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Bioactivity assessment of selected seaweeds from the Persian Gulf, Iran

Soolmaz Soleimani[1], Kiana Pirian[2], Zahra Zarei Jeliani[1], Mitra Arman[3], Morteza Yousefzadi[4*]

[1] *Young Researchers and Elite Club, Bandar Abbas Branch, Islamic Azad University, Bandar Abbas, Iran.*
[2] *Young Researchers and Elite Club, Hamedan Branch, Islamic Azad University, Hamedan, Iran.*
[3] *Department of Biology, Payam Noor University (PNU), P.O. Box 19395-3697, Tehran, Iran.*
[4] *Department of Marine Biology, Faculty of Marine Science and Technology, University of Hormozgan, P.O. Box 3995, Bandar Abbas, Iran.*

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Abstract

Marine algae are known to produce a wide variety of bioactive compounds to be used in pharmaceutical industries. This study aimed to investigate antioxidant and antimicrobial activity, as well as toxicity of algal extracts from some selected seaweeds including *Sargassum angustifolium, Cystoseria myrica* (Phaeophyta) and *Acanthophora muscoides*, *Chondrophycus papillosus* (Rhodophyta). The n-hexane extract of *Sargassum angustifolium* showed potent toxicity against *Artemia franciscana*. Moreover, the ethyl acetate and n-hexane extracts of *Sargassum angustifolium* showed higher reducing power and total antioxidant capacity, respectively. In antimicrobial aspect, the seaweed extracts had the highest antibacterial effect against gram-positive bacteria, *Bacillus subtilis*, and *Staphylococcus aureus*. This study revealed the potential toxicity, antioxidant and antimicrobial activity of the studied seaweeds from the Persian Gulf, which make them suitable candidate as ingredients in pharmaceutical industries.

1. Introduction

There are many kinds of reactive oxygen species (ROS), such as hydrogen peroxide, hydroxyl radical and singlet oxygen. Although ROS at physiological concentration may be essential for normal cell functions, the excessive amount of ROS can damage cellular components such as lipids, protein, and DNA (Circu and Aw, 2010; Zhang *et al.*, 2017). The free

* **Corresponding Author, E-mail**: Morteza110110@gmail.com

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radical theory of aging proposes that the damage produced by the interplay of ROS with cellular macromolecules results in cellular senescence and aging (Wickens, 2001). Most organisms possess antioxidant defense and repair systems to protect them against oxidative damages. However, these systems are insufficient to exclude damages entirely. Therefore, it is essential to extend effective antioxidants to protect the human body from free radicals. Recently, several natural polysaccharides and their formatives have been displayed to possess potent antioxidant activities and potential applications as antioxidants (Jiang *et al.*, 2013; Mahapatra and Banerjee, 2013).

Marine species are increasingly viewed as a significant source of food and health components (Mazarrasa *et al.*, 2014). Global seaweed usage is a multibillion-dollar industry that is enhanced at an annual rate of 7.5% in response to the growing request for food and healthy ingredients (van Hal *et al.*, 2014). In the last few years, natural antioxidants from plant and animal sources have been actively surveyed as substitutes for synthetic antioxidants currently used as food additives. This recent interest in natural antioxidants as food additives has enhanced partly because of the constraint in synthetic antioxidant usage in the food industry due to their long-term toxicological effects, including carcinogenicity (Aruoma *et al.*, 1997; Bandonien *et al.*, 2000; Ito *et al.*, 1986).

Fucoidan is offered to have biological activities including anticoagulant, antithrombotic, antioxidant, anticancer and anti-inflammatory properties (Morya *et al.*, 2012). The biological activity of fucoidan is associated with its structure especially sulfate groups dependent to the fucose monomer of fucoidan (Yu *et al.*, 2014). Phenolic compounds found in brown and red seaweeds are known as phlorotannins which have many biological activities containing antioxidant, angiotensin-I-converting enzyme (ACE-I) inhibition, bactericidal, and anticancer activity (Li *et al.*, 2011).

