

***In vitro* studies on bactericidal activity and sensitivity pattern of isolated marine microalgae against selective human bacterial pathogens**

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Abstract: Ten microalgae cultured under controlled condition were tested for their antimicrobial activity against the selective bacterial pathogens in compliance with paper disk method. Five different solvents were used for the extraction. *Isochrysis galbana* showed overall inhibition of [16.22%] followed by *Chlorella marina* [14.43%], *Nannochloropsis oculata* [14.07%], *Dunaliella salina* [13.91%] and *Pavlova lutheri* [13.17%]. These five microalgal strains were further investigated to examine concentration dependent microbicidal activity using tube dilution method. Microalgal strains were also investigated with agar-well diffusion method to understand the efficacy of antimicrobial principles against various bacterial pathogens. The findings in this study reveal that optimal activity is maintained by butanol extracts on Gram-positive bacteria; ethanol and petroleum ether extracts on both Gram-positive and Gram-negative bacteria; methanol extracts on Gram-negative organisms. Chloroform extracts, on the other hand did not show any significant antimicrobial activity.

Keywords: Bactericidal, disk diffusion, microalgae, antibiotics, pathogens.

Introduction

A study by the World Health Organization (WHO) has shown that about 80% of the world's population still relies on traditional medicine (Farnsworth *et al.*, 1985). Today, over 50% of the marketed drugs are either extracted from natural sources or produced synthetically using natural products as templates or starting materials. Marine environments host a wide range of bio-resources that have tremendous potential to provide new bio-products, including enzymes, antibiotics, anticancer agents, food additives, and pigments. The organisms yielding these bioactive compounds comprised a taxonomically diverse group of marine invertebrate animals, algae, fungi and bacteria (Mayer & Gustafson, 2003). Many bioactive and pharmacologically active substances have been isolated from algae. For instance, extracts of marine algae were reported to exhibit antibacterial activity (Siddhanta *et al.*, 1997). Marine organisms are known to contain a wide range of novel structures (Baker & Murphy, 1981) and compounds with antimicrobial activity are increasingly being isolated from marine algae (Faulkner, 1986). The number of species so far examined is only a small percentage of the estimated total of 30,000 and it is likely that new antibiotics will be isolated. It is also evident from the earlier research that microalgae are significant

resource for bioactive metabolites, particularly cytotoxic agents with applications in cancer chemotherapy (Moreau *et al.*, 1988). Over 20 new drugs launched on the market between 2000 and 2005, originating from terrestrial plants, terrestrial microorganisms, marine organisms, and terrestrial vertebrates and invertebrates (Young *et al.*, 2006). Many authors had found antibacterial activities of microalgae (Cooper *et al.*, 1983; Findlay & Patil, 1984; Viso *et al.*, 1987; Kellam *et al.*, 1988).

With the advent of Molecular biology, the screening of microalgae for antibiotics and active compounds has received considerable attention and a range of pharmacological properties have also been observed in the extracts of microalgae. Most of these bioactive compounds may find application in human or veterinary. However, the antimicrobial activity of microalgae has not adequately studied in India. In this context, the present paper illustrates the bactericidal activity of 10 marine microalgae against selected bacterial pathogens.

Material and methods

Sample collection

Algal samples were collected from sixty one sampling sites from Nerodi to S.P.Pattanam along the south east coast of Tamil Nadu, India with an average sampling distance of 5.42 kms. Marine water samples were collected using micro algal net cone shaped of mesh 20 μ m in size. The water samples were collected in the cup which was tied in the bottom of the algal net and was transferred to 0.1N HCl pretreated, steam sterilized screw cap bottles. The samples were preserved instantly into the ice bucket which was maintained at 5 \pm 1 $^{\circ}$ C and transferred to the laboratory. Methods of isolation and maintenance of microalgae in axenic cultures are based on serial dilution culture techniques and agar plate method as described by (Gopinathan, 1996). Totally 10 microalgal species MA1: *Isochrysis galbana*, MA2: *Nannochloropsis oculata*, MA3: *Dicrateria inornata*, MA4: *Chromulina freibergensis*, MA5: *Pavlova lutheri*, MA6: *Chlorella marina*, MA7: *Chaetoceros calcitrans*, MA8: *Dunaliella salina*, MA9: *Platymonas spp*, MA10: *Synechocystic salina* was isolated from the sampling sites.

