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Screening of Antibacterial, Antifungal and Antioxidant Activity of Methanolic Extract from *Marine algae, Hypnea indica*

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Abstract *Marine algae* have wide applications in food, feed, pharmacy, and industrial fields. They have a wide range of biological activities, such as anti-microbial, anti-inflammatory, and anti-viral. *Hypnea* is a genus of red seaweed under class Florideophyceae, which exhibits seasonal abundance and is highly influenced by seasonal variations in light, temperature, and low tidal levels. The non-flowering, *marine algae Hypnea indica* is the richest source of kappa- carrageenan which has a wide range of applications from food to pharmaceutical uses. The objectives of this research were to evaluate the effect of the methanolic extract of HIE against microbes and to assess its antioxidant property. By using the well diffusion method, (HIE) was evaluated for its antibacterial and antifungal activities against clinical pathogens. HIE showed an anti-fungal effect against A. niger and considerable activity against V. cholera. The results were compared against antibiotics like Gentamycin and Clotrimazole. The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was used to evaluate the antioxidant properties of this extract using ascorbic acid as the reference. Up to 800 mg/mL, HIE did not show any antioxidant activity. The findings of this research demonstrated that HIE has antibacterial potential against infections and that it might be used as a natural alternative to synthetic antimicrobial agents.

Keywords *Marine algae, Hypnea indica,* HIE, Well Diffusion Method, DPPH Assay

1. Introduction

Algae with potential to be utilized in manufacturing and healthcare have been discovered using modern screening techniques. Intense climatic and environmental stressors such salt, light, temperature, and marine chemical diversification enable them to develop unique biologically active secondary metabolites [1, 2, 3, 4, 5]. *Algae*-derived compounds have been shown to possess a diverse range of biological activities, which include antimicrobial [6, 7, 8], antifungal [9, 10, 11], antioxidant [12, 13], anticoagulant [14, 15], antiviral [16], anti-aging [17, 18], antifouling [19, 20], anticancer [21], and anti-inflammatory [22] activities. Sulphated polysaccharides, Acrylic acid, Chlorellin derivatives, Phenolic inhibitors, halogenated aliphatic compounds, Labdane diterpenoids, and Guaiane sesquiterpenes were isolated from them [23, 24].

The genus *Hypnea* has branching, upright, calcareous red seaweeds that naturally thrive close to the coast in intertidal areas. Since it is widely grown as a food source and for the production of phycocolloid, it is an abundant *algae* genus that is commercially viable [25, 26]. Carrageenan, a polysaccharide found in this phycocolloid, is becoming more popular as a texturing agent for both food and non-food applications [26]. Antiviral, antibacterial, antioxidant, and antifungal activities of the

extracts of different *Hypnea* species have been investigated [27, 28, 29]. One of these, *H.indica*, is a red seaweed species that has most recently been found off the coastlines of Daman Diu, Kanyakumari, and Gujarat [30]. Similar to other *Hypnea* species, these tiny, hairy *algae H. indica* have the potential to be used as a source of long- and short-chain chemicals for commercial and medical purposes. They can also be used to make ice cream and jelly.

The present study aimed to investigate the antimicrobial and antioxidant activity of methanolic extract from *marine algae, H.indica*.

2. Materials and Methods

2.1. Chemicals

Mueller-Hinton agar, 2,2-diphenyl-1-1-picrylhydrazyl (DPPH) Nutrient broth and Gentamycin were purchased from Himedia (Mumbai). All other chemicals used in this work were of good quality, commercially accessible, analysis-grade materials.

2.2. Collection of Macroalgae

Rhodophyceae species *H. indica* were collected at low tide along the Vizhinjam coast in Kerala's Trivandrum district (Fig. 1). In order to get rid of epiphytes and other foreign materials, it was carefully cleaned in seawater and deionized water. It was then shade-dried, and an electric blender was used to powder it. Following that, the powdered sample was kept in a refrigerator until the extraction procedures.

