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Phytochemical constituency profiling and antimicrobial activity screening of seaweeds extracts collected from the Bay of Bengal Sea coasts

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ABSTRACT

Seaweeds are able to produce a great variety of secondary metabolites that are characterized by a broad spectrum of biological activities. Two seaweeds species, namely *Hypnea musciformis* and *Enteromorpha intestinalis* were studied to evaluate the phytochemical constituency and antimicrobial activities. First of all, crude extracts of both sea weeds were prepared by two different extraction methods (soaking and water bath) using different solvents. Phytochemicals profiling results revealed the presence of bioactive compounds (flavonoids, alkaloids, tannin, saponin and phenols) in both seaweed extracts. Quantification results for ethanolic extracts of *H. musciformis and E. intestinalis* estimated 51 mg and 43 mg tannins in per gram of dried samples and flavonoids contents were found 67 mg and 57 mg/g (Quercetin equivalents) QE/g, respectively. Total phenolic contents were determined in terms of gallic acid equivalent (GAE). *H. musciformis* exhibited higher amount of phenolics (59 ± 0.0002 mg GAE/g) than *E. intestinalis* extracts (41 ± 0.0002 mg GAE/g).In antimicrobial activity test, ethanol extracts *of H. musciformis* and *E. intestinalis* were found 10 mm of inhibition diameter against all of the bacterial strains. Besides, methanol extracts of *E. intestinalis* were more susceptible to *Staphylococcus aureus and Pseudomonas* which was close to the inhibition diameter (>15 mm) of the mainstream antibiotic, gentamicin. Moreover, *Klebsiella sp.* was found more susceptible to ethanol and methanol extracts of *H. musciformis* as it showed inhibition zone greater than 15 mm. Both Seaweed extracts possessed higher amount of phytochemicals and showed promising antimicrobial activities when compared with the standards.

INTRODUCTION

Millions of people are being afflicted by different infectious diseases induced by pathogenic bacteria. The phenomena of high mortality rate and the emergence of new multi-drug resistance, bacterial strains have become one of the threatening health problems worldwide [1,2]. Different drug molecules are being applied to combat against those microorganisms. Antibiotics, metal ions, and various quaternary ammonium compounds are being used worldwide though these antimicrobial compounds are also being claimed to be associated with antibiotic resistance, complex chemical synthesis, environmental pollution, and high cost [3,4]. However, natural antibacterial agents have been recently identified to overcome these disadvantages [5]. Marine algae are one of the richest sources of

bioactive compounds and chemical diversity [6,7] among them different seaweeds have historical contribution against the predators defense [8,9], and could be a promising antibacterial agents [10,11]. Moreover, algae produce the largest amount of biomass in the marine environmental [12].Seaweeds are plant like ocean organisms that are botanically classified as microphysics marine algae where edible seaweeds are often called sea vegetables, seaweeds come in an amazing variety of beautiful shapes, colors and sizes and found in all of the world's oceans. They are source materials for structurally unique natural products with pharmacological and biological activities [13]. Among the marine organisms, seaweeds occupy an important place as a source of biomedical compounds [14] and the most interesting algae group because of their broad spectrum of biological activities such as antimicrobial [15], antiviral [16], anti-allergic [17], anticoagulant [18], anticancer [19], antifouling [20] and antioxidant activities [21]. Importantly, seaweeds represent a potential source of antimicrobial substances due to their diversity of secondary metabolites with antiviral, antibacterial and antifungal activities [22]. Structurally diverse secondary metabolites of seaweeds offer defense against herbivores, fouling organisms and pathogens and also play role in reproduction, protection from UV radiation and as allopathic agents [23]. The bactericidal agents found in algae include amino acids, terpenoids, phlorotannins, acrylic acid, phenolic compounds, steroids, halogenated ketones and alkanes, cyclicpolysulphides and fatty acids [24]. Some of these metabolites extracted from seaweed such as iodine, carotene, glycerol, alginates, and carrageenans have been used in pharmaceutical industries [25,26].

