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Bio-efficacy of green seaweeds from south east coast of Tamil Nadu, India

Abstract

The present study was aimed to examine the antibacterial potential of *Caulerpa corynephora* Montagne, *Caulerpa scalpelliformis* (R. Br.) *Weber-van Bosse*, *Chaetomorpha antennia* (Bory de Saint-Vincent) Kutzing, *Enteromorpha compressa* (L.) Grev., *Halimeda macroloba* Decsne., *Ulva fasciata* Delile and *Ulva lactuca* Linn. from southern east coast of Tamil Nadu, India. For the bio-efficacy analysis the pathogens viz., *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Serratia marcescens*, *Salmonella typhi*, *Acinetobacter calcoaceticus*, *Enterobacter cloacae*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus pyogenes* and *Bacillus cereus* were procured from Scadder Laboratories. The antibacterial activity was determined by the paper disc diffusion method. 30µg of crude extracts of selected seaweeds were used for the analysis. 30µg and 30µl of the respective solvents were used as positive and negative control. Among the five different extracts examined, antibacterial activities are observed in hexane, petroleum ether, chloroform and ethanolic extracts with varied frequency. Aqueous extracts were failed to show activity against the tested twelve pathogens. In the present study, variability in the antibacterial activity may be related to variability in the bioactive principles present in different seaweeds and organic solvents used for extraction of bioactive compounds. Further purification of the extracts may yield a novel antibacterial drug to treat various diseases typhoid, diarrhoea, pneumonia, meningitis, osteomyelitis, endocarditis, nausea, skin infections etc.

Keywords: antibacterial, seaweeds, bioefficacy

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Introduction

The ancient literatures indicate that seaweed is still employed in folk medicine in many parts of the world as treatments of a variety of diseases. Due to therapeutic potentials of seaweed secondary metabolites (SSM), the seaweeds are employed in the traditional medicines by the traditional healers.[1] Recent research on the marine algae confirmed that seaweeds are credible source for the isolation of diversified novel secondary metabolites.[2,3] A number of biologically active compounds with varied properties viz., antitumour, anticancer, cytotoxic were identified from the seaweeds.[4–9] In addition Villa et al.,[10] Mayer et al.[11] and Blunt et al.,[12] isolated antimicrotubule, antiproliferative photoprotective, as well as antibiotic and antifouling agents from marine sources. Compounds with antioxidant, antiviral, antifungal and antimicrobial activities have been detected in brown, red and green algae.[13–15] The discovery and development of antibiotics are the most powerful and successful achievement of modern science and technology for the control of infectious diseases. Prolonged usage of broad spectrum antibiotics has led to the emergence of drug resistance. There is a tremendous need for novel antimicrobial agents from different sources. Due to the advancement in the analytical chemistry and need for the new antimicrobial agents, the researchers focused their attention on the exploration of seaweeds chemistry and antimicrobial agents.[16–20] Antibacterial efficacy of seaweed extracts against the Gram positive and Gram negative bacteria has been established by several scientists in India and at the global level.[21–34] But very limited studies were conducted on the seaweeds of southern east coast of Tamilnadu, India. Babu et al.,[35] studied the phytochemical constituents of *Caulerpa corynephora* Montagne, *Caulerpa scalpelliformis* (R. Br.) *Weber-van Bosse*, *Chaetomorpha antennia* (Bory de Saint-Vincent) Kutzing, *Enteromorpha compressa* (L.) Grev., *Halimeda macroloba* Decsne., *Ulva fasciata* Delile and *Ulva lactuca* Linn. In addition, they produced the biochemical marker for *C. corynephora*, *C. scalpelliformis*, *C. antennia*, *E. compressa*, *H. macroloba*, *U. fasciata* and *U. lactuca* using UV-vis analysis.[36] To supplement the previous research and continue the rhythm of research, the present study was aimed to examine the antibacterial potential of *Caulerpa corynephora* Montagne, *Caulerpa scalpelliformis* (R. Br.) *Weber-van Bosse*, *Chaetomorpha antennia* (Bory de Saint-Vincent) Kutzing, *Enteromorpha compressa* (L.) Grev., *Halimeda macroloba* Decsne., *Ulva fasciata* Delile and *Ulva lactuca* Linn. from southern east coast of Tamil Nadu, India.

