Analysis of Bioactive Compounds in Some Marine Seaweeds along the Coastline of Bandar Abbas, Iran

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Abstract

Applications of seaweeds as food ingredients and bioactive compounds are increasing these days. Many of seaweeds have to be evaluated for these purposes. This study investigated the biological activities of organic extracts of four green and a red macroalgae collected from the coast of Bandar Abbas, Iran. After species identification, the bioactivities of seaweeds have been studied by antioxidant assay (Ferric reducing power and total antioxidant capacity), total phenolic and flavonoid contents, antibacterial activity, and brine shrimp cytotoxicity activity on model organism, *Artemia salina* (Linnaeus, 1758) and *Artemia franciscana* (Kellogg, 1906) were anaysed. The results revealed the most effective algal extracts by maximum antioxidant capacity were recorded using methanol extracts of *Ulva intestinalis* Linnaeus 1753, and *Ulva clathrata* (Roth) C. Agardh 1811. In addition, the highest content of total phenolic content was recorded by green seaweeds. While, the highest flavonoid content was obtained from *Gracilariopsis persica* Bellorin, Sohrabipour & E.C. Oliveira. Most extracts showed significant antibacterial effects, with disk diffusion method. Among the various extracts, the n-hexane extract was found the highest zone of inhibition against analyzed pathogenic bacteria. *U. intestinalis* and *U. clathrate* have a better antimicrobial activity and are a potential source of antimicrobial compounds. Further, we found a best toxic effect to *A. salina* in n-hexane extract of G. persica, LC50 =125 µg/ml. Generally, the findings can be introduced the green seaweeds as priority species for biological properties and can be subject of isolation of the natural antimicrobials.

Keywords: Antibacterial Activity, Antioxidant, Cytotoxicity, Flavonoid Content, Macroalgae, Phenol Content.

Introduction

Seaweeds applications have been increasing as food ingredients and bioactive compounds. Among the compounds found

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in seaweeds, their antioxidant activities have attracted major industry and consumer interest. As photosynthetic organisms, seaweeds are exposed to a combination of light and high oxygen concentrations which induces the formation of oxidative reagents like free radicals. Seaweeds are able to generate the necessary compounds to protect themselves against oxidation. Therefore, macroalgae can be considered as an important source of antioxidant compounds (Kokabi et al., 2013; Pirian et al., 2017; Soleimani et al., 2018). Furthermore, seaweeds are the rich source of nutritional compounds. These algae are well recognized as a significant part of food chain (especially as wakame, nori, kombu, sushi wrappings, noodles, and vegetables) in some countries of East Asia such as Japan (Ganesan et al., 2008). Besides the importance of seaweeds as a rich source of energy (especially protein, lipid, carbohydrate, mineral, etc.), also attracted attention for their bioactive substances including antioxidant, antibacterial, antifungal, and cytotoxicity properties to develop new medicinal and functional food ingredients (Chandini et al., 2008). The useful action of food to improve human health is due to the well-known correlation between food and health. Nowadays, synthetic antioxidants need to replace with natural antioxidant and also the increasing microbial resistance to pathogenic bacteria against antibiotics, so it is important to identify and utilize new sources of safe, effective, available antioxidants (Plaza et al., 2008; Valifard et al., 2017). The rocky, muddy, and sandy intertidal regions of Persian Gulf coastlines in Bandar Abbas, south of Iran, which create a habitat growing different macroalgae species (Zarei Jeliani et al., 2017b), under various environmental stresses, such as high light levels, high salt concentrations, UV radiation, etc. Furthermore, these conditions are stimulated by Reactive Oxygen Species (ROS) production in seaweeds. The biochemical compounds isolated from algae use making antibacterial drugs. There are several native commercial seaweeds with potential of cultivation, nutritional value, and bioactive properties in tropical waters of the Persian Gulf in the southern coastlines of Iran (Pirian et al., 2017; Pirian et al., 2020; Soleimani et al., 2018; Zarei Jeliani et al., 2017a; Zarei Jeliani et al., 2017b). Although, introducing of seaweeds as available and renewable resource in this region are not completely known yet (Zarei Jeliani et al., 2017a; Zarei Jeliani et al., 2017b). Therefore, this study will attempt to indicate some bioactive compounds (phenol and flavonoid contents), antioxidant, antibacterial and cytotoxic properties using various solvents, such as methanol, ethyl acetate and n-hexane from four common, abundant and available macroalgae *Ulva clathrata, U. intestinalis* (Chlorophyceae), *Gracilariopsis persica* and *Hypnea flagelliformis* Greville ex J. Agardh 1851 (Rhodophyceae) collected from the Persian Gulf.

