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STUDIES ON ANTIMICROBIAL COMPOUNDS FROM MARINE MICROALGAE

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ABSTRACT

The antimicrobial compounds were extracted from marine microalgae such as *Nanochloropsis occulata* and *Chaetocerous calcitrans* using different organic solvents, and studied the efficiency of those extracts on aquatic pathogens such as *Staphylococcus aureus, Pseudomonas aurogenosa, Vibrio harveyi, V. parahaemolyticus and Aeromonas hydrophyilla.* Among the extracts obtained from microalgae, the chloroform + methanol (2:1) extract of *N. occulata* and butanolic extract of *C. calcitrans* had the maximum inhibiting activity against all aquatic pathogens. The best effected crude extracts were again purified through column chromatography and tested the inhibitory activity of eluted fractions against the same pathogens. Then the active fraction was analyzed through FTIR for knowing its functional group. The column purified extracts of antimicrobials obtained from *N. occulata* was found to be light sensitive compound which was activated at the light intensity of 200 lux for 18 h of light exposure. The mode of activity of the compound on the pathogenic bacteria was studied .It was observed that the active compound disturbed the cell wall formation in the pathogen. The identified active functional groups were carboxyl and nitro group in *N. occulata* and the nucleoside compound was the active functional group in *C. calcitrans*.

KEYWORDS: Active compounds, functional group, *Nanochloropsis occulata*, *Chaetocerus calcitrans*. mode of activity.

INTRODUCTION

Marine organisms are rich source of structurally novel and biologically active metabolites. So far, many chemically unique compounds of marine origin with different biological activity

have been isolated and a number of them are under investigation and/or are being developed as new pharmaceuticals.[1, 2] Secondary or primary metabolites produced by these organisms may be potential bioactive compounds of interest in the pharmaceutical industry. In recent years, use of sea plants (macro algae, sponges, micro algae) as an effective alternative to antibiotics has gained importance especially to combat disease problem. Bacterial infection causes high rate of mortality in human population and aquaculture organisms.[3] Moreover, the development of multidrug resistant bacterial species are creating a huge economic loss and exhaust more labour in research in pharmaceutical industry. Prokaryotic and eukaryotic microalgae produce a wide array of compounds with biological activities. These include antibiotics, algicides, toxins, pharmaceutically active compounds and plant growth regulators. Toxic microalgae, in this sense, are common only among the Cyanobacteria and Dinoflagellates. The microalgal toxins are either important as material for useful drugs or one of the great mysteries in the world of bio toxicology.[4, 5]

Many microalgae have been recognised as potential source of antibacterial and antifungal substances. The antimicrobials from microalgae often have a very wide antibacterial range and are likely to act on germs that are resistant to classic antibiotics. The bactericidal effects of cellular extract of *Asterionella japonica* on eight resistant pathogenic *Staphylococci* were studied.[6] The studies carried out on antibiotic fractions of some cellular extracts have enabled some of them to be isolated and identified. One of the active fractions of *Chaetoceros lauderi* is an acid polysaccharide with a high molecular weight, which might include uronic group.[7] An antibiotic substance which proved to be a peptide were isolated from Diatom (*Fragillaria prinata*) and Chrysophycea (*Stichochysis immobilis*).[8] The cellular extract from *Asterionella japonica* has active fractions of the extract which has a low molecular weight and contains ribosome that could be a nucleoside. Another active fraction has been isolated from the cellular extract of *Asterionella japonica*.[9] The microalgal extract was found to have remarkable bactericidal properties after exposure to light and was especially active on *Sarcina lutea*, *Staphylococcus aureus*, *Clostridium tetani*, *Clostridium botulinum*, *Clostridium septicum*, and *Clostridium histolyticum*. The light active antimicrobial compound was identified as a fatty acid. Those fatty acid was C_{20} fatty acid with 5 malonic double bonds in position 5, 11, 14 and 17[10] and also isolated another active fraction from *Chaetoceros lauderi* which was found to be fatty acid with the same bactericidal properties after exposure to light.[11] The active fraction of *Skeletonema costalum* was also found fatty acid.[12] The antimicrobial activity of a marine diatom *Asterionella notata* has been studied.[6]

The author reported that an aqueous extract from this diatom inhibited the growth of several bacteria and of some fungi such as *Candida albicans*, *Penicillum* and *Aspergillus* sp.[12]

