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Antibiotic potentials of red macroalgae *Hypnea musciformis* (Wulfen) Lamouroux and *Hypnea valentiae* (Turner) Montagne

V.S. PRAMITHA[1] AND A.P. LIPTON
Marine Biotechnology Laboratory, Vizhinjam Research Centre of Central Marine Fisheries Research Institute, Vizhinjam - 695 521, Kerala, India
[1]Department of Biotechnology, Udya College of Arts & Science
Udya Nagar, Vellamodi - 629 204, Tamil Nadu, India

ABSTRACT

The methanol extract of *Hypnea musciformis* inhibited the growth of 66% of the Gram +^{ve} bacteria tested. The crude extracts inhibited the growth of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Vibrio alginolyticus* by producing highest inhibitory zone range of 113.04 mm^2. In the aqueous extract, the activity was comparatively less specifically towards Gram -^{ve} bacteria. It was also noted that the Hexane-Benzene (80:20%) fraction (HB5a) of *H. musciformis* exhibited potent activity not only against Gram -^{ve} bacteria but also against Gram +^{ve} *Staphylococcus aureus*. The higher bacterial inhibition zones (>78.5 mm^2) were recorded in the methanol extracts of macroalgae for *Staphylococcus aureus*, *Vibrio alginolyticus* and *Pseudomonas aeruginosa* and also compared to the commercial antibiotics. The *Hypnea valentiae* extract at 0.1% was most active against *Staphylococcus aureus, Vibrio fischeri*, *V. alginolyticus*, *Pseudomonas aeruginosa* and *Micrococcus luteus* at 20^{o}C.

Introduction

The oceans have extensive biodiversity of marine organisms and huge resource potential to provide many complex chemical compounds with functional materials including polyunsaturated fatty acids (PUFA), polysaccharides, essential minerals and vitamins, antioxidants, enzymes and bioactive peptides (Murty and Agrawal, 2010). Among marine organisms, macroalgae are rich sources of structurally diverse bioactive compounds with different bioactivity spectra and biomedical value (Yuvaraj *et al*., 2011). Research and utilization of marine algal community have increased markedly that directly offers enormous untapped reservoir of novel drug leads endowed with ingenious structures and potential biological activities (Kolanjinathan and Stella, 2011).

The use of antibiotics increased significantly due to heavy infections and the pathogenic bacteria becoming resistant to drugs necessitated the development of new antibacterial compounds from marine macroalgae. Many of these could be

developed into antiseptics and cleansing agents, but their antibiotic activity *in vivo* is often only achieved at toxic concentrations. A promising antibacterial agent is a halogenated furanone or fimbrolide from *Delisea pulchra* and has been examined for its effectiveness as a possible treatment for chronic *Pseudomonas aeruginosa* infection. A study on the 17 different macroalgae collected from southern India coast indicated that about 94% exhibited antibiotic activity towards shrimp and fish pathogens (Smith and Kitts, 1994).

Red algae, especially many species of the genus *Hypnea* (order *Gigartinales*, family *Hypneaceae*) are proven to be rich sources of halogenated secondary metabolites. Many of these metabolites have been found to possess a variety of biological activities such as antifeedant (diterpenes), antihelmintic (β-chamigrane-type sesquiterpenes), antimalarial (brominated sesquiterpenes), antifouling (sesquiterpes) and antimicrobial (Chlorellin derivatives, halogenated compounds such as haloforms, halogenated alkanes and alkenes, alcohols, aldehydes, hydroquinones, sterols, ketones, allolaurinterol) activities (Garg *et al*., 1992). In our previous studies, antifouling, anticoagulant, glucosidase inhibitory properties (Pramitha *et al*., 2008), antimicrobial activities of aqueous extract of macroalgae, growth responses of microalgae (Pramitha and Lipton, 2011) and selective cytotoxic activities of macroalgae from the Indian coast have already been reported (Pramitha and Lipton, 2012). Though there are diverse studies on bioactivity to marine flora against several pathogens, our work on testing the antibacterial efficacies of macroalgae *Hypnea musciformis* and *Hypnea valentiae* mainly on human multidrug resistant pathogen and also on fish pathogens is relatively a new concept, as few attempts had been made earlier in this line. Considering such potentials, the marine macroalgae *Hypnea musciformis* and *Hypnea valentiae* collected from Southeast and Southwest coast of India were examined and the findings are presented in this paper.

