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Antibacterial and antioxidant effects from seaweed, *Sargassum wightii* (Greville, 1848) against marine ornamental fish pathogens

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PEER REVIEW

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Comments

This study has described the preliminary screening of *S. wightii* extracts and their bioactivities. It can be a potential source of developing natural products. Authors have analyzed the two types of bioactivities such as antibacterial and antioxidant using common bioassays. The subject of manuscript is appropriate for the journal.
Details on Page 781

ABSTRACT

Objective: To screen seaweed *Sargassum wightii* (*S. wightii*) for bioactive natural substance against marine ornamental fish bacterial pathogens, and also study the antioxidant properties, brine shrimp toxicity effect.

Methods: Crude extract was made using three solvents (acetone, ethanol and methanol) and screened for antibacterial activity and purified by column chromatography, purified fractions obtained were tested for the activity. The 1st fraction of acetone extract showed maximum activity, this was again subjected for purification and obtained three sub-fractions also tested for the activity. Total phenols and flavonoid contents, reducing power, free radical scavenging activities (DPPH and H_2O_2) and brine shrimp toxicity were also studied using purified acetone extract followed by standard methods.

Results: The purified acetone extract showed maximum activity against eight pathogens among ten. GC-MS results revealed two major compounds such as 24-methylene cholesterol (79.9%) and methyl oleate (30.3%) which presented in higher percentage in purified extract and had highest phenols and flavonoid contents, reducing power, free radical scavenging activities, and also showed less toxicity effect. In he present study, the purified extract of *S. wightii* had potential antibacterial activity against *Aeromonas hydrophila* [(22.25±0.35) mm] and minimum activity against *Streptococcus* sp. [(10.00±0.00) mm]. The purified extract of *S. wightii* also had potential total antioxidant activity of (3.87±0.04) μg at 100 μg/mL concentration and the lowest activity was exhibited (1.52±0.01) μg at 25 μg/mL.

Conclusions: The present study concluded that the brown seaweed, *S. wightii* has potential antimicrobial and antioxidant activities, which can be used in aquaculture industry for treated bacterial diseases in infected fishes.

KEYWORDS

Seaweed, Bioactive compounds, Antibacterial activity, Silica gel column chromatography, GC-MS analysis, Antioxidant activity assays, Brine shrimp toxicity

1. Introduction

Over the past two decades, marine ornamental fish industry has undergone a significant transformation globally and market expands day by day due to their high commercial value. Marine ornamental fishes are most popular attractions worldwide due to their beauty and adaptability to live in confinement[1]. However, the success of marine ornamental fish culture and breeding depends on health status of entrant species[2]. Bacterial diseases are most common problem in fish farming and marine ornamental fishes are not exceptional for the bacterial infections caused largely by Gram-negative organisms. Over the past 20 years, various chemotherapeutics, vaccines, immunostimulants

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and probiotics have been used to treat bacterial infections in culture systems but the emergence of drug-resistant bacteria has become a major problem[3].

Natural products are used medicinally and are the vital sources for potent and powerful drugs[4]. Plants are rich in a wide variety of secondary metabolites such as tannins, alkaloids and flavonoids[5]. In India, 500 species of plants exhibit medicinal properties and they are used to control the pathogenic bacteria[6]. In addition, plant-derived phytomedicines provide a cheaper source for treatment and greater accuracy than the chemotherapeutic agents in this field[7]. Marine natural products are also used for treatment and control of bacterial diseases besides using plant extracts to reduce bacterial pathogens in culture systems[8]. There are many reports describing marine plants having more potential bioactive substances are exhibited the antibacterial, antifungal, antiviral, anti-inflammatory, antidiabetic and antioxidant activities[9].

Antioxidant compounds are important for marine ornamental fish culture to inhibit the growth of pathogenic bacteria, which reduce the organic load and toxic chemical residues in water, protect the fishes against environmental stress and offer bio-security to aquarium systems[10]. Natural antioxidants are classified as phenolic compounds (tocopherols, flavonoids and phenolic acids), nitrogen compounds (alkaloids, chlorophyll derivatives, aminoacids and amines) or carotenoids as well as ascorbic acid[11]. The brine shrimp, *Artemia salina* (*A. salina*) used as a food for larval fish in marine ornamental culture systems are also used as a bench-top bioassay for the discovery and purification of bioactive natural products. The shrimp lethality assay is based on ability to kill laboratory cultured brine shrimp nauplii[12].

Seaweeds are the excellent source of bioactive compounds and also play an important role in economy of maritime countries as a source of food, fodder, fertilizers, chemicals, drugs and various other commercial algal products such as agar-agar, algin and carrageenan[13]. The prior studies reported that the maximum yield of alginic acid present in *Sargassum wightii* (*S. wightii*) was 26.32% and 21.71%[14,15]. Sobha revealed that *S. wightii* could be considered as a good raw material for commercial extraction of alginic acid in Kerala[16]. Selvin and Lipton tested bioactivity potential of seaweeds, *Ulva fasciata* (*U. fasciata*) and *Hypnea musciformis* (*H. musciformis*), both species showed potent antibacterial, brine shrimp cytotoxicity and larvicidal activity[17]. Hanniffy and Kraan reported strong antibiotic activity against fish and human pathogens by using macro algal species *Ulva*, *Porphyra* and *Palmararia palmata*[18]. The development of antibacterial, antifungal, antiviral, antitumor, antihypercholesterolemic, anticoagulant, antioxidant, immunomodulating and immunosuppressive activities and antiulcer substances from seaweeds is still in a growing stage of research[19]. Pharmacological properties of several seaweed species are still unexplored and unidentified. By keeping the bioactive potential of seaweeds, the present study was carried out to evaluate the brown seaweed, *S. wightii* against marine ornamental fish bacterial pathogens and also analyze the antioxidant properties and brine shrimp toxicity effect.

