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Antioxidant, antibacterial and cytotoxic activity of *Caulerpa racemosa* (Forsskål) J. Agardh and *Ulva (Enteromorpha) intestinalis* L.

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Abstract

Seaweeds are fresh sources of phytochemical compounds with immense medicinal potential, which have attracted the attention of agriculture, aquaculture and the pharmaceutical industry. The aim of this study was to test the antioxidant, antibacterial and cytotoxic activity and screening the phytochemical properties of methanol crude extract from two green seaweeds *Caulerpa racemosa* (Forsskål) J.Agardh and *Ulva (Enteromorpha) intestinalis* L. The antioxidant and cytotoxic activity of these seaweeds were assessed by 2,2diphenyl, 1-picryl hydrazyl (DPPH) free radical scavenging technique and brine shrimp lethality bioassay method, respectively. The antibacterial activities against *Vibrio fluvialis* was determined using the standard disc diffusion method. The *U. intestinalis* showed higher total phenolic content with the value of 149.87 ± 18.17 mg of GAE/g than 73.95 ± 16.09 mg of GAE/g of *C. racemosa*. However, the recorded inhibition concentration (IC50) to corresponding standards ascorbic acid of *C. racemosa* and *U. intestinalis* were 119.62 and 34.274 µg/mL, respectively. The values were statistically significant ($p < 0.05$) compared to the reference antioxidative agent ascorbic acid. In antibacterial assay, *C. racemosa* extract showed higher inhibition zone (22.65 ± 0.58 mm) than *U. intestinalis* (17± 0.28 mm). Moreover, *U. intestinalis* exhibited a lower LC_{50} value than *C. racemosa* in cytotoxic activity. Phytochemical analysis revealed that these two seaweeds have alkaloids, flavonoids, glycosides, phenols, saponins, steroids and tannins.

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Introduction

Marine macroalgae, commonly known as seaweeds, are one of the major living resources of the ocean and are attached to solid substrate bottoms such as stems, rocks, shells, dead corals and plants (Sahayaraj *et al.,* 2014). In several Asian countries, consumption of seaweeds as marine vegetables for human diets has been a common practice (Kumer *et al.,* 2011). They protect marine and coastal biodiversity (Christie *et al.,* 2009) and a source of ecosystem goods and services such as medicines, food and storm protection (Ronnback *et al.,* 2007).

Antioxidants are compounds that protect cells against harmful free radicals that can attack molecules in biological membranes and tissues and therefore cause oxidative stress that damages living cells and leads to cancer, aging, inflammation, neurodegenerative diseases, hypertension (Devasagayam *et al.,* 2004; Grassi *et al.,* 2009; Sharma *et al.,* 2013). Many antioxidant compounds, such as vitamin C, carotenes, phenolic acids, phytoestrogens, phytate, tocophenols, etc., derived from plant sources (Chu *et al.,* 2000; Mantle *et al.,* 2000). Seaweeds have a low calorie nutritional value and are abundant in vitamins, minerals, proteins, polyphenols and food fibers (Burtin, 2003; Macartain *et al.,* 2007). Several studies have shown that antimicrobial and antioxidant activity is present from seaweed derived polyphenols and flavonoids (Heo *et al.,*

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2005; Chandini *et al.,* 2008). However, the content of bioactive compounds of seaweed varies by species of seaweed (Kumbhar *et al.,* 2014). The bioactive composition of seaweeds also depends on environmental and geographical or agricultural factors (Chithra and Chandra, 2014).

In this study, an effort was made to assess the antioxidant, cytotoxic activity, antibacterial effects and phytochemical screening of methanol crude extract from two green seaweeds *Caulerpa racemosa* and *Ulva intestinalis* collected from the intertidal zone of St. Martin's Island, Bangladesh.

