PHYTOCHEMICAL SCREENING, CYTOTOXIC ACTIVITY, ANTIBACTERIAL ASSAY AND PROXIMATE ANALYSIS OF *ULVA INTESTINALIS* ALGAE FROM THE BAY OF BENGAL, BANGLADESH

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ABSTRACT

Seaweed is widely popular across the globe for its nutritional and biological properties. After delimiting the maritime boundary on Bangladesh, research on seaweeds is gaining momentum. *Ulva intestinalis* is one of the seaweeds found in the St. Martin's Island of Bangladesh, and the present study investigated the presence of potentially bioactive compounds through phytochemical screening and proximate analysis of *Ulva intestinalis* along with cytotoxicity test and antibacterial activity. Phytochemical screening of ethanolic extract of *Ulva* revealed the presence of alkaloids, steroids, flavonoids and coumarins. The cytotoxicity and antibacterial activity was also found to be significant by the algal extract. From the quantitative test of nutrients, it demonstrated that the seaweed contains 15.41% of protein, 51.79% of carbohydrate, 1.21% of fat, 3.74% of fiber, 18.81% ash, and 12.78% moisture. Additionally, the presence of vitamin B12 was promising (1300 µg/100 g). Interestingly, heavy metals were not detected in *Ulva intestinalis* whereas other micronutrients were present. Comparatively, the higher ZOI were showed in *Klebsiella pneumoniae, Salmonella sp* and *E.coli* than *micrococcus sp* which indicates higher antibacterial activity of the species. The result of the brine shrimp lethality test, greater cytotoxicity of crude algal extract showed a significant LC_{50} value with higher concentrations. As a result, it is plausible that *Ulva intestinalis,* from the Bay of Bengal, has the potential antibacterial and cytotoxic activities and might also serve as a source of new bioactive compounds. Moreover, further research is needed to perform for validation of the seaweed to introduce its medicinal activity or nutritional supplement potentiality in industrial scale.

KEYWORDS: Seaweed, *Ulva intestinalis,* Phytochemical, Cytotoxicity, Antibacterial activity, Proximate

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Introduction

During the last few years, seaweeds have gained significant attention worldwide, and Bangladesh is quickly becoming one of the promising seaweed hubs. Until now, 193 species of seaweed have been identified in the coastlines of the Bay of Bengal in Bangladesh (Hossain et al. 2021). Seaweed is one of the most important organisms in the marine ecosystem. It is a very primitive plant that has no proper roots, stems or leaves. There are thousands of different kinds of seaweed, which are macroscopic, multi-cellular, and marine algae. They stick to rocks in the middle of the beach, wash up on shore, and float around on the surface of the ocean. Seaweeds can be divided into three groups: chlorophyta, rhodophyta, and phaeophyta, which are green, red, and brown algae (Babich et al., 2022, Nazarudin et al., 2021). There are over 20,000 different kinds of seaweeds around the world, but only about 1% of them are commercially used. Of those, 145 are used for food, like raw salad and curry, soup, cucumbers, cucumbers, cookies, etc. Among them about 110 are used for phycokolloids, like agar, agars, carrageenans, alginin, mannitol, etc (Xavier and Jose, 2020). They can also be used as medicines, cosmetics, animal feeds, fish feed, fertilizer, and soil conditioners (Magdugo., 2021, Salehi et al., 2019, Qin, 2018, Guevara, 2005). Seaweed is one of the most versatile products in the world and is used as a human food. Seaweed can be made into a functional food due to its low energy content and high polysaccharide and protein content and also a good source of dietary fiber (Babich et al., 2022). Algae are known to contain a variety of bioactive compounds, many of which have beneficial applications in cosmetics, nutrition, food and agriculture approved antioxidants are found in several groups of algae with important bioactive properties compounds that play an important role in various diseases, including antiaging, anticancer etc (Gotteland et al., 2020, Maray et al., 2023). Most flavonoid compounds exhibit antipyretic, analgesic, antiinflammatory, antioxidant and immunomodulatory properties (Gill et al., 2011).