2. Materials and methods

2.1. Collection of sample

Fresh seaweed 2 kg of *S. wightii* (Phylum: Ochrophyta, Class: Phaeophyceae) was collected from intertidal regions of Mandapam coast of Gulf of Mannar (Latitude 9°17′ N; Longitude 79°08′ E), Tamil Nadu, India and was brought to the laboratory by keeping them in plastic bags with seawater. The sample was washed thoroughly with seawater to remove epiphytes, followed by tap water and distilled water so as to remove the salts and other extraneous materials. The sample was shade dried for 15 to 20 d and ground in an electric mixer for 2 h. Finally 640 g of powdered seaweed sample was obtained and stored in refrigerator (4 °C) for further use.

2.2. Extraction of bioactive compounds

Seaweed extract was made by following the method of Manilal using three solvents[20]. The sample (600 g) was taken and weighed 200 g in three times, then the sample was soaked in 300 mL of ethanol, methanol and acetone individually. After 21 d of dark incubation, crude extracts were filtered by using muslin cloth and the filtrate extracts were concentrated by rotary vacuum evaporator (>45 °C) and then freeze-dried (-80 °C) to obtain solid residue and were stored in individual sterile glass container for further use. The percentage of extraction was calculated using the following formula:

$$\text{Percentage of extraction (\%)} = \frac{\text{Weight of the extract}}{\text{Weight of the plant material}} \times 100$$

2.3. Test organisms

Bacterial fish pathogens *viz.*, *Aeromonas hydrophila* (*A. hydrophila*), *Enterobacter aerogens* (*E. aerogens*), *Flavobacterium* sp., *Micrococcus* sp., *Pseudomonas fluorescens* (*P. fluorescens*), *Streptococcus* sp., *Vibrio parahaemolyticus* (*V. parahaemolyticus*), *Vibrio alginolyticus* (*V. alginolyticus*), *Edwardsiella tarda* (*E. tarda*) and *Proteus* sp. isolated from infected part of marine ornamental fishes was obtained from microbiology laboratory of marine ornamental fish hatchery,

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2.4. Antibacterial activity of crude compounds

Antibacterial activity against fish pathogens was performed by Chakraborthy[21]. Muller Hinton agar (Himedia, Mumbai) medium was prepared, sterilized and poured into sterile Petri dishes. After solidification, 24 h old bacterial broth cultures were inoculated by using a sterile cotton swab and the wells (5 mm size) were made on surface of the agar plate by using sterile cork borer. Antibacterial property of crude extract was tested separately for each selected pathogen. About 75 μL (2 mg of crude extract dissolved in 1 mL of dimethylsulfoxide) was placed in different wells and then allowed to diffuse for 2 h. Plates were incubated at 37 °C for 24 h and the activity was determined by measuring the zone of inhibition in diameters.

2.5. Purification and screening of purified compounds

The crude extracts were purified by using column chromatography as described by Emmanuel *et al*[22]. Activated 10 g of silica gel (230-400 mesh size) (MERCK, Germany) was packed onto a glass column with the maximum height of 30 cm using hexane solvent. The crude extracts were loaded on top of the silica gel and eluted successively with 50 mL of acetone, ethanol and methanol and obtained five fractions from each solvent extract and each fraction (10 mL) eluted time taken for 20 to 25 min. The obtained fractions were concentrated by rotary vacuum evaporator (>45 °C) and then freeze-dried (-80 °C) to get solid residue. The purified fractionated samples were screened for antibacterial activity against selected fish pathogens as discussed above.

2.6. Identification of active fraction

The five different fractions collected from each extract were screened for antibacterial activity against fish pathogens. Among them, 1st fraction of acetone extract showed a better activity against most of the pathogens compared to other fractions of acetone, ethanol and methanol. Based on these results, 1st fraction of acetone extract was again purified and three sub-fractions were obtained. Sub-fractions were concentrated by rotary vacuum evaporator (>45 °C) and then freeze-dried (-80 °C) to obtain solid residue. The samples were dissolved in dimethylsulfoxide and tested against same pathogens as discussed above. In all the cases, inhibition zone measuring 20 mm and above are considered as good, 15-20 mm are considered as moderate and below 15 mm are considered as the low activity. Potential sub-fraction was identified and subjected for gas chromatography and mass spectrometry (GC-MS) analysis for characterizing the bioactive compounds.

2.7. Characterization of bioactive compounds

2.7.1. GC-MS analysis

The potential sub-fraction (3rd fraction) of acetone extract was analyzed using an Agilent 6890 series high temperature GC-MS, fitted with auto-injector and high-temperature column (DB-5ht; 30 m×0.25 mm id×0.25 μm film thickness). GC-MS analysis was performed by adopting the method proposed by Yuvaraj *et al*[23]. The compounds were identified by comparison of their mass with NIST library and data found in literature and authentic standards.

2.7.2. Determination of total antioxidant activity

The antioxidant activity of crude and purified extract of acetone was determined by Prieto *et al*[24]. The samples were taken at different concentrations (25-100 μg/mL) and mixed with 3 mL of reagent solutions (0.6 mol/L sulfuric acid, 28 mmol/L sodium phosphate and 4 mmol/L ammonium molybdate). Reaction mixtures were incubated at 95 °C for 90 min, under water bath. Absorbances of all the sample mixtures were measured at 695 nm. Ascorbic acid is used as a standard, total antioxidant activity was expressed as the number of equivalence of ascorbic acid.

2.7.3. Total phenols content (TPC)

The phenols content in purified extract was determined according to Folin-Ciocalteu method of Antolovich *et al*[25]. To different concentrations (25-100 μg/mL) of purified extract, 2 mL of 7.5% (w/v) sodium carbonate solution was added and vortexed vigorously. After 5 min, 1 mL of 1:10 diluted Folin-Ciocalteu's phenol reagent was added and vortexed again. The same procedure was followed for the standard solution of gallic acid. All the tubes were incubated at room temperature for 30 min and the absorbance was measured at 765 nm. The TPC in extract was expressed as gallic acid equivalent.