The Bay of Bengal is the northeastern part of the Indian Ocean, bounded on the west and northwest by India on the north by Bangladesh. In Bangladesh, potential seaweeds are being reported in terms of food staffs and pharmaceutical agents from the south-eastern part of the mainland and offshore island [27,28]. Moreover, Bangladesh is rich with around 133 species of seaweeds and eight of them are commercially important. *Caulerpa racemosa, Enteromorpha sp, Gelidiella tenuissima, Gelidium pusillum, Halymenia discoidea, Hypnea pannosa, Hydroclathrus clathratus and Sargassum sp* are are the commercially important seaweeds in Bangladesh which usually provide low-cost, whole some nutrition and therapeutic protection [29]. Seaweeds may produce a wide variety of secondary metabolites which are characterized by a wide range of biological activities. Still now, there are scanty information regarding the potentiality of these species. This evaluation of *H. musciformis*, *E. intestinalis* extracts directly covers the medicinal properties against different pathogenic bacterial organism. That is why the objectives of the present study were toevaluate antimicrobial activity and phytochemical profiling of two widely available seaweed species collected from the Bay of Bengal sea coast.

MATERIALS AND METHODS

Seaweed material and description of study area

Two seaweeds species; *H. musciformis* and *E. intestinalis* were used in this study. They were freshly collected from North Nuniar Chor, Cox's bazar (21°35′0″N 92°01′0″E) (Figure 1 and 2). Collected samples were washed in running water for 10 minutes and then transported to the laboratory and shade dried at 35±3 °C for 36 h. The shade dried seaweeds were preliminarily powdered using electronic blender machine and used for further experiments.

Preparation of seaweed extracts and fractions

The extracts were prepared by two different methods such as water bath and soaking method [30,31,32]. In both cases, each of 20 grams of powdered seaweed samples were soaked in a conical flask containing 160 ml of the distilled water and different other solvents such as ethanol, methanol and acetone. In soaking method, the samples were gently mixed by shaking and left for 72 hours at room temperature. In water bath method, the samples were mixed gently using the same solvents and left at 65°C for 4 hours. After the soaking at room temperature or by using water bath at specific temperature, the liquid phase was filtered with whatman no. 1 filter paper and allowed to be concentrated at reduced pressure to give specific solvent extracts. The crude extracts were used to test qualitative and quantitative analysis for secondary metabolites and antimicrobial activities against different bacterial strains.

Preliminary qualitative phytochemical analysis

The presences of secondary metabolites are usually preliminarily traced by the different qualitative

phytochemical analysis. Here, several tests had also been conducted for identifying the secondary metabolites present in the alcoholic extracts of *H. musciformis* and *E. intestinalis*. The Sodium hydroxide test and the shinoda test were conducted for screening of flavonoids. After the addition of 2 ml of 10% aqueous sodium hydroxide solution into the crude extracts, yellow color precipitation indicated the presence of flavonoid. When yellow color turned into colorless, diluted hydrochloric acid was added. In case of shinoda test, 2 drops of conc. HCl followed by a few fragments of magnesium ribbon were added. Formation of pink, reddish or brown color usually specifies the presence of flavonoids [33]. Few drops of Wagner's reagents or Dragendorff's reagent in crude extracts was used for the confirmation of the presence of alkaloids. Appearance of a reddish-brown or orange red precipitation was considered as the positive test for alkaloids [33-36]. Addition of 2-3 drops of 5% ferric chloride to the crude extracts turned into brownish green or a blue-black color results the presence of tannin [35]. A fresh mixture was produced from equal amount of 1% ferric chloride solution and 1% potassium ferrocyanide, 3 drops of the mixture was further added to the extract and filtered this solution, presence of phenol obtained from the formation of a bluish-green color [36]. For the saponin test, 2.5 ml of extract was added to 10 ml of sterile distilled water in a test tube, and then test tube was sealed with cap and shaken vigorously for about 30 seconds. It was then allowed to stand for 30 minutes; formation of honeycomb froth indicated the presence of saponins [35,36].