Figure 1. *Hypnea indica*

2.3. Preparation of *Hypnea indica* Extract (HIE)

Using the Soxhlet equipment, the dried *H. indica* powder (5 g) was extracted with Methanol (1:4 w/v) for 4 days at 65°C. A rotary evaporator was then used to concentrate the extract. Thereafter, the residues were diluted with 2 mL of methanol [31]. Further testing was performed using this HIE preparation.

2.4. Bacterial Strains

Antibacterial activity of HIE was determined against bacterial strains such as *S.marcescens, B. subtilis, M. smegmatis, K. pneumonia, V. cholera, E. coli, S. aureus, P. aeruginosa*.

2.5. Inoculums Details

The Microbial Type Culture Collection (MTCC), in Chandigarh, provided the inoculums. The bacteria used in this study are listed in Table 1.

Table 1. Bacterial inoculums details

Name of organism	MTCC No	cubation condition
V. cholerae	3906	37°C for 24 hr
E. coli	443	37°C for 24 hr
S. aureus	87	37°C for 24 hr
P. aeruginosa	741	37°C for 24 hr
S. marcescens	86	37°C for 24 hr
B. subtilis	2413	37°C for 24 hr
M. smegmatis	6	37°C for 24 hr
K. pneumoniae	3384	37°C for 24 hr

2.6. Fungal Culture

Fungal strains are cultured on Potato Dextrose Agar (PDA) MH096 (Himedia). A concentration of 31.5 grams of PDA was heated in 1000 ml of distilled water until it completely dissolved. The medium was then autoclaved for 15 minutes at 121°C and 15 lbs of pressure to sterilise it. After that, it was cooled to 45–50°C. Poured into sterilised Petri plates after thoroughly mixing.

2.7. Fungal Strains

Fungal strains used in this study are *C. albicans, A. niger, P. chrysogenum, M. Indicus* (Table 2).

Table 2. Fungal inoculums details:

Fungi	MTCC No	Incubation condition
C. albicans	*227*	27^0C for 24 hours
A. niger	872	27°C for 48 hours
P. chrysogenum	5108	27°C for 48 hours
M. indicus	3513	27°C for 48 hours

2.8. Antibacterial Assay

The agar well diffusion method, previously reported by Valgas [32], is used to evaluate the antibacterial activity of HIE. On glass petri plates, MH- agar (15–20 mL) was solidified and left to do so. A sterile cotton swab was used to evenly distribute the test organism from the standard inoculum on the surface of the plates. Using a sterile cork borer, four wells on each plate—each measuring 9 mm in

diameter and spaced 20 mm apart-were aseptically bored. HIE (40 and 80 L) from 10 mg/ml stock was injected into wells T1 and T2. Then, gentamycin (40 l from a 4 mg/ml stock) was used as the positive control and DMSO was used as the negative control. The plates were incubated for 24 hours in an aerobic environment at 36°C and 1°C. The plates were examined after incubation, and the length of the zone that prevented bacterial growth was noted.

2.9. Antifungal Assay

The antifungal activity of HIE was tested using the agar well diffusion method. PDA (15–20 mL) was placed on identical-sized glass petri plates and particular time to solidify. Using a sterile cotton swab, the test organism's standardised inoculum was evenly distributed throughout the plates. A sterile cork borer was used to aseptically punch four wells with diameters of 8 and 20 mm. The wells T1 and T2 received HIE (40 and 80 L) from 10 mg/ml stock. Clotrimazole (40 l from a 300 mcg/ml stock) was added as a positive control, and DMSO was used as a negative control. The plates were incubated for 24 hours aerobically at 27°C and 1°C. After incubation, the plates were examined, and the millimeter-sized zone that inhibited fungus growth was observed [33].

2.10. Antioxidant Activity

The method of Brand-William [34] was slightly modified in order to assess the antioxidant activity of HIE against 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH). A freshly made 60 M solution of DPPH in methanol was used to mix 3.9 mL of HIE at different concentrations (1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 g/ml) with 1 mL of the solution. The tubes were kept in the dark at room temperature for 15 minutes before the decline in absorbance at 515 nm was seen. Distilled water containing DPPH solution was chosen as the control. 95% methanol was used to make the blank.