2.7.4. Total flavonoids content (TFC)

TFC was determined by a colorimetric method described by Liu *et al*[26]. The absorbance of samples and standard against the blank was recorded at 510 nm. TFC in extract was expressed as standard quercetin equivalent.

2.7.5. DPPH radical scavenging assay

The free radical scavenging activity of purified extract was measured by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) according to the method of Zhang *et al*[27]. A total of 2 mL of DPPH (0.1 mmol/L) solution in methanol was added to different concentrations of purified extract (25-100 μg/mL),

shaken vigorously, allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm. A low absorbance of the reaction mixture indicated a high free radical scavenging activity. Sample blank and positive control was performed according to the method. Scavenging effect of DPPH radical was calculated using the following equation:

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

Where A_{sample} is the absorbance of DPPH solution and test sample, $A_{sample\ blank}$ is the absorbance of the sample only without DPPH solution. Synthetic antioxidant ascorbic acid was used as positive control.

2.7.6. Total reducing power

Reducing power of purified extract obtained from seaweed was estimated by Oyaizu[28]. Briefly, different concentrations (25-100 μg/mL) of sample was mixed with 2.5 mL of phosphate buffer (0.2 mol/L, pH 6.6) and 2.5 mL potassium ferricyanide (1%). Reaction mixtures were incubated at 50 °C for 20 min. After incubation 2.5 mL of trichloro acetic acid (10%) was added and centrifuged (650 *g*) for 10 min. From the upper layer 2.5 mL solution was mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ (0.1%). Absorbance of all the sample solutions was measured at 700 nm. Increased absorbance indicates increased reducing power.

2.7.7. H_2O_2 radical scavenging activity

The ability of seaweed to scavenge H_2O_2 was determined with slight modification[29]. Briefly, 40 mmol/L H_2O_2 was prepared in phosphate buffer (pH 7.4) and the H_2O_2 concentration was determined spectrophotometrically. Different concentrations of purified extract (25-100 μg/mL) and ascorbic acid (25-100 μg/mL, positive control) were added to 0.6 mL of 40 mmol/L H_2O_2 solution and the absorbance of H_2O_2 was determined at 230 nm after 10 min incubation against a blank solution containing phosphate buffer without H_2O_2. The percentage of scavenging of H_2O_2 was calculated using the following formula:

H_2O_2 radical scavenging activity (%)= $[(A_0-A_1)/A_0]\times100$

Where A_0 is absorbance of control, A_1 is absorbance of sample.

2.7.8. Brine shrimp toxicity assay

About 3 g of *A. salina* (Linnaeus) cysts (Sanders Great Salt Lake, Brine Shrimp Company L.C., USA) was aerated in 15 L capacity white bucket containing 12 L filtered estuarine water (salinity 28‰). The air stone was placed in bottom of the bucket to ensure complete hydration of the cysts. After 24 h incubation at room temperature (28-29 °C), hatched free swimming pink coloured nauplii were harvested and tested for toxicity assay as described by Ayesha *et al*[30].

The collected 40 numbers of *Artemia* nauplii were transferred in to Petri dishes containing different concentrations (20-100 μg/mL) of purified extract. The mortality percentage was recorded after 24 h of interval. Control group was treated without addition of sample and triplicate experiments were carried out and the results were expressed as mean±SD values. At the end of experimental period, the numbers of mobile and dead nauplii in each Petri dish was counted with a hand lens. Nauplii were considered as dead if they were lying immobile at the bottom of the Petri dishes.

2.7.9. Data analysis

All the data were expressed as mean±SD statistical analysis was calculated by One-way ANOVA (P>0.05). A statistical package (Origin 61) was used for the data analysis.

3. Results

3.1. Extraction yield and screening of crude extracts

The yield percentage of extracts of *S. wightii* was 15.2%, 14.3% and 12.8% in ethanol, acetone and methanol respectively. The crude extracts from three different solvents were screened for antibacterial activity against fish pathogens are shown in Table 1. Among three extracts, acetone extract exhibited the maximum activity against seven pathogens among ten, *A. hydrophila* (23.20±0.28) mm followed by *P. fluorescens*, *Micrococcus* sp., *V. parahaemolyticus*, *V. alginolyticus*, *E. aerogens*, *Streptococcus* sp. and the minimum activity against *Flavobacterium* sp. (10.00±0.00) mm compared to other two extracts. The zones of inhibitions made by different pathogens were non-significant (P>0.05).

Table 1
Antibacterial activities of crude extracts of the seaweed against bacterial fish pathogens.

Test organisms	Zone of inhibition (mm)		
	Ethanol extracts	Methanol extracts	Acetone extracts
A. hydrophila	14.50±0.70	0.00±0.00	23.20±0.28
E. aerogens	10.00±0.00	14.10±0.14	15.15±0.21
Flavobacterium sp.	9.00±0.00	13.40±0.56	10.00±0.00
Micrococcus sp.	15.25±0.35	16.25±0.35	19.80±0.28
P. fluorescens	18.60±0.56	18.00±0.00	20.50±0.70
Streptococcus sp.	14.65±0.49	0.00±0.00	13.50±0.70
V. parahaemolyticus	20.15±0.21	15.20±0.28	16.00±0.00
V. alginolyticus	15.40±0.56	12.00±0.00	15.10±0.14
E. tarda	14.25±0.35	14.90±0.14	12.00±0.00
Proteus sp.	15.00±0.00	13.00±0.00	11.25±0.35

Values are means of three replicate determinations±SD.

3.2. Screening of purified extracts

The crude extracts of acetone, ethanol and methanol were

purified and obtained different fractions were screened for antibacterial activity against fish pathogens are given in Tables 2, 3 and 4 respectively. The five fractions from ethanol extract were screened for antibacterial activity against fish pathogens (Table 2). Among five fractions, 1st fraction showed the maximum activity against *E. aerogens* (17.15±0.21) mm followed by *P. fluorescens*, *E. tarda*, *Micrococcus* sp., *A. hydrophila*, *Proteus* sp. and the minimum activity against *V. alginolyticus* (9.80±0.28) mm and no activity was observed against *Flavobacterium* sp. and *Streptococcus* sp. The zones of inhibitions made by different pathogens were significant ($P<0.05$).

