowest concentration of the extract at which the tested bacterial strains do not demonstrate any visible growth after incubation.

Statistical analysis

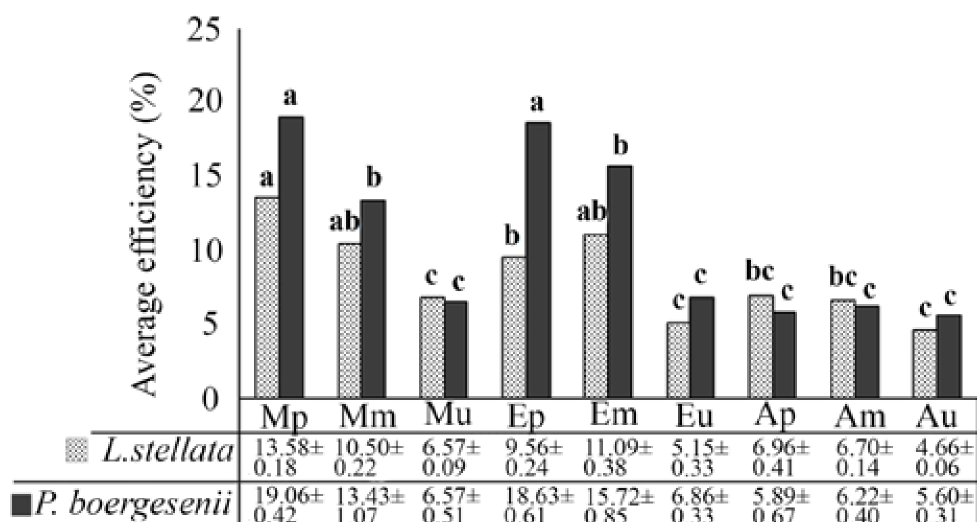
All experiments were performed in triplicates and expressed as mean± Standard deviation. A one-way ANOVA and LSD post hoc test were used to analyze the difference between groups. Data were analyzed statistically by using Statistix 10.0 and Origin 9.0 (Origin lab Corporation, Hampton, USA) software, and values P≤ 0.05 were considered significant.

Results

Crude extraction yield

Among the different methods and solvents, methanol and ethanol through the percolation method exhibited higher yields of 16.32% and 14.09%, respectively, followed by ethanol and methanol extractions of the maceration method (13.41% and 11.97%, respectively). The least efficiency belonged to the ultra-sonication method with all three solvents (Fig. 3).

Total phenolic (TPC) and flavonoids (TFC) contents



	Mp	Mm	Mu	Ep	Em	Eu	Ap	Am	Au
L.stellata	13.58±0.18	10.50±0.22	6.57±0.09	9.56±0.24	11.09±0.38	5.15±0.33	6.96±0.41	6.70±0.14	4.66±0.06
P. boergesenii	19.06±0.42	13.43±1.07	6.57±0.51	18.63±0.61	15.72±0.85	6.86±0.33	5.89±0.67	6.22±0.40	5.60±0.31

Fig. 3. Average efficiency (%) of extraction from two brown algae of Persian Gulf, Iran Expressed as percentage of extract to dry seaweed matter of triplicate. Solvent M: methanol; E: ethanol; A: acetone; method m: maceration; p: percolation; u: ultrasonication. Different letters (a, b, and c) represent significant differences

The measured values of total phenolic content (TPC) of *L. stellata* and *P. boergesenii* crude and semi-purified fractions are presented in Table 1.

Table 1 shows that a higher amount of TPC was obtained with acetone fraction for both *L. stellata* and *P. boergesenii* (126.04±8.65 and 110.22±6.14 mg GAE/g, respectively), which were significantly greater than ethanol and methanolic fractions as well as crude extract.

It was also found that the TFC was the highest (94±0.13) in the ethanolic fraction of *L. stellata* followed by the ethanol fraction of *P. boergesenii* (82±0.09). The remarkable point is that although acetone fractions showed the highest TPC content in both seaweeds, on the contrary, the lowest amount of flavonoids was measured in this fraction. Moreover, *L. stellata* crude extract and all fractions were observed to have a significantly higher amount of TPC and TFC as compared to *P. boergesenii.* The highest concentration of flavonoids in both *L. stellata* and *P. boergesenii* (94±0.13 and 82.00±0.09 mgQE/g) were measured in ethanolic fractions and the lowest one in acetone fractions (Table 1).

