Isolation of *Enteromorpha* species and analyzing its crude extract for the determination of in vitro antioxidant and antibacterial activities

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Abstract
The extract of green algae (*Enteromorpha* species) was prepared by the cold extraction technique. The prepared algal extract exhibits a high antioxidant potential due to the presence of sulfated polysaccharides (SPs). The extract of *Enteromorpha* species was analyzed to identify the presence of significant biochemical composition. The extract of *Enteromorpha* species was evaluated to assess the DPPH-free radical scavenging activity, total antioxidant activity by phosphomolybdenum assay, in vitro anti-bacterial by agar diffusion method, and cell viability by MTT assay. It was found that the extract of *Enteromorpha* species contains the various chemical composition such as carbohydrates (0.13 g/ml), xylose (0.0819 g/ml), sulfate (0.0153 g/ml), and proteins (0.0363 g/ml). Phytochemicals such as flavonoids and phenolic compounds were found in the extract. The antioxidant potential of the crude extract was investigated by the total antioxidant assay (400 µl/ml) and DPPH-free radical scavenging assay (5 µl/ml). The prepared green algal extract produced the highest inhibitory zone up to 18 mm, 13 mm, and 18 mm at 200 µl/ml concentrations against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*, respectively. The above results revealed that the extract of *Enteromorpha* species exhibited strong antioxidant and antibacterial activities due to the presence of sulfated polysaccharides.

Keywords Green algae · Kovalam · Sulfate moieties · Antioxidant · Antibacterial · Cytotoxicity

Abbreviations

| Abbreviation | Definition                                      |
|--------------|-------------------------------------------------|
| MTT-3        | (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| DPPH         | 2,2-Diphenyl-1-picrylhydrazyl                   |
| MCF-7        | Michigan Cancer Foundation-7 (human breast cancer cell line) |
| COVID-19     | Coronavirus disease 19                         |
| SARS-CoV-2   | Severe acute respiratory syndrome coronavirus 2 |
| DMSO         | Dimethyl sulfoxide                             |

1 Introduction

The *Enteromorpha* species (*Enteromorpha* sp.) are edible seaweed that can grow abundantly in littoral zones of polluted and eutrophicated coastal marine waters. A green alga, *Enteromorpha*, has been used as a food diet in East Asia. It is grown and cultivated along seashores throughout the world, most notably in Japan, Korea, and China [1]. Another ancient report says that *Enteromorpha* is not only used as food but also as a natural medicine for treating fever, epistaxis, inflammation, and hydrops fetalis [2]. The cell wall of filamentous green macroalgae contains sulfated polysaccharides [3, 4]. *Enteromorpha* contains abundant nutrients, such as polyunsaturated fatty acids, dietary fiber, vitamins, and minerals [5]. The phytochemicals in *Enteromorpha* were reported as carotenoids, chlorophyll, phycocyanin, phenolic compounds, and flavonoids [6]. Sulfated
polysaccharide possesses antioxidants [7–12, 15], anti-cancer, anti-coagulant, anti-hyperlipidemic, and antiviral activities. [13]. The algal extract of sulfated polysaccharide showed in vitro action against COVID-19 (SARS-CoV-2) [14]. It is having a high industrial demand owing to its potential biological applications [15–17]. In a study conducted by Baek et al. [18], it was demonstrated that an ethyl acetate extract of Enteromorpha prolifera (EAEP) exhibited the strongest antioxidant activity. Interestingly, the chemical composition of sulfated polysaccharides and their activity depends on the geographic location [19, 20].

The antioxidant activity of the extracted sulfated polysaccharides (SPs) from red algae was studied by Souza and his research team [21]. The cell walls of marine algae are mainly composed of sulfated polysaccharides (SPs). The isolated SPs from red, brown, and green algae are classified into carrageenans, fucoidan, and ulvan respectively based on the chemical composition [22]. Chen et al. [23] isolated the SPs from Grateloupia filicina (red algae) and studied its anticoagulant property. The SPs in aqueous extract of Ulva americana (green algae) were evaluated to assess their antibacterial activity against gram-positive and negative bacterial species by Berri et al. [24]. The immune-modulatory activity of the derived SPs from the Enteromorpha prolifera was also reported in the literature [25, 26]. The extracted SPs from Janiaruhens (red algae) and Ulva lactuca (green algae) were evaluated to analyze their antioxidant and phenolic contents by Essa et al. [27]. The promising biological applications of the polysaccharides were extensively reviewed by Vavilala et al. [28] and Chunyan and his coworkers [29].