Seaweeds tissues are the determinate provenance of compounds which display antitumor and molluscicidal activities (Rapado *et al.*, 2011; Wu and Bao, 2013). The lethality assay handling the microcrustacean *Artemia salina* is widely used as a screening method for the plant compounds that display cytotoxicity against human tumor cell lines, and also for ecotoxicity tests in aquatic environment (Anderson *et al.*, 1991; Meyer *et al.*, 1982; Nunes *et al.*, 2006). Macroalgae are available, safe, cheap, and due to their bioactive properties, with positive effects on human health have received considerable attention. Seaweeds distribution and possible of their cultivation on coastlines of Hormozgan provinces have already been studied (Jeliani *et al.*, 2017; Kokabi and Yousefzadi, 2015), and spite of some scattered studies in this area are not completely known yet (Kokabi *et al.*, 2013; Zarei Jeliani *et al.*, 2017). In the present study, the toxicity, antioxidant and antibacterial, activity of brown (*Sargassum angustifolium*, *Cystoseria myrica*) and red (*Acanthophora muscoides, Chondrophycus papillosus*) seaweeds from the Persian Gulf of Iran were evaluated.

2. Material and methods

[DOR: 20.1001.1.23222751.1396.7.3.3.2]

2-1. Reagents

Ascorbic acid (Vit C) and ferrous chloride were purchased from Sigma-Aldrich. All the other reagents were obtained from Merck Chemical Co. and all the chemicals used in the experiments were of analytical grade.

2-2. Sampling

Algal samples were collected from the intertidal zone of Bandar Abbas (N27○9.56'E56○14.26'), Bandar Lengeh (N26°33.46'E54°53.57') and Asaluyeh (N27°28.82'E52°35.86') from the Persian Gulf and transferred to the laboratory.

2-3. preparation of extracts

The studied seaweeds were identified using standard keys (Kokabi and Yousefzadi, 2015; Sohrabipour and Rabei, 2008). The voucher specimen of the algae (Voucher nos. 3020, 3021, and 3022) was deposited at the research center of agriculture and natural source of Hormozgan, Bandar-Abbas. The collected samples were washed in seawater to remove sand and all epiphytes, rinsed with distilled water and dried at room temperature. The dried samples were grounded to a fine powder and kept in 4^{o}C for further analysis.

The n-Hexane, ethyl acetate, and methanol extracts of all seaweeds were prepared. The first extraction of each ground sample was soaked in hexane while shaking for 24h at room temperature. Then, the extract was separated from the residue by filtration through Whatman No. 1 filter paper and Ethyl acetate was added to the residue for 48h. After that, the sample was 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^{o}C using a rotary evaporator (Strike 102, Italia) and kept in –4^{o}C until the experiment commenced.

2-4. Evaluation of bioactive compounds

2-4-1. Total Phenolic Content (TPC)

The amount of total phenolic compounds in the crude extracts was determined using the Folin-Ciocalteau phenol reagent (Singleton and Rossi, 1965), and was expressed as mg Gallic acid equivalent per gram dried seaweed (mg GAE/g DW).

2-4-2. Total Flavonoid content (TFC)

The TFC was determined by a colorimetric method described by Bahorun (1996) (Bahorun *et al.*, 1996). TFC was described as mg quercetin equivalents (QE)/ g DW.

2-5. Determination of antioxidant activity

2-5-1. Reducing power assay

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The reduceing power was determined according to the procedure described by (Oyaizu, 1986). Briefly, 0.4 mL of sample solution (dissolved in 99% DMSO) with concentration of extract (3 mg/mL) was mixed with 1 mL of phosphate buffer (0.2 M, pH 6.6) and 1 mL of potassium ferricyanide (1%w/v). The mixture was incubated at 50 °C for 20 min. 1 mL of trichloroacetic acid (TCA, 10%w/v) was added and mixed with 1 mL of distilled water and 0.2 mL of ferric chloride (0.1%,w/v). The absorbance of this mixture was recorded spectrophotometrically at 700 nm. Increased absorbance of the reaction mixture indicated increasing reducing power. The analyses were performed in triplicate. Ascorbic acid was used as the standard.

