Biological Investigations of Three Marine Algae *Enteromorpha intestinalis*, *Rhizoclonium riparium* and *Ceratophyllum demersum* Collected from the Bay of Bengal

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ABSTRACT: The ocean consists of versatile domain of lives which can be exploited for human health benefits because of diversified secondary metabolites. Seaweeds, generally called macroalgae, are commonly found in the coastal region of the Bay of Bengal. But none of them were investigated properly for their biological activities. Hence, this study was performed to investigate the antioxidant, antimicrobial, cytotoxic and membrane stabilizing activities along with total phenolic content of the crude methanolic extract of three seaweeds namely, *Enteromorpha intestinalis, Rhizoclonium riparium* and *Ceratophyllum demersum* using standard protocols. Among the methanolic extracts of three seaweeds, *C. demersum* (52.09 ± 1.30 mg of GAE) was found to have the highest phenolic content, followed by *E. intestinalis* (35.98 ± 1.27 mg of GAE) and then *R. riparium* (29.94 ± 0.54 mg of GAE). The extractives of these seaweeds also showed promising antioxidant and membrane stabilizing properties compared to the standard. *E. intestinalis* extract displayed greater reducing power with increasing concentration (EC_{50} value 7.70 ± 0.27 µg/ml). The same seaweed *E. intestinalis* was found to have significant free radical (DPPH) scavenging potential (IC_{50} value 23.46 ± 0.54 µg/ml) compared to the reference standard ascorbic acid (IC_{50} value 5.76 ± 0.13 µg/ml). However, the findings of antimicrobial activity test demonstrated mild antibacterial effects of these seaweeds against *Escherichia coli* and *Aspergillus niger* and none of these seaweeds exhibited cytotoxicity when tested against HeLa cell line. These findings will aid in future studies attempting to explore medicinal and therapeutic agents from these readily available seaweeds of Bangladesh.

Key words: Marine algae, *Enteromorpha intestinalis, Rhizoclonium riparium, Ceratophyllum demersum*, DPPH, cytotoxicity, membrane stabilizing activities**.**

INTRODUCTION

Bangladesh is the largest deltaic country in the world. It features a lengthy coastline in the northeast and southeast, as well as a smaller shoreline in the north and northwest, along with the Bay of Bengal. Marine algae acts as a significant source of dissolved organic carbon in these coastal areas. These are multicellular eukaryotic algae, which are regarded as one of the most important natural resources necessary for maintaining proper balance between the chemical and biological environment of oceans. They have the capacity to accumulate enormous quantities of bioactive metabolites, which enables them to serve as useful resources in the pharmaceutical and pesticide industries.[1] In recent years, extracts of these seaweeds have received immense attention since many of these function as the golden origin of numerous bioactive compounds including polysaccharides, proteins, polyunsaturated fatty acids, pigments, polyphenols,

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minerals and hormones. In addition, some of these compounds have been found to possess significant therapeutic values as a result of their antimicrobial, antifungal, antioxidant, and anti-inflammatory properties.[2] Various research studies have shown that bioactive compounds were derived from seaweeds with a broad range of biological activities, such as antibacterial, antioxidant, antiviral, antitumor, cytotoxicity and induce apoptosis in cancer cells.[3] These seaweeds are also used medicinally in China for the treatment of liver diseases, swelling, cysts, phlegm and enlarged thyroid glands.

Marine macroalgae could be a potential source of diversified secondary metabolites. Our Bangladesh serves as a habitat for diversified species of seaweeds that may act as a promising source of potent therapeutics and prominent natural antibiotics against various pathogens.[4] These diversified marine organisms has become an inspiration for researchers to identify novel marine natural products that could eventually be developed into therapeutics or pharmaceutical products. In fact, many structurally diverse natural products isolated from diverse marine organisms are reported to exhibit an astounding array of bioactivities, particularly anticancer activity against multiple tumor types, as well as antibiotic, antiviral, antioxidant and anti-inflammatory activities.[5] *Ulva lactuca* and *Padina gymnospora* are two diversified marine organisms that showed remarkable activities against human pathogens.[6]