There were 117 species of seaweed found on the coast of Bangladesh, 62 of which were red, 31 of which were brown, and 24 of which were green, all reported from the southeast and southwest coasts. The red seaweed made up 52.99% of the species, the brown seaweed made up 26.50%, and the green seaweed made up 20.51%. (Islam Md. et al. 2019). The species *Ulva intestinalis* is bright grass green seaweed can be found in a variety of habitats and in a variety of forms. The fronds are usually unbranched and can reach a length of 10-30 cm and a diameter of 6-18 mm.

Marine algae are used as a food ingredient in Japan, Korean, China, the Far East, and Hawaii. The seaweed industry of Bangladesh is still in its early stages and is not widespread because of socio-economic as well as technological barriers (Ahmed and Taparhudee 2005). Recently, seaweeds have been gaining more attention as a source of important nutrients. However, the development of the seaweed industry does not only depend on natural environmental conditions and workable technical methods, but also on receptive and supportive socio-economic conditions. However, the marine algae in Bangladesh have not yet been able to replace the traditional seafood dishes of the local population, although many types of edible seaweed can be found in the coastal areas. Due to its economic importance and its health-promoting potential, the macroalgae needs to be scientifically studied for its efficient and eco-friendly use. In order to achieve this objective, this study looked at the nutritional value, phytochemical profile and antioxidant properties of *Ulva intestinalis* seaweed found along the coast of St. Martin's Islant. The present study focused on the following parameters: proximal composition (crude protein, crude fat, crude ash, and fiber) mineral content phytochemical profiling.

To the best of our knowledge, this study is the first to report the phytochemical screening and evaluation of cytotoxicity activity, antibacterial properties and proximate analysis of ethanolic extracts of *Ulva intestinalis* found in Bangladesh. The *Ulva intestinalis* species can potentially be used as raw materials or ingredients for enhancing nutritive value and functional properties in human diets as well as in other industrial applications which can play important role in blue economy of Bangladesh.

Materials and Methods

Sample collection and preparation

The seaweed sample *Ulva intestinalis* has been collected from St. Martine Island. St. Martin's Island (coordinates: 20°36′47″N 92°19′36″E) is a small island administered by Bangladesh that covers only 36 km^2. Seaweeds was cleaned from epiphytes and rock debris then gave a quick freshwater rinse to remove surface salts. Primary identification carried out according to morphological characteristics. Sample was stored in icebox and 70% ethanol solution to carry to the laboratory facility. In laboratory samples were dried in the shade and dried samples were finely grinded with the help of mortar and pestle to get powder and stored at -20^{o}C until use.

(a) **(b)**

Figure 1. *U. intestinalis* seaweed (a: wet, b: dry)

Preparation of Extracts

Powdered sample of *Ulva intestinalis* was taken in conical flasks with 50% ethanol as solvent. The flasks were kept for 5 days for maturation at 25°C in a tabletop shaking incubator at 150 rpm. The extracts were then filtered through filter paper, and the filtrates were kept separately at -20°C until further use.

Phytochemical Screening

A phytochemical screening process involves evaluating, and detecting plant-derived compounds that have therapeutic potential. In accordance, phytochemical methods described by Guevara, B. Q. (2005), algal extracts were tested for secondary metabolites such as flavonoids, tannins, saponins, phenolics, sterols and terpenoids (Guevara, 2005).

Cytotoxic activity

A brine shrimp lethality bioassay was carried out to investigate the cytotoxicity of the seaweed extracts against *Artemia salina* nauplii (brine shrimp) according to the method with minor modifications (Sarah et al., 2017). Mortality below 50% will consider non cytotoxic; mortality higher than 50% but below 75% will consider mildly cytotoxic; and mortality higher than 75% will be considered highly cytotoxic.

To prepare artificial sea water, 34 g sodium chloride was dissolved in 1L of sterile distilled water. The pH of the artificial seawater was adjusted to be between 8.25 and 8.5 by adding 1.0 N sodium hydroxide (NaOH). Then, 0.5 g of brine shrimp (*Artemia salina*) eggs were added to the sterile artificial seawater. A 60-watt bulb was used to heat the vessel.