Materials and methods

Preparation of extracts

The dried and powered seaweed materials (30 g) were extracted successively with 180 ml of hexane, petroleum ether, chloroform and ethanol by using Soxhlet extractor for 8 hrs at a temperature not exceeding the boiling point of the solvent. The aqueous extract was prepared by directly boiling the powder with distilled water. The extracts were filtered using Whatman filter paper (No.1) and then concentrated in vacuum at 40ºC using Rotary evaporator. The residues obtained were stored in a freezer -20º C until further tests.

Preparation of the test organisms

The pathogens *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Serratia marcescens*, *Salmonella typhi*, *Acinetobacter calcoaceticus*, *Enterobacter cloacae*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus*

pyogenes and *Bacillus cereus* were procured from Scadder laboratories, Nagercoil, Tamil Nadu and confirmed by conventional microbiology procedure. The procured pathogens were used for the antibacterial activities. Stock cultures of different bacteria were grown in nutrient broth at 30°C and were sub-cultured and maintained in nutrient broth at 4°C. Before swabbing, each culture was diluted (1:10) with fresh sterile nutrient broth.

Antibacterial assay

The antibacterial activity was determined by the paper disc diffusion method.[37] Sterile disc of diameter 5 mm (made from Whatman No. 1 filter paper previously sterilized in autoclave) was dipped in test solution of each extract (30µg of crude extracts of selected seaweeds) prepared by dissolving separately in respective solvents. Then the sterile disc containing test solution of the plant extract was placed over the seeded agar plates in such a way that there is no overlapping of zone of inhibition. The antibiotic amikacin (30µg/disc) was used as standard for bacteria to compare its effect on test organisms with the plant extracts. The plates were kept at room temperature for 2h to allow diffusion of the test solution into the agar; they were incubated for 24h at 37°C. After the incubation period was over, the plates were observed for zone of inhibition measured in millimeters (mm).

Results

The antibacterial activity of five crude extracts (aqueous, ethanol, chloroform, petroleum ether and hexane) of *U. lactuca*, *U. fasciata*, *H. macroloba*, *E. compressa*, *C. antennia*, *C. scalpelliformis* and *C. corynephora* against twelve human bacterial pathogens were determined by paper disc diffusion method and the results were tabulated in Table 1 and their zone of inhibition compared with standard antibiotic amikacin (Table 1). Among the five different crude extracts of *U. lactuca*, antibacterial activities were observed only in hexane, petroleum ether, chloroform and ethanolic extracts of *U. lactuca*. In *U. lactuca*, among the tested crude extracts, highest antibacterial activity was observed in ethanolic extracts followed by petroleum ether, chloroform and hexane. Ethanolic extracts not only produced bigger inhibitory zones, but also active against all the pathogens tested, followed by petroleum ether 4, chloroform 3 and hexane 1. Ethanolic and chloroform extracts of *U. lactuca* were active against, *S. typhi*, *A. calcoaceticus* and *S. aureus* (Table 1). Ethanolic and petroleum ether extract of *U. lactuca* were active against *P. aeruginosa*, *S. aureus*, *S. pyogenes* and *B. cereus*. Ethanolic and hexane extracts produce activity only against *B. cereus*. Compared to the standard (amikacin), the percentage of inhibition varied. Ethanolic extracts of *U. lactuca* was effective against all the tested organisms. However the percentage of inhibition varied with test organisms, highest inhibition was observed against *S. pyogenes* (Gram positive, 118.18%), and lowest against *P. mirabilis* (Gram negative, 35%). Compared to amikacin, 50% and higher inhibitory activities were observed in ethanolic extract against a) *E. coli*, *K. pneumoniae*, *S. typhi*, *A. calcoaceticus*, *S. aureus*, *E. faecalis* and *S. pyogenes*. b) Chloroform extract against S. typhi and A. calcoaceticus.