DPPH radical scavenging capacity

DPPH is a stable free radical with red color which turns yellow when scavenged by polyphenols and anthocyanins through the donation of hydrogen, forming the reduced DPPH-H (Luo et al., 2010). In this study, algae crude and semi-purified extracts with antioxidant activity to various degrees are shown in Figure 4.

As depicted in Figure 4, *L. stellata* extracts (1 mg/ml) had higher DPPH radical scavenging activity than the equivalent concentration of

Table1. Total phenol (TPC) and flavonoid (TFC) contents of *L. stellata* and *P. borgoensii* crude extract and its semi-purified fractions

Extract	seaweed	TPC^{BC} (mg GAE/g)	TFC^{DC} (mg QE/g)
Crude extract	*L. stellata*	66.36±5.86^{b}	73.01±0.08^{bc}
	P. boergesenii	50.60±1.69^{a}	65.20±0.07^{b}
Ethanol fraction	*L. stellata*	88.21±4.22^{c}	94.14±0.13^{d}
	P. boergesenii	61.73±2.56^{ab}	82.08±0.09^{c}
Methanol fraction	*L. stellata*	56.24±3.04^{a}	78.22±0.06^{bc}
	P. boergesenii	40.45±1.51^{a}	60.00±0.05^{b}
Acetone fraction	*L. stellata*	126.04±8.65^{d}	38.35±0.04^{a}
	P. boergesenii	110.22±6.14^{d}	26.18±0.06^{a}

^{C} Mean of 3 determination ± SD. Mean within each seaweed for each parameter with different letter (a to d) differ significantly ($P \leq 0.05$)

^{B} TPC = Total Phenolics Content, expressed as milligram GAE per g dry seaweed matter

^{D} TFC = Total Flavonoid Content, expressed as milligram QE per g dry seaweed matter

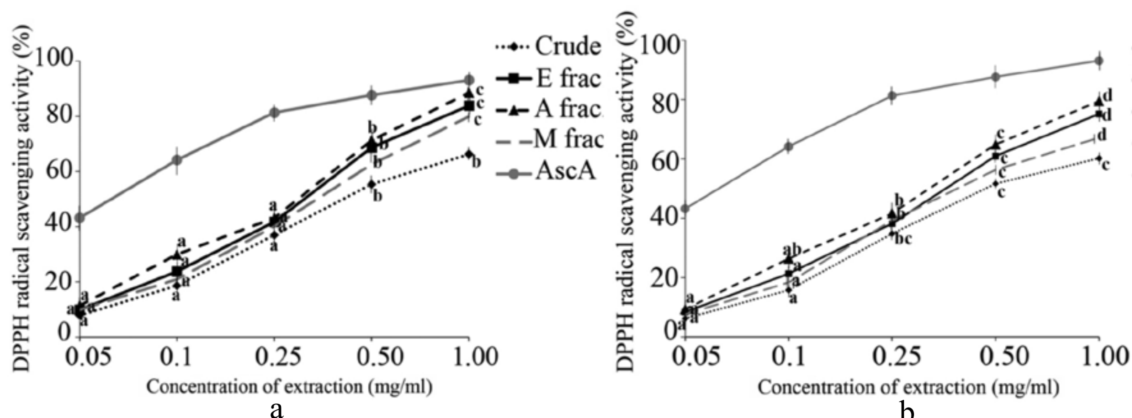


Fig 4. DPPH radical scavenging activity (%) of *Lyengaria stellata* (A) and *Padina boergesenii* (B) crude extract and its semi-purified fractions, from the Persian gulf, Iran (n = 3). AscA: Ascorbic acid. Values are the mean ± standard deviation of triplicate. Different letters (a to d) show a difference significantly (P ≤ 0.05)

P. boergesenii extract. The highest DPPH scavenging activity (88.5%) was observed in the acetone fraction of *L. stellata* (1 mg/ml), followed by an ethanol fraction of this algae (83.83%) at the same concentration (1 mg/ml). Acetone and ethanol fractions of *P. boergesenii* at the concentration of 1 mg/ml also revealed the highest DPPH scavenging activity (79.46% and 75.18%) among other samples. The scavenging activity of crude extracts of both algae was significantly lower than acetone and ethanol fractions at 0.5 and 1 mg/ml concentrations. However, no significant difference was observed between crude extracts and semi-purified fractions at concentrations lower than 0.5 mg/ml/ (P≤0.05).