An airline tip from an air pump was inserted into the bottom of the container to ensure optimum aeration. Hatched nauplii were collected with a dropper after 24 hours for the cytotoxicity experiment. On the otherhand, *Ulva intestinalis* extract in 50% ethanol was used to prepare different concentrations for cytotoxic test. Potassium dichromate ($K_2Cr_2O_7$) used as a positive control. Newly born nauplii were transferred to each test tube with a micropipette. This setup was allowed tocontinue for 24 hours and dead nauplii from each test tube were counted after 24 hours. Magnifying glass was used to facilitate counting and transferring of nauplii. Here as negative controls each solvent used in extraction was taken in separate test tubes. For each concentration of extracts, positive controls, and negative controls, the experiment was repeated three times.

After 24 hours of exposure, the lethal concentrations (LC_{50}) of the test samples have been obtained by using a Probit regression analysis for the positive control: Potassium dichromate ($K_2Cr_2O_7$) and crude extract of *U.intestinalis* respectively. The following formula was used to calculate mortality (Sarah, Anny and Misbahuddin, 2017).

$$\text{Mortality (\%)} \ \frac{\text{Number of nauplii taken} - \text{number of dead nauplii}}{\text{number of nauplii taken}} \times 100$$

Antimicrobial activity

A single colony from the nutrient agar plate was picked with sterile toothpick. Sterile Luria-Bertani (LB) broth was inoculated with it and incubated overnight at 37°C in shaking incubator (Model JSSI-070C, JSR, Korea). The resulting bacterial culture was transferred to fresh LB broth and incubated at 37°C for 15 hours in shaking incubator until enough growth capable of producing confluent culture on agar medium was obtained. The inoculums will be indicated and adjusted to be 1.5 at OD_{660} nm before plating. 500 ml Luria-Bertani semi solid media was prepared following the standard composition. Then, 25-30 mL of inoculated LB agar was poured in each plate. Agar wells were cut into agar using the back of sterile blue tips and 100 µL of each extract of two different concentrations i.e., 100 µg/mL and 50 µg/mL was poured into the Petridis wells. The plates were kept at room temperature for at least one hour to facilitate diffusion. Then incubated at 37°C for 30 hr. The diameter of the zone of inhibition was measured with a ruler. Here Ampicillin, Tetracycline, Kanamycin and Ciprofloxacin antibiotic paper discs were used as positive controls. All experiments were done in triplicates.

Proximate composition determination

The qualitative test was done to confirm the presence of carbohydrate, protein. After that quantitative test were done. All experiments were done in triplicates.

Qualitative test

The qualitative test for carbohydrate was done by Molisch test which indicates reddish-purple coloured complex. To confirm the protein content, two drops of freshly prepared 5% Ninhydrin reagent was added into algal extract and heated for 10 mins. The appearance of bluish or purple color indicates that the presence of amino acids. For carbohydrate presence, Molisch's test was performed. The formation of a purple or a purplish-red ring confirms the presence of carbohydrates in the analyte.

Quantitative test

The mass of the dried seaweed was used for analysis. Protein, minerals, dietary fiber, carbohydrate, ash, minerals and vitamins were determined from a commercial research institute, Waffen Research Laboratory Limited, Tejgaon, Dhaka. A proximate analysis (protein content, carbohydrate content, and fat content), was carried out followed by the standard method (OAAC, 1990). From the samples, dry matter (AOAC 934.01), crude protein (AOAC 984.13, A-D), crude fiber (AOAC 978.10), fat crude content (AOAC 920.39 A) was determined using standard methods. Vitamins A and B12 were measured by High Performance Liquid Chromatography (HPLC) and Atomic Absorption Spectroscopy (AAS) was used to detect minerals and heavy metals. The ash content of the seaweeds was determined by incinerating 1g of the sample taken in a silica crucible and kept in a muffle furnace at 600°C. After incineration, the net content was cooled and weighed and expressed in terms of percentage.

Results and Discussion

Phytochemical Screening

The aqueous extracts of *Ulva intestinalis* was qualitatively analyzed for their phytochemical contents. Characteristic tests for alkaloids, terpenoids, steroids, tannins, saponins, flavonoids, phenol, coumarins, quinones and glycosides were performed with the respective samples. In qualitative phytochemical screening the presence and the absence of a certain class of compounds can be determined by observing changes in color or formation of precipitation upon addition of certain reagents to extracts. The presence of different phytochemicals in ethanolic solvents showed in Table 1. and Table 2.