2-5-2. Total antioxidant capacity assay (TAC)

The antioxidant capacity of the seaweed extracts was evaluated by the phosphomolybdenum method (Prieto *et al.*, 1999). The assay is based on the reduction of Mo (VI) to a green phosphate complex of Mo (V) at acid pH by antioxidants ability of extracts (Concentration of extracts= 3mg/ml). Briefly, 0.1 mL of the sample at a concentration of 1.5 mg/ml was combined with 1 mL of reagent solution (0.6 M sulfuric acid, 28mM sodium phosphate, and 4mM ammonium molybdate). In the case of the blank, DMSO was used in place of extract. The tubes were incubated at room temperature for 15 minutes, after that the absorbance of the solution was measured at 695 nm using a spectrophotometer against blank. The antioxidant activity was expressed as an equivalent of Ascorbic acid (mg ASA/g DW.).

2-6. Cytotoxicity assay by *A. salina, A. franciscana* bioassay

The cytotoxicity assay was performed using the method of (Lincoln *et al.*, 1996) With *Artemia salina* and *Artemia franciscana*. Cysts were hatched in 1000 mL flasks, containing seawater treated as indicated in the Seawater section, with continuous aeration, under continuous light exposure (fluorescent light was used, intensity 100 µE/m2/s) and at a temperature of 30±1◦C. After hatching, nauplii with age below 24 h were transferred to another container with treated seawater and maintained during 24 h at 25±1◦C, with a photoperiod of 16 h L/8 h D. These organisms were used as test organisms. Test protocol using 10 nauplii per microplate well. Tests were conducted in a photoperiod (16 h L/ 8 h D) and temperature (25±1◦C) controlled room. Stock solutions of algae extracts were prepared in DMSO. Test concentrations used were between 1000- 125 µg/mL algae extracts. Treated seawater and DMSO used as a control. Three replicates per treatment were used. The test is therefore focused on mortality of brine shrimps versus extracts concentration in time. The number of dead animals in each treatment was recorded at 24 h intervals.

2-7. Anti-microbial screening

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The anti-microbial activity of the test of the seaweed extracts was determined by the disk diffusion method outlined by NCCLS (1997). Briefly, 0.1 ml of a suspension of a test micro-organism (108 cells/ml) was spread over Mueller-Hinton Agar (bacteria) plates. Immediately after the spreading, a sterile 6mm disk containing 20 µL (neat) of the seaweed extracts was placed in the center of the dish; this amount of oil was prepared so as to deliver 10 mg/mL of the parent extracts. Other plates/discs run in parallel with the standard anti-microbial agent ampicillin (at 10 mg/disc). The plates were incubated at 37 ^{0}C for 24 h for each bacterium. Diameters of zones of inhibition were then measured and reported in millimeters. Triplicate tests were carried out in all experiments.

2-8. Data analysis

The data were expressed as the mean ± SD of three replicates. The analysis was performed using SPSS 21 and Excel 2013. One-way analysis of variance (ANOVA) and Duncan's new multiple-rang test was used to determine the possible differences amongst the means. *P* values ≤ 0.05 were considered as significant differences.

3. Results and Discussion

Phenolic compounds are very important that are produced by most plants because they exert antioxidant activity by inactivating free radicals. Marine macroalgae are valuable in bioactive compounds that human used them for a long time (mainly in Asian countries) to treat some diseases (Pádua *et al.*, 2015). It is known that seaweeds are rich sources of natural antioxidant compounds. Moreover, phenolic compounds have been found in macroalgae showed excellent biological activities (Kuda *et al.*, 2007). The Folin-Ciocalteu method was applied to study the total phenolic content of the seaweeds. The total phenol and flavonoid contents of methanolic extract of various seaweeds are given in Table 1. In this study, methanolic extracts of seaweeds were rich in phenolic and flavonoid compounds. Considerable amounts of phenolic compounds was detected in *C. papillosa* (36.49±0.59 mg GA/g DW.). However, it contains minimum value of flavonoid compounds (1.2±0.02 mg QE/g DW.). According to ANOVA analyses, the red seaweed *A. muscoides* was shown the lowest phenolic and flavonoid compounds (25.87±2.86 and 1.42±0.02, respectively).