Materials and Methods

Collection sites and sampling

Algal samples were collected from the intertidal zone in Bandar Abbas, during April

and November 2018. Samples transferred to the laboratory, collection site data are shown in Table 1. Identification of the seaweeds were carried out with standard keys (Kokabi and Yousefzadi, 2015; Sohrabipour and Rabei, 2008). The collected samples were washed in seawater to remove sand and all epiphytes, rinsed with distilled water to remove extra salt on seaweeds and shade-dried at room temperature. The dried samples were grounded to a fine powder and kept in -4 ºC for further analysis.

Preparation of seaweed extracts

N-hexane, ethyl acetate, and methanol extracts based on polarity were prepared. The first extraction of each ground sample was soaked in n-hexane with shaking for 72 h at room temperature. Then, the extract was separated from the residue by filtration, and ethyl acetate was added to the residue for 72 h. After that, the samples were filtered and the residue was re-extracted by methanol for 72 h and filtrated. Finally, the residual solvent of three filtered extracts was removed under reduced pressure at 40-45ºC using a rotary evaporator (Strike 102, Italia), and kept in -4 ºC until the experiment commenced.

Determination of antioxidant activity Total antioxidant capacity (TAC)

The antioxidant activity of the macroalgae extracts (n-hexane, ethyl acetate, and methanolic extracts in 3 mg/ml concentration) was evaluated by the phospho-molybdenum method according to the procedure of Prieto et al. (1999). The assay was based on the reduction of Mo (VI) to a green phosphate complex of Mo (V) at acidic pH by antioxidants ability of extracts. The absorbance of the solutions was measured at 695 nm using a spectrophotometer against blank in triplicate. DMSO was used as ablank. The antioxidant activity was expressed as an equivalent of Ascorbic acid (mg Ascorbic acid/g dry weight) (Prieto et al., 1999).

Reduction power

Reduction power of the extracts (n-hexane, ethyl acetate, and methanolic extracts in 3 mg/ml concentration) was determined based on the ability of antioxidant to form a coloured complex with Potassium ferric cyanide, trichloroacetic acid, and ferric chloride. The reduction power of the extracts was determined according to Oyaizu method (1986), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reduction power. The analyses were performed in triplicate. Ascorbic acid was used as standard (Oyaizu, 1986).

Total phenolic content (TPC) and total flavonoid content (TFC)

The total phenolic content of seaweed samples was assessed based on the Folin-Ciocalteu method described by Singleton and Rossi (1965). A standard curve was plotted using a different concentration of Gallic acid (Sigma Aldrich). TPC of seaweeds was expressed in milligram Gallic acid equivalent to gram dry weight (Singleton and Rossi, 1965). Total flavonoid content was determined based on Aluminum chloride calorimetric method described by Willett (2002). A standard curve was plotted using a different concentration of quercetin. Results were

expressed in milligram quercetin equivalent to gram dry weight of the samples (Willett, 2002). The TPC and TFC values were conducted in triplicate.

Determination of antibacterial activity

In this work, the n-hexane, ethyl acetate, and methanol extracts from seaweed samples were selected for antibacterial activity. The antibacterial activity of extracts was evaluated using the disc diffusion method against bacterial pathogen strains, *Bacillus subtilis* (ATCC 465), *Staphylococcus aureus* (ATCC 25923), *Shigella flexneri* (PTCC 1234) and *Salmonella typhimurium* (ATCC 19430). The pathogens were obtained from the Pasteur institute in Tehran. Bacteria were in oculated in Muller Hinton broth at 37° C for 24 h. The turbidity of the suspension was adjusted to 10^8 CFU/ml by comparison with 0.5 Mc Farland standard. The concentration 10 mg/ml of the extracts were prepared and loaded on the sterile discs which were placed on the surface of the agar medium. The disks containing Ampicillin and DMSO were used as positive and negative control, respectively (Bauer et al., 1966). The antibacterial activity was determined by measuring the diameter of the inhibition zone (in mm) on the surface of Muller Hinton Agar. The mean was reported in millimeters by performing the experiments in triplicates.

