

***In vitro* effects of *Sargassum latifolium* (Agardeh, 1948) against selected bacterial pathogens of shrimp**

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Abstract

During the last decade, shrimp aquaculture has developed rapidly in many developing countries. Antibiotic resistance is a major problem in shrimp aquaculture. Seaweeds are considered as potent source of bioactive compounds that are able to produce biological activities. The aim of this study was to evaluate the antibacterial activity of ethanolic and chloroformic crude extracts of the brown algae, *Sargassum latifolium* derived from Persian Gulf waters. The extractions against shrimp selective pathogen bacteria including *Vibrio alginolyticus*, *V. parahaemolyticus* and *V. harveyi* were used by disk diffusion agar method. Results revealed that all mentioned bacteria were sensitive to both ethanolic and chloroformic crude extracts of *S.latifolium*. The minimum inhibitory concentration of chloroformic extract of *S. latifolium* against the bacteria was performed by tube dilution technique. MICs were 5, 10 and 10 mg ml⁻¹ for *V. alginolyticus*, *V. parahaemolyticus* and *V. harveyi*, respectively while minimum bactericidal concentrations (MBC) were 5, 15 and 20 mg ml⁻¹. This study recommended that *S.latifolium* isolated from Persian Gulf waters has antibacterial activity potential against shrimp pathogen bacteria.

Keywords: Antibacterial activity, *Sargassum latifolium*, Seaweed extract, *Vibrio*, Persian Gulf

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Introduction

During the last decades, shrimp aquaculture has rapidly expanded in many developing countries (FAO, 2006). Shrimp culture has started in Iran in the 1990's in Bushehr and extended to other areas besides the Persian Gulf in southern Iran (Kakoolaki, 1997). Massive mortality has been reported from hatcheries due to diseases (Afsharnasab et al., 2007; Kakoolaki et al., 2011; Pazir et al., 2011). The origination of major shrimp mortalities is virus but the opportunistic bacterial infections also causing serious losses in marine shrimp hatcheries (Lightner and Redman, 1998). Antibiotics and chemicals were being used in some hatcheries without proper scientific investigation. The environmental pollution and development of antibiotic-resistant pathogens were being then raised (Karunasagar et al., 1994). Antibiotic therapy in shrimp hatcheries and farms has been used for many years. The antibiotic resistant bacteria in shrimp hatcheries and farms is a worldwide problem in aquaculture that has caught the attention of many researchers (Tsoumas et al., 1989; McPhearson et al. 1991; Song and Sung, 1993; Hektoen et al., 1995; Mahasneh et al., 1995; Herwing et al., 1997; Rahim et al., 1998; Miranda and Zemelman 2002).

It has been reported that luminous strains of *Vibrio harveyi* and *V. splendidus* isolated from shrimp larvae are resistant to some antibiotics such as kanamycin, penicillin, erythromycin and streptomycin (Baticados et al., 1990). The use of oxytetracyclin has caused an increase of bacterial resistance in shrimp farms (Nash et al., 1992) and also increasing resistance

to chloromphenicol has been reported in shrimp hatcheries in Ecuador and the Philippines (Baticados and Padihare, 1992). The occurrence of antibiotic resistance of pathogens in aquaculture necessitates new efficient antibiotics to treat maricultured species (Braithwaite and McEvoy 2005; Bansemir et al., 2006). A large variety of seaweeds has been assayed for antimicrobial activity worldwide and has showed different results due to the kind of seaweeds, season and area of collection, solvents and so on. Hornsey and Hide (1974) demonstrated that crude extracts of more than 150 seaweed species have antibacterial activity. Some efforts have been done to find new antibiotics from marine organisms such as macroalgae to control bacterial pathogens (Bansemir et al., 2004; Immanuel et al., 2004; Vineela and Elizabeth 2005). Many types of seaweed (macroalgae) have potent defense- activity against microbial epibionts, e.g. bacteria, viruses and fungi (Hellio et al., 2001; Bansemir et al., 2006). Concerns over human health and environmental safety due to some chemicals have prompted increasing interest in more "natural-green" alternatives such as antibiotics. Seaweeds are considered as potent source of bioactive compounds that are able to produce different important secondary metabolites described with great biological activities. Researchers have shown that these compounds have cytostatic, antiviral, anthelmintic, antifungal and antibacterial activities (Newman et al., 2003; Toney et al., 2006; Taskin et al., 2007). Srinivasa Rao and Parekh (1981) revealed that crude

extracts of seaweeds from India waters were active only against Gram positive bacteria. Toney et al. (2006) examined antibacterial effects of 11 seaweeds from the Mediterranean Sea against *E. faecalis*, *P. aeruginosa*, *E. coli* and yeast and they found that only 6 seaweeds had antibacterial effects. But only a few studies were conducted about the effects of mentioned compounds against aquatic pathogens (Mahaseneh et al., 1995; Imanuel et al., 2004; Chouhury et al., 2005).