On the other hand, the maximum value of flavonoid compounds was obtained by brown seaweed of *S. angustifolium* (3.79±0.01). Cox *et al*. (2010), reported that brown seaweeds exhibited highest phenolic content as compared to other species (Cox *et al.*, 2010). *Hypnea muscifurmis* possesses low phenolic compounds (4.79±0.016 % DW.), (Alves *et al.*, 2012), but red seaweeds in this study are shown the high content of phenolic compounds. The total flavonoid content of the methanolic extract was reported 1.35 and 2.02 mg GAE/g in *Ulva lactuca* and *Sargassum wightii*, respectively, by (Meenakshi *et al.*, 2011).

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Table 1. Total Phenol Content (TPC) and Total Flavonoid Content (TFC) of seaweed samples.

seaweed	TPC (mg GA/g DW.)	TFC (mg QE/g DW.)
C. myrica	27 ± 0.81^{bc}	2.87 ± 0.02^{b}
S. angustifolium	29.2 ± 0.46^{b}	3.79 ± 0.01^{a}
A. muscoides	25.87 ± 2.86^{c}	1.42 ± 0.02^{c}
C. papillosa	36.49 ± 0.59^{a}	1.2 ± 0.02^{c}

Values are mean ± SD (n = 3).

The results of this study indicated that the n-hexane extracts of all of the seaweeds showed the highest total antioxidant capacity. Also, the methanolic extracts didn't show any antioxidant activity in *C. mayrica* and *C. papillosa* (Fig. 1). Similarly, Chandini *et al*. (2008), has reported lowest total antioxidant activity in methanolic extracts of three brown algae (Chandini *et al.*, 2008). In fact, it is now clear that preparations process and solvents used for extraction also have a dramatic effect on the chemical changes, that they make different pieces of information (Yuan *et al.*, 2005).

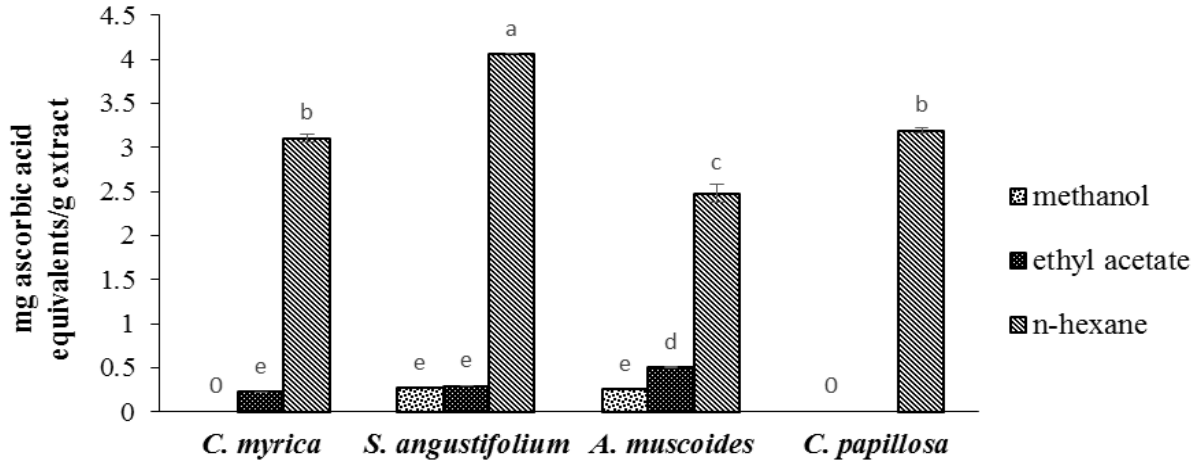

Fig. 1. Total antioxidant capacity (mg ascorbic acid equivalents/g extract) of three extracts obtained from seaweed samples (Concentration of extracts used=3mg/ml) (n=3) and values with different letters are significantly different ($p<0.05$).