Materials and Methods

The red macroalgae, *Hypnea valentiae* (Turner) Montagne was collected from the intertidal rocks of Vizhinjam coast (Lat. 08° 22'N; Long. 76° 59'E) southwest India and *Hypnea musciformis* (Wulfen) Lamouroux was collected from Rameswaram/Mandapam coast (79° 20'E Long; 09° 25' N Lat), southeast India. The collected algal samples were cleaned and washed with tap water to remove the associated debris and shade dried at room temperature (28±2°C) for 5-8 days or until they are brittle easily by hand. After completely drying, plant materials were powdered and extracted with methanol. In this process, 500 g of finely powdered algal material was refluxed with methanol in a 5 l capacity round bottom flask. The extract was filtered and concentrated to recover the excess solvents in another distillation system. Finally, it was reduced to a thick viscous crude extract in a rotary vacuum evaporator (Buchi) at 40°C and the yield of extract was recorded as 0.085 g dry^{-1} weight. Aqueous extracts was prepared by homogenizing the fresh collection with required quantity of phosphate buffered saline (pH 7.4) and the extract was strained using a strainer. Crude extract (200 mg) was further fractionated using normal phase column chromatography, C-40 silica columns (length-600 mm, bore-30 mm) of mesh size (60-120 μ) with sintered disc and screw cock. The eluted fractions were once again evaporated and concentrated using rotary vacuum evaporator.

The American Type Culture Collections of *Staphylococcus aureus* (ATCC– 25923 through Sree Chithra Thirunal Institute of Science & Technology,

Trivandrum), *Micrococcus luteus* (MTCC-106), *Pseudomonas aeruginosa* (MTCC-741), *Aeromonas hydrophila* (MTCC-646), *Bacillus subtilis* (MTCC-736) and Marine Ornamental Fish isolates (MOF) from Marine Biotechnology laboratory, CMFRI, Vizhinjam such as *Serratia marcescens, Vibrio fischeri, Vibrio alginolyticus,* MOF 1 (shrimp isolate) and MOF 2 (Clown fish isolate) were initially activated in nutrient broth and subsequently purified by agar streak plate method were used as test bacterial strains.

Disc diffusion method (Bauer *et al*., 1996) was carried out on Muller Hinton agar plates (Hi-media) to determine the *in vitro* antibacterial susceptibility of the extracts against bacterial isolates. About 1/3rd portion of petri dishes (100 mm) were poured with Muller-Hinton agar up to 2 mm thickness as a single layer and allowed to solidify for 15 minutes. After solidification, 0.1 ml of 18 h bacterial shake culture were surface inoculated using sterile cotton swabs and allowed to set for 5 minutes. Sterile discs (6 mm diameter, Hi-media) which were impregnated with suitable aliquots of desired concentration of test compound at 30 µl and were placed on the inoculated agar surface using sterilized forceps. All the assays were carried out in triplicate sets with suitable controls. After incubation at 20°C or 30°C in a BOD incubator (ROTEK) for 24 h, the area of inhibition zone (mm^2) was determined using a Hi Antibiotic Zone Scale-C (PW-297, Hi-media). The area of the inhibition zone was calculated as Cross diameter of the inhibition zone = m; Net diameter of the well = n; Net diameter of the inhibition zone, x = m-n; Net radius of the inhibition zone, r = x/2; Area of the inhibition zone = πr^2 (π = 3.14).

Results

The methanol extract of *Hypnea musciformis* showed highest activity range of 113.04 mm^2 at 20°C against *Staphylococcus aureus, Vibrio alginolyticus* and *Pseudomonas aeruginosa*. The aqueous extract of *H. musciformis* produced same highest activity towards *Staphylococcus aureus, Psaudomonas aeruginosa* and *Micrococcus luteus* (113.04 mm^2). It was noteworthy that *S. aureus* was totally susceptible at both the incubated temperatures. The methanol extract of *H. musciformis* also found to be active against *Serratia marcescens, Vibrio alginolyticus* and *Aeromonas hydrophila* (63.58 mm^2) at 30°C and *Serratia marcescens* (63.58 mm^2), *Vibrio fischeri* (78.5 mm^2), *Aeromonas hydrophila* (94.98 mm^2), MOF 1 (63.58 mm^2) and MOF 2 (78.5 mm^2) at 20°C. The methanol and aqueous extract of *H. musciformis* exhibited highest range of inhibitory potential (113.04 mm^2) towards Gram +^{ve} strain of *Staphylococcus aureus* (Table-1).