Many compounds responsible for antimicrobial activity was identified and evaluated their optimum concentration and the effects were only estimated in a few decades by the development of highly sophisticated equipments like NMR, DEAE cellulose and sephadex G 200 chromatography. The important compounds identified as antimicrobials are fatty acids, halogenated aliphatic compounds, terpens, sulphur containing hetrocyclic compounds etc.[13] Water cellular extracts from diatom (*Asterionella notata* and *Asterionella japonica*) have been used in therapeutic test in dermatology for localized treatment of infectious skin diseases and of metabolic lesions. Indescriminate use of innumerable drugs, antibiotics and chemicals supplied by the marketers to farmers without knowing its harmful effect to the consumers had led to the total rejection of Indian cultured shrimp by shrimp importing countries G3 and G8 nations.[6] The present work was carried out to explore the antimicrobial potentials of marine microalgae such as *Nanochloropsis occulata* and *Chaetocerus calcitrans* against aquatic pathogens and to characterize the active compounds obtained from them.

MATERIALS AND METHODS

(i) Collection of Marine Microalgae

The micro algae *N. occulata* and *C. calcitrans* were collected from Central Marine Fisheries Research Institute (CMFRI) at Tuticorin, Tamilnadu, India. The collected algal samples were then brought to the laboratory for further studies.

(ii) Culture of Marine Microalgae

The autoclaved or boiled sea water after cooling and aeration was taken in to clean conical flasks and required nutrients were added and about 20 % of the inoculum in the growth phase of culture was inoculated in to the culture flasks, and then incubated at 28ºC under 1000 lux light illuminated room.

(iii) Separation of Algal Cells

When the algal growth reached to exponential phase, the biomass was harvested from culture by batch centrifugation at 8000 rpm for 10 min. The resulted algal pellets were collected.

(iv) Extraction of Bioactive Compounds

Algae 0.5 g was mixed with 5 ml solvent. The solvents used in this experiment were n-butanol, n-hexane, acetone, methanol and chloroform: methanol (2:1 ratio). The solvent extract was centrifuged at 10,000 rpm for 15 min to remove cellular debris. The supernatant was collected and kept at 4ºC.

(v) Collection of Aquatic Pathogenic Strains

Test organisms such as *Vibrio harveyi*, *V. Parahaemolyticus*, and *Aeromonas hydrophilla* were collected from the microbiology unit of our Laboratory of Centre for Marine Science and Technology, Manonmanian Sundaranar University, Rajakkamangalam, Tamil Nadu, India. The other two strains *Staphylococcus aureus*(ATCC9144), *Pseudomonas aeruginosa* (ATCC25619) were purchased from Microbial Type Culture Collection Centre, Chandigar, India.

(vi) Antibacterial Assay

Algal extracts were assayed for their inhibitory activity against the test organisms by Kirby-Bauer disc diffusion method. The Muller Hinton agar medium was poured in to the plates. After solidification, the swabs were prepared from various stock cultures of pathogens and spreaded over the medium. The plates were allowed to dry for 20 min. Then, the prepared antimicrobial extract loaded discs were placed over the Muller Hinton agar, using sterile forceps. After incubation, the antimicrobial activity was observed in the form of inhibition of zone development.

(vii) Phytochemical Analysis of *N. occulata* and *C. Calcitrans*

The algal extracts were screened for the presence of biologically active compounds. Chemical tests were carried out using the aqueous extracts of the powdered algae, using standard procedures described by Trease and Evans, Harborne.[7,8,9]

Test for Alkaloids

Mayer's Test

To a few ml of the filtrates, drops of Mayer's reagent were added on the side of the test tube. A creamy or white precipitate indicates the test is positive.

Test for Carbohydrates

Benedict's Test

To 0.5 ml of the filtrates, 0.5 ml of Benedict's reagent was added. The mixture was heated in boiling water bath for 2 min. A characteristic red coloured precipitate indicates the presence of sugar.

Test for Saponins

The extract was diluted with distilled water and made up to 20 ml. The suspension was shaken in a graduated cylinder for 15 min. A foam layer of 2 cm indicates the presence of saponins.

Test for Phytosterols

Libermann-Buchard's Test

The extract was mixed with 2 ml of acetic anhydride. To this, 1 or 2 drop of concentrated sulphuric acid was added slowly along the sides of the test tubes. An array of color change showed the presence of phytosterols.