Materials and methods

Collection and identification of seaweeds

Fresh green seaweeds i.e. *Caulerpa racemosa* (Forsskål) J.Agardh and *Ulva (Enteromorpha) intestinalis* Linnaeus were collected from the western side of Cheradip of the St. Martin's Island, Bangladesh (Fig. 1 and 2). The collected samples were thoroughly washed with clean seawater for a couple of minutes to remove debris and other external materials and then packed in plastic bags and kept in an ice box. The samples were transferred to the laboratory of

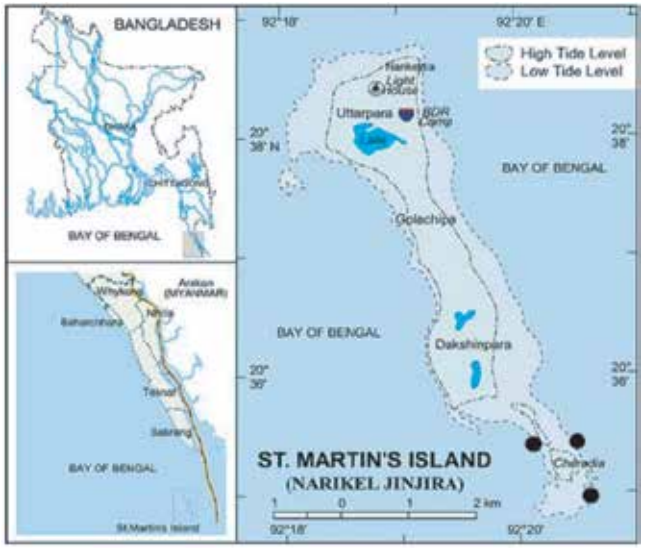

Fig. 1. Location of the sample collection area of St. Martin's Island, Bangladesh

Institute of Marine Sciences, University of Chittagong, Bangladesh for further processing and the species were identified by morphological and microscopic characteristics according to AftabUddin (2019) and Ahmed *et al.* (2008). Antioxidant activity was performed at the department of Biochemistry and Molecular Biology, University of Chittagong.

Preparation of seaweed crude extracts

In the laboratory, epiphytic and superfluous elements and surface salt were removed by washing samples with distilled water and then blotting to remove excess water. Extraction of seaweed was carried out by a slightly modified method of AftabUddin *et al.* (2017). The collected seaweeds were cut into small pieces, shade dried at room temperature for about 3 days and crushed into powdered in a mortar and stored in an airtight container. The subsequent seaweeds powder (400g) were allowed to soak with pure methanol (1:2 w/v) in Erlenmeyer flasks for 48h at room temperature, occasional stirring was performed. After soaking the extracts were filtered through filter paper (Whatman 1). The whole filtrate was evaporated under reduced pressure at 50-55° C through a vacuum evaporator (RE, 200, Bibby Sterling Ltd.) until a dry residue was obtained. The dry residues were dissolved in deionized distilled water at a concentration of 1 g/ml and stored at 4° C for further use. The extracts were weighted separately by a digital balance and the yield (%) was determined by using the following formula:

Yield determination = (weight of particular crude extract / total amount of crushed powder) x 100.

Bioactive compounds (phytochemical content) analysis

Phytochemical analysis was performed to identify the secondary metabolites present in the methanolic extracts of *C. racemosa* and *U. intestinalis* according to the standard method (Harborne 1998).

In vitro assay for antioxidant activity of seaweed extract

Total phenol content (TPC)

Total content of phenolic compounds in two green seaweed extracts were determined spectro-photometrically at 765 nm using Folin-Ciocalteu reagent method (Kumer *et al.*, 2011). All determinations were carried out in triplicate. The TPC was expressed as gallic acid equivalent (GAE) in mg/g sample.

DPPH radical scavenging assay

The antioxidant activity of *C. racemosa* and *U. intestinalis* extract were determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a free radical, in comparison to standard antioxidant ascorbic acid (Sigma, Germany) based on scavenging effect of the stable activity according to the recognized procedure of Brand-Williams et al. (1995). Standard ascorbic acid solution (1ml) and different concentrations (25, 50, 100, 200 and 400 µg/ml in methanol)

Fig. 2. Freshly collected seaweeds a) *C. racemosa* (Forsskål) J.Agardh b) *U. (Enteromorpha) intestinalis* L. from St. Martin's Island, Bangladesh