Quantitative analysis of phytochemical constituency

Quantitative analyses for phytochemicals were performed for flavonoids, tannin, and phenolics.

Determination of total flavonoids content

Total flavonoids content was determined by following the method by Wang [37]. About 0.2 ml of 10% AlCl3, 0.2ml of 1M potassium acetate and 5.6 ml of distilled water followed by 1 ml of methanolic extract at a concentration of 1 mg/ml was added in a test tube to estimate flavonoid content. Blank was prepared without methanol. A series of standard (20, 40, 60,80, 100 µg/ml) was prepared using Quercetin followed by 0.2 ml of AlCl3, 2ml of 1M potassium acetate, 5.6 ml of distilled water. The mixtures were kept at room temperature for 30 minutes. Absorbance was taken at 415 nm using UV-vis spectrophotometer. The result was expressed in mg QE/g of the dried plant extractives.

Determination of total tannins content

The tannins content was estimated by Folin-Ciocaltue's method for both seaweed species as described by Kavita and Indira [37,38]. About 0.1 ml of the sample extract was added to a test tube containing 7.5 ml of distilled water and 0.5 ml of Folin-Ciocaltue phenol reagent. Approximately 1 ml of 35% Na2CO3 diluted to 10 ml with distilled water. The mixtures were shaken well and kept at room temperature for 30 minutes. A set of reference standard solutions of tannic acid (20, 40, 60, 80, 100 µg/ml were prepared. Absorbance for test and standard solutions were measured against the blank at 700 nm with an UV/ Visible spectrophotometer. The estimation of the tannin content was carried out in triplicate. The tannin content was expressed in terms of mg of tannic acid equivalent per g of dried sample.

Determination of total phenolics content

The total phenolic content of dry extract was performed with Folin-Ciacaltue assay with slight described by Singleton et al., (1999) with slight modification [36,37]. About 1 ml of methanolic extract sample (1mg/ml) was mixed with 5 ml of 10% Folin Ciaocalteu Reagent and 5 ml of 7.5% Na2CO3. Blank was concomitantly prepared, containing 1 ml methanol, 5 ml of 10% Folin Ciocalteu's reagent and 5 ml of 7.5% of NaHCO3. The mixtures were incubated for 20 minutes at 25ºC followed by measuring absorbance taken at 760 nm against blank. Gallic acid was used as standard and reactions were performed as triplicates and mean value of absorbance was obtained. Calibration line was constructed using gallic acid as standard. The total content of phenol in extract was expressed in terms of Gallic acid equivalent (mg of GAE/g of extract).

Antibacterial activity of seaweeds extracts

Microorganisms and media

Five bacteria species obtained from the Laboratory of Microbiology, Department of Genetic Engineering and Biotechnology, Shahjalal University of Science and

Technology and used as the antimicrobial test strains: There were four gram negative (*Escherichia coli, Klebsiella sp., Pseudomonas sp., Salmonella sp*.) and one gram positive (*Staphylococcus aureus*) bacteria. The bacterial strains were maintained on the nutrient agar medium [39].

Agar disk diffusion assay

The screening of antimicrobial activity of the seaweed extracts was carried out with agar disk-diffusion method using Muller Hinton Agar (MHA) medium [34,40]. Bacterial culture (50 µL) was taken from the nutrient broth culture using 100 µL micropipette and poured into the sterile plate containing Muller-Hinton agar medium. Sterile cotton was used for streaking the dried surface of plates. Under aseptic condition, prepared discs (5 mm round filter paper soaked with test solution at a concentration of 1mg/ml) were air dried, placed into center of an agar plate by using a sterile forceps and pressed down. Then discs were employed to be incubated at 37°C within 15 minutes. After 24 & 48 hours of incubation, each plate was examined. There was uniformly circular zone of inhibition on the surface. The diameter of the complete zone of inhibition was measured. All tests were performed in triplicate manner.