Cytotoxicity assay

The procedure described below was performed with modification of previously published method by Lincoln et al. (1996), using *A. salina* and *A. franciscana*. Cysts were hatched in 1000 ml flasks, containing artificial seawater with continuous aeration under light exposure at 25 ºC. After hatching, nauplii with age below 24 h were transferred to another container with fresh artificial seawater and maintained for 24 h at 25 ºC, with a photoperiod of 16 h L, 8 h D. These organisms were used as test organisms. The protocol was performed using10 nauplii per microplate well. Stock solutions of seaweed extracts (125-1000 µg/ml) were prepared in DMSO. Seawater and DMSO were used as control. Mortality was assessed by scoring the number of dead nauplii in each microplate well using a stereo microscope after 24 h (Lincoln et al., 1996). Three replicates per treatment were used.

Statistical analysis

Data were expressed as means ± standard deviation (SD) of three replicate determinations. All statistics analyses were carried out using SPSS 21 for Windows. To determine whether there were any differences among the means, one-way analysis (ANOVA) and Duncan's new multiple range tests were applied to the result. The p-values<0.05 were regarded as the significant difference ($p<0.05$).

Results

Antioxidant Activity: TAC and Reduction Power

The total antioxidant activity of seaweed extracts was evaluated by the phosphomolybdate method and expressed as mg Ascorbic acid equivalents/g extract. As shown in Table 2, methanol extract of red seaweeds didnot show any total antioxidant activity.

Table 1. Seaweed samples with collection details, all sites are in the Persian Gulf, Iran, Bandar Abbas.

Macroalgae	Taxonomy	Latitude and Longitude	physicochemical parameters of seawater		
			pH	Salinity (%)	Temperature (°C)
Ulva intestinalis Linnaeus	Chlorophyta, Ulvophyceae,Ulvales, Ulvaceae	N 27° 10′ 59" E 56° 19' 09" (Ghadir Blvd)-November	8.6±0.2	36.8±0.3	22.4±2.3
Ulva clathrata (Roth) C. Agardh	Chlorophyta, Ulvophyceae,Ulvales, Ulvaceae	N 27° 10′ 59" E 56° 19' 09" (GhadirBlvd)-November	8.6±0.2	36.8±0.3	22.4±2.3
Gracilariopsis persica A.M. Bellorin, J. Sohrabipour & E.C. Oliveira	Rhodophyta, Florideophyceae, Gracilariales, Gracilariaceae	N 27° 09′ 15" E 56° 13' 49" (souroo beach)-April	8.4±0.1	37.4±0.1	29.8±0.5
Hypnea flagelliformis Greville ex J. Agardh	Rhodophyta, Florideophyceae, Gigartinales, Cystocloniaceae	N 27° 09′ 15" E 56° 13' 49" (souroo beach)-April	8.4±0.1	37.4±0.1	29.8±0.5

Physicochemical parameters of seawater are presented as Means±SD; SD: Standard Deviation (n=5).

While the green seaweeds showed the highest value by their methanol extract. Moreover, in n-hexane extract *G. persica* did not show total antioxidant activity, while all of thesamples in this work showed total antioxidant activity in ethyl acetate extract. As shown in Figure 1, Ascorbic acid as a positive control (1.1 Absorbance (Abs)) showed significantly the highest reduction power activity compared to other extracts ($p<0.05$). In general, the extract with more reduction power activity in each species was obtained with ethyl acetate, and the most activity was observed in *U. intestinalis* (5.9 Abs) by its methanol extract.