Test for Phenolic Compounds and Tannins

Ferric chloride Test

The extract was diluted to 5 ml with distilled water. To this a few drops of neutral 5% ferric chloride solution was added. A dark green colour indicated the presence of phenolic compounds.

Test for Tannins

About 0.25 mg of dried powdered samples was boiled in 20 ml of water in test tubes then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or bluish black coloration.

Test for Flavonoids

To 5 ml of the diluted ammonia solution, a portion of the aqueous extract was added, followed by addition of concentrated sulphuric acid. Appearance of yellow colour indicated the presence of flavonoids.

Test for Terpenoids

A 5 ml of the extract was mixed with 2 ml of chloroform and concentrated sulphuric acid to form a layer. A reddish brown coloration of the interface showed the presence of terpenoids.

Test for Phlobatannins

Formation of red precipitate when aqueous extract of plant sample was boiled with 1% aqueous hydrochloric acid indicated the presence of phlobatannins.

Determination of Total Phenols

The fat free sample was boiled with 50 ml of ether for the extraction of the phenolic compound for 15 min. A 5 ml of the extract was pipette out into a 50 ml flask and 10 ml of distilled water was added. It was followed by the addition of 2 ml of ammonium hydroxide solution and 5 ml of concentrated amyl alcohol. The samples were made up to mark and left to react for 30 min for colour development and the intensity was measured at 505 nm.

Alkaloid Determination

To 5 g of the sample was taken in 250 ml beaker, 200 ml of 10% acetic acid in ethanol was added, covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.

(Viii) Purification of Crude Extracts Using Column Chromatography

To purify the compounds and get maximum number of active fractions from the crude extracts, the silica gel was used as absorbent stationary phase. The absorbent (stationary phase) was prepared into slurry using Hexane. The mobile phase used was ethyl acetate and hexane. From dried crude extracts which was dissolved in chloroform: methanol and 10 ml of suspension was taken and placed at the top of the column, and elute the compounds with different solvent polarities. The eluted fractions were collected in separate vials at regular time intervals and stored at 4ºC.

(ix) Spectrophotometric Analysis of Fractions to Determine Maximum Absorbency (λ max): The photometric analysis was made using spectrophotometer scanning and optical density of each fraction was determined with wavelength range of 350-750 nm to determine the maximum absorbance of the eluted fractions.

(x) Secondary Antibacterial Screening for Different Active Fractions by Disc Diffusion Method

The active fractions obtained from column chromatography were secondary screened by agar disc diffusion method. The swabs were prepared from various stock cultures of pathogens such as *S. aureus, P. aeruginosa, V. harveyi, V. paraheamolyticus, A. hydrophila* and were spreaded over the medium. The plates were allowed to dry for 10 min and the prepared discs were placed over the agar medium. The bactericidal activity was observed in the form of inhibition zone development which was determined by measuring the diameter of the zone.

(Xi) Microbial Activity Relation with Light Intensity

This activity was determined by disc diffusion method. The Muller Hinton agar medium was poured in sterilized plates. Then swabs were prepared from various stock cultured pathogens and spread over the medium. The plates were allowed to dry and then the prepared discs were placed over the agar using sterile forceps. After the process, the plates were incubated at different light intensities like 200, 400 and 800 lux. After incubation, the effect of light intensity on increasing the antimicrobial activity of the light sensitive antimicrobial was determined by measuring inhibition zone development.

(xii) Study on Mode of Actions of the Compound

To study the mode of action of compound on pathogens, the overnight culture of pathogens were treated with different concentration of active fractions like 10, 20, 40, 80 and 100 µg for 2-3 h. The pathogen cultured without treatment of compound was considered as control and positive control was prepared with the pathogen culture mixed with penicillin antibiotic at 0.3 µg and incubated for 2-3 h. After that the cultures were smeared and subjected to Gram's staining.

(Xiii) FT-IR Analysis

The coloumn purified fraction obtained from *N. occulata and C. calcitrans* showing the maximum antibacterial activity and were analyzed qualitatively for the active compounds by Fourier Transform Infra Red (FT-IR) method described by Kemp, 1991.

RESULTS

1. Primary Screening of Marine Microalgae Extracts against Aquatic Pathogens by Agar Well Diffusion Method

The antimicrobial compounds extracted with different solvents had different activities, against different pathogens.