The methanol extracts of *Hypnea valentiae* exhibited highest activity (113.04 mm^2) at 20°C against *Staphylococcus aureus, Vibrio alginolyticus, Pseudomonas aeruginosa* and *Micrococcus luteus*. The Gram -^{ve} bacterium *Serratia marcescens* was totally resistant at both the tested incubation temperatures. Similarly, MOF 1 isolate was resistant at 30°C and regained a nearly active range of 63.58 mm^2 at 20°C. The Gram +^{ve} *Bacillus subtilis* showed active range of inhibition of 94.98 mm^2 at both temperatures (30°C and 20°C). *Hypnea valentiae* aqueous extract produced highest activity against *Vibrio alginolyticus* at 20°C and the same was found to be reduced at 30°C (63.58 mm^2). However, methanol and aqueous extracts of *H. valentiae* produced complete inhibitory effect upon *Vibrio alginolyticus* at 20°C. In the case of *H. musciformis, Bacillus subtilis* isolate and in *H. valentiae, Aeromonas hydrophila* were found to be resistant at 30°C. It was also noted that in *H. valentiae,* MOF 1

Table-1. *Zone of inhibition (mm²) of methanol and aqueous extracts of Hypnea musciformis*

Test organisms		Methanol		Aqueous	
		30°C	20°C	30°C	20°C
Clinical pathogens					
Staphylococcus aureus	(Gram +ve)	113.04	113.04	63.58	113.04
Micrococcus luteus	(Gram +ve)	38.46	50.24	94.98	113.04
Pseudomonas aeruginosa	(Gram -ve)	50.24	113.04	78.5	113.04
Aeromonas hydrophil	(Gram -ve)	63.58	94.98	50.24	63.58
Bacillus subtilis	(Gram +ve)	38.46	50.24	R	50.24
Fish pathogens					
Serratia marcescen	(Gram -ve)	63.58	63.58	63.58	63.58
Vibrio fischeri	(Gram -ve)	50.24	78.5	50.24	78.5
Vibrio alginolyticus	(Gram -ve)	63.58	113.04	94.98	94.98
MOF 1 (Shrimp isolate)	(Gram -ve)	50.24	63.58	50.24	50.24
MOF 2 (Clown fish isolate)	(Gram -ve)	50.24	78.5	R	R

R - Resistant

Table-2. *Zone of inhibition (mm²) of methanol and aqueous extracts of* Hypnea valentiae

Test organisms		Methanol		Aqueous	
		30°C	20°C	30°C	20°C
Clinical pathogens					
Staphylococcus aureus	(Gram +ve)	94.98	113.04	63.58	78.5
Micrococcus luteus	(Gram +ve)	113.04	113.04	R	78.5
Pseudomonas aeruginosa	(Gram -ve)	78.5	113.04	50.24	94.98
Aeromonas hydrophila	(Gram -ve)	3.14	7.06	R	R
Bacillus subtilis	(Gram +ve)	94.98	94.98	50.24	63.58
Fish pathogens					
Serratia marcescens	(Gram -ve)	R	R	3.14	R
Vibrio fischeri	(Gram -ve)	50.24	78.5	50.24	78.5
Vibrio alginolyticus	(Gram -ve)	63.58	113.04	63.58	113.04
MOF 1 (Shrimp isolate)	(Gram -ve)	R	63.58	R	63.58
MOF 2 (Clown fish isolate)	(Gram -ve)	50.24	50.24	3.14	R

R - Resistant

isolate was resistant at 30°C and produced an active range of inhibition (63.58 mm^2) at 20°C (Table-2). The results also indicated that the aqueous extracts of macroalgae successfully inhibited *Vibrio alginolyticus* isolate followed by *Pseudomonas aeruginosa* and *Micrococcus luteus* at 30°C and 20°C.

Most of the Gram -^{ve} strains, did not exhibit a higher range of inhibition except *Vibrio alginolyticus* and *Pseudomonas aeruginosa*. In the case of *H. musciformis*, the least inhibited strains were *Micrococcus luteus* and MOF 2 isolate at 30°C at 1.0% level. The methanol extract of *H. musciformis* exhibited 100% inhibition towards the Gram +^{ve} and Gram -^{ve} strains at 10% level at 20°C. The antibacterial activity of *H. musciformis* extracts was found to be effective even at 0.01% giving 66.66% inhibition of tested Gram +^{ve} bacteria at 30°C and 33% at 20°C. It was also observed that crude *H. musciformis* extracts were less effective (1 to 30 mm^2) at 30°C in lower concentrations. Among the fish pathogens, MOF 2 isolate was found to be sensitive (Table-3).