Enteromorpha intestinalis (Fam: Ulvaceae) a green filamentous alga commonly named as sea lettuce, is widely distributed in the rocky and stone containing coastal area of the Bay of Bengal, Thailand, Indonesia etc. Hydroalcoholic extracts of *E. intestinalis* collected from Caspian Sea coast of Iran showed potential antimicrobial and antihemolytic activities.[7] Another chlorophyll rich filamentous seaweed *Rhizoclonium riparium* (Fam: Cladophoraceae) is a habitat in the near coastal region of Sundarban, the largest Mangrove Forest in the World. Osuna-Ruiz *et al.* previously showed the highest antimutagenic activity of *R. riparium* acetone extract against aflatoxin B1 (AFB1) using *Salmonella typhimurium* strains in the Ames test.[8] *Ceratophyllum demersum* (Fam: Ceratophyllaceae), commonly known as hornwort, is a submerged marine macrophyte, a free-floating aquatic plant found in Sundarban area. A total of 78 phytochemicals have been reported from this plant using GC-MS analysis of the whole plant dried extract.[9]

Present study was conducted to evaluate the antioxidant, antimicrobial, cytotoxic and membrane stabilizing potentials of the crude methanolic extracts of one species of *Ulva*, namely *Enteromorpha intestinalis* and two species of *Padina*, namely *Rhizoclonium riparium* and *Ceratophyllum demersum* collected from the Bay of Bengal for future applications in medicinal and pharmaceutical industries.

MATERIALS AND METHODS

Sample collection and processing. The samples were collected from three different stations near the shore of the Bay of Bengal. The sample of *Enteromorpha intestinalis* was collected from the St. Martin Island at low tide in the month of February, 2019 while the sample *Rhizoclonium riparium* was collected from Kotka point of Sundarban mangrove forest tidal shore of the Bay of Bengal. The sample of *Ceratophyllum demersum* was collected from another point of Sundarban, near the Bay of Bengal. These three seaweeds were identified by Dr. Moniruzzaman Khondker, Professor, Department of Botany, University of Dhaka. After collection, these samples were first washed with seawater, followed by freshwater. Then these seaweeds were dried at room temperature for five days. After drying, the samples were powdered by using mortar and pestle.

Preparation of methanol extract. The air-dried seaweed powders were extracted (at the ratio of 6:1 (v/w)) with methanol and left at room temperature for 7 days. The mixtures were shaken every 24 hours. The extracts were subjected to successive filtering and the filtered extracts were concentrated under reduced pressure in a rotary evaporator.

Quantitative assessment of phenolic contents of crude methanolic extracts of seaweeds

Analysis of total phenolic content. Total phenolic contents of the plant extracts were determined spectrophotometrically employing the method as described by Wolfe *et al.*[10] involving Folin-ciocalteu reagent as the oxidizing agent and gallic acid as the standard. The absorbances were measured at 760 nm by UV spectrophotometer and the standard curve was prepared using solutions of varying concentrations of gallic acid**.**

Analysis of total flavonoid content. Total flavonoid contents of the plant extract was determined spectrophotometrically employing the method as described by Kumaran and Karunakaran *et al.*[11] The absorbance was measured at 415 nm by UV spectrophotometer and the standard calibration curve was prepared using solutions of varying concentrations of quercetin.

Evaluation of antioxidant activities of crude methanolic extracts of seaweeds

DPPH antioxidant assay. This method was developed by Blois with the viewpoint to determine the antioxidant activity in an identical manner using a stable free radical DPPH. [12] The assay is based on the measurement of the scavenging capacity of antioxidants towards it. The absorbance readings of extracts were taken at 517 nm. The antioxidant activities of extracts were expressed as IC_{50} and their antioxidant potentialities were determined by comparing with the reference standard ascorbic acid.

Analysis of reducing power assay. The reducing power assay of seaweed extracts and the reference standard were analyzed employing the method described by Oyaizu.[13] The EC_{50} (median effective concentration) values were used to express the reducing power potentials of seaweed extracts.