Table 2

Antibacterial activities of five purified fractions of ethanol extract of *S. wightii* against bacterial fish pathogens.

Test organisms	Zone of inhibition (mm)				
	1st	2nd	3rd	4th	5th
A. hydrophila	13.90±0.14	0.00±0.00	10.35±0.49	12.15±0.21	11.25±0.35
E. aerogens	17.15±0.21	10.30±0.42	15.00±0.00	15.20±0.28	10.00±0.00
Flavobacterium sp.	0.00±0.00	0.00±0.00	0.00±0.00	12.00±0.00	13.15±0.21
Micrococcus sp.	15.00±0.00	13.00±0.00	10.00±0.00	10.25±0.35	13.00±0.00
P. fluorescens	15.85±0.21	9.90±0.14	12.15±0.21	15.00±0.00	14.90±0.14
Streptococcus sp.	0.00±0.00	0.00±0.00	11.20±0.28	0.00±0.00	0.00±0.00
V. parahaemolyticus	10.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
V. alginolyticus	9.80±0.28	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
E. tarda	15.25±0.35	12.00±0.00	11.10±0.14	13.30±0.42	14.00±0.00
Proteus sp.	13.00±0.00	11.25±0.35	9.25±0.35	11.90±0.14	12.30±0.42

Values are means of three replicate determinations±SD.

Table 3

Antibacterial activities of five purified fractions of methanol extract of *S. wightii* against bacterial fish pathogens.

Test organisms	Zone of inhibition (mm)				
	1st	2nd	3rd	4th	5th
A. hydrophila	16.15±0.21	14.85±0.21	13.10±0.14	14.00±0.00	12.25±0.35
E. aerogens	0.00±0.00	7.00±0.00	16.85±0.21	10.95±0.07	13.15±0.21
Flavobacteruium sp.	17.10±0.14	10.20±0.28	7.00±0.00	12.20±0.28	11.00±0.00
Micrococcus sp.	16.00±0.00	15.00±0.00	14.15±0.21	10.00±0.00	9.85±0.21
P. fluorescens	18.00±0.00	16.10±0.14	11.00±0.00	13.25±0.35	14.90±0.14
Streptococcus sp.	11.25±0.35	9.00±0.00	9.90±0.14	11.85±0.21	14.20±0.28
V. parahaemolyticus	15.90±0.14	0.00±0.00	14.00±0.00	15.10±0.14	10.00±0.00
V. alginolyticus	12.00±0.00	10.80±0.28	14.85±0.21	12.00±0.00	9.00±0.00
E. tarda	13.20±0.28	12.25±0.35	11.15±0.21	9.00±0.00	12.30±0.42
Proteus sp.	11.00±0.00	14.90±0.14	8.25±0.35	7.80±0.28	14.10±0.14

Values are means of three replicate determinations±SD.

Table 4

Antibacterial activities of five purified fractions of acetone extract of *S. wightii* against bacterial fish pathogens.

Test organisms	Zone of inhibition (mm)				
	1st	2nd	3rd	4th	5th
A. hydrophila	19.15±0.21	16.85±0.21	12.20±0.28	14.15±0.21	12.50±0.70
E. aerogens	17.25±0.35	15.15±0.21	13.00±0.00	8.90±0.14	14.00±0.00
Flavobacterium sp.	16.00±0.00	4.00±0.00	14.30±0.42	8.00±0.00	13.15±0.21
Micrococcus sp.	17.20±0.28	4.25±0.35	7.00±0.00	6.20±0.28	9.00±0.00
P. fluorescens	15.15±0.21	12.85±0.21	11.90±0.14	13.10±0.14	14.10±0.14
Streptococcus sp.	4.50±0.70	14.00±0.00	9.00±0.00	9.00±0.00	10.25±0.35
V. parahaemolyticus	15.00±0.00	10.00±0.00	13.25±0.35	10.00±0.00	12.00±0.00
V. alginolyticus	13.10±0.14	8.25±0.35	11.15±0.21	13.85±0.21	15.85±0.21
E. tarda	15.90±0.14	14.10±0.14	4.30±0.42	5.25±0.35	13.20±0.28
Proteus sp.	12.00±0.00	9.10±0.14	5.00±0.00	13.00±0.00	14.25±0.35

Values are means of three replicate determinations±SD.

The five fractions from methanol extract were screened for antibacterial activity against fish pathogens (Table 3). Among 5 fractions, 1st fraction showed the maximum activity against *P. fluorescens* (18.00±0.00) mm followed by *Flavobacterium* sp., *A. hydrophila*, *Micrococcus* sp., *V. parahaemolyticus*, *E. tarda* and the minimum activity against *Proteus* sp. (11.00± 0.00 mm) and no activity was observed against *E. aerogens*. The zones of inhibitions made by different pathogens were non-significant ($P>0.05$).

The five fractions from acetone extract were screened for antibacterial activity against fish pathogens (Table 4). Among 5 fractions, 1st fraction showed the maximum activity against *A. hydrophila* (19.15±0.21) mm followed by *E. aerogens*, *Micrococcus* sp., *Flavobacterium* sp., *E. tarda*, *P. fluorescens*, *V. parahaemolyticus*, *V. alginolyticus* and the minimum activity against *Streptococcus* sp. (4.50±0.70) mm. The zones of inhibitions made by different pathogens were non-significant ($P>0.05$).