Culture of microalgae

Culture medium (Walne's medium), modified by (Laing, 1991) was taken in a series of test tubes and each inoculated with the isolated algal colonies in various concentrations. These tubes were kept under sufficient

light (1000 lux) and incubated in the algal culture room under room temperature (22- 28⁰C) with a pH of 8.2 ± 1. After 15 - 18 days, some discoloration was seen in the culture tubes due to the growth of microalgae. These were examined under the microscope and successful cultures were diluted and sub cultured in 20 ml and subsequently to 250 ml; 1 litre Erlenmeyer flask and maintained as stock culture under a luminosity of 1000 lux. The cultured flasks were shaken thrice a day to ensure proper growth. The algae were further mass cultured in Haufkin's flask using Walne's medium and harvested in the exponential phase for experimental purpose.

Separation of algal cells

Microalgal cells in exponential growth phase were recovered from culture by batch centrifugation at 3000 rpm for 10 minutes. The cells were repeatedly washed in normal saline (0.85% sodium chloride) for three times by centrifugation at low speed. Microalgal samples were also extracted by ultrafiltration technique using KMS ultra-filtration tubular membrane of 0.1 µm pore size. Extracted biomass were transferred to a pre-weighed dry filter paper using a clean spatula then placed in an oven at 60⁰C overnight to reach a fixed weight. The dry weight of the microalgal cells was weighed and the results were tabulated. Further microalgae were stored in refrigerator at + 4⁰ C till extraction process.

Preparation of algal extract

Five different solvents were used in this experiment. They were Petroleum ether, Butanol, Ethanol, Methanol and Chloroform. By using these solvents algal cells were homogenized, extracted with respective solvents and filtered after extraction in accordance with (Khan *et al.*, 1988). The extracts were dried under reduced pressure. All the extracts were preserved at + 4⁰C for further investigation.

Collection of human bacterial pathogens

The pathogens such as BP1- *E. coli*, BP2- *Klebsiella pneumoniae*, BP3- *Proteus vulgaris*, BP4- *Pseudomonas aeruginosa*, BP5- *Pseudomonas fluorescens*, BP6- *Staphylococcus aureus*, BP7- *Streptococcus pyogenes*, BP8- *Vibrio cholerae*, BP9- *Salmonella typhi* and BP10- *Bacillus subtilis* were collected from a local clinical Laboratory. These bacteria's were cultured in L-broth medium (Bacto tryptone 1%; yeast extract 0.5% and NaCl 0.5%).

Antibiotic sensitivity test with commercially available antibiotics (Kirby- Bauer method)

Agar Disk Diffusion method (Chabbert, 1963) was followed to perform the susceptibility test. The Muller-Hilton agar plates were prepared (Beef

infusion form - 30 (g.l⁻¹), Casein acid hydrolysate- 17.5 (g.l⁻¹), Starch- 1.5 (g.l⁻¹), Agar- 17(g.l⁻¹), pH- 7.3). A swab of the test culture was taken aseptically and inoculated to the surface of the Muller-Hinton agar plate so as to make a lawn. This was allowed atleast 5 minutes for the agar surface to dry before applying disc. The forceps was sterilized by dipping in alcohol, then flamed and allowed to cool. The commercially available antibiotic disc was carefully taken and placed over the agar plate atleast 15mm from the edge of the plate. The disc was pressed gently to give a better contact with agar. The plates were incubated for 16 to 18 hours at 37⁰C. The zone of inhibition was observed around the antibiotics discs. The indication whether test organisms is resistant (no zone or inhibition) or sensitive (clear zone of inhibition) to the antibiotics was observed and tabulated.

Screening of marine algal extracts against selective bacterial pathogens (Agar disk diffusion method)

The human bacterial pathogens were sub-cultured in L-broth medium. About 15ml of sterile molten Muller Hilton (Beef infusion form - 30 (g.l⁻¹), Casein acid hydrolysate- 17.5 (g.l⁻¹), Starch- 1.5 (g.l⁻¹), Agar- 17(g.l⁻¹), pH- 7.3) was introduced aseptically into sterile petridishes and after solidification; 12 hr old L-broth culture of the test organisms were spread uniformly on the surface of the agar plates with the help of a sterile cotton swab (lawn culture). Then the empty sterile paper discs were dipped in the respective extracts and air dried in the room temperature and placed on the agar medium in petridishes, pre-spread with the bacterial pathogens using sterilized forceps. Then the plates were incubated at 37⁰C for 24 hrs. The antibacterial activity of the marine microalgal extract was observed through zone of inhibition around the disc. Then this zone of inhibition was measured in mm using a ruler and tabulated. Control discs soaked with the respective solvents were also run simultaneously.