In 50% ethanolic extract, sample showed the presence of alkaloids, coumarin compounds, flavonoids, glycosides, quinones, saponins, steroids, and terpenoids. However, only phenolic compounds and tannins were absent in 50% ethanol extracts of *Ulva intestinalis.* These two important phytochemicals might be the found in other solvents or with other contentrations of ethanol. That could be carried out in further investigation. Saponins are one of the most important and useful metabolites of marine macroalgae that have various applications in the cosmetic, food industry, and health section. In living organisms, seaweed saponins prevent or reduce many diseases due to their biological and physicochemical features (Abd El Hafez and et al., 2022).

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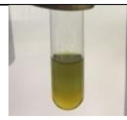
Table 1. Phytochemical analysis of 50% ethanol extracts of *Ulva intestinalis*

Attributes	Tested tube	Observation	Results
Alkaloids Test		White colour precipitation	Alkaloids found
Terpenoids Test		Reddish –brown colour seen at the interface	Terpenoids found
Steroids Test		Reddish-brown ring seen at the interface	Steroids found
Tannins Test		Dark blue/Greenish black colour was not present	Tannins not found
Saponins Test		A layer of foam was present	Saponins found
Flavonoids Test		Bright yellow colour	Flavonoid found
Phenols Test		Blue/Green colour was not present	Phenols not found
Coumarins Test		Yellowish colour	Coumarins found
Quinones Test		Slightly red colour was not present	Quinones found
Glycosides Test		Reddish-brown colour was present	Glycosides found

Antibacterial activity test

The antimicrobial activities of the tested extracts of *Ulva intestinalis* were evaluated by agar well diffusion methods and the results were expressed as the diameter of inhibition zone. The crude sample (ethanolic extract), was used for antimicrobial activity in different concentrations such as 50 ug/mL and 100 ug/mL and the antibiotic discs concentrations were tetracycline 30 µg/disc, Ampicillin 10 µg/disc, Ciprofloxacin 5 µg/disc and Kanamycin 30 µg/disc. The Antibacterial activities in 50% ethanol extracts of *Ulva intestinalis* against *Miicrococcus, E.coli, Salmonella sp*. and *Klebsiella* sp. are showed in Table and the values are expressed as mean ± SD, where n=3. *Ulva* species have antibacterial action against bacteria such as *Staphylococcus aureus, Escherichia coli, Candida albicans, and Aspergillus niger*, according to recent investigations using the Broth-dilution method (Srikong et al., 2017).

In this study, zone of inhibition (ZOI) showed a prospective activity against all the selected bacteria where algal

Table 2. Phytochemical analysis of *Ulva Intestinalis* extract

SI	Test name	Observation
1.	Alkaloids Test	+++
2.	Terpenoids Test	+
3.	Steroids Test	++
4.	Tannins Test	-
5.	Saponins Test	+
6.	Flavonoids Test	++
7.	Phenols Test	-
8.	Coumarins Test	++
9.	Quinones Test	+
10.	Glycosides Test	+

NB: (-) not detectable, (+) low quantities, (++) moderate quantities, (+++) high quantities

extract concentration was 100 µg/mL. The higher ZOI were showed for *Klebsiella pneumoniae*, *Salmonella sp* and *E.coli*, which indicates the algal extract can play role in diarroreal or intestinal disorders. In another study, *U. intestinalis* still did not show more or less zone of inhibition against *Shigella* sp. (Rizwan et al. 2020). The green *U. intestinalis* algae extracts tested were found to be effective against *Salmonella typhi* (Jayalakshmi et al. 2021).

Table 3. Antibacterial activity of *Ulva intestinalis* extract against the selected bacteria

Bacteria		Zone of Inhibition (ZOI)	
	Name of bacteria	**Positive control**	**Algal extract (250 µg/mL)**
Gram Positive	*Micrococcus sp.*	Yes	Yes
	Staphylococcus sp.	Yes	Yes
Gram Negative	*Klebsiella Pneumoniae*	Yes	Yes
	Escherichia coli	Yes	Yes
	Salmonella sp.	Yes	Yes

Cytotoxicity assay

Brine shrimp (*Artemia salina*) can be used as a model organism to preliminary screen extracts. The cytotoxic effect of the bioactive compound present in ethanol extracts of *Ulva intestinalis* has been studied using Brine Shrimp Lethality Assay (BSLA). From this study, the examined crude extract showed 50% mortality at 100 µg/mL algal extract concentration for brine shrimps. The LC_{50} value for the algal extract, and the positive control ($K_2Cr_2O_7$) was 92.38 µg/mL and 125.28 µg/mL respectively which indicates comparatively mild cytotoxic activity of the algal extract than the control.