The methanol extract of *H. valentiae* extracts was found to be most active at 0.01% against *Staphylococcus aureus*, *Vibrio fischeri*, *V. alginolyticus*, *Pseudomonas aeruginosa* and *Micrococcus luteus* at 20°C. It was also assumed that *Hypnea valentiae* produced less inhibitory potential towards *Aeromonas hydrophila* at 30°C. Gram -^{ve} bacterium *Vibrio alginolyticus* was much sensitive towards *H. valentiae* methanol extract at a low concentration of 0.01% at both the incubated temperatures (Table-4). In the case of *H. musciformis*, the Gram +^{ve} strains were more susceptible in methanol extract compared to their respective aqueous extract. In the aqueous extract of *H. valentiae*, the Gram +^{ve} strains had 66.66% and 100% inhibition at 30°C and 20°C respectively, while the Gram -^{ve} strains produced 71.42% and 85.71% at 30°C and 20°C respectively. *Aeromonas hydrophila* was found to be resistant in the aqueous extract of *H. valentiae*.

Among the eluted 5 fractions of *Hypnea musciformis*, the active fractions noted were H_4 (against *Pseudomonas aeruginosa* and *Micrococcus luteus*), HB1a (against *Vibrio alginolyticus* and *A. hydrophila*) and HB2b (against *Staphylococcus aureus*) with an inhibitory potential of >100 mm^2. The Gram +^{ve} strain of *Micrococcus luteus* (109.3 mm^2) and Gram -^{ve} strain of *Pseudomonas aeruginosa* (113.04 mm^2) exhibited highest inhibitory potential towards H_4 (Hexane -100%) fraction. Among the very active fractions, the resistant noted were H_4 against *Vibrio fischeri* and HB1a against *Staphylococcus aureus* and MOF 2. The 1.0% of crude extract of *H. musciformis* exhibited an active range of inhibition against the bacterial isolates of *Pseudomonas aeruginosa*, *Serratia marcescens*, *Micrococcus luteus* and MOF 2 isolates. Fractionated same extracts also showed more or less same range of $\leq$ 6.54 mm^2 inhibition towards the same bacterial strains. Similarly, an active range of $\leq$ 60 mm^2 inhibition was exhibited by the crude as well as the fractionated extracts towards the isolates of *Staphylococcus aureus*, *Serratia marcescens*, *Vibrio alginolyticus* and *Aeromonas hydrophila*. Among the Gram +^{ve} strains, highest inhibition was produced by *Staphylococcus aureus* (86.54 mm^2) against H_2 fraction and *Micrococcus luteus* (86.54 mm^2) and *Bacillus subtilis* (96.71 mm^2) against HB1a fraction. In the case of Gram -^{ve} strains, *Vibrio alginolyticus* (96.71 mm^2), *Pseudomonas aeruginosa* and *Aeromonas hydrophila* (86.54 mm^2) were sensitive against H_4 while *Serratia marcescens* was sensitive against H_4 and MOF1 (86.54 mm^2) was sensitive against HB1a.

Table-3. *Antibacterial activity in different concentrations of* Hypnea musciformis