Concentration dependent bactericidal activity

The tube dilution technique was performed to determine the minimal amount of microalgal active principles required to inhibit the growth of selective bacterial pathogens under laboratory conditions. In the present technique ten promising microalgal extracts from five microalgae MA1, MA2, MA5, MA6, and MA8 [2 extracts from each MA's], which shows optimal activity in the agar disk diffusion method were tested against respective bacterial pathogens. MA samples were added in an increasing

concentration of 20 µg in a series of test tube of Muller Hinton broth freshly inoculated with one ml of corresponding bacterial pathogens (1×10^5 cfu/ml). The experiment set up was incubated at 37° C for 24 hours. After incubation the culture samples were scanned at 620 nm and the results were recorded and tabulated.

Bacterial susceptibility testing using agar well diffusion method

The agar plate well-diffusion method was used as described by (Desta, 2005). Standardized inoculums from 12 hour old selective bacterial culture ($1-2 \times 10^5$ cfu/ml) were introduced onto the surface of sterile agar plate, and evenly distributed the inoculums by using a sterile glass spreader. Simultaneously 8 mm wells were cut from the plate using a sterile cork borer. 70µl of extract at a concentration of 10 mg/ml were introduced into each well. The agar plates were incubated aerobically at 37°C and the inhibition zones measured with a ruler and compared with the control well (well containing only the respective solvent) after 24 hr.

Table 1. Quantitative determination of dry weight of microalgae

Microalgal species examined	Comparative determination of dry weight [g/L] of microalgae extracted from 1 litre of exponential phase culture using centrifugation / ultrafiltration method					
	Centrifugation			Ultrafiltration		
	Day 10	Day 12	Day 14	Day 10	Day 12	Day 14
MA-1	2.166	2.666	2.867	2.237	2.873	3.017
MA-2	2.933	3.033	4.126	3.126	3.237	4.237
MA-3	3.356	3.343	3.363	3.849	3.627	3.787
MA-4	1.526	2.166	2.563	1.437	2.176	3.016
MA-5	3.266	3.279	3.126	3.473	3.337	3.231
MA-6	2.033	2.633	2.663	2.633	2.836	2.137
MA-7	1.216	1.543	1.769	1.267	1.332	1.679
MA-8	2.133	1.766	2.337	2.367	1.832	2.446
MA-9	0.866	0.986	1.436	0.976	0.996	1.332
MA-10	2.666	2.966	3.234	2.763	3.016	3.743

Results and discussion

The dry weight of microalgal samples extracted from 1 litre of culture medium using centrifugation and ultrafiltration method was analyzed using paired sample statistical analysis (Table 1). The mean difference of micro algal samples extracted on day 10 by centrifugation method was 2.216 ± 0.847 & SEM of 0.267, on day 12 was 2.438 ± 0.794 & SEM of 0.251, on day 14 was 2.748 ± 0.786 & SEM of 0.248 and the yield of dry microalgae by ultra-filtration method on day 10 was 2.412 ± 0.956 & SEM of 0.302, on day 12 was 2.526 with a standard

deviation of 0.894 & SEM of 0.282, on day 14 was 2.862 with a standard deviation of 0.951 & SEM of 0.300. It was also observed from the result of Pearson correlation method of all 3 paired samples shows significant correlations of C10:U10 0.981, C12:U12 0.993 and C14: U14 0.954 respectively. The result clearly indicates ultra-filtration as a preferable extraction procedure.

Ten bacterial pathogens were screened with different 16 commercially available discs. It was observed that Furazolidone shows maximum bacteriocidal activity across all selected bacterial pathogens with a mean value of 24.1 ± 2.601 . Subsequently Nalidixic acid shows activity with a mean value of 19.9 ± 2.806 followed by Netilmycin which shows activity with a mean value of 19.1 ± 1.911 . Cephalexin, a first-generation cephalosporin antibiotic shows an overall activity of 18 ± 3.464 . Amikacin and Ampicillin shows activity with a mean value of 16.5 ± 3.689 and 15.9 ± 3.604 respectively. Oxytetracycline shows activity with a mean value of 14.3 ± 5.812 . Interestingly streptomycin shows activity towards *B subtilus* shows an inhibition zone of 9 mm. The antibiotics like Vancomycin, Tetracycline, Kanamycin, Erythromycin, Gentamycin, Chloramphenicol, Amoxycillin and Cloxacillin were resistant towards the experimented bacterial strains.