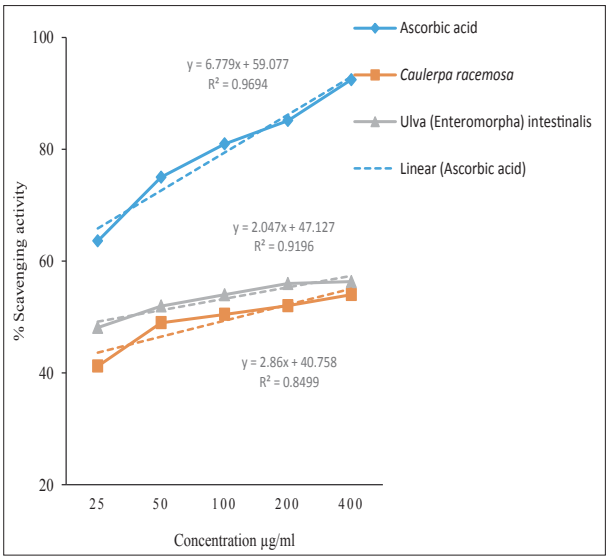

Fig. 3. Comparison of DPPH scavenging activity of two green seaweeds extract with standard ascorbic acid (25-400 µg/ml)

of 1ml of two green seaweeds solution were mixed with 3 ml of 0.4 mM DPPH solution (Rahman *et al.,* 2010). Each mixture was kept in the dark for 30 min and the absorbance was measured at 517 nm using UV-Visible Spectrophotometer (Cintra, Australia) and ascorbic acid was used as a positive control. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. Experiment was performed in triplicate and a control reaction was carried out without the test sample. Absorbance values were corrected for radicals' decay using a blank solution. The inhibitory effect of DPPH was calculated according to the following formula:

Scavenging (%) = [1-(A sample–A sample blank / A control)] × 100

Where,

A sample = The absorbance of the sample (DPPH solution with sample)

A sample blank = The absorbance of the sample only (sample without DPPH solution)

A control = The absorbance of the control (DPPH solution without sample)

The antioxidant activity of each sample was expressed in terms of IC_{50} (the concentration of sample required to scavenge 50% DPPH free radical) was calculated from the plot of inhibition (%) against the concentration of the extract (Fejes *et al.,* 2000).

In vitro cytotoxicity assay

Brine shrimp, newly hatched *Artemia sailina* nauplii were collected from a local shrimp hatchery and used for cytotoxicity assay according to the method of Caldwell *et al.* (2003). Each test vial (10 ml capacity) contained 10 Artemia nauplii at various concentrations 10, 50, 100, 200, 400 and 600 µg/ml) of methanol extracts of *C. racemosa* and *U. intestinalis*. The methanolic extracts of the seaweeds were dissolved in 0.1 ml of dimethyl

sulfoxide (DMSO) solution and supplemented with 10 ml of sterilized seawater. The negative control consisted of 0.1 ml of DMSO, and the positive control comprised 0.1 ml of 10 mg/ml $K_2Cr_2O_7$ solution, which is known to be toxic to Artemia. The LC_{50} values are reported as mg/ml. The toxicity was determined after 24 h of exposure by counting the number of dead nauplii and comparing with the control group using a 4X magnifying glass. Tests were carried out in triplicate. The lethal concentration of the extract was defined as that which caused 50% mortality of the nauplii (LC_{50}) after 24 h. The 24-h LC_{50} values were calculated from Probit chart using computer software "BioStat - 2009".