Antimicrobial activity by disc diffusion method. The disc diffusion method was performed in accordance with the process of Bauer *et al.*[14] Four different microorganisms were utilized for investigating the antimicrobial potentials of seaweeds. The test microorganisms included one Gram-positive bacterium (*Staphylococcus aureus*), two Gram-negative bacteria (*Escherichia coli* and *Salmonella typhi*) and one fungus (*Aspergillus niger*). Standard antibiotic (chloramphenicol) and blank discs were used as positive and negative control. Finally, the antimicrobial activity of the test extracts were determined by measuring the diameter of the zone of inhibition expressed in millimeters.

Determination of cytotoxic activities of crude methanolic extracts of seaweeds

Analysis of cytotoxic effect against HeLa cell Line. Cytotoxic effects were examined in the Centre for Advanced Research in Sciences by following an established protocol.[15] In brief, *HeLa*, a human cervical carcinoma cell line was maintained in DMEM (Dulbecco's Modified Eagles' Medium) containing 1% penicillin-streptomycin (1:1) and 0.2% gentamicin and 10% fetal bovine Serum (FBS). Cells (200×10^2/100 µl) were seeded onto a 96-well plate and incubated at 37°C + 5% CO_2. Next day, 25 µl samples (filtered) were added to each well. Cytotoxicity was examined under an inverted light microscope after 48h of incubation. Duplicate wells were used for each sample.

Brine shrimp lethality bioassay. The brine shrimp lethality bioassay was performed according to the method of Meyer *et al.*[16] The concentration–mortality relationship of methanolic extracts was expressed as the values of median lethal concentration (LC_{50}).

Determination of membrane stabilizing activities of crude methanolic extracts of seaweeds. The membrane stabilizing activities were investigated using hypnotic solution and heat induced hemolysis. These tests were performed in accordance with the procedures of *Shinde et al.*[17] The absorbances were measured at 540 nm using Shimadzu UV spectrophotometer.

RESULTS AND DISCUSSION

Assessment of total phenolic and flavonoid contents. The total phenolic content was found to vary in different species, ranging from 29.94 to 52.09 mg of gallic acid equivalent (Table 1). Among three

extractives of seaweeds, the highest phenolic content was observed in the methanolic extract of *Ceratophyllum demersum* (52.09 ± 1.30 mg of GAE), signifying its remarkable antioxidant activity as phenolic compounds have been found to exhibit promising action in neutralizing free radicals.[18] On the other hand, the methanolic extract of *Rhizoclonium riparium* (29.94 ± 0.54 mg of GAE) was found to possess lowest phenolic content among the three seaweeds. However, in terms of the flavonoid content, *Ceratophyllum demersum* was again observed to possess the highest amount compared to other seaweeds, indicating its powerful antioxidant potentialities compared to the others two algae tested (Table 1).

Table 1. Total phenolic and flavonoid contents in methanolic extracts of seaweeds.

Methanolic extracts of seaweeds	Total phenolic content (mg GAE/g DW)	Total flavonoid content (mg QE/g DW)
Enteromorpha intestinalis	35.98 ± 1.27	223.43 ± 7.91
Rhizoclonium riparium	29.94 ± 0.54	261.47 ± 4.69
Ceratophyllum demersum	52.09 ± 1.30	267.53 ± 6.68

*Data represents mean ± standard deviation of n=3 experiments, GAE-Gallic acid equivalent, DW-Dry weight, QE-Quercetin

Evaluation of antioxidant activities. This study evaluated the free radical scavenging potentials of seaweeds through the reducing power assay and DPPH tests. The reducing power assay test is usually performed to determine the electron donating potential of an antioxidant, which is regarded as a crucial mechanism of action of a phenolic antioxidant.[19] Numerous investigations have also discovered a close association between the reducing power abilities of certain plant extracts and their antioxidant potentials.[20, 21] In the current study, the ferric reducing capacities of seaweeds were evaluated in comparison to ascorbic acid, which served as the standard (Figure 1). The higher absorbance values of seaweed extracts denoted greater reducing power. According to figure 1, the *E. intestinalis* extract showed greater reducing power with increasing concentration (EC_{50} value of 7.70 ± 0.27 µg/ml). However, *Ceratophyllum demersum* was found to possess the second highest reducing power (EC_{50} value of 30.82 ± 0.77 µg/ml) among the methanolic extracts of three seaweeds, indicating the presence of remarkably abundant phenolic contents that led to the reduction of ferric form to its ferrous form. In DPPH test (Table 2), lower value of IC_{50} indicated greater antioxidant potentials. In accordance to this test, *Enteromorpha intestinalis* was found to have lower IC_{50} value (23.46 ± 0.54 µg/ml) compared to the other two seaweeds (*Rhizoclonium riparium* and *Ceratophyllum demersum*), indicating significant free radical (DPPH) scavenging potential in contrast to the reference standard ascorbic acid (5.76 ± 0.13 µg/ml).