The aim of this study was to evaluate the antibacterial activity of a brown seaweed *Sargassum latifolium* obtained from Persian Gulf waters against three shrimp pathogen- bacteria named *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus*.

Materials and methods

63 Petri dishes including three groups (2 extractions and 1 antibiotic) along with 3 replicates were used against 3 selected bacteria to test the hypothesis of the study. The groups were 1-*Sargassum latifolium* in two form of extractions named ethanolic extraction and chloroformic extraction as three concentrations of 50,200 and 500 mg^l⁻¹ 2- oxytetracyclin.

Sargassum latifolium were collected from a marine rocks area of the Persian Gulf coastal line named Alafdoon in north of Bushehr port in southern Iran during low tide November 2008.

Algal samples cleaned of epiphytes, debries and extraneous matters were removed along with the necrotics parts. The surface of algal samples were washed carefully with sea water and then in fresh water. Seaweeds were dried under

a shade for 5 days and cut to small pieces. Afterwards they formed powder in a mixer grinder. 30 g of that mixed in ethanol (300ml) and in chloroform (300ml) separately for 72 hr at room temperature. Then the different extracts were filtered through Whatman filter paper No. 1 and concentrated under vacuum condition on a rotary evaporate at 45°C to get ethanolic and chloroformic crude extracts. They were then stored at -20°C until use.

The *Vibrio spp.* including *V. harveyi*, *V. parahaemolyticus*, and *V. alginolyticus* were used in this study. Bacterial strains were cultured overnight at 30 °C in Tryptic Soy agar (TSA, Merk, Germany) with supplement of 2% NaCl (Merk,Germany) at 4°C until use.

The method of disk diffusion was done for screening antibacterial activity of the ethanol-chloroform extracts. The minimal inhibitory concentrations (MICs) of the samples against the test microorganisms were determined by the tube dilution method, and minimum bactericidal concentration (MBC) was assayed according to Fazli et al. (2007).

The agar diffusion method was assayed as described by Murry et al. (1995) previously with some modifications. A 18-hr-old culture loopful isolated from the selected organisms that were previously diluted to 0.5 index of McFarland turbidity standard (in order to produce approximately 1.5×10^8 colony forming units (cfu) per ml) was introduced to Muler Hinton agar (MHA, Merck, Germany) along with a supplement of 2% NaCl (Merck, Germany). The ethanol-chloroform extracts were dissolved in DMSO (dimethyl sulfoxide) to perform

different concentrations from 50 mg/ml to 500mg/m. They were sterilized by filtration by 0.45 µm Millipore filters and then discs (6 mm in diameter) were impregnated with those and finally placed on the MHA agar plates. Negative controls were prepared using DMSO and Oxytetracyclin (20µg/disc) as positive reference standards to determine the sensitivity of each bacterial species. The inoculated plates were incubated at 25 °C for 24 hr. Antibacterial activity was evaluated by measuring the zone of inhibition against the test organisms.

The minimum inhibitory concentration (MIC) of the ethanol-chloroform extracts was determined for each of the selected organisms as described by Doughary and Manzara (2008) with some modification, at 100, 80, 50, 30, 20, 15, 10 and 5 mg ml⁻¹. To achieve these concentrations, 1 ml of different concentrations of the extracts including double strength of the concentrations (200, 160, 100, 60, 40, 30, 20 and 10 mg ml⁻¹) in a test tube and 1 ml of nutrient broth with a supplement of 2% NaCl (Merck, Germany) were added and then a loopful of the selected organism previously diluted to 0.5 McFarland turbidity standard to produce 1.5×10^8 cfu per ml was introduced to the tubes. The procedure was repeated over the selected organisms using Oxytetracyclin. A tube containing nutrient broth was only used with the selected organisms to serve as negative control. All the tubes were then incubated at 30°C for 24 hr and then examined for growth by screening for turbidity. The minimum bactericidal concentration (MBC) of the ethanol-

chloroform extracts on the clinical bacterial isolates was performed according to Fazli et al. (2007). Briefly, 0.2 ml bacterial culture was pipetted from the highest dilution tubes that did not show any growth obtained for determination of MIC and subcultured on to MHA and incubated at 30°C for 24 hr. After incubation the concentration at which there was no single colony of bacteria was expressed as MBC (Fazli et al., 2007).

