Antibacterial studies on *Hypnea musciformis* against selected pathogens

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***Abstract*: *Hypnea musciformis* was evaluated for the production of antimicrobial compounds against Gram positive and Gram negative bacteria. The current study reveals that Gram positive bacteria are sensitive to *H. musciformis* at lower concentrations, whereas Gram negative bacteria showed high resistance against the tested bacterial strains.**

***Keywords*: Antimicrobial activity, *Hypnea musciformis*, Gram positive, Gram negative.**

Introduction

Understanding bacterial cell division is believed to be critical in the development of new antibiotics because cell division is an essential process for bacterial survival and the bacterial divisome possesses a complex set of biochemical machinery that contains many proteins as potential drug targets [Erickson, 1997; Margolin, 2000; Addinall and Holland, 2002]. Bacterial resistance to antibiotics is one of the most serious challenge to global public health, as drug resistance has been found for all classes of antibiotics used in clinical practice [Arias and Murray, 2015]. Managing this situation will require extensive search for new drugs and elucidation of their mechanisms of action [Kon and Rai, 2012]. The marine environment is clearly the last greatest frontier of natural sources for drugs. Among marine organisms macroalgae are rich source of structurally diverse bioactive compounds with different bioactivity spectra and biomedical value [Yuvaraj *et al*., 2011].

Materials and methods

Cleaning and drying

Samples of the selected seaweed were collected from coastal areas of Rameswaram. *Hypnea musciformis* was rinsed with fresh water to eliminate foreign materials such as sand and shells. The selected seaweed was air dried at room temperature 27±2ºC below 30ºC to avoid decomposition of thermolabile compounds.

Primary isolation

About 10 g of the finely powdered alga was loaded in Soxhlet apparatus (SUNBIM 250 mL) to prepare crude methanolic extract, finally reduced in rotary evaporator (Buchi) at 40ºC.

Antimicrobial studies of algal extract

Test pathogens

The following test microbes were used for the present study. Gram positive bacteria: *Bacillus subtilis* MTCC 2756, *Staphylococcus aureus* MTCC 902, *Staphylococcus epidermis* MTCC 435 and *Staphylococcus simulans* MTCC 3610; Gram negative bacteria: *Escherichia coli* MTCC 2622, *Klebsiella pneumoniae* MTCC 109, *Proteus mirabilis* MTCC 425, *Vibrio cholerae* MTCC 3905, *Pseudomonas aeruginosa* MTCC 2642 and *Salmonella typhi* MTCC 3216. All the test microorganisms were purchased from Microbial Type Culture Collection Centre (MTCC), IMTECH, Chandigarh, India. The test bacteria were maintained on nutrient agar slants.

Antibacterial activity

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of the crude extract was determined according to the method described by the Clinical and Laboratory Standards Institute [CLSI, 2012a], with some modifications. Two fold serial dilutions of the extract and antibiotics were made with Mueller Hinton Broth (MHB) to give concentrations ranging from 4 to 4000 µg/mL for crude extract and 0.12 to 1000 µg/mL for antibiotics. Hundred microliters of test bacterial suspension were inoculated in each tube to give a final concentration of 1×10^5 CFU/mL. The tubes were incubated for 24 h at 37ºC. The control tube did not have any antibiotics or crude extract, but contained the test bacteria and the solvent used to dissolve the antibiotics and extract. The growth was observed both visually and by measuring OD at 600 nm. The lowest concentration of the crude extract showing no visible growth was recorded as the MIC. Triplicate set of tubes were maintained for each concentration of the test sample. Ciprofloxacin and ampicillin were used as positive control.

Minimum Bactericidal Concentration (MBC)

Minimum bactericidal concentration was determined according to the method of Smith-Palmer *et al.* [1998]. About 100 µL from the tubes not showing bacterial growth in the MIC test were serially diluted and plated on nutrient agar. The plates were incubated at 37ºC for 24 h. Minimum bactericidal concentration is defined as the concentration at which bacteria failed to grow on nutrient agar inoculated with 100 µL test bacterial suspensions.