Among the five different crude extracts of *U. fasciata*, activities were observed only in hexane, chloroform and ethanolic extracts of *U. fasciata* (Table 1). However the size of the inhibition zone varied with test organisms and extracts. Highest activity was observed in ethanolic extracts followed by chloroform and hexane. Ethanolic extracts not only produced bigger inhibitory zones, but also active against 11 different pathogens followed by chloroform (7) and hexane (1). Ethanolic and chloroform extracts of *U. fasciata* were active against *E. coli*, *K. pneumoniae*, *P. mirabilis*, *S. typhi*, *E. cloacae* and *S. aureus*. *S. marcescens* was inhibited only by the chloroform extract of *U. fasciata*. Similarly ethanolic extract alone inhibited *P. aeruginosa*, *A. calcoaceticus*, *E. faecalis* and *S. pyogenes*. There was negligible difference on the size of the inhibition zone between ethanolic and hexane extracts against *B. cereus*. Compared to the standard (amikacin), the percentage of inhibition varied. The highest zone of inhibition was observed in ethanolic extracts. The above results showed that ethanolic extracts showed 92% activity, chloroform 58.33% and hexane 8.33%. Ethanolic extract showed 100% activity against Gram positive pathogens, and 87.5% activity against Gram negative pathogens.

In *H. macroloba*, 3 crude extracts showed antibacterial activity, highest was observed in ethanolic extracts followed by chloroform and hexane (Table 1). Ethanolic extracts not only produce bigger inhibitory zones, but also active against different pathogens. *P. aeruginosa* and *S. typhi* were inhibited by both ethanolic and chloroform extracts. Ethanolic and hexane extracts of *H. macroloba* were active against *K. pneumoniae* and *B. cereus*. All the tested bacterial pathogens except *S. aureus*, all the Gram positive and negative pathogens were susceptible to the ethanolic extract of the *H. macroloba*. However the size of the inhibition zone varied. Ethanolic extract of *H. macroloba* was effective against 92% of the tested organisms, highest inhibition was observed against *S. pyogenes* (Gram positive, 73%) and lowest against *P. aeruginosa* (Gram negative, 29.16%). 50% and more than 50% of inhibition was observed in the ethanolic extract of *H. macroloba* against six different bacterial pathogens namely *E. coli* (Gram negative, 65%), *P. mirabilis* (Gram negative, 50%), *S. marcescens* (Gram negative, 50%), *A. calcoaceticus* (Gram negative, 50%), *E. faecalis* (Gram positive, 64%) and *S. pyogenes* (Gram positive, 73%). Chloroform and hexane extracts were active against 17% of the tested organisms. The size of the inhibition zone was less than 40%. Chloroform extract showed activity only against two different Gram negative pathogens (*P. aeruginosa* and *S. typhi*). Hexane extract showed activity against *K. pneumoniae* (Gram negative, 38.09%) and *B. cereus* (Gram positive, 26%) bacteria.

Petroleum ether, chloroform and ethanolic extract of *E. compressa* showed antibacterial activity against 11 of the tested 12 bacterial pathogens. The size of the inhibitory zone varied with test organism and type of solvent used for extraction (Table 1). Compared to control, petroleum ether extracts showed 50% and higher inhibitory activity against *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. marcescens*, *A. calcoaceticus*, *E. cloacae*, *S. aureus* and *S. pyogenes*. Similarly chloroform extract showed 50% and higher activity against: *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *P. mirabilis*, *S. typhi*, *A. calcoaceticus*, *S. aureus*, *S. pyogenes* and *B. cereus*. Ethanolic extract showed 50% and higher inhibitory activity against *P. aeruginosa*, *P. mirabilis*, *S. marcescens*, *S. typhi*, *S. aureus*, *S. pyogenes* and *B. cereus*. An important observation was noted that 100% of inhibition was exhibited by the ethanolic extract against *S. pyogenes*.