Reducing power

The reducing power of the extracts of two macroalgae, expressed in OD_{700} values, was analyzed by determining the color changes of the extract solution from yellow to various degrees of green and blue. The highest reducing power was observed in the acetone fractions of both *L. stellata* and *P. boergesenii* at 100 mg/ml concentration (1.130±0.040 and 0.839±0.010, respectively), followed by ethanol fractions of both seaweeds (0.828±0.020 and 0.794±0.070). It must be noted that the reducing power of the acetone fraction of *L. stellata* was greater than that of ascorbic acid, which was more evident in concentrations of 50 and 100 mg/ml. The lowest reducing power was observed in crude fractions of both seaweeds, which was more noticeable at a concentration of 50 mg/ml. In general, according to the results, the reducing power of *L. stellata* extracts was higher than that obtained for *P. borgoensii* extracts in all concentrations. Moreover, the reducing power of crude extracts and all fractions of both tested seaweeds showed a dose-dependent activity. In a concentration above 50 mg/ml, a sharp rising of curves is obvious (Fig. 5).

Antibacterial activities

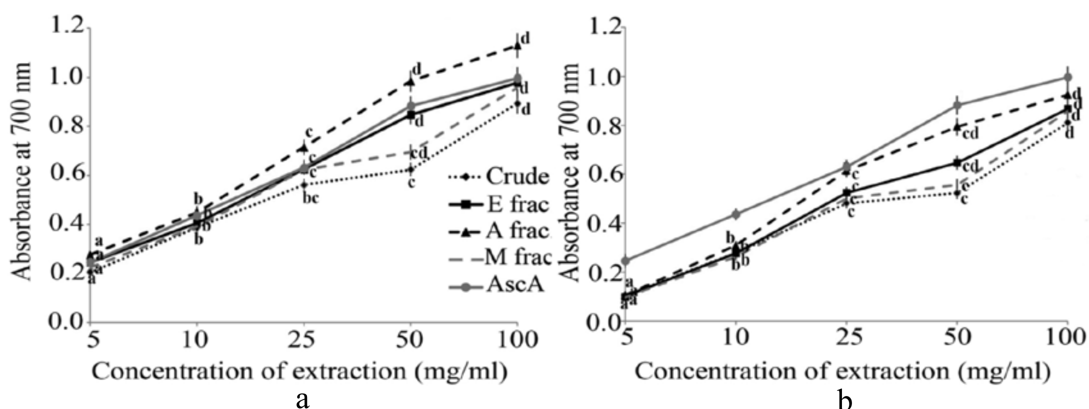


Fig. 5. Reducing power of extracts of *Lyengaria stellata* (A) and *Padina boergesenii* (B) crude extract and its semi-purified fractions from the Persian gulf, Iran (n = 3). AscA: Ascorbic acid. Values are the mean ± standard deviation of triplicate. Different letters (a to d) are showing a difference significantly ($P \leq 0.05$).

The Agar disk diffusion method was carried out to test the antibacterial activities of crude extracts and different fractions from two species of marine seaweeds, and the results are presented in Table 2. The greatest IZ (inhibition zone) diameters were observed in ethanolic and acetone fractions of *L. stellata* (29.6±0.5 and 28.0±0.7 mm, respectively) against *S. aureus*, and the lowest activity was recorded for crude extract (8.6±0.1) followed by ethanolic fraction extract (9.0±0.6) of *P. borgoensii* against *S. enterica* (Table 3).

According to the results from two tested seaweeds, ethanolic and acetone fractions showed maximum activity (according to IZ diameters) against all tested bacteria in comparison to crude extracts. There was no significant difference between the Inhibition activity of acetone and ethanolic fractions of both seaweeds against Gram-positive tested bacteria (*S. aureus* and *B. circulance*).