RESULTS

Preliminary qualitative phytochemical analysis

The present study exposed that ethanolic extracts of *H. musciformis* and *E. intestinalis* contained different plant secondary metabolites *viz* alkaloids, flavonoids, tannins, phenols, and saponins. Preliminary phytochemicals study revealed that *E. intestinalis* extracts contained flavonoids and saponins in higher extent on the other hand alkaloids and tannins were present in *H. musciformis* in greater content. Other secondary metabolites were moderately present in two seaweed extracts (Table 1).

Quantitative determination of the phytochemical constituency

Table 2 showed the quantitative determination of the tannin, flavonoid and phenolic content. Folin-Ciocaltue's method was used to determine the total tannin content in different seaweed extracts. The tannin content was expressed in terms of mg of Tannic acid equivalent per g of dried sample. Ethanolic extracts of *H. musciformis and E. intestinalis* estimated 51 ± 0.0002 and 43 ± 0.0002 mg/g, respectively. However, total flavonoid contents of the extracts were expressed in terms of quercetin equivalent (QE). *H. musciformis* and *E. intestinalis* estimated 67 ± 0.0002 and 57 ± 0.0002 mg/g respectively. Total phenolic contents of the extracts were determined in terms of gallic acid equivalent (GAE). *H. musciformis* exhibited higher amount of phenolics (59 ± 0.0002) present in the crude extracts rather than *E. intestinalis* extracts (41 ± 0.0002).

Table 1. Phytochemical screening of *H. musciformis* and *E. intestinalis* collected North Nuniar Chor, Cox's bazaar, Bangladesh.

Name of Test	Method of Test	Seaweed	
		H. musciformis	*E. intestinalis*
Flavonoids	Sodium Hydroxide test	++	++
	Shinodas test	++	+++
	Wagner's test	+++	+
Alkaloids	Dragendorff's test	++	+
	Potassium dichromate test	+++	++
Tannins	Ferric chloride test	++	++
Phenols	-	++	++
Saponins	Froth test	++	+++

[+++: Higher presence, ++: Moderate presence, +: Low presence]

Table 2. Quantitative determination of phytochemical constituency

Seaweeds Extracts (1 mg/ml)	Quantitative determination of selected phytochemicals		
	Tannic content (mg TAE/g sample)	Phenolics content (mg GAE/g sample)	Flavonoid content (mean ± SD) (mg QE/g sample)
H. musciformis	51 ± 0.0002	59 ± 0.0002	67 ± 0.0002
E. intestinalis	43 ± 0.0002	41 ± 0.0002	57 0.0002

Figure 1. Sample collection site; North Nuniar Chor, Cox's bazaar, Banglades (21°35′0″N 92°01′0″E)

Figure 2. The pictorial view of *Enteromorpha intestinalis* and *Hypnea musciformis* at North Nuniar Chor, Cox's bazaar, Bangladesh.

Table 3. Screening of antibacterial activity of *H. musciformis* and *E. intestinalis* extracts by soaking method