Among the five crude extracts of *C. antennia*, highest antibacterial activity was observed in ethanolic extracts followed by hexane and chloroform (Table 1). Ethanolic extracts not only produced bigger inhibitory zones, but also active against 10 different pathogens followed by hexane (5) and chloroform (4). *K. pneumoniae* was inhibited by the ethanolic, chloroform and hexane extracts of *C. antennia*. The size of the inhibitory zone in chloroform extracts were comparatively bigger (9, 8, 7 mm) than those of hexane and ethanolic

extracts of *C. antennia*. Ethanolic and chloroform extracts of *C. antennia* were active against, *K. pneumoniae*, *S. marcescens*, and *E. cloacae*. Ethanolic and hexane extracts of *C. antennia* were active against *K. pneumoniae*, *P. aeruginosa*, *P. mirabilis*, *A. calcoaceticus* and *S. aureus*. *S. typhi* was inhibited only by the chloroform extract of *C. antennia*. Similarly ethanolic extract alone inhibited *E. faecalis*, *S. pyogenes* and *B. cereus*. Ethanolic extracts of *C. antennia* was effective against 83% of the test organism, except *E. coli* and *S. typhi* (Gram negative). However the percentage of inhibition varied with test organisms. Highest inhibition was observed against *S. pyogenes* (Gram positive, 145.45%) and lowest against (33.33%) *K. pneumoniae*, *P. aeruginosa* and *A. calcoaceticus* (Gram negative). 50% and more than 50% of inhibition was observed against two bacterial pathogens namely *S. pyogenes* (145.45%) and *E. faecalis* (73%) and three pathogens (Gram negative) viz., *P. mirabilis*, *S. marcescens* and *A. calcoaceticus* 50% respectively. Chloroform extract was active only against 33.33% of Gram positive pathogens. No inhibitory activity was observed against any of the Gram positive bacteria tested. A noted observation was that the activity of ethanolic extract of *C. antennia* against *S. pyogenes* was 45% more than the control. Hexane extracts of *C. antennia* was effective against 42% of the test organisms.

Out of five different crude extracts *C. scalpelliformis*, antibacterial activities were observed only in chloroform and ethanolic extracts of *C. scalpelliformis* (Table 1). However the size of the inhibition zone varied with test organisms and extracts. Highest activity was observed in ethanolic extracts of *C. scalpelliformis* followed by chloroform extracts. In *C. scalpelliformis*, ethanolic extracts produced the bigger inhibitory zones, and also activity against 10 different pathogens. The chloroform extract inhibited only two pathogens. *K. pneumoniae* and *P. aeruginosa* were inhibited by both ethanolic and chloroform extracts of *C. scalpelliformis*. The size of the inhibitory zone in ethanolic extracts was bigger (10, 9 mm) than chloroform extract (8, 7 mm) against *K. pneumoniae* and *P. aeruginosa* respectively. Ethanolic extracts of *C. scalpelliformis* was effective against 90% of the test organisms. Except *S. typhi* (Gram negative) and *E. faecalis* (Gram positive) all the other pathogens were inhibited by ethanolic extracts. However the percentage of inhibition varied with test organisms. Highest inhibition was observed against *S. pyogenes* (Gram positive, 82%) and lowest against *S. marcescens* (Gram negative, 32%). 50% and more than 50% of inhibition was observed against two bacterial pathogens viz. *E. coli* (Gram negative 50%) and *S. pyogenes* (Gram positive, 82%). Chloroform extract produced less than 50% of inhibition against *K. pneumonie* (38%) and *P. aeruginosa* (29%). Chloroform extract was active only against 17% of Gram negative pathogens. No inhibition was observed against any of the Gram positive bacteria tested.

In *C. corynephora*, 2 crude extracts showed antibacterial activity, highest was observed in ethanolic extract, followed by chloroform (Table 1). Among the 12 bacterial pathogens tested, 10 were susceptible to the ethanolic and chloroform extracts of C. corynephora. However the size of the inhibition zone varied. *S. pyogenes* was inhibited only by the ethanolic extract. *E. faecalis* was not susceptible to the ethanolic and chloroform extract of *C. corynephora*. Ethanolic extracts of *C. corynephora* was effective against 92% of the test organisms. Except *E. faecalis* (Gram positive), all the other pathogens were inhibited by the ethanolic extract. However the percentage of inhibition compared to the control varied with test organisms. Highest inhibition was observed in *S. marcescens* and lowest against *S. aureus*. Chloroform extracts of *C. corynephora* was effective against 83.33% of the test organisms (Table 1) except *E. faecalis* and *S. pyogenes* (Gram positive). Compared to the control, the ethanolic extract of *C. corynephora* produced more than 50% of inhibition against 11 different bacterial pathogens. The screened Gram negative were susceptible to the ethanolic extract. Chloroform extracts were comparatively similar to ethanolic extracts in inhibiting 10 different bacterial pathogens.