Discussion

The different methods of extraction of brown algae samples and the assays used for the analysis make it difficult to compare our results with others reported previously. The comparative extraction results showed a higher yield of total extracts for *Padina boergesenii* and *Lyengaria stellata* than other brown algae such as *Eisenia bicyclis* (9.91%, 1.80%), *Kjellmaniella crassifolia* (7.25%, 1.87%) as reported by Airanthi et al., 2011. It is also seen that the yields for methanol/chloroform-based extraction of *Sargassum fusiforme* and *S. hornery* were 5.83% and 5.42%, respectively (Luo et al., 2010), which might be due to different extraction methods or different species. Our results (in Fig. 3) are in agreement with the study performed by Airanthi et al. (2011) on *Alaria crassifolia* (14.61% extraction yield). Furthermore, significant differences were observed between the yield of methanol percolation and methanol maceration extracts,

Table 2. Overview of the phenolic content of some brown algae

Brown algae species	Solvent	Total phenolic contents (TPC) (mg GAE/gr)	Total flavonoid contents(TFC) (mg QE/g)	Reference
Sargassum siliquastrum	Methanol crude extract	51.00	Not measured	(Lim et al., 2002)
	Ethyl acetate	64.10		
Dictyota asiatica	Methanol (70%)	13.71±1.20		
Padina arborescens		21.28±0.13		
Eklonia cava		168.38±4.12		
Colpomenia sinusa		7.06±0.25		
Sargassum thunbergii		19.51±0.42		
Colpomenia crispus	Methanol	62.33±1.04	Not measured	(Cox et al., 2010)
	Ethanol	61.00±2.82		
Padina palmata	Methanol (60%)	42.83±3.26		
	Ethanol (60%)	30.00±0.00		
Padina sp.	Ethanol (70%) + ultrasonication	124.65±0.78	20.74±0.49	(Cox et al., 2010, Dang et al., 2018a)
Sargassum linearifolium		47.06±0.05	13.93±0.41	
Phyllosporea cemosa		67.78±1.01	9.89±0.41	
Dictyopteris membranacea	Methanol/Dichlor omethan(1/1)	24.00±0.07	65.00±0.03	(Akremi et al., 2017)
Gracilaria gracilis	Ethylacetate	35.53±1.47	66.48±1.87	(Ebrahimzadeh et al., 2018)
	Mathanol	29.39±2.01	26.47±1.203	
Padina antillarum	Methanol(50%)	24.30	Not measured	(Generalić Mekinić et al., 2019)
Padina pavonica	Methanol	10.55		
Cystoseria crinita		261.53		
Fucus saratus	Methanol(80%)	80.70		

Table 3. Comparing antimicrobial activity measured by agar disk diffusion assay (Inhibition zone diameter or IZ), and Minimum inhibitory concentration (MIC) of the crude extract and its semi-purified fractions from *Lyengaria stellata* and *Padina borgoensii*

	Microbial strains	Ampicillin	seaweed	Inhibition zone (mm)±SD			
				Crude ext.	Ethanol frac.	Methanolic frac.	Acetone frac.
Gram +	*S. aureus*	30.0±0.4	*L. stellata*	20.5±0.4[a]	29.6±0.5[b]	19.5±0.4[a]	28.0±0.7[b]
			P.	18.0±0.0[a]	23.5±0.4[a]	16.6±0.4[a]	23.0±0.4[a]
	B. circulance	18.3±0.7	*L. stellata*	18.7±0.4[a]	18.2±0.6[a]	NA	19.6±0.6[a]
			P. boergesenii	14.6±0.8[a]	14.6±0.6[a]	NA	16.4±0.1[a]
Gram -	*E. coli*	20.4±0.6	*L. stellata*	14.5±0.8[a]	16.0±0.4[a]	NA	19.8±0.4[a]
			P. boergesenii	10.7±0.6[a]	12.5±0.4[a]	NA	16.6±0.3[a]
	S. enterica	17.0±0.6	*L. stellata*	10.6±0.4[a]	11.4±0.4[a]	NA	15.5±0.0[a]
			P. boergesenii	8.6±0.1[a]	9.0±0.6[a]	NA	11.4±0.1[a]
				MIC (µg/ml)			
Gram +	*S. aureus*		*L. stellata*	51.3±1.0[a]	65.8±2.4[a]	100.2±4.4[b]	50.8±1.7[a]
			P. boergesenii	62.2±2.4[a]	52.7±3.1[a]	125.4±1.0[b]	50.7±1.2[a]
	B. circulance		*L. stellata*	65.3±1.8[a]	63.2±2.0[a]	NA	65.3±2.0[a]
			P. boergesenii	75.4±0.9[a]	75.4±1.1[a]	NA	75.8±2.3[a]
Gram -	*E. coli*		*L. stellata*	100.0±0.0[b]	75.3±0.8[a]	NA	75.6±0.6[a]
			P. boergesenii	123.1±4.2[a]	100.4±2.0[a]	NA	100.5±3.5[a]
	S. enterica		*L. stellata*	151.3±2.0[a]	125.2±4.1[a]	NA	125.4±0.6[a]
			P. boergesenii	256.1±3.8[b]	255.4±3.7[b]	NA	206.3±3.1[a]

Values are the mean ± standard deviation of triplicate. Different letters (a and b) are showing the different significantly ($P \leq 0.05$)

while no significant difference was observed between methanolic and ethanol extracts. This suggests that the methods employed might significantly influence the extraction yield and the solvent type. Therefore, the solvents and procedures should be carefully selected for maximum yield.