Test organisms		Concentrations (%) 10		1		0.1		0.01		0.001		Control
		30°C	20°C	30°C	20°C	30°C	20°C	30°C	20°C	30°C	20°C	
Clinical pathogens												
Staphylococcus aureus	Mtl	+++	+++	+	++	+	+	-	+	-	-	-
	Aqs	+++	++++	++	++	+	+	+	+	-	-	-
Micrococcus luteus	Mtl	+++	++++	+	+	-	+	-	+	-	-	-
	Aqs	+++	++++	+	+	-	+	-	+	-	-	-
Pseudomonas aeruginosa	Mtl	+++	++++	+	++	+	+	+	+	-	-	-
	Aqs	+++	++++	+	++	+	+	+	+	-	-	-
Aeromonas hydrophila	Mtl	++	+++	+	+	+	+	+	-	-	-	-
	Aqs	++	+++	+	+	+	+	+	-	-	-	-
Bacillus subtilis	Mtl	-	++	-	-	-	-	-	-	-	-	-
	Aqs	-	++	-	-	-	-	-	-	-	-	-
Fish pathogens												
Serratia marcescens	Mtl	+	+	-	+	-	-	-	-	-	-	-
	Aqs	+++	+++	-	-	-	-	-	-	-	-	-
Vibrio fischeri	Mtl	++	+++	+	+	-	-	-	-	-	-	-
	Aqs	++	+++	+	+	-	-	-	-	-	-	-
Vibrio alginolyticus	Mtl	+++	+++	+	++	+	-	-	-	-	-	-
	Aqs	+++	+++	+	+	+	-	-	-	-	-	-
MOF1(Shrimp isolate)	Mtl	++	+++	-	++	-	++	-	+	-	-	-
	Aqs	++	++	-	++	-	++	-	+	-	-	-
MOF2(Clown fish isolate)	Mtl	+++	+++	+	+	+	+	+	+	-	-	-
	Aqs	-	-	-	-	-	-	-	-	-	-	-

Mtl - Methanol extract; Aqs - Aqueous extract; ++++ Maximum active; +++ More active; ++ Active; + Minimum active; - No active

Table-4. *Antibacterial activity in different concentrations of* Hypnea valentiae

Test organisms		Concentrations (%) 10		1		0.1		0.01		0.001		Control
		30°C	20°C	30°C	20°C	30°C	20°C	30°C	20°C	30°C	20°C	
Clinical pathogens												
Staphylococcus aureus	Mtl	+++	++++	+	++	-	+	+	+	-	-	-
	Aqs	+++	+++	+	+	-	-	-	-	-	-	-
Micrococcus luteus	Mtl	++++	++++	+++	+++	++	++	-	+	-	-	-
	Aqs	-	+++	-	++	-	++	-	++	-	-	-
Pseudomonas aeruginosa	Mtl	+++	++++	+	++	-	++	-	+	-	-	-
	Aqs	++	+++	+	+	+	+	-	-	-	-	-
Aeromonas hydrophila	Mtl	+	+	-	+	-	-	-	-	-	-	-
	Aqs	-	-	-	-	-	-	-	-	-	-	-
Bacillus subtilis	Mtl	+++	+++	+	+	-	+	-	-	-	-	-
	Aqs	++	+++	+	+	+	+	+	+	-	-	-
Fish pathogens												
Serratia marcescens	Mtl	-	-	-	-	-	-	-	-	-	-	-
	Aqs	+	+	-	+	-	-	-	-	-	-	-
Vibrio fischeri	Mtl	++	+++	+	+++	-	+	-	+	-	-	-
	Aqs	++	+++	+	++	+	+	-	-	-	-	-
Vibrio alginolyticus	Mtl	+++	++++	++	+++	+	++	+	+	-	-	-
	Aqs	+++	++++	++	+++	+	++	+	+	-	-	-
MOF1 (Shrimp isolate)	Mtl	-	+++	-	+	-	-	-	-	-	-	-
	Aqs	-	+++	-	+	-	+	-	+	-	-	-
MOF2(Clown fish isolate)	Mtl	++	++	+	++	+	+	-	-	-	-	-
	Aqs	+	+	+	+	-	-	-	-	-	-	-

Mtl - Methanol extract; Aqs - Aqueous extract; ++++ Maximum active; +++ More active; ++ Active; + Minimum active; - No active

Among the Gram +ve strains, the H_4 fraction moderately inhibited *Staphylococcus aureus* (56.71 mm^2) and *Bacillus subtilis* (58.05 mm^2), while HB5a and HB2b fractions inhibited *Staphylococcus aureus* (56.71 mm^2) and *Bacillus subtilis* (31.15 mm^2) isolates respectively. In case of Gram -ve strains, HB5a and HB2b moderately inhibited *V. fischeri*, *Vibrio alginolyticus*, *Aeromonas hydrophila* and MOF 2. The *Pseudomonas aeruginosa* was moderately inhibited only by HB2b fraction. The results also revealed that *Micrococcus luteus* and MOF 1 isolates were less inhibited by HB5a and HB2b fraction (Table-5).