Table 1 Antibacterial activity of green seaweeds from south east coast of Tamil Nadu

Organisms	Zone of Inhibition in mm																				
	C. corynephora		*C. scalpelliformis*		*C. antennia*			*E. compressa*			*H. macroloba*			*U. fasciata*			*U. lactuca*				Amikacin in 30 µg
	C	E	C	E	H	C	E	P	C	E	H	C	E	H	C	E	H	P	C	E	S
E. coli	12	23	-	10	-	-	-	11	12	7	-	-	13	-	7	13	-	-	-	10	20
K. pneumoniae	6	17	8	10	8	9	7	11	12	10	8	-	7	-	7	11	-	-	-	14	21
P. aeruginosa	14	26	7	9	7	-	8	14	13	16	-	8	7	-	-	9	-	10	-	9	24
P. mirabilis	6	22	-	9	7	-	10	9	12	16	-	-	10	-	7	14	-	-	-	7	20
S. marcescens	16	31	-	7	-	7	11	14	9	14	-	-	11	-	12	-	-	-	-	8	22
S. typhi	11	25	-	10	-	9	-	7	13	11	-	7	9	-	7	15	-	-	12	11	23
A. calcoaceticus	9	17	-	-	7	-	9	15	11	8	-	-	9	-	-	8	-	-	15	12	18
E. cloacae	8	26	-	8	-	7	8	13	11	6	-	-	8	-	9	8	-	-	-	9	24
S. aureus	11	13	-	11	9	-	7	13	14	15	-	-	-	-	9	7	-	11	8	15	24
E. faecalis	-	-	-	-	-	-	8	-	-	-	-	-	7	-	-	7	-	-	-	12	11
S. pyogenes	-	12	-	9	-	-	16	9	8	11	-	-	8	-	-	7	-	11	-	13	11
B. cereus	17	30	-	9	-	-	10	12	14	16	7	-	11	8	-	9	10	10	-	14	27

Note: C, chloroform; E, ethanol; H, hexane; P, petroleum ether; S, standard amikacin; mm, millimeter

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Discussion

The seaweeds known as medicinal are rich in secondary metabolites which include alkaloids, glycosides, flavonoids, saponins, tannins, steroids, related active metabolites are of great medicinal value and have been extensively used in the drug and pharmaceutical industries. The results of the phytochemical analysis of aqueous, ethanolic, chloroform, petroleum ether and hexane extracts of seven green seaweeds viz., *Ulva fasciata*, *Caulerpa scalpelliformis*, *Halimeda macroloba*, *Enteromorpha compressa*, *Caulerpa corynephora* and *Ulva lactuca* revealed the presence of a good number of secondary metabolites.[35] Antibacterial efficacy of seaweed extracts against the Gram positive and Gram negative bacteria has been established by several scientists.[31–33] Mostly the availability of active substances is dependent on the type of the extraction solvent used. The differences in the activity of various solvents have been reported earlier.[38–39] In the disc diffusion antibacterial assay, the various extracts of *C. corynephora*, *C. scalpelliformis*, *C. antennia*, *E. compressa*, *H. macroloba*, *U. fasciata* and *U. lactuca* were effective against Gram positive and Gram negative strains. Bacterial infection causes high rate of mortality in human population and aquaculture organisms.[40] Infection of *S. typhi* leads to the development of typhoid or enteric fever. Other symptoms include constipation or diarrhea, enlargement of spleen and possible development of meningitis.[41] *S. aureus* can cause a range of illnesses from minor skin infections to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, chest pain, bacteremia and sepsis.[42] *S. pyogenes* is the cause of many important human diseases, ranging from mild superficial skin infections to life threatening systemic diseases.[43] *B. cereus* is responsible for a minority of food borne illnesses (2-5%), causing severe nausea, vomiting and diarrhea.[44] *K. pneumoniae* can cause pneumonia and destructive changes to human lungs inflammation and hemorrhage with cell death that sometimes produces thick, bloody, mucoid sputum.[45] *E. coli* and *P. aeruginosa* cause diseases like mastitis, abortion and upper respiratory complications. *Proteus mirabilis* can be found throughout the stones, and these bacteria lurking in the kidney stones and can also cause wound infections, septicemia and pneumonias. *S. marcescens* may cause extrinsic staining of the teeth. *S. marcescens* is involved in nosocomial infections particularly catheter-associated bacteremia, urinary tract infections and wound infections.[46] *E. cloacae* and *A. calcoaceticus* can cause the disease urinary tract and respiratory tract infections. *E. faecalis* can cause endocarditis and bacteremia, urinary tract infections (UTI) meningitis and other infections in humans. The production of antibacterial activities was considered to be an indicator for the capability of the seaweeds to synthesize bioactive compounds. It is because; marine natural products contain a wide range of novel bioactive compounds or antibiotics with distinctive complex structures because they developed unique metabolic and physiological capability. The marine macroalgae have an effective antibacterial activity against most of the human bacterial pathogens. It was reported that 151 species of macroalgal crude extracts showed inhibitory activity against pathogenic bacteria.[47] There has been a number of reports that demonstrating the antimicrobial activity of marine plants,[48] marine algae or seaweeds.[49–56] Seaweeds belonging to red, brown and green algae exhibit inhibitory action against both gram-positive and gram-negative bacteria.[24,57,58] In the present study also the green seaweeds exhibited the antibacterial efficacy with varied frequency (Table 1).