3.3. Identification of active fraction

The three sub-fractions from acetone extract were screened for antibacterial activity against fish pathogens (Table 5). Tetracycline used as positive control and acetone as negative control. Among three sub-fractions, 3rd fraction exhibited the maximum activity against eight pathogens, *A. hydrophila* (22.25±0.35) mm followed by *Micrococcus* sp., *P. fluorescens*, *V. parahaemolyticus*, *E. aerogens*, *E. tarda*, *Proteus sp.*, *V. alginolyticus* and the minimum activity against *Streptococcus* sp. (10.00±0.00) mm compared to other two fractions. Tetracycline exhibited the maximum activity against five pathogens, *Streptococcus* sp. (16.15±0.21) mm followed by *A. hydrophila*, *P. fluorescens*, *V. alginolyticus*, *Micrococcus* sp. and the minimum activity against *Proteus* sp. (9.00±0.00) mm. The zones of inhibitions made by different pathogens were non-significant ($P>0.05$). No activity was observed in negative control. So, 3rd sub-fraction of acetone extract selected as an active fraction and was characterized by GC-MS analysis.

Table 5

Antibacterial activities of three sub-fractions of purified acetone extract of *S. wightii* against bacterial fish pathogens.

Test organisms	Zone of inhibition (mm)				
	1st	2nd	3rd	Positive control	Negative control
A. hydrophila	19.10±0.14	16.80±0.28	22.25±0.35	15.85±0.21	0.00±0.00
E. aerogens	15.00±0.00	14.00±0.00	17.10±0.14	10.25±0.35	0.00±0.00
Flavobacterium sp.	12.90±0.14	15.15±0.21	12.00±0.00	12.80±0.28	0.00±0.00
Micrococcus sp.	15.15±0.21	16.90±0.14	19.80±0.28	14.00±0.00	0.00±0.00
P. fluorescens	15.85±0.21	15.00±0.00	19.15±0.21	15.10±0.14	0.00±0.00
Streptococcus sp.	10.00±0.00	13.10±0.14	10.00±0.00	16.15±0.21	0.00±0.00
V. parahaemolyticus	16.20±0.28	14.80±0.28	18.00±0.00	11.00±0.00	0.00±0.00
V. alginolyticus	12.00±0.00	10.00±0.00	14.20±0.28	15.00±0.00	0.00±0.00
E. tarda	13.25±0.35	15.20±0.28	16.00±0.00	12.20±0.28	0.00±0.00
Proteus sp.	9.00±0.00	12.00±0.00	15.85±0.21	9.00±0.00	0.00±0.00

Values are means of three replicate determinations±SD.

3.4. GC-MS analysis

In the present study, active fraction afforded three unsaturated fatty acids methyl esters and three sterols (Tables 6 and 7). They have been analysed through mass and their fragmentation pattern. The results revealed that the occurrence of methyl oleate was found to be a major compound (30.3%) followed by methyl hiragonate (27.7%) and methyl tetradecatrienoate (24.5%). The active fraction also yielded three sterols, 24-methylene cholesterol was found to be a major compound (79.9%) followed by cholesterol (25.3%) and 24-methyl cholesterol (16.3%).

3.5. Total antioxidant activity

The antioxidant activity of seaweed acetone extracts which was determined are shown in Figure 1. The maximum activity was exhibited by crude extract (3.64±0.02) μg at 100 μg/mL and the minimum activity was exhibited (1.35±0.04) μg at 25 μg/mL, comparatively, the purified extract was exhibited the highest activity of (3.87±0.04) μg at 100 μg/mL and the lowest activity was exhibited (1.52±0.01) μg at 25 μg/mL. The antioxidant activity is expressed as the number of equivalents of ascorbic acid, the maximum activity was exhibited (3.95±0.03) μg at 100 μg/mL and the minimum activity was exhibited (1.69±0.01) μg at 25 μg/mL.

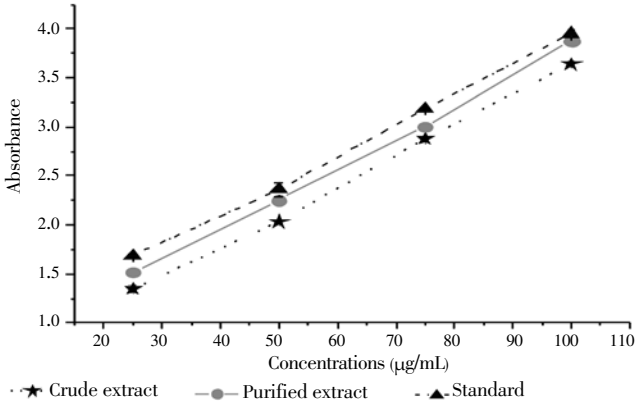

Figure 1. Total antioxidant activity of acetone extracts of *S. wightii* and standard ascorbic acid (25-100) μg/mL.
Results are expressed as mean±SD.

3.6. TPC

Phenolic compounds are commonly found in seaweeds and have been reported to have several biological activities including antimicrobial and antioxidant activity[31]. The present study, TPC was studied in *S. wightii* and results are shown in Figure 2. The maximum TPC value was (3.79±0.01) μg obtained in purified extract at 100 μg/mL and the minimum value was (1.35±0.03) μg obtained at 25 μg/mL. Comparatively, standard gallic acid, the maximum TPC value was (3.89±0.01) μg obtained at 100 μg/mL and the minimum value was (1.53±0.04) μg obtained at 25 μg/mL.

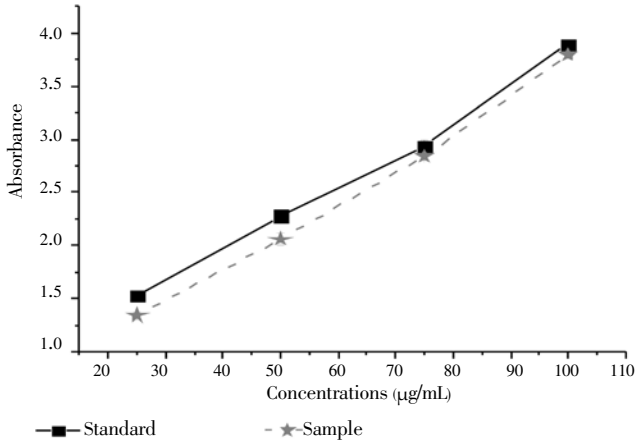

Figure 2. Total phenolic contents of purified extract and standard gallic acid (25-100) μg/mL.
Results are expressed as mean±SD.