The immunological activity of extracted SPs from Caulerpa lentillifera (green algae) was studied by Zhang et al. [30]. The study of Wang and his coworkers [31] reported that the aqueous extract of Enteromorpha linza (green algae) showed excellent anticoagulant properties due to the presence of SPs in the extract. The antimicrobial potential of extracted SPs from Chaetomorpha linum (green algae) was evaluated by Hamzaoui et al. [32]. The various marine algae such as Caulerpa lentillifera [33], Enteromorpha intestinalis [34], Codium divaricatum [35], Caulerpa racemosa var. peltata [36], Caulerpa sertularioidea [37], and Caulerpa racemosa [38] were also evaluated for their wide range of biological activities. The SPs were also extracted from Lithothamnion muelleri (red algae) to study its antipheres activity by Malagoli et al. [39]. Salehi et al. reported that Anacardium exhibited antioxidant, antimicrobial, and anticancer effects [40]. Similarly, another study conducted in Bangladesh proved that the presence of bioactive phytochemicals like phenols, tannins, and flavonoids in Amaranthus lividus and Amaranthus hybridus species exhibited strong antioxidant, anticancer, and antimicrobial activities [41]. This study intends to analyze the biological activities of SPs in the aqueous extract of Enteromorpha species (green algae) against pathogenic strains. Covelong (Latitude 12.7898° N, Longitude 80.2542° E) is the unexplored marine hotspot for the isolation of new and potential aquatic organisms [42, 43]. Hence, the current study was planned to isolate the Enteromorpha sp. from the Covelong seashore area, and its crude extract was also screened for antioxidant and antibacterial properties.

2 Materials and methods

2.1 Collection of marine algae

The green algae of Enteromorpha sp. were collected and stored in a sterile glass bottle. The collected algae sample was identified by a standard seaweed manual [44]. From the sample, algal epiphytes and necrotic parts were removed by rinsing them with sterile water. After rinsing, Enteromorpha sp. was shade dried at room temperature for 5 days before the extraction process.

2.2 Crude extract preparation

Enteromorpha sp. powder was subjected to a cold acidic extraction. In brief, the sample was defatted and decolorized in methanol:acetone solvent mixture (3:7) followed by 2-day stirring in 1 N HCl. An equal volume of ethanol was added to the final extract (Fig. 1). This ethanol suspension was left overnight at –20 °C. The precipitate was separated by centrifugation (3840 g for 60 min at 4 °C) and stored at 4 °C. This extract was further subjected for bio-chemical analysis and screened for antioxidant, anti-bacterial, and cytotoxicity effects.

2.2.1 Biochemical analysis

The presence of sulfate, total carbohydrates, xylose and protein was identified in the Enteromorpha sp. sp., extract. In brief, the total carbohydrate level was estimated by the phenol–sulfuric acid method [45]. The barium chloride–gelatin protocol was used to determine the sulfate content, and the potassium sulfate was used as standard. The monosaccharide xylose was estimated using the orcinol method [46]. The protein level was estimated by Lowry’s method [47].

2.3 Phytochemical test

The fresh extract of Enteromorpha sp. was examined to analyze the presence of phytochemicals such as flavonoid and phenolic compounds. The method of analysis is discussed here.
2.4 Flavonoids

An alkaline reagent test was used to determine the presence of flavonoids in algal crude extracts. Ten milligrams of aqueous crude extract was mixed with 3 ml of 2% sodium hydroxide solution to carry out this analysis. The presence of flavonoids in the extract was identified by the formation of intense yellow color (Fig. 2a).

2.5 Phenolic compounds

Lead acetate test was used to identify the presence of phenols in algal crude extracts. In this study, the crude ethanolic extract of Enteromorpha sp. produced a bulky white precipitate (Fig. 2b) upon the addition of 10% lead acetate solution. This result confirms the presence of phenolic compounds in the extract of Enteromorpha sp.