Antibacterial assay by disc diffusion technique

The antibacterial activity of the extract was determined by the disc diffusion method [CLSI, 2012b] against human pathogenic bacteria. The test cultures maintained in nutrient agar slant at 4°C were sub-cultured in nutrient broth to obtain the working cultures approximately containing 1×10^6 CFU/mL. The MIC concentration of the crude extract was incorporated in a 6 mm sterile disc. Mueller Hinton (MH) agar plates were swabbed with each bacterial strain and the test discs were placed along with the control discs. Ciprofloxacin discs (5 µg/disc) were used as positive control. Plates were incubated overnight at 37°C for 24 h. Clear, distinct zone of inhibition was visualized surrounding the discs. The antimicrobial activity of the test agents (extract and antibiotics) was determined by measuring the zone of inhibition measured in mm and expressed as diameter in millimeter (mm).

Results

Antibacterial activity of crude extract

Minimum Inhibitory Concentration (MIC)

MIC indicated that the tested crude methanolic extract of *H. musciformis* inhibited *E. coli*, *S. epidermis* and *S. simulans* at 500 µg/mL (Table 1). *S. aureus*, *B. subtilis* and *K. pneumoniae* were moderately active and the MIC was between 1000-2000 µg/mL. In *P. aeruginosa* the MIC was recorded at 4000 µg/mL. In *P. mirabilis*, *V. cholerae* and *S. typhi* no MIC was observed up to 4000 µg/mL. In case of standard antibiotics tested, ciprofloxacin was most active and all the bacterial strains tested were very sensitive with MIC ranging between 1-4 µg/mL (Table 1).

Minimum Bactericidal Concentration (MBC)

The crude methanolic extract of *H. musciformis* showed an MBC of 500-4000 µg/mL (Table 1). No MBC was observed in *P. mirabilis*, *V. cholerae* and *S. typhi*. The MBC values of ciprofloxacin ranged between 2-8 µg/mL against all the tested bacterial strains. In ampicillin the MBC values were between 2-16 µg/mL against the tested bacteria.

Table 1 MIC, MBC and MFC of crude extract of *H. musciformis* against test bacteria

Sl. No.	Test organisms	Activity (µg/mL)					
		Extract		Ampicillin		Ciprofloxacin	
		MIC	MBC/ MFC	MIC	MBC	MIC	MBC
1.	*B. subtilis*	2000	4000	8	16	2	2
2.	*S. aureus*	1000	2000	4	8	2	2
3.	*E. coli*	500	500	4	4	1	2
4.	*P. aeruginosa*	4000	4000	8	16	2	4
5.	*S. epidermis*	500	1000	2	4	2	4
6.	*P. mirabilis*	-	-	2	2	4	8
7.	*V. cholerae*	-	-	4	8	2	4
8.	*K. pneumoniae*	2000	4000	2	4	2	4
9.	*S. simulans*	500	1000	2	2	4	8
10.	*S. typhi*	-	-	1	2	4	4

values represents mean of three replications (-) no MIC up to 4000 µg/mL (*) not tested

Antibacterial activity by disc diffusion method

Table 2 Antibacterial activity of crude extract of *H. musciformis* against test bacteria

Sl. No.	Test organisms	Zone of inhibition (diameter in mm)	
		Extract	Ciprofloxacin
1.	*B. subtilis*	13±1	26±0
2.	*S. aureus*	15±1	27±1
3.	*E. coli*	16±1.52	32±1.15
4.	*P. aeruginosa*	10±1	30±0
5.	*S. epidermis*	17±1.12	29±1
6.	*P. mirabilis*	-	31±0.57
7.	*V. cholerae*	-	31±0
8.	*K. pneumoniae*	16±0	28±1.52
9.	*S. simulans*	14±1.2	25±0.57
10.	*S. typhi*	-	30±1.52

(-) not tested as the MIC value is above 4000 µg/mL, (*) not tested

The antimicrobial activity of crude methanolic extract of *H. musciformis* against the tested bacteria were shown in Table 2. *P. aeruginosa, B. subtilis, S. simulans, S. aureus, K. pneumoniae, E. coli* and *S. epidermis* were sensitive to crude extract of *H. musciformis* and the inhibition zone ranged between 10±1 to 16±1.12 mm. *P. mirabilis, V. cholera* and *S. typhi* was not tested as the MIC value was above 4000 µg/mL. All the tested bacterial strains were susceptible to ciprofloxacin and the zone of inhibition ranged between 25±0.57 to 32±1.15 mm.