The results of the reducing power assays of this study indicated that only ethyl acetate extract of *S. angustifolium* had higher reducing power than ascorbic acid (Fig. 2). It can be due to the existence of fucoxanthin, phyllo pheophytin, phlorotannins and fucoidan as antioxidant compounds in brown algae (Holdt and Kraan, 2011). Also, it was found that the ethyl acetate extracts were the best source of antioxidant compounds in compared to the other solvents. Despite algae species, solvents used for extraction can be the effect on antioxidant activity. A necessity for antioxidant activities in algae is implicit as intertidal organisms require protection against UV irradiation (Swanson and Druehl, 2002). Apparently, in this study, the antioxidant activity of seaweeds could be due to phenol and flavonoid compounds.

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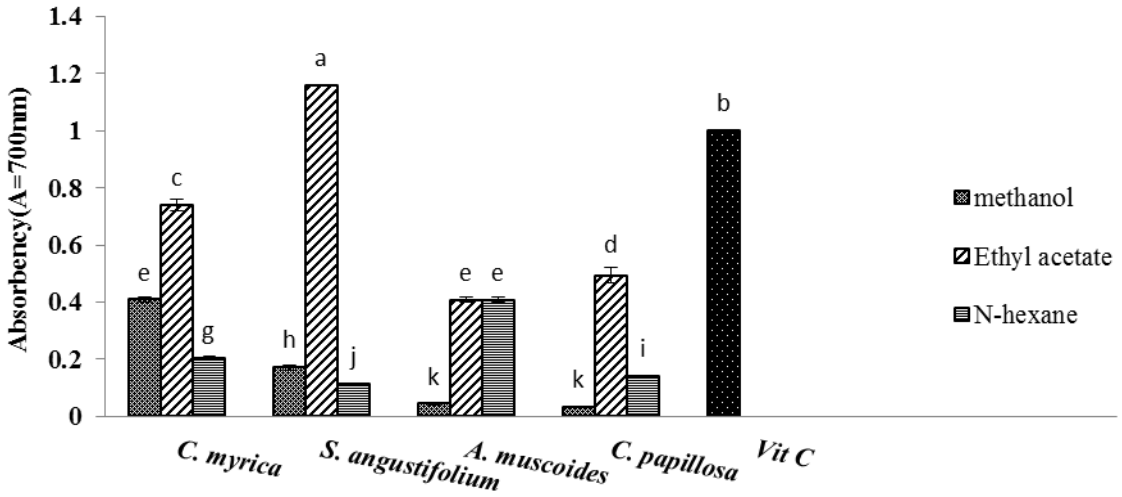

Fig. 2. Reducing power activity of three extracts from seaweed samples at the concentration of 3 mg/ml. Values are mean±SD (n=3) and values with different letters are significantly different ($p<0.05$). Vit C= ascorbic acid.

The brine shrimp cytotoxic assay is considered to be a convenient research for primary evaluation of toxicity, the discovery of fungal toxins, heavy metals, pesticides (Du *et al.*, 2017; WANG *et al.*, 2011). It can as well as be extrapolated for cell line toxicity and antitumor activity (Anderson *et al.*, 1991). Cytotoxicity assay on both species brine shrimp (*Artemia salina*, *Artemia franciscana*) showed that the mortality was directly relevant to the concentration of the different extracts of seaweeds tested.