2.6 In vitro antioxidant activities

In vitro antioxidant activity of Enteromorpha sp. was proved by determining the total antioxidant capacity and free radical scavenging activity. The determination of total antioxidant potential was done by phosphomolybdenum assay [48]. The assay principle involves the reduction of Mo (VI) to produce a green complex at lower pH conditions. One hundred microliters, 200 µl, 300 µl and 400 µl of algal extracts were mixed with 1 ml of DMSO and incubated in a water bath at 95 °C for 90 min. After incubation, the absorbance values of the mixture were read at 695 nm. The ascorbic acid (10 mg/ml DMSO) was used as a standard. The % phosphomolybdenum reduction potential (PRP) was calculated by the following formula:

\[
\text{Phosphomolybdenum reduction potential (\%) } = \frac{[\text{Control}] - [\text{Sample}]}{[\text{Control}]} \times 100 \tag{1}
\]
where Abs (control) is the absorbance value of the control and Abs (sample) is the absorbance value of the extracts.

### 2.7 Free radical scavenging determination

The DPPH free radical–scavenging assay was carried out to examine the antioxidant potential of the crude extract of *Enteromorpha* sp. [49]. This assay may prevent the oxidation of the substrate. The experiment was carried out with the addition 5 μl, 10 μl, 15 μl, and 20 μl of algal extract to the mixture of 40 μl DMSO and 2.96 ml of DPPH (0.1 mM). The reaction mixture was then incubated under dark condition for 20 min at room temperature to record the readings at 517 nm. Three milliliters of DPPH was used as a control.

\[
\% \text{DPPH scavenging activity} = \frac{\text{Abs (control)} - \text{Abs (sample)}}{\text{Abs (control)}} \times 100
\]

(2)

where Abs (control) is the absorbance value of the control, and Abs (sample) is the absorbance value of the extracts/standard.

### 2.8 In vitro antibacterial activity

The agar disc diffusion method was used to determine the antibacterial efficacy. One hundred microliter, 150 μl, and 200 μl concentrations of *Enteromorpha* sp. extracts were saturated in the sterile paper disc and tested against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*. After 48 h of incubation, the inhibitory zones were measured. The agar diffusion method was done according to the guidelines of the Clinical and Laboratory Standard Institute (CLSI) [50, 51].

### 3 Results and discussion

The extract of *Enteromorpha* sp. was investigated to analyze its antioxidant, antibacterial, and anticancer properties. All the obtained results were compared and discussed critically to conclude the antioxidant and antibacterial potentials of SPs.

#### 3.1 Chemical analysis of sulfated polysaccharides

The sulfated polysaccharides of *Enteromorpha* sp. are an excellent source of sulfate and xylose [52]. In this current study, the extract of *Enteromorpha* sp. was quantitatively examined for carbohydrate, sulfate, xylose, and proteins, respectively. The existence of these essential ingredients was determined at a significant level in the extract. They are as follows: carbohydrate up to 0.13 g/ml (phenol–sulfuric acid method) (Fig. 3a and c); sulfate up to 0.0153 g/ml (Barium chloride-gelatin method) (Fig. 3b and d); xylose up to 0.0819 g/ml (Orcinol method) (Fig. 4a and c), and protein up to 0.0363 g/
ml (Lowry’s method) (Fig. 4b and d). This analysis was highly correlated with the previous studies on *Corallina officinalis*, *Pterocladia capillacea* [53], *Laminaria japonica* [54], and *Fucus vesiculosus* [55]. Hence, it confirms the presence of sulfated polysaccharides in the extract.

### 3.2 Antioxidant activity of extract of Enteromorpha spsces

The antioxidant efficacy of *Enteromorpha* sp. was examined by performing total antioxidant and DPPH-free radical scavenging assays. SPs derived from marine seeds are known for antioxidant activity [56]. In both the assays, ascorbic acid was used as a standard. In the total antioxidant assay, the scavenging potential of the extract of *Enteromorpha* sp. was determined. The *Enteromorpha* sp., extract effectively reduced the molybdenum [Mo (IV)] into phosphomolybdenum which paved the way for the formation of dark green color (Fig. 5a). It was confirmed with the development of a dark green color appearance. The extract of *Enteromorpha* sp. showed a free radical scavenging effect of 23 ± 0.35, 41 ± 0.05, 60 ± 0.52, and 81 ± 0.5% at the various extract concentrations of 100 μL, 200 μL, 300 μL, and 400 μL respectively (Fig. 5c). This result proved that Mo (IV) reducing activity is dose-dependent. Sulfated polysaccharides present in the *Enteromorpha* sp. may act as the reducing agents to scavenge the free radicals [57]. But the activity is higher compared to the studies done with other algal extracts [58].
3.3 DPPH-free radical scavenging assay