Seaweed	Solvent	Zone of inhibition (mm)				
		Gram positive bacteria	Gram negative bacteria			
		S. aureus	*E. coli*	*Klebsiella sp.*	*Pseudomonas sp.*	*Salmonella sp.*
H. musciformis	Ethanol	10 ± 0.75	10 ± 0.28	13 ± 0.30	12 ± 0.80	10 ± 0.35
	Methanol	7 ± 0.50	11 ± 0.80	8 ± 0.75	10 ± 0.90	10 ± 0.85
	Acetone	14 ± 0.26	6 ± 0.38	8 ± 0.90	9 ± 0.57	6 ± 0.63
	Distilled water	10 ± 0.45	12 ± 0.25	10 ± 0.39	10 ± 0.47	13 ± 0.77
E. intestinalis	Ethanol	13 ± 0.88	14 ± 0.76	13 ± 0.45	13 ± 0.86	11 ± 0.28
	Methanol	15 ± 0.35	12 ± 0.90	13 ± 0.36	15 ± 0.65	14 ± 0.63
	Acetone	15 ± 0.55	8 ± 0.50	10 ± 0.32	13 ± 0.28	10 ± 0.30
	Distilled water	11 ± 0.44	7 ± 0.57	12 ± 0.90	12 ± 0.32	14 ± 0.58
Gentamicin (Control)		18 ± 0.25	19 ± 0.50	15 ± 0.41	17 ± 0.34	18 ± 0.40

*Study followed as triplicate manner

Table 4. Screening of antibacterial activity of *H. musciformis* and *E. intestinalis* extracts by water bath method

Seaweed	Solvent	Zone of inhibition (mm)				
		Gram positive bacteria	Gram negative bacteria			
		S. aureus	*E. coli*	*Klebsiella sp.*	*Pseudomonas sp.*	*Salmonella sp.*
H. musciformis	Ethanol	12 ± 0.28	14 ± 0.57	16 ± 0.25	13 ± 0.56	14 ± 0.45
	Methanol	13 ± 0.89	12 ± 0.76	15 ± 0.23	12 ± 0.43	10 ± 0.27
	Acetone	11 ± 0.48	12 ± 0.37	13 ± 0.65	13 ± 0.39	10 ± 0.43
	Distilled water	12 ± 0.38	13 ± 0.46	14 ± 0.28	12 ± 0.67	11 ± 0.19
E. intestinalis	Ethanol	11 ± 0.77	12 ± 0.45	11 ± 0.66	10 ± 0.89	11 ± 0.52
	Methanol	10 ± 0.35	12 ± 0.83	10 ± 0.64	12 ± 0.86	08 ± 0.56
	Acetone	10 ± 0.47	11 ± 0.67	12 ± 0.84	12 ± 0.11	10 ± 0.66
	Distilled water	09 ± 0.43	10 ± 0.56	10 ± 0.34	11 ± 0.24	12 ± 0.50
Gentamicin (Control)		18 ± 0.25	19 ± 0.50	15 ± 0.41	17 ± 0.34	18 ± 0.40

*Study followed as triplicate manner

Antibacterial activity of seaweeds extracts

Two extraction methods, for instances, soaking and water bath method were applied for generating seaweeds extracts by using ethanol, methanol, acetone and distilled water. The results of antibacterial activities of the seaweeds extracts against selected bacterial strains are summarized in Table 3 in terms of socking method and Table 4 for water batch method.

In case of soaking method, ethanol extracts of *H. musciformis* and *E. intestinalis* were found more than 10 mm inhibition zone against all of the bacterial strains. But, methanol extracts of *E. intestinalis* were found more active against *Staphylococcus aureus Pseudomonas* as these showed inhibition diameter greater than 15 mm, which was similar to our studied control; Gentamicin (Figure 3). In soaking method ethanol extract of *H. musciformis* showed highest zone of inhibition (14 ± 0.76 mm) against *E. coli* and methanol extract of *E. intestinalis* showed highest zone of inhibition against *S. aureus* (Figure 3). Acetone extract of *H. musciformis* show highest zone of inhibition against *S. aureus* but which is less than *E. intestinalis*.

In case of water bath method, *Klebsiella sp.* was found more susceptible to ethanol and methanol extracts of *H. musciformis* as it showed inhibition zone greater than 15 mm (Figure 3). However, in terms of *E. intestinalis*, inhibition zone was found more than 10 mm in maximum antimicrobial tests against all bacterial strains. Again, *H. musciformis* exhibited more active zone of inhibition against different microbes rather than *E. intestinalis*.