Among the fractionated extracts of *Hypnea valentiae*, the H_2 showed very active inhibitory potential towards *Pseudomonas aeruginosa* (109.3 mm^2). Dcm/methanol (1:1) fraction exhibited maximum inhibitory potential of ≤ 96.71 mm^2 and 86.54 mm^2 towards Gram +ve strain of *Bacillus subtilis* and Gram -ve strain of *Vibrio alginolyticus* respectively. *Staphylococcus aureus, Vibrio alginolyticus*, *Bacillus subtilis* and MOF 2 isolate were found to be resistant towards ether fraction. The most resistant isolate noted was MOF 2 (Clown fish isolate) against H_2 (Hexane-100%), ether and acetone fractions. Gram +ve strain of *Micrococcus luteus* exhibited moderate range of inhibition towards H_2 and Dcm/methanol (1:1) fractions. The maximum and minimum moderate range of inhibitory response was produced by the Gram +ve isolates of *Staphylococcus aureus* (58.05 mm^2) and *Micrococcus luteus* (3.14 mm^2) against Dcm/methanol (1:1) and Dcm fractions respectively. The Gram +ve strains of *Staphylococcus aureus* (58.05 mm^2) and *Micrococcus luteus* (31.15 mm^2) and Gram -ve strains of *Vibrio fischeri* (33.16 mm^2), *Pseudomonas aeruginosa* (56.71 mm^2) and *Aeromonas hydrophila* (33.16 mm^2) were moderately sensitive towards Dcm/methanol (1:1) fraction.

In the case of Gram -ve strains very weak response was produced by *Serratia marcescens* against H_2 (3.14 mm^2), Dcm/methanol (1:1) (4.90 mm^2) and ether (3.14 mm^2) fractions. While the maximum moderate range of inhibitory response was produced by the Gram -ve isolates of *Pseudomonas aeruginosa* (56.71 mm^2) against Dcm/methanol (1:1) and ether and *Vibrio alginolyticus* against Dcm fractions (Table-6). The commercial antibiotics tested produced greater inhibitory response towards *Serratia marcescens* isolate at 20°C. When compared with standard chloramphenicol, the methanol extract of *Hypnea musciformis* and *H. valentiae* produced 21.42% more inhibitory potential towards Gram +ve strain of *Staphylococcus aureus*. The methanol and aqueous extract of *Hypnea musciformis* extract exhibited very active range of inhibition towards *Staphylococcus aureus* and *Pseudomonas aeruginosa* compared to the standard antibiotics.

Discussion

The results of antibacterial potential of macroalgae towards *Staphylococcus aureus* and *Pseudomonas aeruginosa* is of particular significance because of their known bacterial resistance to various antibiotics (UNDP, 1994). A series of small molecular volatile halogenated compounds (halomethanes, haloether and haloacetales) also account for the antimicrobial activity. Padmakumar (1988) reported that the bacterial flora associated with *Ulva lactuca* and *Hypnea musciformis* showed a significant negative correlation with the antibacterial activity. The cell extracts and active constituents of various algae have been shown to produce antibacterial activity *in vitro* against the Gram +ve and Gram -ve bacteria (Tuney *et al*., 2006). Earlier reports suggest that extracts of Indian macroalgae were active against only Gram +ve bacteria (Sreenivasa Rao and Parekh, 1981).

Table-5. *Antibacterial study on* Hypnea musciformis *fractions at 30^{0}C (Inhibition zone in mm^2)*

	Gram +^{ve} strains				Gram -^{ve} strains					
Fractions	*S.a*	*M.l*	*B.s*	*S.m*	*V.f*	*V.a*	*P.a*	*A.h*	MOF1	MOF2
H_2	86.54	24.61	R	86.54	3.14	23.74	28.26	34.19	24.61	3.14
H_4	56.71	109.3	58.05	41.83	R	96.71	113.04	86.54	109.3	9.61
HB1a	R	86.54	96.71	28.26	19.62	113.04	86.54	143.06	86.54	R
HB5a	56.71	12.56	12.56	12.56	33.16	44.15	R	44.15	12.56	33.16
HB2b	113.04	19.62	31.15	31.15	41.83	36.29	38.46	36.29	19.62	44.15

S.a - Staphylococcus aureus; S.m - Serratia marcescens; V.f - Vibrio fischeri; V.a - Vibrio alginolyticus; P.a - Pseudomonas aeruginosa; M.l - Micrococcus luteus; B.s - Bacillus subtilis; A.h - Aeromonas hydrophila; MOF 1 - Shrimp isolate; MOF2 - Clown fish isolate; H2&H4 - Hexane (100%); HB1a & HB5a : Hexane - Benzene (80:20%); HB2b: Hexane-Benzene (60:40%)