During the DPPH assay, the free radical scavenging activity was confirmed by the formation of yellow color (Fig. 5b). At the algal extract concentrations of 5, 10, 15, and 20 μg mL⁻¹, the free radical scavenging capacity was measured as 69 ± 0.52, 75 ± 0.34, 79 ± 0.82, and 87 ± 0.82% respectively (Fig. 5d). When the concentration was increased from 5 to 20 μg mL⁻¹, the scavenging capability also increased. Hence, the scavenging activity is dose-dependent for the prepared extract of Enteromorpha sp. It was observed that 20 μg mL⁻¹ showed the highest percentage of activity. The obtained results of algal extract were compared with the ascorbic acid standard. The current results are in good agreement with a previous study [59, 60].

3.4 Antibacterial determination

The extract of Enteromorpha sp. had been tested against P. aeruginosa, S. aureus, and E. coli to analyze its antibacterial activity by disc diffusion method (Fig. 6). At the concentration of 150 mL⁻¹ and 200 mL⁻¹, it produced a zone of clearance with the diameter of 11 ± 0.2 mm and 13 ± 0.2 mm, respectively against Pseudomonas aeruginosa (Fig. 6a). It exhibited inhibitory zones of 10 ± 0.2 mm, 16 ± 0.2 mm, and 18 ± 0.2 mm at the concentration of 100 mL⁻¹, 150 mL⁻¹, and 200 mL⁻¹, respectively for Staphylococcus aureus (Fig. 6b) and 11 ± 0.2 mm, 15 ± 0.2 mm, and 18 ± 0.2 mm respectively against Escherichia coli (Fig. 6c). The difference in the inhibitory zones is due to the resistance pattern of the organisms. The
antibacterial activities of marine algal extract are mainly due to the presence of sulfated polysaccharides [61]. However, it showed less activity against gram-negative bacteria [62]. The results of the disc diffusion assay proved the broad-spectrum antibacterial efficacy of *Enteromorpha* sp. Srikonga et al. studied the effects of green seaweed, *U. intestinalis*. The algal extract demonstrated antimicrobial activity against gram-positive bacteria, with inhibition zones ranging from 6.85 ± 0.17 to 16.4 ± 2.4 mm [63]. *U. intestinalis* was also found to possess strong antioxidant activity. It was reported that the methanolic extract of *U. intestinalis* exhibited the highest DPPH scavenging activity (48% inhibition) and a lower IC50 value of 2.32 mg/ml [64]. Kim and Jeong [65] investigated the antimicrobial and antioxidant activities of *Enteromorpha intestinalis*. Three solvents were used by them to obtain the extracts of *Enteromorpha intestinalis*. The obtained results proved that the extracts exhibited strong antimicrobial and antioxidant activity [65].

**4 Conclusion**

The extract of *Enteromorpha* sp. showed significant biological activities. The presence of major constituents in the extract of *Enteromorpha* sp. was identified as sulfate, xylose, carbohydrate, and proteins. Phytochemicals like phenolic compounds and flavonoids were also present in the marine algal extract. The radical scavenging activity of the *Enteromorpha* sp. was found to be increased from 23 ± 0.35 to 81 ± 0.5% with the increasing concentration of the algal extract. Therefore, the radical scavenging activity of *Enteromorpha* sp. was concluded as dose-dependent. The diameter of the inhibitory zone was increased from 10 ± 0.2 to 18 ± 0.2 mm for *Staphylococcus aureus* and from 11 ± 0.2 to 18 ± 0.2 mm for *Escherichia coli* while increasing the concentration of algal extract from 100 to 200 mL⁻¹. This demonstrated the excellent antioxidant and antibacterial properties of the *Enteromorpha* sp. The extract of *Enteromorpha* sp. may be used for the betterment of mankind owing to its promising biological activities.
**Fig. 6** Antibacterial activity of Enteromorpha sp. extract against bacterial strains. 
a Pseudomonas aeruginosa.  
b Staphylococcus aureus.  
c Escherichia coli

**Declarations**

**Conflict of interest**  The authors declare no competing interests.

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