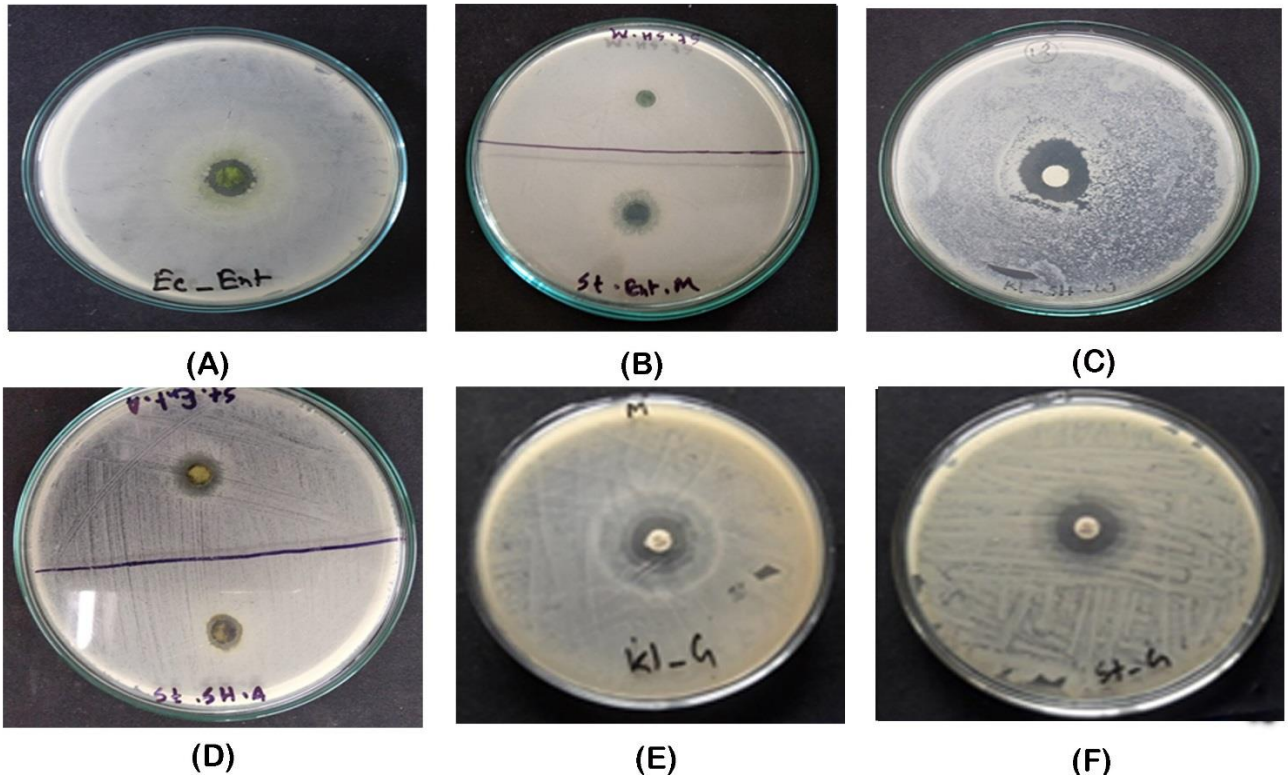

Figure 3. Growth inhibition zone of different extracts of *H. musciformis* and *E. intestinalis*. (A) *E. intestinalis* ethanol extract against *E. coli* (soaking method). (B) *E. intestinalis* and *H. musciformis* methanol extract (soaking method) against *S. aureus*. (C) *H. musciformis* ethanol extract against *Klebsiella* sp. (Water bath method). (D) *E. intestinalis* and *H. musciformis* acetone extracts against *S. aureus* (soaking method). (E) Gentamicin against *Klebsiella* sp. (F) Gentamicin against *Staphylococcus aureus.*