3.7. TFC

Flavonoids are the largest class of polyphenols and are thought to exert beneficial health effects through their antioxidant and chelating properties and are the major contributor to the antioxidant capacity of plants. They act either by blocking the generation of hypervalent metal forms by scavenging free radicals or by breaking lipid peroxidation chain reactions[32]. In this study, TFC was estimated in *S. wightii* and results are given in Figure 3. Among all the concentrations, the value of TFC (3.64±0.02) μg increased from purified extract at a concentration of 100 μg/

Table 6
Three major compounds of unsaturated fatty acids methyl esters were analyzed from purified extract of *S. wightii*.

Common name	Systemic name	Retention time (min)	Molecular formula	Molecular weight	Peak area (%)
Methyl tetradecatrienoate	Methyl-2,4,5- tetradecatrienoate	17.36	$C_{18}H_{28}O_2$	276	24.5%
Methyl hiragonate	Methyl-6,10,14- hexadecatrienoate	19.23	$C_{17}H_{26}O_2$	262	27.7%
Methyl oleate	Methyl-9- octadecenoate	16.45	$C_{19}H_{30}O_2$	290	30.3%

Table 7
Three major compounds of sterols were analyzed from purified extract of *S. wightii*.

Common name	Systemic name	Retention time (min)	Molecular formula	Molecular weight	Peak area (%)
24-Methyl cholesterol	24-Methyl-cholest-5-en-3β-ol	12.49	$C_{27}H_{44}O$	384	16.3%
Cholesterol	Cholest-5-en-3β-ol	15.69	$C_{27}H_{46}O$	386	25.3%
24-Methylene cholesterol	24-Methylene-cholest-5-en-3β-ol	19.52	$C_{28}H_{46}O$	398	79.9%

mL and the lower value (1.27±0.04) μg was noticed at 25 μg/mL concentration. Compared with standard quercetin, the increased TFC value (3.81±0.04) μg was noticed at 100 μg/mL and the lower value (1.32±0.04) μg was noticed at 25 μg/mL.

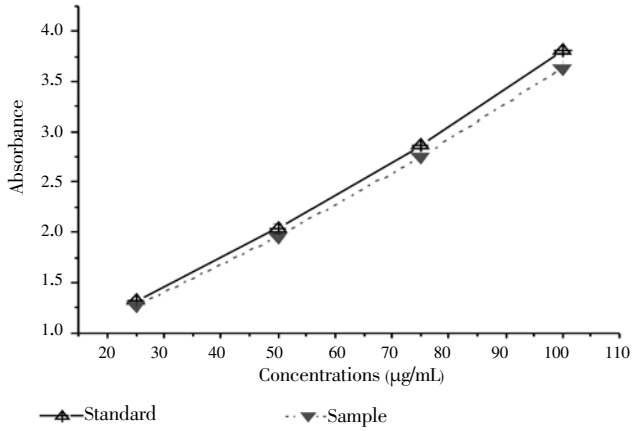

Figure 3. Total flavonoid contents of purified extract and standard quercetin (25-100) μg/mL.
Results are expressed as mean±SD.

3.8. Reducing power

Reducing capacity is considered as a significant indicator of potential antioxidant activity of a compound or sample. The presence of reductants (*i.e.* antioxidants) causes reduction of Fe^{3+}/ferricyanide complex to the ferrous form. Higher absorbance indicated higher reducing power. Increase in OD determines the increase in reducing power[33]. In the present study, purified extract of *S. wightii* possessed a good reducing power (3.85±0.03) μg at 100 μg/mL followed by the minimum reducing power (1.91±0.06) μg at 25 μg/mL. Compared with standard ascorbic acid, the maximum reducing power (3.86±0.03) μg was recorded at 100 μg/mL and the minimum reducing power (2.05±0.06) μg was recorded at 25 μg/mL (Figure 4).

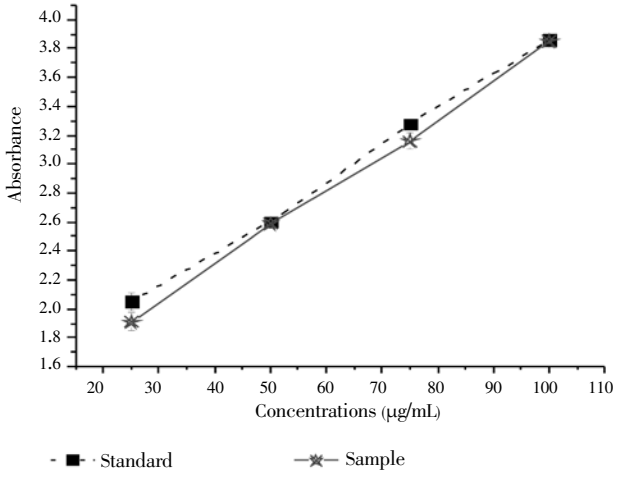

Figure 4. Reducing ability of purified extract and standard ascorbic acid (25-100) μg/mL.
Results are expressed as mean±SD.

3.9. DPPH radical scavenging assay

DPPH has been used extensively as a free radical to evaluate reducing substances[34]. A freshly prepared DPPH solution exhibits a deep purple color with absorption maximum at 517 nm. This purple colour generally disappears when an antioxidant is present in the medium. Thus, antioxidant molecules can quench DPPH free radicals and convert them to a colourless product resulting in a decrease in absorbance at 517 nm. Hence, the more rapidly absorbance decreases, the more potent antioxidant activity of the extract. The present study, DPPH radical scavenging assay was performed with purified extract of *S. wightii* and standard ascorbic acid (Figure 5). Among four concentrations, the highest scavenging effect (57.51±0.16)% inhibition was shown by 100 μg/mL and the lowest scavenging effect (23.17 ±0.21)% inhibition was shown by 25 μg/mL. For standard, the highest inhibition (57.91±0.061)% was shown by 100 μg/mL and the lowest inhibition (23.53±0.04)% was shown by 25 μg/mL.