The crude extract of 10 microalgal species were screened against ten bacterial pathogens and the results were illustrated in (Table 2). In the present study, microalgae were extracted with petroleum ether, methanol, ethanol, butanol and chloroform and tested against three gram positive and seven gram negative bacteria. Earlier researchers also used different solvents to determine the antibacterial activity of seaweed extracts such as methanol, toluene, diethyl ether, petroleum ether (Padmini *et al.*, 1986), ethanol (Padmakumar & Ayyakannu, 1986) and toluene: methanol mixture (Cassamese *et al.*, 1981).

Petroleum ether [PE] extract was treated against ten bacterial pathogens and the result illustrates that *Pavlova lutheri* [MA5] shows total inhibitory zone of 36.8 mm 26.41% [3.68 ± 4.4361] with maximum inhibition against *Vibrio cholerae* [BP8] 31.5% and *E. coli* [BP1] 25.5%. *Platymonas sp* [MA9] shows

Table 2. Sensitivity pattern of microalgal extracts against the bacterial pathogens

Microalgae used	Solvent Used	Bacterial pathogens used; Mean Inhibition zone in mm										Mean Total
		BP1	BP2	BP3	BP4	BP5	BP6	BP7	BP8	BP9	BP10	
<i>Isochrysis galbana</i>	Petroleum ether	9.6	0	4.6	0	0	3.3	0	0	2.6	0	2.01
	Butanol	3.6	NG	5.3	7.3	3.2	13.3	NG	0	6.6	2.3	4.16
	Ethanol	12.3	0	12.6	2.3	0	2.6	0	NG	10.6	6.3	4.67
	Methanol	0	2.6	2.6	6.3	3.2	0	0	NG	3.6	2.6	2.09
	chloroform	0	0	0	0	4.2	0	4.6	0	1.6	0	1.04
<i>Nannochloropsis oculata</i>	Petroleum ether	0	0	6.3	0	0	NG	NG	0	0	0	0.63
	Butanol	13.3	12.6	3.3	0	6.6	3.3	0	7	0	8.6	5.47
	Ethanol	2.3	12.3	0	4.6	0	4.6	0	5.3	2.3	6.3	3.77
	Methanol	0	0	0	0	0	2.3	0	0	0	0	0.23
	chloroform	0	9.6	0	0	3.3	0	NG	3.3	0	4	2.02
<i>Dicrateria inornata</i>	Petroleum ether	0	8.6	3.3	2.6	0	0	0	4.6	0	0	1.91
	Butanol	4.6	4	0	0	8.6	NG	6.3	0	2.6	1.6	2.77
	Ethanol	8.3	5.3	0	0	7.6	0	3.3	7.6	2.3	0	3.44
	Methanol	0	0	0	3.3	0	NG	0	NG	0	N	0.33
	chloroform	0	0	0	0	0	NG	0	0	0	0	0
<i>Chromulina freibergensis</i>	Petroleum ether	0	0	0	0	0	0	0	0	0	0	0
	Butanol	0	0	0	NG	NG	0	0	0	6.6	2.3	0.89
	Ethanol	NG	0	0	NG	6.3	0	NG	0	3.6	3	1.29
	Methanol	0	2.3	0	0	1.6	0	0	0	0	0	0.39
	chloroform	0	0	1	0	0	0	0	0	0	0	0.1
<i>Pavlova lutheri</i>	Petroleum ether	9.4	0	3.2	0	0	6.3	6.3	11.6	0	0	3.68
	Butanol	0	0	8.6	NG	3.2	13.2	0	2.3	11.3	6.6	4.52
	Ethanol	11.6	3.3	0	NG	0	3.6	0	0	0	2.6	2.11
	Methanol	0	0	0	0	3.2	0	0	0	NG	0	0.32
	chloroform	2.3	0	0	0	0	0	2.6	2.3	0	0	0.72
<i>Chlorella marina</i>	Petroleum ether	2.3	0	0	0	0	0	0	0	1.6	2.6	0.65
	Butanol	0	9.3	11.6	8.6	7.3	0	0	8.6	0	0	4.54
	Ethanol	0	0	0	7.6	13.3	0	0	6.3	3.6	0	3.08
	Methanol	0	13.3	6.3	6.6	4.6	0	0	0	2.6	0	3.34
	chloroform	6.6	0	0	0	0	0	0	NG	0	1.6	0.82
<i>Chaetoceros calcitrans</i>	petroleum ether	0	0	0	0	0	0	1.6	0	NG	0	0.16
	Butanol	NG	0	0	6.6	0	8.6	11.3	3.3	0	2.6	3.24
	Ethanol	NG	3.6	0	10.6	6.3	2.3	0	6.6	0	0	2.94
	Methanol	NG	0	0	0	0	4.3	0	0	0	0	0.43
	chloroform	0	0	0	0	0	0	0	0	NG	0	0
<i>Dunaliella salina</i>	petroleum ether	0	3.3	6.6	0	7.3	0	0	NG	0	0	1.72
	Butanol	0	0	0	6.6	0	0	12.6	11.3	6.6	0	3.71
	Ethanol	0	0	0	0	3.6	NG	9.6	13.6	6.3	7.3	4.04
	Methanol	0	0	0	0	3.3	NG	0	0	0	0	0.33
	chloroform	0	2.6	5.6	0	0	0	11.3	2.3	0	0	2.18
<i>Platymonas sp.</i>	petroleum ether	0	6.6	3.6	0	0	9.6	9.6	2.3	0	0	3.17
	Butanol	1.6	0	0	0	0	0	0	3.6	0	0	0.52
	Ethanol	3.3	0	0	NG	NG	0	2.3	2.6	0	0	0.82
	Methanol	2.3	0	NG	0	0	0	0	4.6	0	0	0.69
	chloroform	0	0	0	0	0	0	0	0	0	0	0
<i>Synechocystic salina</i>	petroleum ether	0	0	0	0	0	0	0	0	0	0	0
	Butanol	0	1.6	0	0	NG	2.3	0	0	0	NG	0.39
	Ethanol	0	0	0	NG	0	3.6	0	NG	0	0	0.36
	Methanol	4.3	0	NG	0	0	0	0	0	0	0	0.43
	chloroform	0	0	0	0	0	0	0	0	NG	0	0