Determination of antimicrobial activities. The findings of the experimental data revealed that none of the three test sample possesses antimicrobial activity against the Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *Salmonella typhi.* However, all the seaweeds demonstrated very mild antimicrobial activities against the Gram-negative bacteria *Escherichia coli* and fungi *Aspergillus niger* (table 3). Among the three test species, *Enteromorpha sp.* (400 µg/disc) exerted highest zone of inhibition (8.4 mm) against *Escherichia coli.* This mild antimicrobial activity of seaweeds may be attributable to the presence of flavonoids which have been reported to provide antimicrobial action through blockage of DNA gyrase enzyme.[22]

Determination of cytotoxic activities. The crude methanolic extracts of *Enteromorpha intestinalis, Rhizoclonium riparium and Ceratophyllum demersum* were screened for their cytotoxic activity against HeLa Cell line. The

findings demonstrated that none of the three seaweeds possess cytotoxic effect against human cervical cancer cell HeLa cell line, since the survival rate of HeLa cell was found more than 95% for all three methanolic extracts of seaweeds at varying concentration (data not shown).

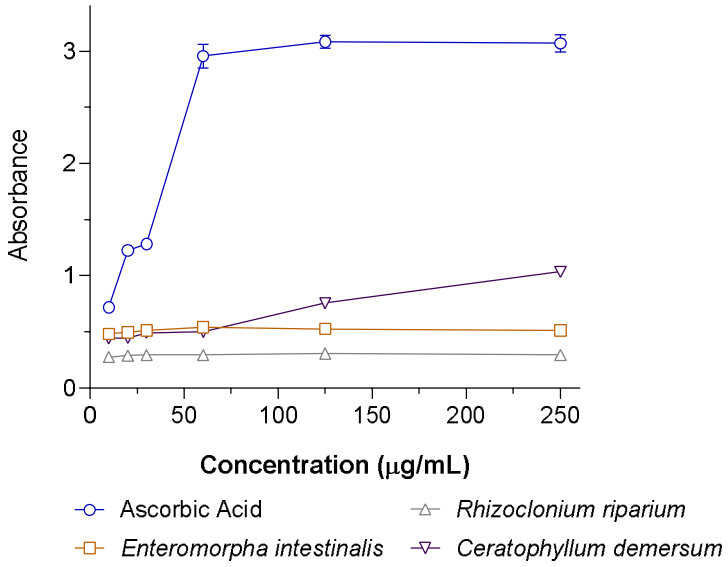

Figure 1. Reducing power effects of methanolic extracts of seaweeds. Data represents mean ± S.D. of 3 experiments.

Table 2. Free radical scavenging activities as demonstrated by the DPPH and reducing power test for the methanolic extracts of the seaweeds.

Sample code	IC_{50} in DPPH test (µg/ml)	EC_{50} in reducing power (µg /ml)
Enteromorpha intestinalis	23.46 ± 0.54	7.70 ± 0.27
Rhizoclonium riparium	87.97 ± 3.12	248.78 ± 4.46
Ceratophyllum demersum	41.89 ± 0.83	30.82 ± 0.77
Ascorbic acid	5.76 ± 0.13	3.37 ± 0.12

* Data represents mean ± Standard Deviation of n=3 experiments

Table 3. Results of the antimicrobial activities of methanolic extracts of seaweeds.