Table-6. *Antibacterial study on* Hyupnea valentiae *fractions at 30°C (Inhibition zone in mm^2)*

	Gram +^{ve} strains				Gram -^{ve} strains					
Fractions	*S.a*	*M.l*	*B.s*	*S.m*	*V.f*	*V.a*	*P.a*	*A.h*	MOF1	MOF2
H_2	86.54	31.15	7.06	3.14	33.16	86.54	109.3	R	R	R
Dcm	56.71	3.14	R	R	28.26	56.71	86.54	33.16	109.3	9.61
Dcm/methanol (1:1)	58.05	31.15	96.71	4.9	33.16	86.54	56.71	33.16	19.62	23.74
Ether	R	12.56	R	3.14	23.74	R	56.71	3.14	R	R
Acetone	R	19.62	19.62	R	R	28.26	36.29	R	R	R

S.a - Staphylococcus aureus; S.m - Serratia marcescens; V.f - Vibrio fischeri; V.a - Vibrio alginolyticus; P.a - Pseudomonas aeruginosa; M.l - Micrococcus luteus; B.s - Bacillus subtilis; A.h -Aeromonas hydrophila; MOF1 - *Shrimp isolate;* MOF2 - *Clown fish isolate;* H_2 - Hexane (100%); Dcm - Dichloromethane

It was also reported that the Gram +^{ve} strains were more susceptible to macroalgal crude extracts than Gram -^{ve} bacterial strains (Rosell and Srivastava, 1987). But Shanmughapriya *et al*. (2008) while discussing about the results of antimicrobial activity of the fourteen species of macroalgae collected from the intertidal zone of southwest coast of India pointed out that the macroalgae were highly active against Gram -^{ve} bacteria than Gram +^{ve} bacteria. The results of the present study indicated successful inhibition of both Gram +^{ve} and Gram -^{ve} strains at higher concentrations. These results are in agreement with the earlier findings of Selvi *et al*. (2001) for the methanol extracts of *Hypnea valentiae,* exhibiting higher inhibition against Gram +^{ve} and Gram -^{ve} bacteria. In the present

study, methanol extract of *Hypnea musciformis* inhibited 66% of Gram +ve bacteria, although the activity was narrowed towards Gram -ve bacteria in aqueous extract of *H. musciformis*. The extract of *Enteromorpha compressa*, *Cladophoropsis zollingeri*, *Padina gymnospora*, *Sargassum wightii* and *Gracilaria corticata* were active against Gram +ve *Bacillus* (Rao, 1991). According to Gonzalez del Val *et al.* (2001), the algal extracts of *Enteromorpha ramulosa* and *Dictyopteris membranacea* were active against both Gram +ve and Gram -ve strains.

Kandhasamy and Arunachalam (2008) studied the methanol extract of *H. musciformis* extract and indicated their less activity towards *Staphylococcus aureus* (12 ± 0.69 mm) and *Pseudomonas aeruginosa* (12 ± 0.95 mm). In contrast, the results of antibiotic screening gave active inhibitory range of 113.04 mm^2 towards *S. aureus* and *P. aeruginosa*. It was also noted that the methanol extract of *H. valentiae* extracts was found to be most active at 0.01% against *Staphylococcus aureus*, *Vibrio fischeri*, *V. alginolyticus*, *Pseudomonas aeruginosa* and *Micrococcus luteus* at 20°C. According to Hornsey and Hide (1974), volatile compounds such as fatty acids, terpenes and carbonyls from Rhodophyceae (*Porphyra*, *Digenia*, *Hypnea*), Phaeophyceae (*Sargassum*, *Laminaria*) and Chlorophyceae (*Enteromorpha*, *Codium*, *Ulva*) were active at very low concentration on pathogenic bacteria. The present results revealed that the incubation temperature considerably influenced the antibiosis of extracts. In certain cases, the inhibitory activity was significantly increased at 20°C. At the lower incubation temperature, the bacterial growth could have been reduced and therefore the activity of extracts was increased at 20°C. In the present study also the MOF 1 isolate was found to be resistant at 30°C and regained a nearly active inhibitory range of 63.58 mm^2 at 20°C in *Hypnea valentiae* methanol extract. However, among the Gram +ve bacteria, not all the target strains tested were equally susceptible to the antimicrobial metabolites produced. In the present study, the absence of very active range of inhibitory response was noted among the Gram +ve strains of *Micrococcus luteus* and *Bacillus subtilis* towards the methanol extract of *H. musciformis*. This could indicate the presence of some masking factors as reported by Sreenivasa Rao and Parekh (1981).