Vibrio fluvialis isolation

Diseased *Penaeus monodon* juveniles (weighted 5-10 g) showing clinical signs of bacterial disease were aseptically collected from the culture pond of Cox's Bazar and processed according to AftabUddin *et al.* (2017). The samples were plated on Thiosulphate Citrate Bile Salt Sucrose (TCBS, Oxoid, UK) agar (pH 8.5 ± 0.2) supplemented with 2% NaCl and incubated for 48 h at 27°C (Lightner, 1996) and subculture in tryptic soy broth (TSB; Difco) supplemented with 1% NaCl at 28º C for 24 h. The *Vibiro fluvialis* isolates were screened using triple sugar iron agar, motility media, cytochrome oxidase production, sensitivity to vibrio static agent 0/129 (2,4-diamino-6-7-di-isopropyl pteridine), Arginine dihydrolase (ADH) and ONPG (O-nitrophenyl-betaD-galactosidase). The detailed bacterial examination of *V. fluvialis* was performed following the methods of Alsina and Blanch (1994a,1994b). In addition, API 20E and API 20NE (BioMérieux, France) test strips were used for further confirmation. The broth cultures were centrifuged at 4000 rpm for 4 min and the supernatant was removed and the bacterial pellets were re-suspended in phosphate-buffered saline (PBS) at pH 7.5. The optical density (OD) was measured in a spectrophotometer; the solution was adjusted to 0.5 at 456 nm which corresponded to 1 million (10^7) cells/ml.

Antibacterial assay of seaweeds

The antibacterial assay of *C. racemosa* and *U. intestinalis* seaweeds methanolic extracts were tested by the disc diffusion technique (Ismail *et al.,* 2016). The *V. fluvialis* bacterium (0.1 ml) were incorporated on Muller Hinton Agar petri dishes by using a cotton swab (Hellio *et al.,* 2000). Then, methanolic crude extracts of two green seaweeds were dissolved in 20 µl dichloromethane solvent under aseptic conditions. Sterile HiMedia paper disc (6mm) were impregnated with 20 µl of different concentrations (125, 250 and 500 µg/disc) of crude extracts. Then the paper discs were dried overnight at room temperature (28-30º C). After inoculation (swab), the discs with extract were placed on the surface of the MHA plate with sterile forceps. Negative control was prepared simultaneously using the respective solvents, while ciprofloxacin (5 µg/disc), gatifloxacin (5 µg/disc), penicillin (10 µg/disc) (HiMedia, Bombay) were used as positive controls. The plates were incubated at 37º C for 24 h (Immanuel *et al.,* 2004). The antimicrobial activity of the test materials was observed through zone of inhibition (in mm) on the plates. Each test was performed in triplicate and the three values obtained were averaged.

Statistical analysis

All data were stated as mean ± standard deviation of three replicates. The data were analysed using Microsoft Excel 2016.

Results and discussion

Seaweed extraction and phytochemical analysis

The yield percentage of extracts of *C. racemosa* and *U. intestinalis* were 15.4%, 16.3% respectively. The phytochemical characteristics of both green seaweeds are summarized in Table I. The qualitative phytochemical (bioactive compound) tests showed that *C. racemosa* possess alkaloids, flavonoids, glycosides, phenols, saponins and steroids, however, *U. intestinalis* have not glycosides and steroids.

Antioxidant activity of green seaweed extracts

Total phenolic content

Phenolic compounds sometimes called polyphenolic compounds are considered being one of the most important classes of natural antioxidants (Machu *et al.,* 2015). The total phenolic contents of the tested *C. racemosa* and *U. intestinalis* were 73.95 ± 16.09 and 149.87 ± 18.17 mg of GAE/g respectively (Table II).

DPPH radical scavenging activity

Various methods are used to determine the free radical scavenging effects of antioxidants (Aksoy *et al.,* 2013). The DPPH method is a favorite because it is quick, easy and reliable, without any special reaction or device. DPPH is a stable synthetic radical which is not decay into water, methanol or ethanol (Shimada *et al.,* 1992). A newly prepared DPPH solution has a profound purple colour, with a maximum absorption of 517 nm. This purple colour usually disappears if the medium has an antioxidant. Antioxidant molecules can therefore remove DPPH-free radicals and convert them to a colourless product that reduces absorption

Table I. The analysis of bioactive compounds in the methanol extract of *C. racemosa* (Forsskål) J.Agardh and *U. (Enteromorpha) intestinalis* L.