Total four seaweeds, two belong to Phaeophyta (brown algae), two other belong to Rhodophyta (red algae) were screened for the cytotoxic activity. All of the extracts of four seaweeds showed LC50 value below 1000 µg/mL against *A. salina* and *A. franciscana*, except n-hexane extract of *S. angustifolium*, the methanol extract of *C. myrica*, ethyl acetate extract of *C. papillosus* against *A. salina* and methanol extract of *C. papillosus* against *A. franciscana*. Ethyl acetate extracts of four seaweeds against *A. franciscana* demonstrated more activity.

Table 2. LC50 values (µg/mL) of seaweed extracts in brine shrimp toxicity assay by *A. salina* and *A. franciscana.*

Extraction	Methanol		Ethyl acetate		N-hexane	
	A. salina	***A. franciscana***	***A.salina***	***A. franciscana***	***A.salina***	***A. franciscana***
S. angustifolium	986±0.3	781±0.2	980±0.1	245±0.2	>1000	>125
C. myrica	>1000	357±0.3	781±0.1	223±0.1	877±0.3	769±0.1
A. muscoides	291±0.1	462±0.2	281±0.1	260±0.2	532±0.2	876±0.1
C. papillosus	>1000	>1000	>1000	246±0.2	403±0.1	478±0.2

Values shown are means ± SD (n = 3)

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The extracts of seaweed were tested for cytotoxicity versus *A.salina* and *A. franciscana* larvae. The brine shrimp assay is considered as a stable indicator for the elementary evaluation of toxicity. As displayed in Fig. 3 cytotoxicity of the different extracts of seaweed increased by increasing concentration of samples (125-1000 µg/mL). Moreover, highest cytotoxicity in a concentration of 1000 µg/mL of n-hexane extract of *S. angustifolium* against *A. franciscana* and lowest in a concentration of 125 µg/mL of ethyl acetate *C. myrica* against *A.salina* observed. All in all, the graph in Fig. 3 shows that all extracts of seaweed have high cytotoxicity against *A. franciscana* to *A. salina*.

Cytotoxic property by plant material is due to the attendance of antitumor compounds (Manilal *et al.*, 2009). Many of the secondary metabolites produced by the marine red algae are well known for their cytotoxic property. As noted by Harada and Kamei, the extract from a red alga, *Amphiroa zonata* displays potent cytotoxicity to human leukemic cell line (Harada and Kamei, 1997). El-Baroty *et al*. display the cytotoxic activities of powdered *Asparagopsis taxiformis* and its water extract on *Daphna magna* (El-Baroty *et al.*, 2007). Sreejamole and Greeshma display the brine shrimp cytotoxic activities of ethanolic extract of red alga *Gracilaria corticata* (Sreejamole and Greeshma, 2013). Ayesha *et al* showed cytotoxicity of ethanol extracts of seaweeds that among the seaweeds of Phaeophyta group (brown seaweed) attracted the cytotoxic activities (Ayesha *et al.*, 2010). Many investigations have revealed that bioactive compounds containing fucoidans, terpenes, stypoldione, sterols, polyunsaturated fatty acids and phenolic compounds have anticancer and cytotoxic activity (Carte, 1996; Gerwick, 1994; Kim *et al.*, 2011; Manilal *et al.*, 2009). The present study supports that brine shrimp bioassay as a dependable method for the assessment of bioactivity of seaweeds and lends support for their use in pharmacology.