BP- Bacterial Pathogens , NG- No Growth

total inhibitory zone of 31.7 mm, 22.75% [3.17 ± 4.0227] with maximum inhibition against gram positive strains BP6, BP7 and *Klebsiella pneumoniae* [BP2] 20.8%. *I. galbana* [MA1] had shown maximum inhibition against *E. coli* [BP1] 47.76%. *D. inornata* [MA3] shows maximum inhibition against *K. pneumoniae* [BP2]. *D. salina*

[MA8] shows inhibitory activity against *Pseudomonas fluorescens*. Interestingly no growth was observed in *V. cholerae* [BP8] Petri plates. *C. marina* [MA6], *N. oculata* [MA2], *C. calcitrans* [MA7], *C. freibergensis* [MA4] and *S. salina* [MA10] extracts has shown less impact over the selective bacterial pathogens and registers less inhibition zone.

aureus [BP6] plate. *C. freibergensis* [MA4], *Platymonas sp.* [MA9] and *Synechocystis salina* [MA10] extracts had shown less bactericidal activity. Richard *et al.* (1988) showed *B. subtilis* as inhibited by the methanol extracts of *Chlorella spp.* This can be related to the present study where ethanol and butanol extracts of *C. marina* showed inhibitory activity against BP2, BP3, BP4, BP5, BP8, BP9

Table 3. Agar plate well diffusion method - mean bacterial susceptibility test with hole diameter subtracted

Microalgae used	Solvent Used	Bacterial pathogens used										
		BP1	BP2	BP3	BP4	BP5	BP6	BP7	BP8	BP9	BP10	Total
<i>Isochrysis galbana</i>	Butanol	6.6	10.6	6.6	9.6	4.3	14.6	12.3	0	5.6	2.6	7.28
	Ethanol	14.3	0	13.3	2.6	0	6.3	0	8.6	11.3	9.6	6.6
<i>Nannochloropsis oculata</i>	Butanol	14.6	13.3	5.6	0	7.6	3.6	0	6.3	0	9.3	6.03
	Ethanol	3.3	13.3	0	4.3	0	4.6	0	6.3	3.6	7.6	4.3
<i>Pavlova lutheri</i>	petroleum ether	11.6	0	4.6	0	0	8.6	7.6	12.3	0	2.3	4.7
	Butanol	0	0	7.6	11.3	6.3	14.6	0	3.6	10.6	8.1	6.21
<i>Chlorella marina</i>	Butanol	0	10.6	11.3	8.3	7.6	0	3.6	7.3	0	0	4.87
	Methanol	4.3	14.3	7.6	6.3	6.3	0	0	0	4.3	0	4.31
<i>Dunaliella salina</i>	Butanol	7.6	0	0	7.3	0	10.3	11.6	4.6	3.6	0	4.5
	Ethanol	3.3	6.3	0	0	5.6	3.6	11.3	5.6	8.3	7.6	5.16