Bioactive compound	Test name	Observation	Qualitative result	
			C. racemosa	*U. intestinalis*
Alkaloids	Dragendorff's	Orange red precipitate	++	++
	Mayer's	Cream colored precipitate	++	++
	Wagner's	Brown colored precipitate	++	++
Flavonoids	General test	Reddish pink color	++	++
Glycoside	Borntrager's test	Rose pink to red color in the aqueous layer	++	--
Phenols	Ferric chloride	Red or blue color	++	++
Saponins	Frothing test	A foam layer	++	++
Steroids	Salkowski test	Reddish brown coloration	++	--
Tannins	Ferric chloride	Blue-green coloration	--	++

"++" stands for the presence and "--" indicates the absence of bioactive compounds

at 517 nm. The decreasing amount of DPPH radicals in the environment leads to discoloration. The faster the absorption, the stronger the antioxidant activity of the extract. The methanolic crude extract of *C. racemosa* and *U. intestinalis* with DPPH radical scavenging test and standard ascorbic acid is shown in Fig. 3. Of the five concentrations, the highest scavenging effect was 54.16±0.14% and 56.83±0.81% inhibition at 400 µg / ml and the lowest scavenging effect was 41.23±0.17% and 48.11±0.78% inhibition at 25 µg / ml for *C. racemosa* and *U. intestinalis* respectively. For the standard, the highest inhibition (92.43 ± 0.02 %) was at 400 µg/ml and the lowest inhibition (63.60 ± 0.07%) was seen by 25 µg/ ml.

Cytotoxicity assay

The cytotoxicity assay for six different concentrations (10, 50, 100, 200, 400 and 600 µg/ml) of *C. racemosa* and *U. intestinalis* in methanol extracts were estimated after 24-h exposure time. Among six concentrations, the toxicity level after 24 h of exposure caused 40% mortality at 600 µg/ml followed by 30% mortality at 400 µg/ml, 10% mortality at 100 µg/ ml for *C. racemosa* seaweed extract, while 50% mortality at 600 µg/ml followed by 40% mortality at 400 µg/ml, 20% mortality at 100 µg/ml and 10% mortality at 50 µg/ml for *U. intestinalis* extract and no mortality was observed in negative control group. The lethal concentration (LC_{50}) of two green seaweeds, *C. racemosa* and *U. intestinalis* were 1012.86 and 695.41 µg/ml respectively while positive control shows 49.19 µg/ml (Table III). According to our protocol, seaweed extracts with LC_{50}<300 µg/ml were classified as toxic, while those with LC_{50}>300 µg/ml were considered non-toxic. Based on this classification, these two-green seaweeds, *C. racemosa* and *U. intestinalis* did not show any cytotoxic effects on brine shrimp larvae.

Antibacterial sensitivity test

In this study, the methanolic crude extracts of *C. racemosa* and *U. intestinalis* were screened for antibacterial activity against *V. fluvialis* bacteria. Among these two green seaweeds extracts, *C. racemosa* (500 µg/disc) exhibited maximal antibacterial activity against *V. fluvialis* bacteria with zones of inhibition 22.65 ± 0.58 mm (Table IV). However, the zones of inhibition made by *C. racemosa* and *U. intestinalis* extracts of 125 (µg/disc) and 250 (µg/disc) were not significantly different ($p > 0.05$).

Phenolic compounds are used for their antioxidant ability as important components of human and animal diets. To date, most polyphenols are of seaweed or macro algae origin, isolated from marine sources and referenced in the literature (Li *et al.,* 2011; Devi *et al.,* 2014). The TPC of the tested *C. racemosa* and *U. intestinalis* seaweeds were 73.95 ± 16.09 and 149.87 ± 18.17 mg of GAE/g respectively (Table 1) which is slightly higher than that of brown seaweed extracted with ethyl acetate by Chakraborty *et al.* (2013), they found 105.97±1.47 mg of GAE/g. Machu *et al.* (2015) reported that the edible brown seaweed *Eisenia bicycle* extracted with 80

Table II. Amounts (mg/g GAE) of total phenolic content of two green seaweeds after methanol extraction

Sample	Amount of sample (µg/mL)	Total phenolic content (mg of GAE/g)
C. racemosa	200	73.95 ± 16.09
U. intestinalis	200	149.87 ± 18.17

Table III. Antimicrobial activity of *C. racemosa* and *U. intestinalis* against *Vibrio fluvialis*