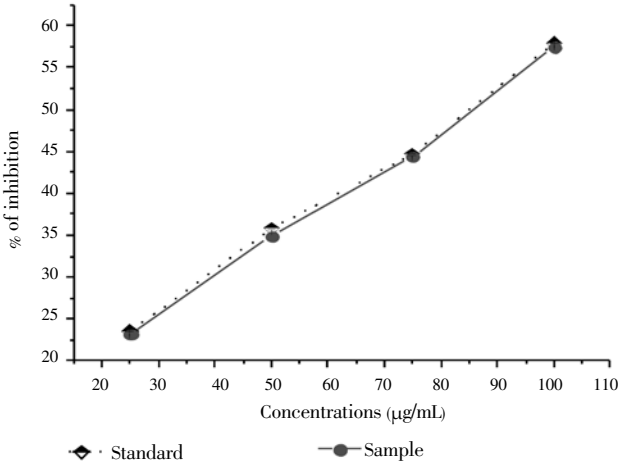

Figure 5. Comparison of DPPH scavenging activity of purified extract with standard ascorbic acid (25-100 μg/mL).
Results are expressed as mean±SD.

3.10. H_2O_2 radical scavenging assay

Many species of seaweed possess scavenging ability of hydrogen peroxide[35]. Hydrogen peroxide itself is not very reactive, but sometimes it can be toxic to cells because of rise in hydroxyl radicals in the cells. The present study, H_2O_2 radical scavenging assay was also performed with purified extract of *S. wightii* and standard ascorbic acid (Figure 6). The maximum scavenging activity was found by purified extract (57.33±0.86)% inhibition at 100 μg/mL and the minimum activity was found (29.33±0.18)% inhibition by 25 μg/mL. Compared with standard, the highest inhibition was showed (57.14±0.17)% at 100 μg/mL and the lowest inhibition was showed (29.16±0.22)% at 25 μg/mL.

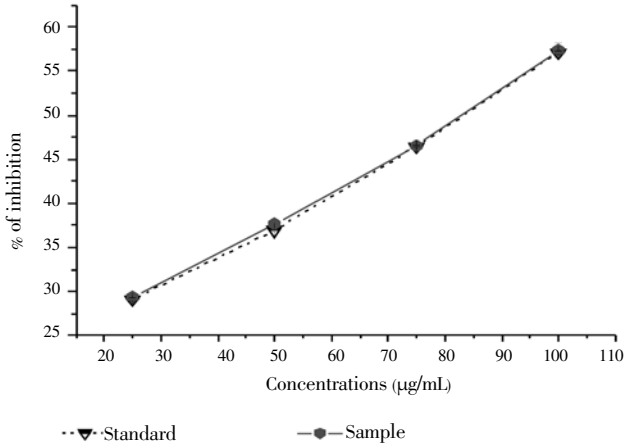

Figure 6. H_2O_2 radical scavenging activity of purified extract and standard ascorbic acid (25-100 μg/mL).
Results are expressed as mean±SD.

3.11. Brine shrimp toxicity assay

In the present study, active fraction of purified acetone extract tested for toxicity assay using *A. salina* nauplii (Figure 7). Among five concentrations, the toxicity level after 24 h of exposure caused 41% mortality at 100 μg/mL followed by 32.18% mortality at 80 μg/mL, 24.65% mortality at 60 μg/mL, 15.25% mortality at 40 μg/mL and 8% mortality at 20 μg/mL and no mortality observed in control group. Finally, lethal concentration of 50% mortality (LC_{50}) was determined by counting the dead nauplii after 24 h of incubation period but less than 50% mortality was occurred during the toxicity study.

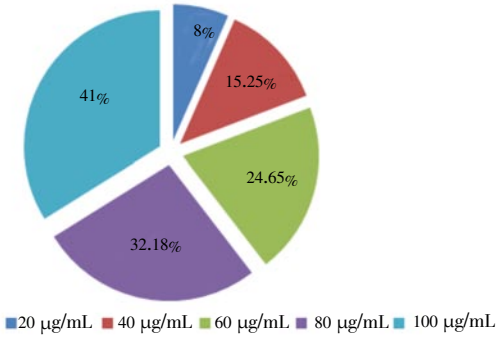

Figure 7. Mortality percentage of *A. salina* nauplii after 24 h immersion in different concentrations (20-100 μg/mL) of purified extract.

4. Discussion

While culturing the marine ornamental fishes, bacterial diseases are quite common and are responsible for heavy mortality. With the establishment of hatcheries for breeding marine ornamental fishes, particularly on a commercial basis, disease outbreak may become a major threat. Once, these diseases occur within a very short period, the survival rate is also reduced[36]. The continuous use of antimicrobial agents in aquaculture has resulted in accumulation of more resistant bacterial strains in aquatic environment and may also create threats to consumers[37]. Since ancient times, marine plants extracts have been used for treatments of common infectious diseases, treatments with plants having antibacterial activity are a potential beneficial alternative in aquaculture[38]. Several works have been undertaken on crude and purified compounds obtained from seaweeds for evaluating their bioactive potential. Brown seaweeds are known to contain more bioactive components than either green or red seaweeds[39].