Test organisms	*Enteromorpha intestinalis*		*Rhizoclonium riparum*		*Ceratophullum demersum*		Chloram phenicol
	200 µg/disc	400 µg/disc	200 µg/disc	400 µg/disc	200 µg/disc	400 µg/disc	
Gram-positive bacteria							
Staphylococcus aureus	-	-	-	-	-	-	38.2 ± 1.4
Gram-negative bacteria							
Escherichia coli	6.7 ± 0.24	8.4 ± 0.15	6.7 ± 0.17	6.9 ± 0.24	6.8 ± 0.12	6.9 ± 0.17	39.2 ± 1.48
Salmonella typhi	-	-	-	-	-	-	40.4 ± 1.01
Fungi							
Aspergillus niger	6.4 ± 0.04	7.1 ± 0.03	6.2 ± 0.13	6.3 ± 0.06	-	-	37.7 ± 0.34

The value was expressed as diameter of zone of inhibition (mm). Data represents mean ± S. D. of 3 experiments.

However, in case of brine shrimp lethality bioassay, the methanolic extracts of all the seaweeds (*Enteromorpha intestinalis*, *Rhizoclonium riparium*, *Ceratophyllum demersum)* exhibited promising cytotoxic activities with LC_{50} values of 17.76, 37.32 and 47.43 µg/ml respectively (Table 4). According to the results of the brine shrimp lethality test, it can be predicted that the crude methanolic extract of seaweeds possesses cytotoxic principle and may have considerable cytotoxic potency. Hence, further bioactivity guided investigation can be done to find out the potent antitumor and pesticidal compounds of these seaweeds.

Table 4. Results of brine shrimp lethality bioassay for methanolic extracts of seaweeds.

Test sample	Regression line	R^2	LC_{50} values (µg/ml)
Vincristine sulphate	Y=120x+49.97	0.742	0.25
Enteromorpha sp.	Y=0.447x+42.06	0.603	17.76
Rhizoclonium sp.	Y=0.127x+45.26	0.373	37.32
Ceratophyllum sp.	Y=0.115x+44.51	0.404	47.73

Table 5. Effects of methanolic extracts of seaweeds on hypotonic solution and heat induced hemolysis.

Sample	Concentration (mg/ml)	Hemolysis inhibition (%)	
		Hypnotic solution induced	Heat induced
Enteromorpha intestinalis	2	12.45 ± 0.45	10.76 ± 0.25
Rhizoclonium riparium	2	29.82 ± 0.54	21.20 ± 0.75
Ceratophyllum demersum	2	35.77 ± 0.91	29.38 ± 0.58
Acetyl salicylic acid	0.1	59.45 ± 2.14	41.69 ± 0.95

Data represents mean ± S. D. of 3 experiments.

Determination of membrane stabilizing activities. The membrane stabilizing activity assay showed significant abilities of seaweeds in protecting the heat and hypnotic solution induced hemolysis at 2 mg/ml concentration in contrast to the reference standard acetyl salicylic acid at a concentration of 0.1 mg/ml (table 5). In case of hypnotic solution induced hemolytic test, the methanolic extract of *Ceratophyllum demersum* showed higher hemolysis inhibitory potential (35.77 ± 0.91%) compared to the other two seaweeds (*Rhizoclonium riparium* and *Enteromorpha intestinalis*), which exhibited 29.82 ± 0.54% and 12.45 ± 0.45% hemolysis protecting abilities respectively. However, the haemolysis inhibitory potentials of all three seaweed extracts were quite lower to that of the standard (59.45 ± 2.14%). On the other hand, in case of heat induced hemolytic condition, the methanolic extracts of *Ceratophyllum demersum* showed the highest hemolysis inhibitory potential (29.82 ± 0.54%), followed by *Rhizoclonium riparium* (21.20 ± 0.75%) and *Enteromorpha intestinali* (10.76 ± 0.25%).

CONCLUSION

The methanolic extracts of the studied seaweeds showed promising antioxidant, cytotoxic and membrane stabilizing potentials but they exhibited mild antimicrobial effects. Moreover, the phenolic and flavonoid contents of the seaweeds may serve as potential anti-inflammatory constituent which may regulate the inflammatory responses by antagonizing the effects of mediators responsible for inflammatory responses. These findings support the potential use of marine organisms for therapeutic purposes in order to

combat severe fatal diseases. However, further analysis is needed to identify the key compounds responsible for the cytotoxic and membrane stabilizing activities as well as to understand the precise mode of action of these activities.

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