Seaweeds extract	Concentration (µg/disc)	Zone of Inhibition (mm)	Antibacterial activity score
C. racemosa	125	9 ± 0.50	+
	250	14 ± 0.45	++
	500	22 ± 0.58	+++
U. intestinalis	125	7 ± 0.56	+
	250	12 ± 0.50	++
	500	17 ± 0.28	++
Commercial antibiotic disc			
Ciprofloxacin	5	25 ± 0.48	+++
Gatifloxacin	5	19 ± 0.26	++
Penicillin	10	13 ± 0.20	++
Control	-	0.0	-

(-) no activity; + low activity (6-10 mm halo); ++ medium activity (10-17 mm halo); +++ high activity (18-30mm/halo, or above)

percent methanol had a total phenolic content of 143.2 ± 9.5 mg GAE/ g. On the other hand, flavonoids are the largest class of polyphenols and are known to have beneficial effects on health through their antioxidant and chelating properties and are the major contributors to plant antioxidant capacity (Devi *et al.,* 2014). In this study, both green seaweeds have flavonoids (Table I).

The highest radical scavenging activity was observed in the *U. intestinalis* methanol extract (lowest IC_{50} value= 34.27 µg / ml) (Table II). The high DPPH radical scavenging ability of the *U. intestinalis* extracts might be due to the presence of high contents of phenolic contents. Different research suggests that most of the plant extracts with antioxidant activity are due to the presence of phenolic compounds (Machu *et al.,* 2015; Vertuani *et al.,* 2004). So, to determine the antioxidant capacity of the seaweed plant extracts, the quantity of phenolic compounds is very important.

The cytotoxicity of marine seaweed is considered to be the presence of new drug compounds (Kwak, 2014; Murphy *et al*., 2014). Seaweeds *Undaria pinnatifida*, *Laminaria* and *Sargassum* spp., also possess antitumor activity (Ohigashi *et al.,* 1992). Ara *et al.* (1999) reported cytotoxicity of ethanolic extract of green seaweed *C. racemosa* was less cytotoxic (LC_{50}=929 µg/ml) and their specimen was collected from Karachi coast, Pakistan. Whereas, Ayesha *et al.* (2010) found that the cytotoxicity of green seaweed *Ulva fasciata* was 724 µg/ml against brine shrimp. On the other hand, it was reported that the LC_{50} values of red seaweeds *Amansia multifida* and *Meristiella echinocarpa* were 484.2 µg/ml and 281.9 µg/ml respectively for the ethanolic extracts.

Antibacterial activity of studied green seaweeds was comparable with the activity in *C. racemosa* and *U. compressa* seaweeds reported by Abdalla *et al.* (2016) wherein methanolic extract showed 20.0, 16.0 and 19.0, 16.0 mm inhibition zone against gram negative *Escherichia coli* and *Pseudomonas aeruginosa* bacteria, respectively. However, our results were much higher than compared to the data reported by TÜney *et al.* (2006) wherein green seaweed *E. linza* and *U. rigida* of diethyl ether extract exhibited 7.5, 10.0 and 8.5, 9.0 mm antimicrobial activity against *E. coli* and *P. aeruginosa* microorganisms respectively. This study has showed that the crude methanolic extracts of the investigated two green seaweeds have significant antibacterial effect against *V. fluvialis* bacteria. The inhibition zone produced by the commercial antibiotic disc was larger than that produced by these seaweed extract disc.

Conclusion

It has been shown that methanol extracts of two green seaweeds *C. racemosa* and *U. intestinalis* have significant

Table IV. Lethal concentration (LC_{50}) for two green seaweed extracts, obtained from the brine shrimp cytotoxicity assay

Seaweed extract / treatment	LC_{50} (µg/ml)
C. racemosa	1012.86
U. intestinalis	695.41
Negative control (DMSO)	-
Positive control (DMSO + $K_2Cr_2O_7$)	49.19

antioxidant activity using DPPH. Both seaweeds have been shown to be an interesting source of natural antioxidants and antibacterial compounds. The low cytotoxic activity observed for these species also makes them possible tools for use in the food industry. Further studies are necessary, however, to explain the mechanism. This study can be a step in biological and pharmacological activities of *C. racemosa* and *U. intestinalis*.

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