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Antimicrobial and Phytochemical Screening of Seaweeds: *Enteromorpha intestinalis* and *Ulva lactuca* Collected from Indian Sunderbans Delta Region

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Abstract: *The aim of this study was to evaluate the antimicrobial activities of two green seaweeds: Enteromorpha intestinalis and Ulva lactuca collected from the Matla river of Indian Sunderbans Delta. Both seaweeds showed antibacterial activities against several gram positive, and gram negative human pathogens obtained either clinically or from microbial type collection centre. Several pathogens among the clinically isolated strains were multidrug resistant isolates. Among the different solvents tried, best activity was noted in the n-Hexane fraction. Along with the antimicrobial activities, both seaweeds also showed antifungal activities. The MIC values of the activities were determined by disc diffusion assay and macrodilution assay. The phytochemical screening revealed the presence of xanthoprotein, flavonoid, tannin, alkaloid, glycosides, saponins and cardiac glycosides.*

Keywords: *Antimicrobial, phytochemical screening, seaweed extracts, Enteromorpha intestinalis, Ulva lactuca*

I. Introduction

Seaweeds are the source of important bioactive compounds and various seaweeds possess various properties like antiviral, cytotoxic, antihelminthic, antioxidant, haemolytic, antifungal and antibacterial activities (1-3). They are also used as food, feed and fertilizer (4). They are the active ingredients of many life saving drugs for treatment of cancer, arthritis etc. Marine seaweeds are rich in polyunsaturated acids, carotene, sulphated polysaccharides and sterols. Antimicrobial activity of seaweeds has been studied in different parts of the world (5-7). In India, antibacterial activity of sea weeds (*Sargassum wightii, Stocheospermum marginatum, Gracilaria foliifera, Padina boergesenii, Gracilaria edulis, Ulva lactuca*) collected from various areas like Gulf of Manner, Andaman, South East Coast of India has been shown to possess antibacterial activity (8-12). However, no studies were performed with sea weeds collected from the Indian Sundarbans. In the present study, we describe the anti-microbial characteristics of two marine algae (*Enteromorpha intestinalis* and *Ulva lactuca)* obtained from the Matla river of the Indian Sunderbans. *Enteromorpha intestinalis* is green seaweed with tubular and elongated fronds that may be branched, flattened or inflated. They are found in all levels of shore and salt marshes and are able to withstand low salinities. Where conditions are calm, the seaweeds may detach and survive as free floating clumps. *Ulva lactuca* is known by its common name sea lettuce. It is green algae in the division of chlorophyta. The overall objective of the present study was to ascertain the antimicrobial activities of the *Enteromorpha intestinalis* and *Ulva Lactuca* extract against selected multidrug resistant bacteria and other clinical gram positive and gram negative pathogens known to cause disease in humans and animals. Presence of important phytochemicals was also determined.

II. Materials and Methods

1. Sample collection

The seaweeds (*Enteromorpha intestinalis* and *Ulva lactuca*) were collected from the Matla River of Indian Sunderbans delta region (22˚18'20"N latitude and 88˚40'46"E longitude).

2. Preparation of the seaweed extract

The seaweeds were collected, cut into small pieces and air dried in shade. The dried seaweed materials were grounded to a coarse powder by mortar and pestle. 1 g of these two seaweeds was extracted in 10 ml of n-Hexane, chloroform and ethyl acetate. The conical flasks were kept in shaker for 3 days. Then the samples were filtered using nitrocellulose membrane filter paper. The crude extracts were preserved in sterile and air tight container at -20^{o}C for further analysis.

3. Microorganisms used and their characteristics

Both gram positive and gram negative human pathogens were obtained from Microbial Type Culture Collection. Some strains were obtained clinically from hospital patients. Table 1 shows name of the strains used and their properties.

Table 1: Microbial strains used to determine antibacterial activity of the sea weed extract.

Serial number	Bacteria used	Source	Characteristics
1	*Psychrobacter* sp.	Isolated clinically	Gram negative, Cocci/ Coccobacilli
2	*Delftia* sp.	Isolated clinically	Gram negative, Rod shaped
3	**Citrobacter* sp.	Isolated clinically	Gram negative, Coliform bacteria
4	**Stenotrophomonas* sp.	Isolated clinically	Gram negative, Rod shaped
5	*Klebsiella pneumoniae*	ATCC 700603	Gram negative, Rod shaped & Antibiotics susceptible
6	*Enterococcus faecalis*	ATCC 29212	Gram positive, Rod shaped & Antibiotics susceptible
7	*KPC producing *Klebsiella pneumonia*	ATCC BAA1705	Gram negative, Rod shaped, Carbapenemase producing MDR strain
8	*Escherichia coli*	ATCC 25922	Gram negative, Rod shaped & Antibiotics susceptible
9	*Bacillus subtilis*	ATCC 6051	Gram positive, Rod shaped & Antibiotics susceptible
10	*Staphylococcus aureus* subsp.	ATCC 25923	Gram negative, cocci & Antibiotics susceptible
11	**Pseudomonas* sp.	Isolated clinically	Gram negative, rod & Multiple Antibiotics susceptible