Fraction HB5a (Hexane-Benzene (80:20%) of *H. musciformis* exhibited potent activity not only against Gram -ve bacteria but also against Gram +ve strain of *Staphylococcus aureus*. Similar observations were made by Vlachos *et al.* (1997) that fractionation of crude extracts enhanced their activity against both Gram -ve as well as Gram +ve pathogens. The antibacterial activity found in hexane extracts showed the success of the non-polar hydrophobic extracts independent of diffusion parameters in the assay method employed (Choudhury *et al*., 2005). Higher inhibition activity yielded by methanol extract than n-hexane and benzene fractions indicated that some of the chemical compounds are more soluble in methanol than other solvents (Zineb *et al*., 2004). Methanol as the best solvent for extracting the effective antimicrobial materials from the algae species used in this experiment also coincided with the earlier findings of Selvin and Lipton (2004). They reported the antibacterial activity of the methanol extracts of same algal species. In marine plants, it was observed that the biological activities mainly reside in the polar fractions (Guzman *et al*., 2001). Studies concerning the effectiveness of extraction methods highlighted that chloroform was better than methanol and Benzene (Febles *et al*., 1995). However, higher inhibition rate shown by the *n*-Hexane fraction of *Hypnea valentiae* indicating the

intermediary and non-polar nature of some active compounds. Guzman *et al*. (2001) also reported that chloroform, alcohol and acetone extracts of *H. musciformis* were inhibitory to pathogenic bacteria *viz*. *Pseudomonas*, *Staphylococcus* and *Salmonella*. The present study also revealed the minimum activity of aqueous extract in all the tested Gram $+^{ve}$ strains. This may complement the explanation of the hypothesis developed for the methanol extract, which suggest that the partial inhibition could be due to the partial extraction of the active compounds which are more extractable by methanol than by water (Zineb *et al*., 2004).

The differences in antimicrobial activity observed between the methanol, aqueous and fractionated extracts of macroalgae could be attributed to the intraspecific variability in producing secondary metabolites, influenced by seasonal changes (Lima-Filho *et al*., 2002). Secondly, there could be differences in the capability of the extraction protocols to recover the active metabolites and differences in the assay methods that would result in different susceptibilities of the target strains as suggested by Gonzalez del Val *et al*. (2001). It is clear that organic solvents provided a higher efficiency in extracting compounds for antimicrobial activities compared to water-based extraction methods (Lima-Filho *et al*., 2002). The results of present study revealed that methanol extract of *Hypnea musciformis* produced very active range of inhibitory pattern (113.04 mm^2) towards Gram $-^{ve}$ strain of *Vibrio alginolyticus* while the aqueous extract of same produced only active range of inhibition of 94.98 mm^2 at 20°C.

According to Patra *et al*. (2008), the methanol extract of *Sargassum* sp. possesses strong antimicrobial activity against Gram $+^{ve}$ strain of *Staphylococcus aureus* when compared with Ampicillin as the standard. In the present study also, the methanol extract of *Hypnea musciformis* and *H. valentiae* could be considered as valuable alternative to the tested commercial antibiotics since this extract successfully inhibited both Gram $+^{ve}$ and Gram $-^{ve}$ strains of *Staphylococcus aureus*, *Vibrio alginolyticus* and *Pseudomonas aeruginosa*. Kandhasamy and Arunachalam (2008) reported that the crude extract of *Hypnea musciformis* produced high antibiotic susceptibility against *Pseudomonas aeruginosa*, *Micrococcus luteus*, *Staphylococcus aureus* and *Bacilus subtilis* when compared with standard Chloramphenicol. In the present study, methanol extract of *Hypnea musciformis* exhibited very active range of inhibitory response against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (113.04 mm^2) compared to all the tested commercial antibiotics. The activity of the macroalgal methanol and other solvent extracts will lead to the findings of natural antimicrobial agents as alternatives to the existing antibiotic, which is already resistant to the pathogens, especially in the treatment of Methicillin-Resistant *Staphylococcus aureus* (MRSA). However, systematic investigations regarding toxicity, stability and metabolism of macroalgae and macroalgal compounds need to be undertaken.

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