Total Phenol and Flavonoid Contents: TPC and TFC

As shown in Table 3, significant differences were observed in TPC and TFC between macroalgae ($p <0.05$). The TPC ranged from 76.9 to 10.6 mg GAE/g dry weight of macroalgae, Which the highest was observed in *U. clathrata* (76.9 ± 1.4 mg GA/g dw). Moreover, the TFC ranged from 28.2 to 2.1 mg QE/g dw of seaweeds, which the highest was obtained in the *G. persica* (28.2 ± 0.01 mg QE/g dw). Furthermore, the lowest TPC and TFC value were observed in *H. flagelliformis* (10.7 ± 0.02 mg GA/g and 2.1 ± 0.01 mg QE/g dw, respectively).

Antibacterial activity

As shown in Table 4 the n-hexane, ethyl ac-

Table 2. Total antioxidant activity (mg ASA/g extracts) of three extracts obtained from seaweeds (Concentration of extracts used = 3 mg/ml)

	Methanol	Ethyl acetate	N-hexane
G. persica	-	1.7±0.01^{d}	-
H. flagelliformis	-	1.9±0.02^{c}	1.2±0.00^{d}
U. intestinalis	5.5±0.04^{a}	1.3±0.00^{d}	1.2±0.00^{d}
U. clathrata	5.2±0.00^{a}	2.7±0.04^{c}	4.2±0.02^{b}

Values are mean ± SD; SD: Standard Deviation (n = 3). (-): no activity.
a, b, c, d values with different letters are significantly different ($p<0.05$, ANOVA) when compared between extracts and seaweeds.

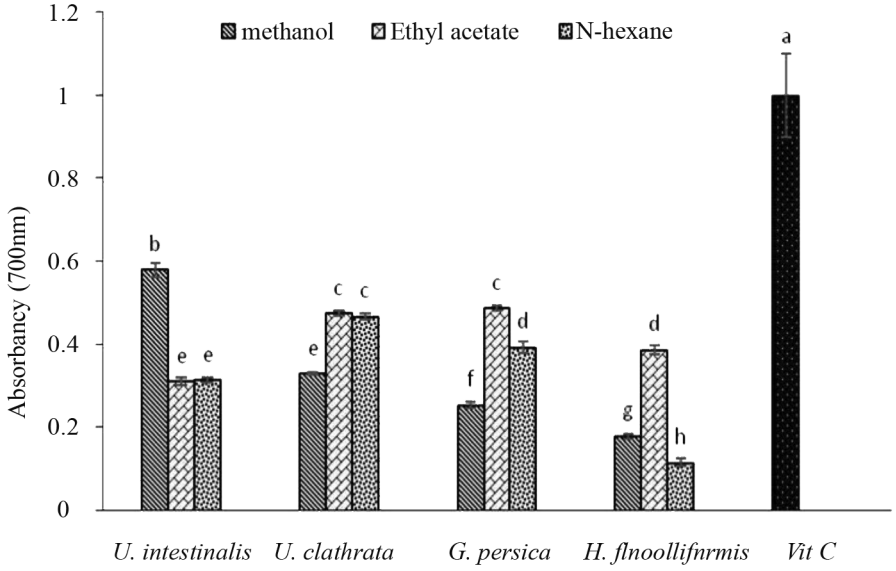

Fig. 1. Reduction power activity of three extracts from seaweeds at a concentration of 3 mg/ml. Values are mean ± SD (n = 3), and values with different letters are significantly different ($p<0.05$: ANOVA). Vitamin C= Ascorbic acid (ASA).

Table 3. Total phenol content (TPC), and total flavonoid content (TFC) of seaweeds.

	TPC (mg GA/g dw.)	TFC (mg QE/g dw.)
G. persica	45.1±0.01[c]	28.2±0.01[a]
H. flagelliformis	10.7±0.02[d]	2.1±0.01[c]
U. intestinalis	52.6±5.53[b]	2.4±0.03[c]
U. clathrata	76.9±1.4[a]	5.5±0.01[b]

Values are mean ± SD; SD: Standard Deviation (n = 3).
[a, b, c]: values with different letters are significantly different ($p<0.05$: ANOVA) when compared in the same column.

Table 4. Antibacterial activity of seaweed extracts against pathogenic bacteria.