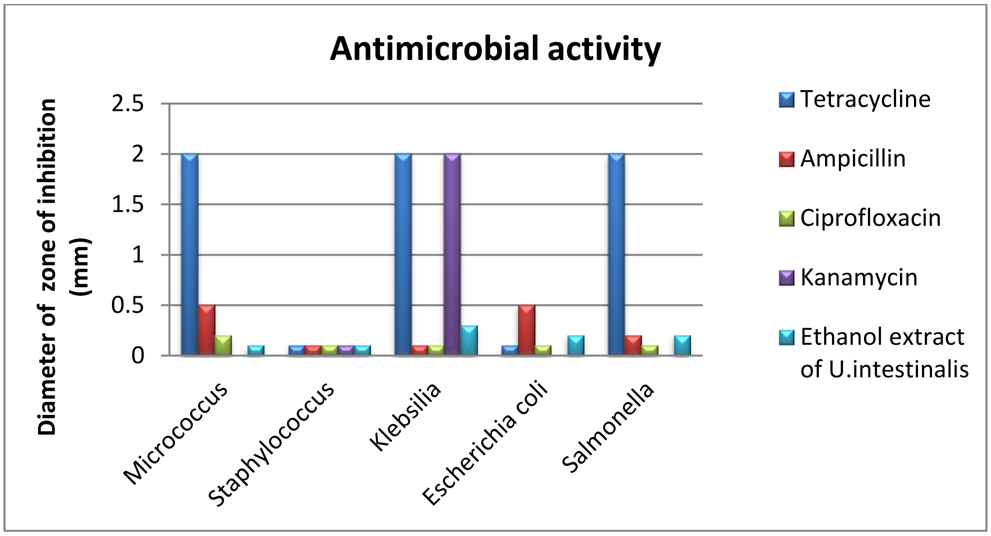


Figure 2. Antibacterial activity of *U.intestinalis* extract and positive control (antibiotic)

Proximate analysis

Food composition includes protein, carbohydrates, fats, fiber, ash, vitamin A, vitamin B12, lead, arsenic, cadmium, zinc and moisture. Qualitative tests help to identify the presence (or absence) of an ion or compound in solution. A colour change is observed if the concentration of the ion or compound is above a specific minimum detection limit. The appearance of purple color of Ninhydrin test indicates that the presence of amino acids and Molisch's test was confirmed for the presence of carbohydrates in *Ulva intestinalis.*

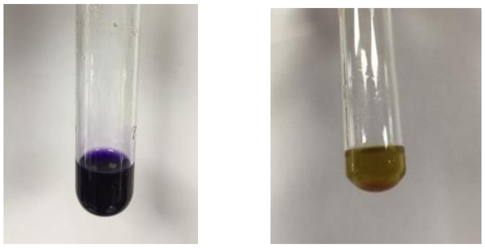
Afterwards, the quantitative test nutrients were performed in *Ulva intestinalis*. The results revealed that the seaweed contains protein 15.41% which is much higher than found in this *Ulva sp*. (Jatmiko et al., 2019). Moreover, it also contains fat 1.21%, fiber 3.74%, ash 18.81%, and moisture 12.78%. The presence of vitamin A was not detected, while the concentration of vitamin B12 was 1300 µg/100g.