*Multi drug resistant bacteria

4. Antimicrobial assay of the seaweed extracts by disc diffusion assay

The antibacterial activity was determined based on the method of (O'Bryan *et al*. 2008) (13) with modifications. Autoclave-sterilized (121 °C for 20 min) Luria Bertini medium (Hi Media) was used in the disc diffusion assay. A 50-ml sample of the filtration-sterilized plant extract was loaded onto a sterile paper disc (6 mm in diameter), which was then placed on the surface of the agar plate previously inoculated with the bacteria. A disc prepared under the same conditions with only 50 ml of solvent was used as a negative control. In addition, a similar disc was loaded with the reference antibiotic standard (ampicillin for gram positive bacteria and kanamycin for gram negative bacteria) at a concentration of 50ug/ml. The plates were incubated at 37 °C for 24 h. Antibacterial activity was determined by calculating the diameter of the growth inhibition zones (mm) around the disc. Each assay was performed in triplicate and the results were expressed as average values. The inhibition zones of the extracts prepared in different solvents were compared and the n-Hexane extract which gave the best results for all the microbes tested were used in later experiments.

5. Determination of the **antifungal activity of the seaweed extract**

In order to check the antifungal activity of the seaweed, the disc diffusion method was used again. 30 ml of YPD broth was prepared. *S.cerevisae* (yeast) was inoculated and kept overnight in shaker. Nystatin was used for reference standard. Antifungal activity was determined by calculating the diameter of the growth inhibition zones (mm) around the disc. Each assay was performed in triplicate and the results were expressed as average values. The inhibition zones of the extracts prepared in different solvents were compared and n-Hexane extract which gave the best results for all the microbes tested were used in later experiments.

6. MIC determination by agar dilution assay (macrodilution)

The Minimal Inhibitory Concentration (MIC) of the extract which inhibits growth of the microbe was determined by macrodilution assay carried out in MHA medium (14). Serial dilutions of n-Hexane extracts were prepared in n-Hexane. Four different types of dilutions were prepared. 50 µl of each diluent was added in filter paper disks in LB ager plates containing all the mentioned bacteria. 50 ml of solvent was used as a negative control and above mentioned antibiotics were used as positive control. The plates were incubated at 37℃ for 24 hours, and the bacterial growth was determined on each plate by comparing the punctual growth zones with those in the controls. The absence of growth was interpreted as due to antimicrobial activity of the extract.

7. Determination of Minimum Bactericidal Concentration (MBC)

The MBC was determined by the method of Vila *et al.* (2010) with slight modifications (15). Approximately, 2 µl of the sample from Minimum Inhibitory Concentration assay was spread onto freshly prepared LB agar plates, incubated at 37℃ for 24 hours and monitored for the presence of bacterial growth. The MBC were taken as the lowest concentration that did not allow bacterial growth on the surface of the agar plates.

8. Phytochemical screening

The n-Hexane seaweed extracts were used for phytochemical screening of protein xanthoprotein, terpenoids, resins, saponins, steroid, Acidic compounds, alkaloids, tannins, flavonoids, cardiac glycosides, phenols, anthocyanin, leukoanthocyanin and coomerin by following standard protocols (16-17).

III. Results and Discussion

Antimicrobial screening (antibacterial and antifungal)

The results of antimicrobial assay of the two seaweed extracts showed formation of zone of inhibition surrounding the discs (Figure 1). The measurements of the zone of inhibitions are summarized in the Table 2 & 3. In our study, it was found that the n-Hexane extract of the seaweeds *Enteromorpha intestinalis* and *Ulva lactuca* showed antibacterial activity against *E.coli, Bacillus* sp., ATCC human pathogenic strains *Klebsiella pneumonia*, *Enterococcus faecalis*, *Staphylococcus aureus* and multi drug resistant bacteria such as *Cytobacter sp.*, *Pseudomonas* sp. and *Strenotrophomonas* sp. as well as multi drug resistant human pathogen *Pseudomonas* sp.. Antifungal activity was observed using the same extract. Maximum inhibitory effects against the above mentioned strains were observed from n-Hexane extracts of *Enteromorpha intestinalis* and *Ulva lactuca.* N-Hexane extract of *Enteromorpha intestinalis* showed better result as compared to *Ulva lactuca.*