The antibacterial activity of seaweeds may be influenced by some factors such as the habitat and the season of algal collection, different growth stages of plant, experimental methods etc. But variation in antibacterial activity may be due to the method of extraction and solvent used in extraction.[60] Although a variety of solvents have been employed in screening seaweeds for antibacterial activity, it is still uncertain which kind of solvent is most effective and suitable for extraction of seaweeds. A few workers used different solvents for screening the antibacterial activity of seaweeds and made comparisons.[61] Kolanjinathan and Stella[62] indicated that acetone was the best solution for extracting the effective antimicrobial compounds. Cordeiro et al.,[63] showed successive extraction with acetone, methanol-toluene, ether and chloroform-methanol. Kim & Lee[64] used methanolic extract to observe strong antibacterial activities. Our results also correlate with the previous observations showing better antibacterial activity in ethanolic extracts. The ethanolic extracts of selected seven seaweeds were most effective and demonstrated a broad-spectrum antibacterial activity against all Gram positive and Gram negative and bacteria followed by chloroform, benzene and petroleum ether extracts. The chloroform and methanolic extracts of some red and brown algae showed maximum activity against certain human pathogenic bacteria.[24] Among the various organic solvents such as methanol, acetone, diethyl ether and ethanol extracts of eleven macroalgae screened for antimicrobial activity against human pathogens, the extracts of diethyl ether was found to possess bioactive compounds.[65] In another study, acetone was found best among several solvents used for extracting antibacterial substances.[66] Sastry & Rao[67] reported chloroform extract exhibited the strongest activity. Similar to this in the present study, the chloroform extract of *E. compressa* against *E. coli*, *K. pneumoniae* and *S. typhi*, *C. corynephora* against *B. cereus* and *U. lactuca* against *A. calcoaceticus* showed more activity than the tested other solvents. Some other studies performed in the extraction of seaweeds using chloroform and ethyl acetate also exhibited good antibacterial activity.[23,68] Similar to previous observations, in the present study also the chloroform extract of *C. corynephora* showed activity against *B. cereus*. This kind of less or more activity could also be attributed to the sequential extraction of marine algae using solvents from low polar to high polar. In the present study also the chlorophyceae from South east coast of Tamil Nadu showed high antibacterial activity.

The results of the present study suggested that the possible exploitation of various extracts of *Caulerpa corynephora*, *Caulerpa scalpelliformis*, *Chaetomorpha antennia*, *Enteromorpha compressa*, *Halimeda macroloba*, *Ulva fasciata* and *Ulva lactuca* in the management of the infectious diseases caused by various tested bacteria. Further purification of the extracts may yield a novel antibacterial drug to treat various diseases typhoid, diarrhoea, pneumonia, meningitis, osteomyelitis, endocarditis, nausea, skin infections etc.

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None.

Conflicts of interest

Author declares that there is no conflict of interest.

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