Statistical analysis

Student's *t*-test was applied to calculate the differences of diameter over inhibition zones performed in concentrations of 50 and 200 (mg ml⁻¹) in two forms of extractions (E.E. & C.E. extraction) and ANOVA test was used for the same aim between 3 groups (E.E. extraction & C.E. extraction in concentration of 500 mg ml⁻¹ and Oxytetracyclin) after exposure of each selected bacteria against them. Meanwhile ANOVA test was applied to show the differences of diameter over inhibition zones performed in the same concentrations within each group (E.E. extraction , C.E. extraction and Oxytetracyclin) while *P*-values < 0.05 were considered to be statistically significant.

Results

The average diameter of inhibition zone of chloroformic extracts of *S. latifolium* in concentration of 500 mg l⁻¹ was (n:3, 15± 1.3 mm) significantly higher (*P*≤ 0.05) than that of the same concentration of ethanolic extracts of *S. latifolium* (n:3, 12±1.2 mm) meanwhile they were significantly lower (*p*≤ 0.05) than that of

Oxytetracyclin against *V. alginolyticus*. These results were repeated for these three groups at the same concentration against two other bacteria, *V. parahaemolyticus* and *V.harveyi*. The diameter of inhibition zone of chloroformic extracts of *S. latifolium* in concentration of 500 mg l⁻¹ (n:3, 15± 1.3 mm) against *V. alginolyticus* was greater than that of same substance against *V. parahaemolyticus* and *V. harveyi* (n:3, 14±0.08 mm and n:3, 12±1.2 mm, respectively). The index of chloroformic extracts of *S. latifolium* in concentration of 200 mg l⁻¹ (n:3, 10± 0.9

mm) had no significant difference (P≥0.05) in comparison to that of ethanolic extracts (n:3, 10± 1.1 mm) against *V. alginolyticus* but showed significantly greater amounts within groups against *V. parahaemolyticus* and *V. harveyi*, respectively (n:3, 8±1.2 mm and 7.8±0.5 mm). The latter data was repeated for concentration of 50 mg l⁻¹ (Table 1). Antibacterial activity of ethanol and chloroform extracts of *S. latifolium* against the test bacteria are summarized in Table 1.

Table1: Antibacterial activity of ethanol and chloroform extracts of *Sargassum latifolium* against the selected bacteria

Bacteria	E. E. (mg ml ⁻¹) and diameter I.Z in mm (Mean±SD)			C.E. (mg ml ⁻¹) and diameter I.Z. in mm (Mean±SD)			Oxytetracyclin 20µg/disk diameter I.Z in mm (Mean±SD)
	50	200	500	50	200	500	
<i>V. alginolyticus</i>	^a 7±0.8 ^a	^a 10±1.1 ^a	^c 12±1.2 ^a	^a 8±1.0 ^a	^a 10±0.9 ^a	^b 15±1.3 ^a	^a 18±0.4 ^a
<i>V. parahaemolyticus</i>	^a 6.2±0.6 ^b	^b 9±0.7 ^b	^c 11±0.9 ^b	^a 5.9±0.4 ^b	^a 8±1.2 ^b	^b 14±0.8 ^b	^a 19.2±0.5 ^b
<i>V.harveyi</i>	^a 5.8±0.5 ^b	^b 9.2±1.1 ^b	^b 12±1.3 ^a	^a 6±1.1 ^b	^a 7.8±0.5 ^b	^b 12±1.2 ^c	^a 19.5±0.5 ^b

E. E: Ethanol extract, C. E: Chloroform extract, I.Z: Inhibition zone

The results also showed that minimum inhibitory concentration of chloroformic extract of *S. latifolium* for *V. alginolyticus*

was 5 mg ml⁻¹ and this amount (MIC) for *V. parahaemolyticus* and *V. harveyi* was 10 mg ml⁻¹ (Table 2).