Extract	*Bacillus subtilis* (ATCC 465)	*Staphylococcus aureus* (ATCC 25923)	*Shigella flexneri* (PTCC 1234)	*Salmonella typhimurium* (ATCC 19430)	Species
methanol	6 ± 0.5	6 ± 0.5	9 ± 0.4	-	*U. intestinalis*[a]
ethyl acetate	13 ± 0.5	10 ± 0.5	11 ± 0.5		
n-hexane	6 ± 0.3	10 ± 0.4	8 ± 0.4	-	
methanol	6 ± 0.6	8 ± 0.5	7 ± 0.3	7 ± 0.5	*U. clathrata*[a]
ethyl acetate	12 ± 0.5	8 ± 0.6	11 ± 0.4	10 ± 0.3	
n-hexane	15 ± 0.5	12 ± 0.4	11 ± 0.3	10 ± 0.3	
methanol	6 ± 0.5	9 ± 0.6	10 ± 0.4	8 ± 0.6	*G. persica*[a]
ethyl acetate	10 ± 0.4	10 ± 0.3	10 ± 0.3	10 ± 0.5	
n-hexane	9 ± 0.3	10 ± 0.6	10 ± 0.6	10 ± 0.5	
methanol	6 ± 0.4	10 ± 0.3	8 ± 0.5	7 ± 0.3	*H. flagelliformis*[a]
ethyl acetate	6 ± 0.4	12 ± 0.5	6 ± 0.5	8 ± 0.5	
n-hexane	12 ± 0.4	10 ± 0.5	9 ± 0.5	10 ± 0.4	
	14 ± 0.5	13 ± 0.4	13 ± 0.6	19 ± 0.5	Ampicillin[a]

[a] Tested at 10 mg extracts/disc, and 10 mg ampicillin/disc. Inhibition Zone includes diameter of disc (6 mm).
Values are mean ± SD; SD: Standard Deviation (n = 3). (-): no activity.

etate and methanol extracts reveal antibacterial activity against the tested bacteria. In contrast, methanol extract of all seaweeds did not inhibit *B. Subtilis* growth. In addition, most of seaweed extracts showed the low antibacterial activity compared to ampicillin, whereas the n-hexane extract of *U. clathrata* restricted *B. Subtilis* growth (15 mm). Generally, among the seaweed extracts, n-hexane extract showed high inhibitory activity compared to other extracts.

Cytotoxic activity

The LC50 of three seaweed extracts on *A. salina* and *A. franciscana* was shown in Table 5. Among the extracts, methanol extract of *G. persica*, *U. clathrata* and ethyl acetate extract of *G. persica* show LC50>1000 µg against *A. salina*. In addition, all of the extracts belong to *U. intestinalis* show LC50>1000 µg against *A. franciscana*. According to the results, n-hexane extract of *G. persica*i llustrate LC50<125 µg against *A. franciscana*. Generally, n-hexane extract of *G. persica* show the best LC50 against *A. salina* and *A. Franciscana* (125 ± 0.2).

Discussion

There is an increasing demand for natural antioxidant molecules to replace the synthetic additives in food industry. This study was designed to evaluate some bioactive compounds and properties in four different seaweeds (*G. persica*, *H. flagelliformis*, *U. intestinalis*, and *U. clathrata*), that are common in Iranian south coasts. Seaweeds are one of the natural sources of bioactive compounds with nutritional, pharmaceutical and biomedical importance. Limited studies on the biochemical constituents and biological activity of some macroalgae carried out from different parts of the Persian Gulf (Farasat et al., 2014; Sadati et al., 2011).

Two different assays were performed to evaluate the antioxidant properties of methanol, ethyl acetate, and n-hexane extracts of samples and they were total antioxidant activity

Table 5. LC50 values (µg/ml) of brine shrimps (*A. salina* and *A. franciscana*) at different extracts of seaweeds after 24 hours.

Extraction	Methanol		Ethyl acetate		N-hexane	
	A. salina	*A. franciscana*	*A. salina*	*A. franciscana*	*A. salina*	*A. franciscana*
G. persica	>1000	489 ± 0.3	>1000	250 ± 0.1	125 ± 0.2	<125
H. flagelliformis	543 ± 0.3	268 ± 0.1	255 ± 0.1	273 ± 0.1	373 ± 0.2	287 ± 0.1
U. intestinalis	417 ± 0.1	>1000	298 ± 0.3	>1000	926 ± 0.3	>1000
U. clathrata	>1000	991 ± 0.2	806 ± 0.2	482 ± 0.2	500 ± 0.3	735 ± 0.3

Values are mean ± SD; SD: Standard Deviation (n = 3).