Many reports exist about the antioxidant activity of polyphenols such as phlorotannins and fucoxanthin of algae extracts (Chandini et al., 2008, Wang et al., 2012, Sathya et al., 2017, Fernando et al., 2016). Considering the difference in structures and molecular weights of phenolic compounds of brown algae, they are highly similar in chemical properties (Lee and Kim, 2015), and it is presumed that phenolic compounds can act as a chemical shield against harmful UV irradiation and grazers (Swanson and Druehl, 2002). It needs to be noted that in many previous seaweed studies, the amount of TPC in the methanolic extract was higher than those assayed in ethanolic or aqueous extracts (Rastian et al., 2007, Airanthi et al.,

2011, Dang et al., 2018a, Neto et al., 2018). We presume that high temperature and long-term extraction may lead to different results in our case. There is a strong relationship between TPC and extraction methods (Tierney et al., 2013, Machu et al., 2015, Dang et al., 2018b). On the other hand, Lee et al. (2015) observed a comparatively higher phenolic content of 168.34 mg GAE/g in the methanolic extract of *Eklonia cava.*

Comparing our results with other reports mentioned in Table 2 also can prove that, total phenolic contents of *L. stellata* and *P. boergesenii* were higher than many other brown algae such as *Padina arborescens*, *Sargassum thunbergii*, *Dictyota asiatica*, and *Colpomenia sinusa*.

It's also known that flavonoids are responsible for a vast variety of biological activities (Cox et al., 2010, Dang et al., 2018a). According to the previous studies (Ganesan et al., 2008, Lee and Kim, 2015, Fellous et al., 2018, El-Sheekh et al., 2020), there was a wide range of TFC from different brown algae species and the highest evaluated amount was 66.48±1.87 mg QE/g in *Gracilaria gracilis;* while the highest value of TFC (94.00±0.13 mg QE/g) from ethanol fraction of *L. stellata* was measured in the present study was 40% more than that. It's known that each extract's TPC and TFC contents strongly depend on the extraction solvent and its polarity (Akremi et al., 2017), as well as the algae species. In our case, in addition to these factors, semi-purification of the extract also played the main role in the enhancement of TFC from the extract compared to other reports about crude extracts. Our results were supported by previous studies by Akremi et al., 2017 and Tierney et al., 2013, who mentioned that purification would increase the polyphenols and flavonoid contents of extracts.

Comparison of total phenolic and flavonoids contents of the extracts for these two brown algae, with the reports of other researchers on some other brown algae extracts, confirms that *L. stellata* and *P. boergesenii* had relatively higher contents of TPC and TFC (Table 2) and could be used as a natural source for bioactive compounds.

DPPH radical-scavenging capacity has been widely used to screen antioxidants from plants and algae (Luo et al., 2010, Ganesan et al., 2008, Lee and Kim, 2015). It is an easy, rapid, and convenient method (Nickavar et al., 2007). It was used in this research to evaluate the antioxidants and radical scavenging capacity of *L. stellata* and *P. boergesenii.* According to our results, *L. stellata* extracts showed higher DPPH radical scavenging activity as than *P. boergesenii* extract (Fig. 4). The scavenging activity of the crude extract of *P. boergesenii* was significantly lower than other extracts ($P \leq 0.05$).

Many studies have been done to determine the antioxidant capacity of seaweeds. Lee and Kim (2015) assayed the antioxidant activity of the methanolic (70%) extracts of more than 50 brown algae species of Korea based on the DPPH free radical-scavenging activity. They reported an activity range between 101.93±1.49 (*Callophyllis crispata*)

and 5.12±0.32 (*Sargassum macrocarpum*). According to the results of this research (Fig. 4), *L. stellata* acetone fraction showed relatively high radical scavenging activity among brown seaweeds. Dang et al. (2017) investigated six brown algae for their antioxidant activity and reported that *Hormosira banksii* and *Sargassum vestitum* showed DPPH levels almost near to ascorbic acid (positive control) at all concentrations, which is in agreement with our results of *L. stellata* (1mg/ml) (Fig. 4). However, Zhang et al. (2007), reported that the antioxidant activity of brown seaweeds was much lower than positive controls of ascorbic acid. The results of the present study also suggest that DPPH scavenging activities of the algae extracts were dose-dependent, which is in agreement with previous studies in the literature (Luo et al., 2010, Farasat et al., 2013, Liu et al., 2017).