In the present study, the results revealed that the active fraction of purified acetone extract exhibited maximum activity against eight bacterial fish pathogens among ten. Similar studies are also reported that the secondary metabolites of seaweeds, *Ulva fasciata* and *Hypnea musciformis* for bioactivity potential. Both species showed potent activity in antibacterial, brine shrimp toxicity and larvicidal assays[17]. Hanniffy and Kraan described that the macro algal species, *Ulva*, *Porphyra* and *Palmararia palmata* showed a strong antibiotic activity against fish and human pathogens[18]. Bansemir *et al.* discussed that dichloromethane, methanol and water extracts of 26 species of cultivated seaweeds were screened for their antibacterial activities against five fish pathogenic strains[40]. Wefky and Ghobrial investigated that *in vitro* screening of organic solvents extracts from five marine macroalgal species showed specific activity against five virulent strains of fish pathogenic bacteria and two fungi[41]. This study provides the potential of red and brown macroalgae extracts for the development of anti-pathogenic agents for use in aquaculture. Kolanjinathan *et al.* studied crude extracts from the seaweeds, *Gracilaria edulis*, *Calorpha peltada* and *Hydroclothres* sp. and screened for their antibacterial activity against six fish pathogens[42]. Lavanya and Veerappan discussed the extracts of six seaweed samples that were screened for antibacterial activity against fish and human pathogens[43]. This study results showed that all the seaweeds extracts have shown moderate activity against all pathogens. Compared to available literatures, the present study investigated that the purified extract of *S. wightii* showed maximum activity against eight fish pathogens. However, variation in antibacterial activity may be influenced by some factors such as habitat, season of collection, physiochemical parameters, different growth stages of plants and based on the solvents used in extraction of bioactive compounds[44].

The present study, GC-MS results unveiled that, two compounds such as 24-methylene cholesterol (79.9%) and methyl oleate (30.3%) are present in higher percentage; our

study suggested that these compounds might be responsible for the antibacterial effect against the pathogenic bacteria. Similarly pharmacologically active compounds were isolated from red algae, the green seaweed and brown seaweeds[23,45,46].

The present study, phenols and flavonoid contents and various antioxidant activities were also tested with purified extract of *S. wightii* and compared with different standards *viz.*, ascorbic acid, gallic acid and quercetin. Finally results are concluded that, 100 μg/mL concentration of purified extract have maximum phenolic and flavonoid contents, total antioxidant activity, reducing power and free radical scavenging activities (DPPH and H_2O_2). Compared with 100 μg/mL concentration of different standards, purified extract showed moderate equivalent only, but synthetic antioxidants caused the some side effects after long period usage at the same time, the cost of those antioxidants are also very high, based on these circumstances, we need to analyze the potential antioxidant compounds from natural sources. Our study suggested that, the antioxidant mechanism of this purified extract having free radical scavenging ability. Previous similar studies are reported that the highest antioxidant properties present in brown alga, *Padina minor* by Amornlerdpison *et al.*[47] and the maximum phenolic content and antioxidant activity obtained from methanol extracts of marine algae *Padina antillarum*, *Caulerpa racemosa* and *Kappaphycus alvarezii* were studied by Chew *et al*[48]. Ganesan *et al*. reported that the *in vitro* antioxidant activities of three selected red seaweeds *viz*., *Euchema kappaphycus*, *Gracilaria edulis* and *Acanthophora spicifera*[49]. Meenakshi *et al*. reported that total flavanoid content and antioxidant activity were higher in *S. wightii* than in *Ulva lactuca*[11]. Furthermore, phylopheophytin, fucoxantine and phlorotannins as antioxidant compounds, which were detected from brown algae[50]. Considered the previous and present reports, seaweeds can be used for a variety of beneficial effects in aquaculture systems.

The present study, purified extract were tested for toxicity assay using *A. salina* nauplii. The 100 μg/mL concentration of extract caused less than 50% mortality and low amount affected the appendages and life span of *Artemia* nauplii. Based on these result, 100 μg/mL of extract is suitable for *Artemia* enrichment for providing feed to marine ornamental fish larvae and juveniles. Our study suggested that 100 μg/mL concentration of purified extract is suitable for *Artemia* enrichment and to reduce the mortality percentage in culture systems. Several reports has also been published based on the use of this organism for environmental studies, screening for natural toxins and as a natural screening for bioactive substances in plant extracts[30,33,51,52]. So it is proved that the brine shrimp toxicity assay is a reliable method for the assessment of bioactivity of seaweeds and lends support for their use in pharmacology. The present study concluded that the brown seaweed, *S. wightii* has potential antimicrobial and antioxidant activities, the purified extract of this seaweed used in aquaculture industry for treated bacterial diseases in infected fishes and also used for pharmacological industry because this seaweed having potential antioxidants. Future research also needs to purify the potential bioactive compound and also study the mechanism of enhanced the growth inhibition of pathogenic bacteria for the successful completion of *in vivo* studies and management of bacterial fish diseases in aquaculture systems.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Isolation of natural bioactive materials from seaweeds has shown promising area of research for pharmaceutical applications. Seaweed diversity is very high and they show different bioactivities depend on season, natural habitat conditions as well as isolation techniques. In order to find novel bioactive materials it is important to screen various sources of seaweeds under specific properties such as antimicrobial, antioxidants, *etc*.

Research frontiers

Authors have extracted the bioactive substances from *S. wightii* using acetone, ethanol and methanol which commonly used methods for screening and identifying novel natural products. Also, this study investigated on use of identified extracts to control ornamental fish bacterial pathogens, antioxidant capacity, and check the toxic effects which could be considered as a new study with seaweed source of *S. wightii*.

Related reports

Bioactivity potential of wide range of seaweeds such as *Ulva fasciata* and *Hypnea musciformis* has been reported and well documented.

Innovations and breakthroughs

Results showed that 24-methylene cholesterol and methyl oleates are present in higher percentage in extract of *S. wightii*. It has shown potential to develop antimicrobial products after identifying specific molecules at structural level.

Applications

This study reveals several new findings of *S. wightii* extracts which could have potential to apply in aquaculture industry. Antioxidant properties of *S. wightii* extracts can be useful to develop seaweed based nutraceuticals. Study has covered the assessment of toxicity of *S. wightii* extracts which supports for its use in pharmacology.

Peer review

This study has described the preliminary screening of *S. wightii* extracts and their bioactivities. It can be a potential source of developing natural products. Authors have analyzed the two types of bioactivities such as antibacterial and antioxidant using common bioassays. The subject of manuscript is appropriate for the journal.

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