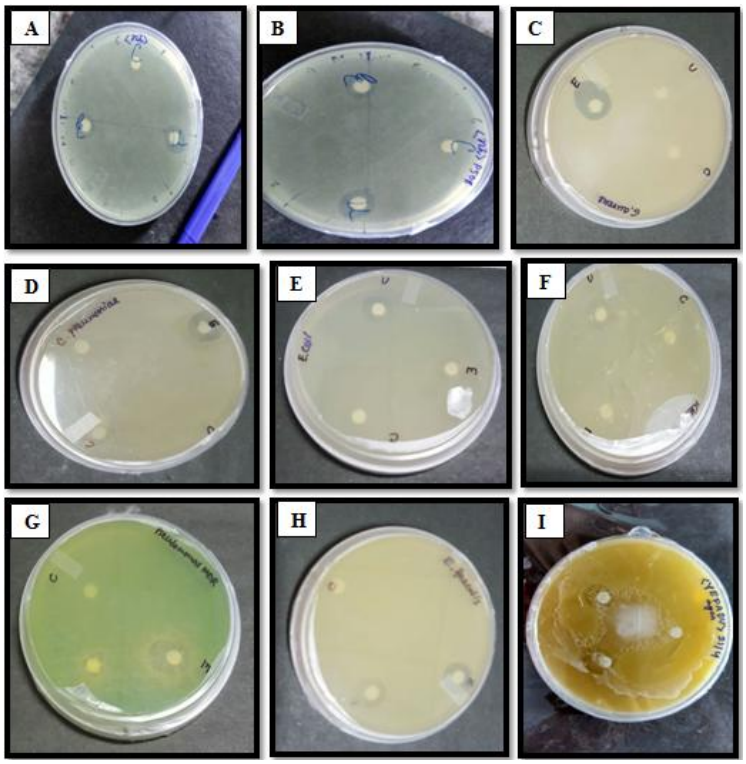

Fig 1: Antibacterial and antifungal activity of n-hexane extracts of the seaweeds. **A-** *Stenotrophomonas sp.*; **B-** *Citrobacter sp.*; **C-** *Staphylococcus aureus subsp.*; **D-** *Klebsiella pneumonia*; **E-** *E.coli*; **F-** KPC roducing *Klebsiella pneumonia*; **G-** *Pseudomonas sp.*; **H-** *Enterococcus faecalis*; **I-** *S.cerevisae*. The three discs in each plate are for E.intestinalis extract, U.lactuca extract and solvent. Disc soaked with solvent was used as the negative control.

Table 2: The results of zone of inhibition of *Enteromorpha intestinalis* are given in tabulated form in the following

Strain	**Zone with n-hexane (in cm)** ± SD	**Zone with Ethyl acetate (in cm)** ± SD
Psychrobacter sp	1 ± 0.02	2.7 ± 0.02
Pseudomonas sp.	1± 0.03	Nil
Bacillus subtilis	1.6 ± 0.05	Nil
Stenotrophomonas sp.	1.4 ± 0.02	Nil
Delftia sp.	1.7 ± 0.04	1 ± 0.04
Citrobacter sp.	1.4 ± 0.04	Nil
E.coli	1 ± 0.03	Nil
Klebsiella pneumonia	1.8 ± 0.07	0.6 ± 0.03
Enterococcus faecalis	1.4 ± 0.05	Nil
**KPC roducing Klebsiella pneumonia*	1.9 ± 0.02	Nil

Escherichia coli	1 ± 0.05	Nil
Bacillus subtilis	1.3 ± 0.08	0.7 ± 0.05
Staphylococcus aureus subsp.	1.7 ± 0.04	0.4 ± 0.05
Pseudomonas sp.	1.7 ± 0.05	Nil
S.cerevisae	1.6 ± 0.10	Nil

Table 3: The results of zone of inhibition of *Ulva lactuca* are given in tabulated form in the following

Strain	**Zone with n-hexane (in cm)** ± SD	**Zone with Ethyl acetate (in cm)** ± SD
Psychrobacter sp	Nil	0.2 ± 0.02
Pseudomonas sp.	1.4 ± 0.04	Nil
Bacillus subtilis	1.5 ± 0.03	0.6 ± 0.04
Stenotrophomonas sp.	0.5 ± 0.08	Nil
Delftia sp.	Nil	Nil
Citrobacter sp.	1.7 ± 0.06	Nil
E.coli	1 ± 0.03	Nil
Klebsiella pneumonia	1.4 ± 0.05	0.3 ± 0.03
Enterococcus faecalis	1.3 ± 0.06	Nil
**KPC roducing Klebsiella pneumonia*	1.3 ± 0.05	0.7 ± 0.04
Escherichia coli	1.3 ± 0.04	Nil
Bacillus subtilis	1.5 ± 0.05	Nil
Staphylococcus aureus subsp.	1.4 ± 0.07	Nil
Psedomonas sp.	0.8 ± 0.05	0.2 ± 0.04
S.cerevisae	1.1 ± 0.07	Nil