In the present study butanol extract showed maximum activity of 41.3% against the bacterial pathogens. Butanol [BT] extract of *N. oculata* [MA2] shares 18.1% of total inhibitory zone with better inhibitory against *E. coli* 24.3%, *K. pneumoniae* [12.6 mm] and *Bacillus subtilis* 15.6%. The results support earlier investigations by Kellar and Walker, (1989) where the extracts of *N. maculata* and *N. oculata* were inhibitory against *Streptococcus faecalis* and *Bacillus subtilis*. *Chlorella marina* [MA6] BT extract registered 15% of total inhibitory activity against gram negative strains *P. vulgaris* 25.5%, *K. pneumoniae* 20.4%, *P. aeruginosa* & *V. cholerae* 18.9% and *P. fluorescens*. BT extract of *P. lutheri* shows inhibitory against *S. aureus* 29.2% and *S. typhi* 25%. No growth was identified in *P. aeruginosa* plate. *I. galbana* had shown activity against *S. aureus* 31.9% and *P. aeruginosa* 17.5%. Remarkably no growth was identified in MA1 treated *K. pneumoniae* [BP2] and *S. pyogenes* [BP7] plates. *D. salina* inhibits *S. pyogenes* up to 33.9% and *V. cholerae* 30.4%. No growth was observed in MA8 treated *S. aureus* plate. *C. calcitrans* inhibited gram positive strains *S. pyogenes* 34.8% and *S. aureus* 26.5%. No growth was observed in *E. coli* [BP1] plate. *D. inornata* inhibits *P. fluorescens* growth up to 31%. Additionally no growth was observed on *S.*

strains.

The growth of gram negative bacterial pathogens *P. vulgaris* 26.9%, *E. coli* 26.3% and *S. typhi* 22.6% were inhibited by the ethanol [ET] extract of *I. galbana*. MA1 also exhibit minimal activity against gram positive strains. Ethanol extract of *D. salina* shows 33.6% of inhibitory activity against *V. cholerae* [BP8] and 23.7% against *S. pyogenes*. No growth was identified on *S. aureus* [BP6] plate. *N. oculata* [MA2] shows inhibitory against *K. pneumoniae* 32.6% and shows minimal activity against *E. coli*, *P. aeruginosa*, *S. aureus*, *V. cholerae*, *S. typhi* and *B. subtilis*. *D. inornata* [MA3] shows inhibitory against *E. coli* 24.1% and 22% against *P. fluorescens* and *Vibrio cholerae*. ET extract of *C. marina* [MA6] shows inhibitory activity against *P. fluorescens* 43.1%, *P. aeruginosa* 24.6 % and *V. cholerae* 20.4% and *S. typhi*, *B. subtilis*. Earlier study by Miura *et al.* (1993) who tested crude extracts of ethanol - methanol from over 100 strains of marine microalgae against antimicrobial activity and found *Bacillus subtilis* as strongly inhibited by *Chlorella sp.* *C. calcitrans* [MA7] shows promising activity against *P. aeruginosa* up to 36%, *Vibrio cholerae* 22.4% and *P. fluorescens* 21.4%. Alternatively no growth was observed in *E. coli* plate. Visco *et al.* (1987) reported the activity of

Chaetoceros affinis against *Aeromonas salmonicida*, *Pseudopiscida sp.* and *Alteromonas rubra*; *Chaetoceros brewis* against *Vibrio sp.* and *Aeromonas salmonicida*, *Chaetoceros danicus* against *K. pneumoniae*, *Proteus vulgaris*, *P. fluorescens*, *E. coli*, *S. typhi* and *V. cholerae*. In the present study *Chaetoceros calcitrans* optimally inhibited the growth of BP1, BP4, BP6, BP7 and BP8. *Pavlova lutheri* [MA5] shows total inhibitory zone of 21.1 mm, 7.9 % with an inhibitory against *E. coli*, *K. pneumoniae* [BP2], *S. aureus* [BP6] and *B. subtilis* [BP10]. No growth was observed in *P. aeruginosa* [BP4], plate. Ethanol extract of *Chromulina freibergensis* [MA4] *Platymonas sp* [MA9] and *Synechocystis salina* [MA10] extracts had shown less bactericidal activity. Earlier study conducted by Regini (2004) who screened *Noctiluca scintillans* against bacterial strains and found the ethanol extract showing antibacterial activity against *Escherichia coli* and acetone extract showing activity against *S. faecalis*. The study also showed variable response in ethanolic extracts by producing various zones of inhibition against different bacteria. Sastry and Rao (1994) showed antibacterial activity against gram positive and gram negative pathogenic strains after successive algal extraction with benzene, ethanol and methanol. This can be related to the present study where ethanol and butanol extracts resulted in various zones of inhibition against both gram positive and gram negative bacteria.