DISCUSSION

Phytochemical profiling of the seaweed samples revealed the presence of different phytochemicals in *H. musciformis* and *E. intestinalis.* Phenol, tannin and flavonoids were found in greater amount in both seaweeds extracts. Comparing the quantitative results of both seaweeds extracts it had been found that *H. musciformis* contained more phytochemicals than *E. intestinalis*. Moreover, *H. musciformis* exhibited higher amount of phenolics (59 ± 0.0002 mg/g), tannin (51 ± 0.0002 mg/g) and flavonoid (67 ± 0.0002 mg/g) present in the crude extracts rather than *E. intestinalis* extracts. These phytochemicals could exhibit antimicrobial

activity, Rojas *et al*. indicated that the antibacterial activity is due to different chemical agents present in the extract such as flavonoids, triterpenoids and other compounds of phenolic nature or free hydroxyl group which was also supported by other study [41,42, 43]. Extraction methods, different solvents used and time frame of sample collections may influence the antibacterial activity [40]. There were several different organic solvents that had already been suggested to screen algae for antibacterial activity [44]. In the present study, we used two different extraction methods; water bath method and soaking method. Among them *H. musciformis* showed better results in water bath extraction method and *E. intestinalis was better* in soaking method. So it could be assumed that *H. musciformis* release more bioactive compound in water bath method and *E. intestinalis* in soaking method.

In current study we used alcoholic and aqueous solvents to generate seaweeds extracts where ethanol extract of *E. intestinalis* showed highest zone of inhibition against *E. coli* and methanol extract of *E. intestinalis* showed utmost zone of inhibition against *S. aureus*. Ethanol extract of *E. intestinalis* and methanol extracts of *H. musciformis* were more effective against selected bacterial strains.

The seaweed extracts were effective against both gram positive and gram negative bacteria. Kolanjinathan and Stella showed that some macro alge effective against *E. coli, P. aeruginosa, S. aureus, K. pneumoniae, E. faecalis* [26,45] which strongly supports the study results. There are some other studies which could strengthen our findings, for example, Tuney *et al.* illustrated that *Enteromorpha sp.* Is effective against *Candida* sp., *E. faecalis, S. aureus, S. epidermidis, P. aeruginosa, E. coli* [46]. Sukatar *et al.* reported that *Enteromorphalinza* is highly susceptible to *S. aureus, S. epidermidis, S. fecalis, B. subtilis, S. typhimurium, P. aeruginosa, E. cloacae, E. coli, C. albicans* [47].

The both studied seaweeds, *H. musciformis* and *E. intestinalis* could possess potential antimicrobial activities though it needs further comprehensive studies. The availability of secondary metabolites in the natural extracts could directly or indirectly influence the induction of antimicrobial approaches [48,49,50].

CONCLUSIONS

The priority for the next decades should be focused in the expansion of alternative drugs. Antimicrobial potentiality and phytochemical screening of seaweeds could be the best solution for many pharmaceuticals questions. Much consideration has been funded to the development of innovative projects for the pharmaceutical applications of seaweed, particularly in the design of novel antimicrobial drugs.

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AUTHOR CONTRIBUTIONS

This work is a collaboration among all the authors. Idea conceptualization, supervision, manuscript finalization and technical support by Mahmudul Hasan. Sample collection, characterization, antibiotic activity screening (Disc Diffusion Method), and manuscript prepared by Md. Abdus Shukur Imran. Sample collection, characterization, antibiotic activity screening (Disc Diffusion Method) by Farhana Rumzum Bhuiyan. Qualitative phytochemical screening by Sheikh Rashel Ahmed and Parsha Shanzana. Tannin by Mahmuda Akter Moli. Flavonoid Screening by Shakhawat Hossain Foysal and Mahmuda Akter Moli. Phenol Screening by Suma Bala Dabi. All authors read and approved the final submitted version of the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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