The following formula was used to determine the percentage of DPPH that the extracts were able to scavenge:

$$\% \text{ of DPPH Radical scavenging} = [(Ac - At) / Ac] \times 100$$

Ac is the absorbance of the control (DPPH),

At is the absorbance of a test sample.

2.11. Statistical Analysis

All experiments were carried out in triplicate. The data were calculated in Microsoft excel and expressed as mean ± standard deviation.

3. Results & Discussion

The development of novel pharmaceutical compounds may be accelerated by secondary metabolites with bioactive principles that are actively produced by marine organisms [35]. Table 3 demonstrated the antibacterial activity of HIE. *V. cholerae* (Fig. 2) showed the highest activity, followed by *B. subtilis* (Fig. 3), and then *S. aureus* (Fig. 4). The utilisation of several organic solvents produced by algae for antibacterial activity against gram-positive and gram-negative bacteria has been extensively documented. More research is needed, since it is presently unknown how well these extracts inhibit and maintain the stability. The antimicrobial activity may be influenced by factors including ecology, the time and year when they are gathered, techniques, etc. The antibacterial response has been the topic of numerous works, according to the literature. There were many works regarding the antibacterial response of *marine algae* from native and abroad researchers [6, 7, 8, 36, 37]. Prasanna [38] assessed *H. valentiae* antibacterial effectiveness against seven common pathogenic bacterial strains. The three extracts of *H. valentiae* are nearly ineffective against the strain of *B. subtilis* in that study. Mahendran [39] reported that the hexane extract of the same species showed a broad spectrum of efficacy against ten human bacterial pathogens. The highest levels of inhibition were seen against *M. luteus*, *V. parahaemolyticus*, and *E. coli*, and the lowest levels were seen against *V. cholerae*. Additionally, similar reports of *H. musciformis* methanolic extract exhibiting high antibacterial activity against all the investigated microorganisms were obtained [40, 41].

Table 3. Antibacterial activity of HIE

Organism	Standard Gentamycin (160 mcg)(mm)	HIE (40µl from 10 mg/ml) (mm)	HIE (80µl from 10 mg/ml) (mm)
Vibrio cholerae	26 ± 1.73	13.65 ± 1.54	25.66 ± 0.58
E coli	29 ± 0.58	0 ± 0	0 ± 0
Staphylococcus aureus	21.33 ± 1.52	5.33 ± 0.58	5.66 ± 0.58
Vibrio cholerae	26 ± 1.73	13.65 ± 1.54	25.66 ± 0.58
Pseudomonas aeruginosa	25± 0	0 ± 0	0 ± 0
Bacillus subtilis	21.33 ± 1.52	0 ± 0	11.67± 0.58
Klebsiella pneumoniae	22 ± 1.0	0 ± 0	0 ± 0
Mycobaterium smegmatis	2367 ± 1.15	0 ± 0	0 ± 0
Serratia marcescens	23.67 ± 1.15	0 ± 0	0 ± 0

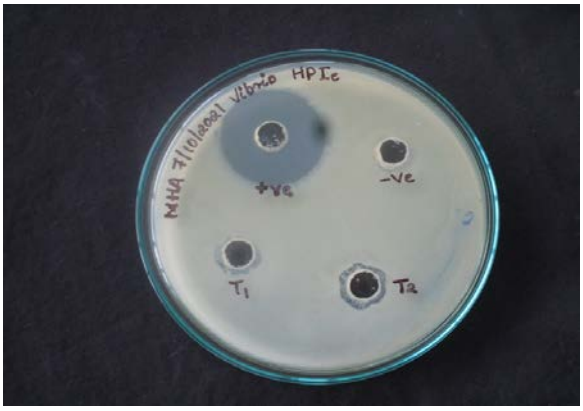

Figure 2. Vibrio cholera

Figure 3. Bacillus subtilis

Figure 4. Staphylococcus aureus

Table 4 depicts the antifungal activity of HIE against various fungi. *A. niger* was vulnerable to moderate antifungal activity from HIE (Fig. 5). Similar antifungal activities were observed by the methanolic extract of *H. pannosa* against *A. flavus*, *C. glabrata*, *T. longifusus*, and *M. canis* [42]. It has been discovered that *R. confervoides* and *P. pavonica*, two *marine algae*, can effectively manage *M. ramaniannus* and *C. albicans*, respectively [43]. In a similar manner, Stein [44] observed that the marine algal red alga genus Laurencia lacks antifungal efficacy against three types of pathogenic fungi, specifically *C. albicans*, *C. parapsilosis*, and *C. neoformans*.