Both seaweeds extracts showed reducing power almost at the same degrees (Fig. 5), and it can be expressed in descending order as acetone> ethanol> methanolic fraction> crude extract. Interestingly, the acetone fraction extract of *L. stellata* presented higher reducing power than the positive control (AscA). Figure 4 reveals that the reducing power of all extracts showed a dose-dependent response at 700 nm. Similar results have been reported in the literature (Ganesan et al., 2008, He et al., 2016, Xu et al., 2018).

The reducing properties are generally associated with reluctant, which exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (He et al., 2016). Comparing TPC and reducing the power of these two seaweeds, demonstrates that higher TPC of extracts leads to higher reducing power. Our results are thus in agreement with Luo et al. (2010), He et al. (2016), and Akremi et al. (2017), who reported a relation between TPC and the reducing power of seaweed extracts.

Crude extract and all prepared fractions showed high inhibition activity against *S. aureus*, and altogether tested gram-negative bacteria, were less negatively affected by the extracts compared to gram-positives.

All the extracts revealed antimicrobial activity but only methanolic fractions, as shown in Table 2. Methanolic fractions of both seaweeds had inhibition activity only against *S. aureus* and did not show any effect against other bacteria (*B. circulance*, *E. coli*, and, *S. enterica*).

The analysis of variance revealed that the effect of MIC was significantly different for gram-positive and gram-negative tested bacteria. At the same time there was no significant difference between the two seaweed extracts ($P \leq 0.05$). The minimum value of MIC was observed in acetone fractions of both seaweeds against *S. aureus* (50.7 and 50.8 µg/ml), while, *S. enterica* showed the highest resistance to seaweeds extracts according to the highest MIC values (> 200 mg/ml) and the smallest IZ diameters (Table 3).

The results also indicated that gram-negative tested bacteria were more resistant to the seaweed extracts than gram-positive ones;

which may be due to their cell wall structure (Eliuz et al., 2019, El-Sheekh et al., 2020, Maadane et al., 2021). The outer membrane of gram-negative bacteria is covered by a slim layer, which in turn hides the antigens of the cell wall and does not allow the penetration of antibiotics into the cells (Vergalli et al., 2020). Generally, acetone fractions with the highest phenolic and flavonoid contents showed the most significant antimicrobial activity. On the contrary, the methanolic fraction showed a lack of antimicrobial effect but only against *S. aureus* despite the relatively high TPC and TFC content levels. This result can be explained by the synergy outlined by Freeman et al. (2010) and Parker et al., (2010). The synergic effect is caused by changes in concentrations of the compounds and their proportions in different fractions (García et al., 2015). So, it can be concluded that antimicrobial solid activity depends not only on the high content of antimicrobial components such as polyphenols and flavonoids but also on the synergy between all components of each extract (Akremi et al., 2017).

Concerning our findings on *Lyengaria stellata* and *Padina borgoensii,* the antimicrobial activity of crude extract can be improved by purification to semi-purified acetone, and ethanol fractions, and our results were supported by previous findings that purification of extracts leads to increasing the antimicrobial potency (García et al., 2015, Parker et al., 2010).

The results of the present study indicated that the percolation method with methanol and ethanol solvent produced the most outstanding extraction efficiency from brown seaweeds *L. stellate* and *P. borgoensii*. Acetone fractions of both analyzed seaweed were the richest fractions of phenolic, while the ethanol fractions showed the highest amounts of flavonoids. *L. stellate* and *P. borgoensii* were found to be potent sources of natural antioxidants and showed good radical scavenging activity and reducing power. Nowadays, the world's human community faces a significant challenge regarding the antimicrobial resistance of bacteria against existing antibiotics. These seaweed extracts also showed good antibacterial activities against pathogenic bacteria, which suggests them as potent source of alternative antibiotics. So identification and characterization of bioactive compounds from natural sources are highly regarded; as a source of producing a new class of therapeutics that might be used as new antibiotics.

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