The results from methanol extract shows that except MA6 and MA1 all microalgal strains had shown less impact of total inhibitory zone <7 mm against the bacterial pathogens. Methanol extract of *Chlorella marina* [MA6] registered maximum inhibitory activity against *K. pneumoniae* [BP2], *P. vulgaris* [BP3] and *P. aeruginosa* [BP4] and *P. fluorescens* [BP5] and *S. typhi* [BP9]. *I. galbana* [MA1] had shown inhibitory against *P. aeruginosa* [BP4], *K. pneumoniae* [BP2], *Proteus vulgaris* [BP3], *P. fluorescens* [BP5], *S. typhi* [BP9] and *B. subtilis* [BP10]. Remarkably no growth was identified in *V. cholerae* [BP8] plate. Earlier studies by (Richard *et al.*, 1988), who screened the extracts of 100 cyanobacteria and 300 eukaryotic fresh petroleum ether algae and found antibacterial activity against *B. subtilis* and *S. aureus* and none against *E. coli*. Keller and Walker (1989) while screening the methanolic and hexane extracts of 132 marine microalgae, against six strains of bacteria, found that methanolic and hexane extracts were more

effective against *S. aureus*, *S. faecalis* and less effective with regards to *Bacillus subtilis*. Chloroform [CF] extract of *Dunaliella salina* [MA8] registered inhibitory activity against *S. pyogenes*, *P. vulgaris* [BP3], *K. pneumoniae* and *V. cholerae*. Microalgae *N. oculata* had shown inhibitory against *K. pneumoniae* and no growth was identified on *S. pyogenes* [BP7] plate. No other MA extracts had shown promising activity against bacterial pathogens.

Pesando and Caram (1984) reported *E. coli* as more sensitive to macroalgae *Laurencia obtuse*. Issac and Hedge (1987) found *E. coli* as more sensitive to *Sargassum johnstoni*. In the present study 80% of screened microalgae had antimicrobial activity against *E. coli*. Padmini (1986) found *S. typhi* as more resistant to *Sargassum johnstoni*. Padmakumar and Ayyakannu (1987) tested 80 species of macroalgae and only 19 species had antibacterial activity against *S. typhi*. In the present study the extracts of MA1, MA5, MA6, showed high antibacterial activity against *S. typhi*. Crude extracts of the microalgae showed activity only against gram negative bacteria as reported by Rao (1991). However, in the present study microalgal extracts showed activity against both gram positive and gram negative pathogens. Issac and Hedge (1987) found *P. vulgaris* as more sensitive to the marine algae *V. pachytenma*. In the present study *Proteus vulgaris* was found to be sensitive (9.8%) to MA1, MA2, MA5, MA6, MA8. Among the 10 microalgae used against the bacterial pathogens, *I. galbana* showed highest inhibition [16.22%] followed by *C. marina* [14.43%], *N. oculata* [14.07%], *D. salina* [13.91%] and *P. lutheri* [13.17%]. These five microalgal strains were further investigated in the present study to understand the concentration dependent bactericidal activity. Recently, Kim *et al.*, 2001 studied hexane extract of *C. ellipsoidea* which inhibited the growth of *B. subtilis* and *S. aureus*: *T. suecica* showed relatively excellent antimicrobial activity against *E. coli*.

Concentration dependent bactericidal activity was performed in the present study and the obtained data were statistically analyzed by paired sample t test to understand the efficacy of solvent extractions and antimicrobial properties. The bactericidal activity shown by butanol extract of MA1 against five bacterial pathogens BP1, BP3, BP4, BP6 & BP9 were compared with the ethanol extract of MA1, which shown optimal activity against bacterial pathogens BP1, BP3, BP6, BP9 & BP10.