Table 4. Antifungal activity of HIE

Fungi	Clotrimazole (300mcg) (mm)	HIE (40µl from 10 mg /ml) (mm)	HIE (80µl from 10mg/ml) (mm)
Aspergillus niger	36.67 ± 1.15	0 ± 0	13.67 ± 1.15
Penicillum chrysogenum	37.33 ± 2.31	0 ± 0	0 ± 0
Mucor indicus	38.67 ± 2.31	0 ± 0	0 ± 0
Candida albicans	36.67 ± 1.15	0 ± 0	0 ± 0

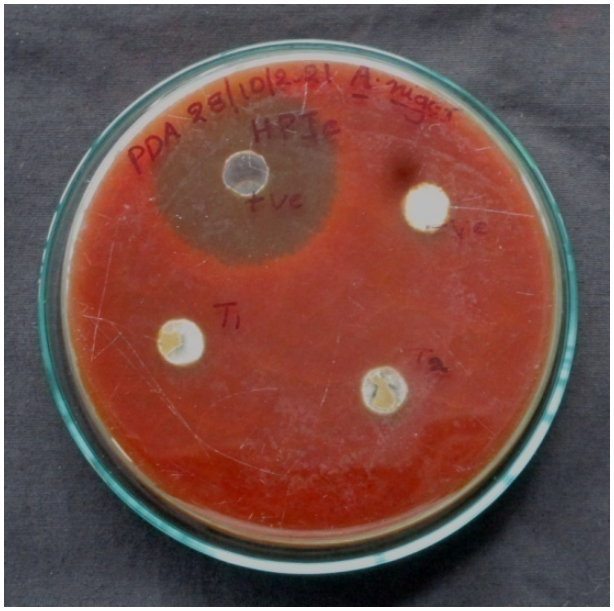

Figure 5. *Aspergillus niger*

The field of research in screening *marine algae* with antioxidant potential is growing rapidly, and many techniques have been used to analyze the *algae*'s antioxidant capacity. The DPPH assay is based on the reagent's capability to decolorize once antioxidants are present as a stable radical. It provides an effective, reliable, and economical technology that has been broadly utilized to evaluate the antioxidant potential of numerous natural compounds. Since it supports multiple models at once and has low component concentrations, it is the most widely used approach.

Antioxidant-activity is not observed in the methanolic extract of *H. indica* (Tables 5 & 6), which can be caused by seasonal factors or inadequate antioxidant compound isolation by methanol. The IC_{50} value of 26.29 for standard ascorbic acid demonstrated higher antioxidant activity. Using the DPPH technique, Rafiquzzaman [45] examined the antioxidant activity of different crude extracts of *H. musciformis*. However, it has been demonstrated that *H. valentiae* [46] and *H. pannosa* [47] have antioxidant activity.

Table 5. Antioxidant activity of Ascorbic Acid (Standard)

Concentration (µg/ml)	% of Inhibition
1.56	6.1 ± 1.62
3.12	6.7 ± 1.62
6.25	12.2 ± 2.63
12.5	25.07 ± 5.62
25	49.23 ± 10.85
50	92.83 ± 19.16
IC50	26.29

Table 6. Antioxidant activity of HIE

Concentration (µg/ml)	% of Inhibition
50	2.28 ± 0.03
100	5.04 ± 0.02
200	5.53 ± 0.11
400	5.91 ± 0.1
800	6.36 ± 0.15

The data suggest that these *algae* are important in the management of infectious disorders, particularly bacterial infections. For the production of new bioactive compounds, physiologically active molecules must be further separated, purified, and characterised using chromatographic and spectroscopic methods.

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