(by the phospho-molybdenum method) and reduction power (measuring the conversion of a Fe^{3+}/ferricyanide complex to the ferrous form). All ethyl acetate extracts of seaweeds showed various level of antioxidant activity (Table 2 and Figure 1). Some studies indicated that methyl or acetone extracts are the best sources of reducing activities (Narasimhan et al., 2013). The results of total antioxidant capacity in this work indicated that the methanol extract of *U. intestinalis* and *U. clathrata* (5.5 and 5.2 mg ASA/g extract) showed the highest total antioxidant activity, followed by n-hexane extract of *U. clathrata* (4.2 mg ASA/g extract). Similar results were previously obtained (El-Din and El-Ahwany, 2016). However, previous studies reported that the highest antioxidant properties present in brown and green algae (Chandini et al., 2008; Meenakshi et al., 2009; Meenakshi et al., 2011). Furthermore, some researchers have reported that the natural Ulvan (a group of sulfated polysaccharides obtained from *Ulva* species) and their derivatives showed much higher scavenging activity on superoxide radical than ascorbic acid (Qi et al., 2010). In this study among red seaweeds *G. persica* was more reactive than *H. flagelliformis* through reduction power assay. Kumar et al. 2008, were reported that the reduction power of *Kappaphycus alvarezii* extracts was concentration-dependent and in 3 mg/ml concentration, the reduction powers were found to be in the following order: BHT (0.650) > methanol (0.250) > ethyl acetate (0.170) > hexane (0.07) (Kumar et al., 2008).

Phenolic compounds (phenolic acids, flavonoids, anthocyanidins, lignin, tannins, Gallic acid, etc.) are commonly found in plants kingdom. Like terrestrial plants, seaweeds contain various inorganic and organic compounds with biological activity like antioxidant activity, which can benefit human

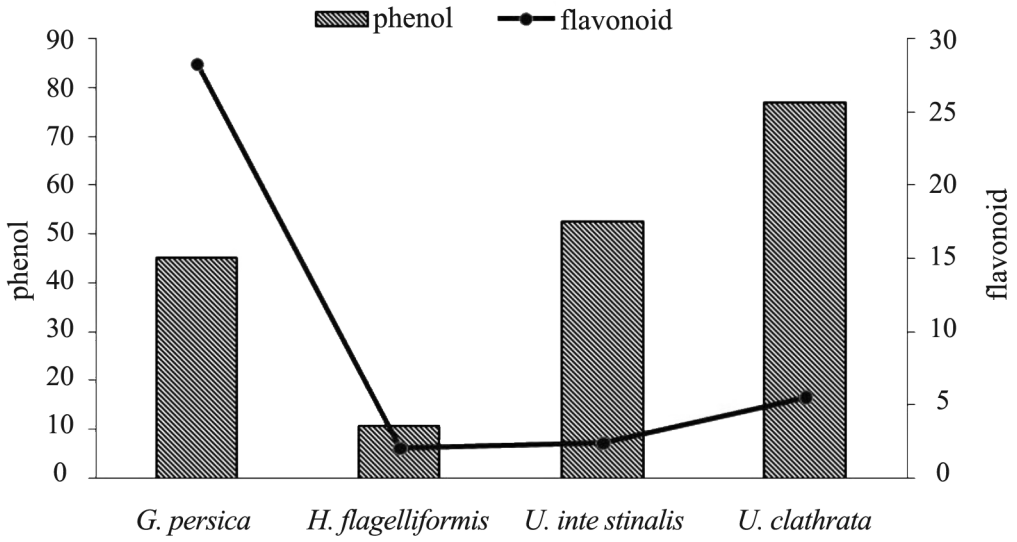

Fig. 2. Total phenolic content (TPC), and total flavonoids content (TFC) measurement in seaweeds.