Discussion

In the last decades, microbial infections have become a huge threat to human health, mainly due to the increase in microbial resistance [Li *et al.,* 2016]. Therefore, research on antimicrobials and development are needed to improve the current therapeutic options against the microbial infections. Reports of Rao and Parekh [1981], Vidyavathi and Sridhar [1991], Nagayama *et al.* [2002] revealed that Gram positive bacterial strains were more susceptible to seaweed extracts than Gram negative bacterial strains. The better results for Gram positives, in general and moderate activity of Gram negative bacterial strains throw a light in this aspect. The present study reveals that crude extract of *H. musciformis* inhibited the growth of bacterial strains like *B. subtilis, S. aureus, E. coli, P. aeruginosa, S. epidermis, K. pneumoniae* and *S. simulans*. The MIC, MBC and zone of inhibition obtained against tested bacteria ranged between 500-4000 µg/mL, 500-4000 µg/mL and 10±1-17±1.2 mm. *P. mirabilis, V. cholerae* and *S. typhi* were resistant to the crude extract of *H. musciformis*.

Studies by Manilal *et al.* [2009] revealed that extracts of *Hypnea* exhibited best results among the members of Rhodophyceae which, is identical to the results of the current study. Yi *et al.* [2001] reported that members of Rhodophyceae showed highest activity against bacteria and fungi which is in conformation with the results of the present study. Rhimou *et al.* [2010] reported that methanolic extract of *H. musciformis* inhibited *S. aureus* and *K. pneumoniae* which is in agreement to the results of the current study. Earlier reports of Christobel *et al.* [2011] revealed the antibacterial activity of *H. musciformis* against *P. aeruginosa* and *P. mirabilis* which is closely related to the current study.

Results of the current study reveals that Gram positive bacteria are sensitive to *H. musciformis* at lower concentrations, whereas Gram negative bacteria showed high resistance and also the concentration at which the extracts have been loaded in the discs contributed to its inhibitory action. Cells of Gram negative bacteria are surrounded by an additional outer membrane rich in lipopolysaccharide molecules, which provide them hydrophilic surface that functions as a permeability barrier for many substances including natural compounds [Hemaiswarya *et al.,* 2008; Briers and Lavigne, 2015]. Additional contribution to intrinsic resistance in Gram negative bacteria is provided by efflux pumps (Eps) which actively pump out a broad spectrum of compounds (such as antibiotics, toxins, β-lactamase inhibitors, dyes, detergents, lipids and molecules involved in quorum sensing) from the periplasm to the outside of the cell. The over expression of EPs is recognized as a major component in the development of the multidrug resistance phenotype in Gram negative bacteria [Opperman and Nguyen, 2015; Venter *et al.,* 2015].

The ineffectiveness of plant compounds toward Gram negative pathogens has been proposed to be strongly related to EPs as the combination of plant antimicrobials with EPs inhibitors leads to a striking increase in antimicrobial activity [Tegos *et al.,* 2002]. Although the mechanisms of action of natural products are distinct, the cytoplasmic membrane ranks as the most common site of action for secondary metabolites. They usually act through cell lysis, triggering the leakage of cellular contents and consequently cell death [Da Silva *et al.,* 2013]. However, the Gram positive bacteria do not possess such outer membrane and cell wall structures [Kalamba and Kanicka, 2003]. Results of the current study reveals that members of Rhodophyceae were highly active. Among the three groups of macroalgae, the class Rhodophyceae produces aplethora of structurally diversified novel halogenated compounds, symbolize the extraordinary wealth of biogenic compounds principally for pharmaceutical leads [Faulkner, 2001].

Conclusion

The present study revealed that the presence of bioactive compounds in *H. musciformis*, which could be a promising source for the discovery of novel bioactive metabolites against wide range of pathogens. Much attention has been paid to the development of innovative projects for the pharmaceutical applications of seaweed, further investigations in the design of novel antimicrobial drugs is the need of the hour.

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