Table 2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of chloroformic extracts against tested bacteria (mg ml⁻¹)

Test organisms	MIC	MBC
<i>V. alginolyticus</i>	5	5
<i>V. parahaemolyticus</i>	10	15
<i>V. harveyi</i>	10	20

Discussion

In the present *in vitro* study we observed the potent antibacterial activity of the seaweed; *S. latifolium* deriving from Persian Gulf waters against shrimp pathogen bacteria that could be applied in shrimp culture industries to control and treat such diseases as a substitute for antibiotics. Both crude ethanolic and chloroformic extracts of *S. latifolium* inhibited all of the selected bacteria. Highest antibacterial activity of chloroformic extracts of *S. latifolium* was presented against *alginolyticus* and least one of the same solvent has effect against *V. parahaemolyticus* and *V. harveyi*, exception of 50 mg/l concentration.

It is revealed that the antibacterial activity of the chloroformic extracts of *S. latifolium* is more efficient than that of ethanolic extracts against *V. alginolyticus* and *V. parahaemolyticus* at concentration of 500 mg/l and caused better halo-zones but no significant difference dealing with the antibacterial effect of two extracts against *V. harveyi* was observed in the same concentration and showed that *V. alginolyticus* is more sensitive to chloroformic extracts of *S. latifolium* in such concentration followed by *V. parahaemolyticus* and *V. harveyi*. Some findings about the efficiency of extraction

methods emphasized that methanolic extraction gives higher antimicrobial activity than n-hexane and ethyl acetate (Moreau et al., 1988; Tuney et al., 2006), while others informed that chloroform is better than methanol and benzene (Febles et al., 1995). Freile-Pelegr and Morales (2004) reported that the hexane extract has greater antibacterial activity. However the result showed the antibacterial effect of Oxytetracyclin at 20µg/disk is greater than that of two selected extracts in concentration up to 500 mg ml⁻¹.

According to our results, in contrary to *V. parahaemolyticus* and *V. harveyi*, no differences were observed between the effect of antibacterial inhibition of both ethanolic and chloroformic crude extracts of *S. latifolium* against *V. alginolyticus*. The antibacterial effect of both ethanolic and chloroformic crude extracts of *S. latifolium* against *V. parahaemolyticus* and *V. harveyi* at 50 mg/l is similar and minimum in comparison to other concentrations. Chellaram et al. (2004) observed that the extracts obtained from acetone and chloroform exhibit higher activity than that of butanol and toluene similar to our study in case of chloroform. It has been reported

that variety of solvents has different solubility capacities for different phytoconstituents (Marjorie, 1999). Imanoel et al. (2004) showed antibacterial properties of the n-butanolic extracts of four ayurvedic herbals and two seaweeds *Ulva lactuca* and *Sargassum wightii* against shrimp pathogen *Vibrio parahaemolyticus*. They reported that seaweeds (*U. lactuca* and *S. wightii*) have a good ability to inhibit shrimp pathogen producing with inhibitory zone 17.5 ± 0.81 and 16.3 ± 0.47 , respectively. These results were approximately similar to our investigation, particular at 500 mg/l concentration. Patar et al. (2008) revealed that the extract of *Sargassum sp.* was more effective against *B. subtilis* and *E. coli*, showing 18 mm and 16 mm zone of inhibition, respectively at 4000 $\mu\text{g}/100 \mu\text{l}^{-1}$ concentration in comparison to *S. aureus* showing 10 mm zone of inhibition at the same concentration. Also Choudhury et al. (2005) indicated that alcoholic extracts of marine algae can inhibit *V. alginolyticus* as a fish pathogen. The results of this work obviously revealed that the macroalgae *S. latifolium* contains bioactive compounds which are related to antibacterial activities. Results of the present study revealed that minimum bactericide concentration (MBC) and MIC for *V. alginolyticus* was the same, but MBCs for *V. parahaemolyticus* and

V. harveyi were higher than their MICs (Table 2).

In addition this study revealed that both ethanolic and chloroformic crude extracts had bacteriostatic and bactericide properties against the examined *Vibrio spp.* The results of the present study showed that macroalgae can be a good substitute for antibiotics in shrimp aquaculture.

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