Table 4a: MIC of multi drug resistant bacteria against n-hexane extract of *Enteromorpha intestinalis*

Dilutions	**Zones obtained for *Enteromorpha intestinalis* (in cm) ± SD**		
	Pseudomonas sp.	***Stenotrophomonas sp.***	***Citrobacter sp.***
3:7	1.45 ± 0.02	Minimal	1.1 ± 0.05
5:5	2.6 ± 0.03	1.3 ± 0.06	2.2 ± 0.04
7:3	1.35 ± 0.02	1.4 ± 0.08	1.6 ± 0.06
1:9	0	0	0

Table 4b: MIC of multi drug resistant bacteria against n-hexane extract of *Ulva lactuca*

Dilutions	**Zones obtained for *Ulva lactuca* (in cm) ± SD**		
	Pseudomonas sp.	***Stenotrophomonas sp.***	***Citrobacter sp.***
3:7	0.5 ± 0.02	Minimal	1.1 ± 0.05
5:5	2.2 ± 0.05	1.4 ± 0.06	2.0 ± 0.07
7:3	1.0 ± 0.02	0.4 ± 0.08	2.1 ± 0.06
1:9	0	0	0.1±0.01

2. Phytochemical screening of *Enteromorpha intestinalis* and *Ulva Lactuca* n-Hexane extracts

A preliminary qualitative estimation of the presence of different phytochemicals in the n-Hexane extract of *Enteromorpha intestinalis* showed the presence of proteins, xanthoproteins, saponins, cardiac glycosides, terpenoids and acidic compounds. Proteins, xanthoproteins, flavonoid, alkaloid, saponins and cardiac glycosides were present in the n-Hexane extract of *Ulva lactuca*. However, we failed to detect the presence of resins, tannins, alkaloids, flavonoid, phenols, anthocyanin, leukoanthocyanin and coomerin in the case of *Enteromorpha intestinalis* and for *Ulva lactuca* we failed to detect the presence of resins, terpenoids, acidic compounds and phenols.

Table 5: shows the presence and absence of different phytochemicals present in the n-hexane extracts of *Enteromorpha intestinalis* and *Ulva lactuca* seaweeds**.**

Tests	**Inference of n-Hexane extracts of *Enteromorpha intestinalis***	**Inference of n-Hexane extracts of *Ulva lactuca***
Protein & Xanthoprotein	Positive	Positive
Resins	Negative	Negative
Tannins	Negative	Positive
Alkaloids	Negative	Positive
Saponins	Positive	Positive
Cardiac glycosides	Positive	Positive
Terpenoids (Salkowskii Test)	Positive	Negative
Flavonoids	Negative	Positive
Acidic compounds	Positive	Negative
Phenols	Negative	Negative
Glycosides (Keller- Kiliani test)	Positive	Positive
Anthocyanin	Negative	Negative
Leukoanthocyanin	Negative	Negative
Coomerin	Negative	Negative

Nowadays, emergence of bacterial resistance poses a significant clinical problem. The development of bacterial resistance to presently available antibiotics has necessitated the search for new antibacterial agents. As marine organisms including marine seaweeds are proven reservoirs of different previously unknown bioactive compounds, the present study was carried out to find out the antibacterial activity of the two selected sea weeds collected from Indian Sundarbans. Both the sea weed extracts prepared in n-Hexane showed antimicrobial activity against multidrug resistant clinical isolates belonging to *Pseudomonas, Stenotrophomonas* and *Citrobacter*. They also showed activities against a broad range of other gram positive and gram negative pathogens. The zone of inhibition ranged from 0.5-2.6cm. The extracts not only showed antibacterial activity but also anti-yeast property too. In future, we would also test its performance on other fungal strains. More studies need to be performed to isolate the bioactive compound responsible for multi drug resistance.

As seaweeds also have been found to contain various phytochemicals not commonly found in terrestrial plants phytochemical screening were also performed. Our studies confirmed the presence of sulphated polysaccharides, polyphenolic compounds and antioxidant enzymes that previously have been found in many marine of edible seaweeds also.

IV. Conclusion

It can be concluded that the n- Hexane extracts of the *Enteromorpha intestinalis* and *Ulva lactuca* has antimicrobial activities against multiple drug resistant bacteria and can be regarded as a new source of antibacterial and antifungal compounds. However, further research needs to be done on the identification of the bioactive compounds present in the extract. Screening of phytochemicals from the two seaweeds showed the presence of different compounds that differed minutely from each other. In future studies, other bioactive compounds of *E. intestinalis* and *U. Lactuca* may be analyzed qualitatively and also quantitatively with different solvent extracts.

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