The result shows that butanol extract of *I. galbana* [MA1] shows respective bacterial clearance with a concentration of BP1 (140µg), BP3 (140µg), BP4 (100µg), BP6 (80µg) & BP9 (120µg), as ethanol extract of *I. galbana* [MA1] shows respective bacterial clearance with a concentration of BP1 (80µg), BP3 (80µg), BP6 (160µg), BP9 (120µg) & BP10 (140µg). Interestingly BP1 and BP3 were cleared by ethanol extract of MA1 at 80 µg concentration when compared with butanol extract of MA1 (140 µg), which is highly significant [$p < 0.01$]. The bactericidal activity of MA2 butanol extract against five bacterial pathogens BP1, BP2, BP5, BP8 & BP10 were compared with MA2 ethanol extract, which had shown optimal activity against bacterial pathogens BP2, BP4, BP6, BP8 & BP10. The results elucidates that butanol extract of *N. occulata* [MA2] shows respective bacterial clearance with a concentration of BP1 (80µg), BP2 (80µg), BP5 (160µg), BP8 (120µg) & BP10 (100µg), as ethanol extract of *N. occulata* [MA2] shows respective bacterial clearance with a concentration of BP2 (80µg), BP4 (140µg), BP6 (140µg), BP8 (140µg) & BP10 (120µg). Interestingly BP2 was cleared by both butanol and ethanol extract of MA1 at 80 µg concentration. MA5 petroleum ether extract was treated against five bacterial pathogens BP1, BP3, BP6, BP7 & BP8 and the results were compared with MA5 butanol extract treated bacterial pathogens BP3, BP5, BP6, BP9 & BP10. The results elucidates that PE extract of *P. lutheri* [MA5] shows respective bacterial clearance with a concentration of BP1 (120µg), BP3 (180µg), BP6 (160µg), BP7 (160µg) & BP8 (100µg). The butanol extract of *P. lutheri* [MA5] shows respective bacterial clearance with a concentration of BP3 (120µg), BP5 (160µg), BP6 (80µg), BP9 (100µg) and BP10 (120µg). It was observed that a significant clearance of BP6 at 60 µg [$p < 0.001$] and BP3 at 120 µg [$p < 0.005$] by butanol extract of MA5.

Concentration dependent bactericidal activity of MA6 butanol and methanol extract against bacterial pathogens BP2, BP3, BP4, BP5, BP8 & BP9 were analyzed. The results elucidates that butanol extract of *C. marina* [MA6] shows respective bacterial clearance with a concentration of BP2 (100µg), BP3 (60µg) [$p < 0.05$], BP4 (100µg), BP5 (140µg) & BP8 (120µg), as methanol extract of *C. marina* [MA6] shows respective bacterial clearance with a concentration of BP2 (80µg), BP3 (100µg), BP4 (100µg), BP5 (120µg) and BP9 (140µg). The bacterial clearance of MA8 butanol extract against

the bacterial pathogens BP4 (120µg), BP6 (20µg), BP7 (60µg), BP8 (80µg) and BP9 (120µg) was compared with ethanol extract BP6 (20µg), BP7 (80µg), BP8 (60µg), BP9 (120µg) and BP10 (100µg). Walter and Mahesh (2000) screened 11 marine diatoms against 13 pathogenic bacteria and found the lipophilic and aqueous extracts of 6 diatoms showed high antibacterial activity. This is in conformity with the present study.

Comparative analysis of bactericidal activity between paper disk and well cut method for ten selective micro algal extracts against bacterial pathogens were also performed in the present study and the obtained data were statistically analyzed by paired sample t test (Table 3). It was observed that the crude butanol extract of *I. galbana* [MA1] had shown a total inhibitory measurement of 64.5 mm, 13.35% in the paper disk method when and 72.8 mm, 13.50% in the well cut method ($p < 0.05$). Similarly the *I. galbana* [MA1] crude ethanol extract experiment in paper disk method had shown a total inhibitory measurement of 55.3 mm, 11.44% against 66 mm, 12.2% by well cut method ($p < 0.05$).

Crude butanol extract of *N. occulata* [MA2] shows total inhibitory activity of 60.3 mm, 11.1% in well cut method and 54.7 mm 11.3% in the paper disk method. Petroleum ether extract of *P. lutheri* [MA5] registered total inhibitory effect of 36.8 mm, 7.61% in the paper disk method where well-cut method registered total inhibitory of 47.0 mm, 8.71% ($p < 0.01$). Methanol extract of *C. marina* [MA6] registered total inhibitory activity of 33.4 mm, 6.91% in the paper disk method: while a total inhibitory of 43.1 mm, 7.99% was registered in the well cut method. The well cut method of *D. salina* [MA8] shows total inhibitory of 45 mm, 8.34% on the crude butanol extract: while it shows total inhibitory of 55 mm 11.38% in the paper disk method. The results clearly indicated significant increment in microbicidal activity when performed using well-cut method.

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