Table 4. Cytotoxicity of potassium dichromate ($K_2Cr_2O_7$) and lethality LC_{50} (µg/mL) value

SI	Concentration (µg/mL)	Total nauplii	No. of Dead nauplii	Mortality (%)	LC_{50} (µg/mL)
1.	200	10	8	80	92.38
2.	100	10	6	60	
3.	50	10	5	50	
4.	25	10	4	40	
5.	12.5	10	2	20	
6.	6.25	10	2	20	
7.	3.125	10	1	10	
8.	1.565	10	1	10	
9.	0.80	10	1	10	
10.	0.40	10	1	10	

Table 5. Cytotoxicity of *U. intestinalis* extract and the lethality LC_{50} (µg/mL) value

Sl.	Concentration (µg/mL)	Total nauplii	No. of Dead nauplii	Mortality (%)	LC_{50} (µg/mL)
1.	100	10	5	50	125.28
2.	50	10	3	30	
3.	25	10	2	20	
4.	12.5	10	2	20	
5.	6.25	10	2	20	
6.	3.125	10	1	10	
7.	1.565	10	1	10	
8.	0.80	10	1	10	
9.	0.40	10	1	10	
10.	0.20	10	1	10	



(a) (b)

Figure 3. Qualitative test of *Ulva intestinalis* (a) amino acid (b) carbohydrate

Carbohydrate content was about 51.79% which was significantly higher than that of *Ulva rigida* (22%), *Chlorella vulgaris* (5.9%), *Sargassum cristaefolium* (7.25%), and many other species of seaweed (Rizwan et al., 2020, Kumar et al., 2021). In this study, heavy metals, Pb, and As was not detected in *Ulva intesinalis* whereas Cd was present at 1.50 ppm. The micronutrient Zn was observed in high amount (7.76 ppm). Due to high carbohydrate content, edible green seaweeds could be used as a food supplement. The student T-test showed that there is a significant difference ($P < 0.05$) between ash content and moisture content. The shelf life of the food products relies on the moisture content present in it. Moisture content has an important role as change in moisture will drastically affect the flavor and texture of the food product, making it inedible and not fit for consumption. It also affects the physical and chemical properties of the food product as the water molecules provide a conducive environment to catalyze the chemical reactions.

Table 6. Proximate composition determination in *Ulva intestinalis*

SL	Test Parameters	Amount
1.	Protein (g/100g)	15.41
2.	Carbohydrate (g/100g)	51.79
3.	Total fat (g/100g)	1.21
4.	Fiber (g/100g)	3.74
5.	Ash (g/100g)	18.81
6.	Vit A (ug/100g)	ND
7.	Vit B12 (ug/100g)	1300
8.	Lead (Pb), ppm	ND
9.	Arsenic (As), ppm	BDL
10.	Cadmium (Cd), ppm	1.50
11.	Zinc (Zn), ppm	7.76
12.	Moisture content (g/100g)	12.78

*ND: Not detected, *BDL: Below detection level

Conclusion

In the present study, the algal extract of *Ulva intestinalis* in 50% ethanol was used to perform phytochemical screening, antibacterial activity, cytotoxicity and proximate composition analysis. *Ulva Intestinalis* extract exhibited presence of promising phytoconstituents such as alkaloids, steroids, flavonoids, coumarins, glycosides, quinones, saponins, and terpenoids. The cytotoxicity assay revealed that crude algal extract showed 50% mortality at 100 μg/mL concentration for brine shrimps. Besides, the LC_{50} value for the algal extract (92.38 μg/mL), was lower than the positive control (125.28 μg/mL) indicates comparatively mild cytotoxicity than the control. In antibacterial assay, the higher zone of inhibition (ZOI) was showed in *Klebsiella pneumoniae, Salmonella sp.* and *E.coli*, using algal extract. From the proximate analysis, it is exhibited that the seaweed contains protein 15.41%, carbohydrate 51.79%, fat 1.21%, fiber 3.74%, ash 18.81%, and moisture 12.78%. The presence of vitamin A was not detected, while the concentration of vitamin B12 was 1300 μg/100g. Interestingly, heavy metals such as lead (Pb) and arsenic (As) were not detected in *Ulva intestinalis.* From the results, it is suggested that the phytochemical properties and proximate composition of *Ulva intestinalis* has potential bioactive ingredients along with its promising biological activity such as antibacterial activity. We can conclude that this seaweed has adequate possibilities to be considered as a good candidate for further use in medicine and food industry which will play significant role in blue economy of Bangladesh.

Competing Interests

We declare that the authors have no competing interests in this paper.

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