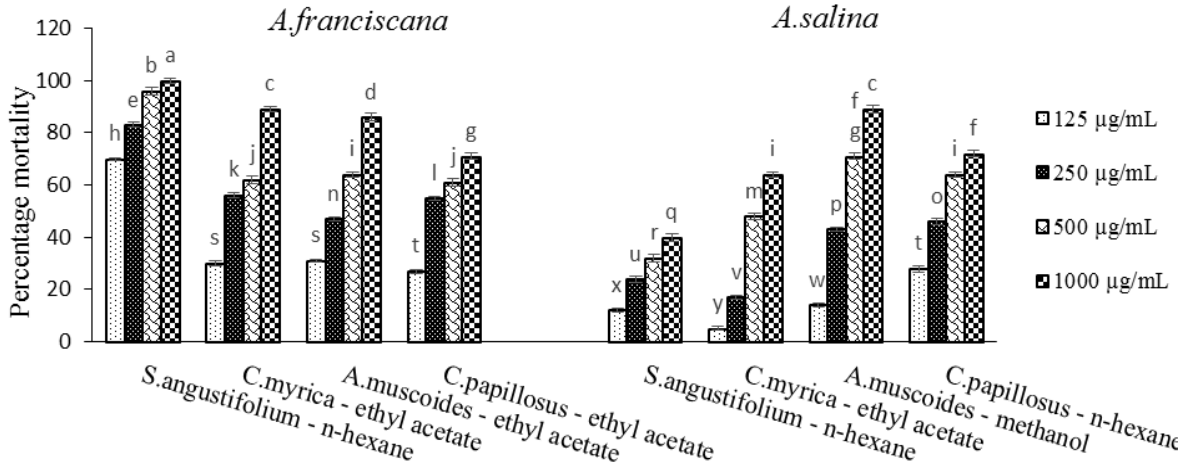

Fig. 3. Comparison of extracts of seaweeds, which possessed more toxicity between the both of *A.salina* and *A. franciscana* larvae. Values are mean±SD (n=3) and different letters are significantly different ($p<0.05$).

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Seaweeds represent a high level of fatty acid variety and many of which have potential bioactivity (Ara *et al.*, 2005). These compounds could be the further prospect as new leads to cancer chemoprevention and supplementary chemotherapy and necessitate further investigation (Ramasubburayan *et al.*, 2015).

The capability of marine algae to produce secondary metabolites of potential interest has been extensively documented (Cabrita *et al.*, 2010). According to earlier reports, anti-bacterial activity appertains on algal species, the efficiency of the extraction method, and the resistor of the tested bacteria (Seenivasan *et al.*, 2010). Results of the evaluation of the anti-microbial properties of the seaweed extracts, using a disk diffusion method, are shown in Table 3. Inhibition zones (IZ) showed a variability of inhibition among the tested bacteria. The results indicated that, in compare to Ampicillin, the n-hexane and methanol extracts of *S. angustifolium* exhibited stronger anti-microbial activity against *S. marcescens* (11±0.3) and *P. aeruginosa* (11±0.5), respectively. The terms strong, moderate, etc. to describe activity were isolate from a scaling method reported by Baron and Finegold (1990), wherein IZ were used to reflect potency of a test agent, i.e. weak (< 7), moderately active (7–14); highly active (> 14).

The seaweed extracts were quite effective against the growth of Gram-positive *Bacillus subtilis* and *Staphylococcus aureus* and Gram-negative *Salmonella typhi*. The data showed that *Bacillus subtilis* were the most sensitive of the micro-organisms tested, with IZ values of 12±0.4, 11±0.5. Of the test pathogens, *Pseudomonas aeruginosa* and *Escherichia coli* were the most resistant to effects of the seaweed extracts. Evaluation of the anti-microbial activity of the seaweed extracts revealed that the inhibitory activity with brown seaweeds was comparable to that of red seaweeds. In comparison, the IZ values for the standard antimicrobial ampicillin ranged from 10–19 overall.