health (Kuda et al., 2007). These polyphenolic compounds have several biological activities including antioxidant (through free radical scavenging activity), antiviral and anticancer (Chandini et al., 2008). Probably flavonoids are the significant natural phenol due to their wide range of chemical and biological activities, including antioxidant and free radical scavenging properties (Kähkönen et al., 1999). Apparently, in this work, the antioxidant activity of seaweeds could be due to phenol and flavonoid compounds. Our results are in line with several reports, for example the amount of TPC in *H. valentiae* (6.91± 0.06 mg GA/g) and *H. musciformis* (9.84± 0.03 mg GA/g) (Chakraborty et al., 2013), and *Gracilariopsis tenuifrons* (20.4± 0.13 mg GA/g) and *Gracilaria* sp. (3.4 to 5.0 mg GA/g) (Zubia et al., 2007), are lower than that in *H. flagelliformis* (10.7± 0.02 mg GA/g) and *G. persica* (45.1± 0.01 mg GA/g) in present study. According to the Table 3 and Figure 2, the phenolic content in the *U. clathrata* and *U. intestinalis* was significantly high ($p<0.05$), compared with other species. In general, the higher total phenolic content resulted in higher antioxidant capacity.

The ability of marine algae to produce secondary metabolites of potential interest has been extensively documented (Cabrita et al., 2010). Previous studies demonstrated that, antibacterial activity depends on algal species, the efficiency of the extraction method, and the resistance of the tested bacteria (Seenivasan et al., 2010; Soleimani et al., 2018). Data obtained in this work indicated n-hexane was the most effective solvent for the extraction of the bioactive compounds followed by ethyl acetate. Furthermore, *U. clathrate* was the most effective seaweeds against tested bacterial species, followed by *U. intestinalis*, *G. persica*, and *H. flagelliformis*. Present results obtained by *U. clathratea* re in agreement with those earlier studies (Pushparaj et al., 2014; Shukla et al., 2014). Ethyl acetate extraction has been reported to result in extracts with higher antibacterial activity than methanol ether, while few other reports indicated n-hexane as better extraction than ethyl acetate and methanol (Pushparaj et al., 2014). Our findings are in agreement which reported earlier extracting antibacterial substances such as hydroquinones, sesterpenoids, phenols, brominated phenols and polyphenols from some Chlorophyceae, Phaeophyceae and Rhodophyceae species (Hu et al., 2015).

Brine shrimp (*A. salina* and *A .franciscana*), which are sensitive to toxic and culture quickly, could be apply as a model organism for an elementary and quick screening of the insecticidal activity (Du et al., 2017; Wang et al., 2011). Iran has a rich algal flora in the coastal and inshore waters of Persian Gulf. Among the all seaweed extracts examined for brine shrimp's cytotoxicity, *G. persica* was found effective and showed considerable activity that its LC50 was 125 µg. Several diterpenes from some red and green seaweeds exhibit significant cytotoxicity that have been reported earlier (Kladi et al., 2014). *U. intestinalis* in this work have shown *cytotoxic activity* with LC50= 298

µg. Alcoholic extracts of *U. fasciata* and *U. lactuca* exhibited antiviral and anti-implantation activities (Mosaddegh et al., 2014). *U. fasciata* has been reported to produce a novel sphingosine derivative with antiviral activity (Parsaeimehr and Chen, 2013). Seaweeds exhibit a different type and level of fatty acids and many of which possess potential bioactivity (Pirian et al., 2017; Pirian et al., 2020). Several cytotoxic compounds such as fucoidans, laminarians, and terpenoids recognized by anticancer, antitumor and antiproliferative properties are reported to be abundant in seaweeds (Selim, 2012). These compounds could be further explored as novel leads to cancer chemoprevention and complementary chemotherapy and necessitates further investigation (Vinayak et al., 2011).

This study demonstrates that the marine macroalgae found in the selected areas of Persian Gulf are rich in bioactive components. Phenolic and flavonoid contents are present in significant quantities, which suggest the species can be used in medicine or cosmetic industry. The current study implies that methanol extraction of green seaweeds may deliver considerable antioxidant, antibacterial, and cytotoxicity benefits which may provide a basis for various pharmaceutical applications.

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