Data obtained in the present study display that, ethyl acetate and n-hexane were the most effective solvent for the extraction of the bioactive compounds compared with methanol. These results are in the settlement with those earlier reports (Isnansetyo *et al.*, 2003; Meyer *et al.*, 1982; Saeidnia *et al.*, 2009; Williamson and Carughi, 2010). A good number of authors (Amico *et al.*, 1981) have documented the antimicrobial potency of organic extracts and of some compounds isolated from the marine algae of the genus of *Sargassum* such as terpenes (Culioli *et al.*, 2000; Demirel *et al.*, 2009). From our results, it seems that the antibacterial actions of the organic extracts were more pronounced with brown algae is the best actives. Researchers reported that the seaweed extracts are reliable for its activity against Gram (+) bacteria, especially *Bacillus subtilis* and *Staphylococcus aureus* and there is some reportage about the antimicrobial activity of seaweeds from the Aegean Sea, Turkey (Ozdemir *et al.*, 2006; Pesando and Caram, 1984). The previous reports showed that the algal extracts were commonly more effective against Gram (+) than Gram (–) bacteria, maybe due to the more complex structure of the cell wall of Gram (–) bacteria. Antibacterial activity found by other authors in Mediterranean seaweeds was considerably larger; it ranged between 13% (Kolsi *et*

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al., 2015) and approximately 50% (Muñoz Ochoa, 2016; Oumaskour *et al.*, 2012) of the species tested.

The diversity in results for antimicrobial activity in these marine algae shows the involvement environmental factors in the metabolism of algae and considerable way affects the attendance of such bioactive compound responsible for these activities. Finally, we conclude that seaweeds from the Persian Gulf coast are potential sources of bioactive compounds and should be surveyed from natural antibiotics. This study has shown that the production antibacterial substance by seaweed is an orderly incidence among those found on the coast of the Persian Gulf. Biochemical analyses are currently undertaker to characterize the structure and nature of these compounds.

4. Conclusions

Seaweeds possess a number of biodynamic compounds of food and therapeutic value. These compounds are providing valuable ideas for the expansion of new drugs against cancer, microbial infections and antioxidant. Many of these secondary metabolites biosynthesised by the marine plants are well known for their cytotoxic property. The results of this study clearly display that the seaweed extract of *S.angustifolium* is a strong anti-microbial mixture that also possesses antioxidant and cytotoxic activities against two types of brine shrimp (*A.salina*, *A.franciscana*). We hope that the present results will process a starting point of research aimed at exploiting new natural pharmaceutical substances present in the extracts of the species prepared from the coast of the Persian Gulf.

Acknowledgments

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Table 3. Anti-microbial activity of the seaweed extracts.

Micro-organisms	*S.angustifolium* [a]			*C. myrica* [a]			*A. muscoides* [a]			*C. papillosus* [a]			Ampicilli [a]
	M	E	N	M	E	N	M	E	N	M	E	N	
B. subtilis	8±0.5	-	10±0.5	10±0.4	12±0.4	9±0.7	-	12±0.6	12±0.3	-	11±0.5	11±0.3	14±0.5
S. aureus	9±0.5	10±0.5	11±0.3	10±0.6	9±0.5	10±0.8	-	10±0.4	11±0.4	8±0.6	10±0.6	10±0.7	13±0.4
S. flexneri	8±0.5	10±0.4	8±0.9	9±0.3	8±0.4	10±0.5	10±0.6	8±0.6	7±0.6	7±0.8	9±0.7	8±0.5	13±0.6
S. typhi	11±0.8	8±0.5	9±0.6	8±05	10±0.3	9±0.4	8±0.5	10±0.7	7±0.7	8±0.8	10±0.6	7±0.4	19±0.5
S. marcescens	9±0.6	9±0.8	11±0.3	9±0.4	8±0.6	9±0.3	-	8±0.4	8±0.6	-	8±0.5	-	10±0.4
P. aeruginosa	11±0.5	-	-	9±0.6	-	9±0.7	-	-	8±0.4	-	-	-	10±0.6
E. coli	-	-	9±0.6	8±0.4	8±0.5	-	-	8±0.6	8±0.8	-	8±0.7	8±0.6	12±0.5

Results (shown as mean±SD) were obtained from three independent experiments, each performed in duplicate.
M= methanol of extract, E= ethyl acetate of extract, and N= n-hexane of extract.
[a] Tested at 10 mg extracts/disc, and 10 mg ampicillin/disc.
Inhibition Zone includes diameter of disc (6 mm).
- = Inactive.

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