Nanochloropsis occulata

Among the four extracts tested on aquatic pathogens, the maximum activity was noticed in chloroform + methanol (2: 1) extract. This extract inhibited the growth of *S. aureus* to the level of 22 mm. The same extract also showed relatively higher inhibition on *A. hydrophilla*. The second solvent hexane, inhibited the growth of different bacteria and expressed the maximum inhibition zone of 18 mm against *V. harveyi*. The n-butanol and acetone extract even possessed the inhibitory response on different bacteria, the level of inhibition was comparatively lower than the chloroform + methanol and hexane. The effect of solvents on extraction of compound's activity was statistically significant the($P < 0.05$). The results are shown in table1.

Chateoceros calcitrans

Among the four tested extracts of *C. calcitrans* obtained from acetone, n-butanol, hexane and chloroform: methanol (2 : 1) the maximum inhibitory activity was observed in n-butanol extract. In this extract, the highest inhibition zone was measured in *A. hydrophila* (24 mm) and followed by 19 mm in *V. harveyi*, and 16 mm in *V. parahaemolyticus.* The *C. calcitrans* extract obtained using n-butanol was found to be more effective in controlling the pathogenic bacteria than the extract obtained from *N. occulata*, obtained the same solvent. The effect of solvents on extraction of compounds and the activity was studied, and it was statistically significant the $P < 0.05$. The results are shown in the table 2.

2. Phytochemical Analysis of Algal Extracts

The qualitative analysis of the crude phytochemical extracts of *Nanochloropsis occulata*, contain the following phytochemical constituents

The extracts reacted with Mayer's reagent and resulted in creamy or white precipitates. The extracts reacted with Libermann-Buchard's test[7] and it was positive reaction of colour change and that indicated the presence of phytosterols. A dark green colour indicated the

presence of phenolic compound. A mixture of blue and green colour showed the presence of steroids. Appearance of yellow colour indicated the presence of flavonoids. A reddish brown colour change indicated the presence of terpenoids.

The qualitative analysis of the crude extract of *Chatocerus calcitrans* contains the following constituents: A yellow precipitate was formed and that indicated the presence of alkaloids in the extract. Appearance of yellow colour indicated the presence of flavonoids. A mixture of blue and green colour showed the presence of sterols. A dark green colour indicated the presence of phenolic compounds. The other reactions like Mayer's, Benedict's, saponins, tannins, terpenoids, phlobatannins, were negative and indicated that the absence of the above compound. The results are shown in table 3.

3. Column Purification and Secondary Screening of Column Fraction Compounds on Aquatic Pathogens

The two microalgae such as *N. occulata* and *C. calcitrans* showed a considerable antibacterial activity against selected shrimp bacterial pathogens. The crude extracts were purified through column chromatography and the elution was collected at the flow rate of 0.5 ml/min. Totally 11 fractions of 15 ml were collected at 30 min intervals. The fractions were read from 300 to 750 nm and assayed (screened) against the same shrimp bacterial pathogens. Five fraction were collected from *N. occulata* and among the tested fractions, fraction no 3 (EA40) had maximum inhibitory activity which controlled all the pathogens. The activities were ranged from 16 to 26 mm. The 100% of ethyl acetate fraction showed no effect on these bacterial pathogens. The minimum zone formation was found in fraction no 2 (EA20). The results are shown in table 4. Six fractions were collected from *C. calcitrans* and among the six tested fraction, EA60 was selected as an active fraction which controlled most of the pathogens. The results are shown in table 5, and fig 1. The obtained data were statistically analysed (two way ANOVA) and are statistically significant ($P < 0.05$).

4. Effect of Light Intensity on Antimicrobial Compounds

In this experiment, fractions obtained from the *N. occulata* and *C. calcitrans* when exposed to light the activity was found increased. Among the three light intensities, 200, 400 and 800 lux tested, the 800 lux incubated compounds of both microalgae *N. occulata* and *C. calcitrans* showed maximum activity. The results are summarized in table 6, 7, and 8. The effect of light on antimicrobial activity was analysed and it is statistically significant ($P < 0.05$).

5. Mode of Activity

The mode of activity of active compound on shrimp bacterial pathogens was studied. The fresh inoculums of pathogenic bacteria were treated with the compound of different concentration for 2 to 3 h. After incubation, the bacterial suspension was observed for the cell wall destruction. An 8 µg/ml concentration of *N. occulata* compound fraction damaged the cell wall and hence the cell wall of the pathogen formed irregular rod when compared with the negative control. It indicates the active compound which destroyed the bacteria by defective cell wall synthesis.(Fig. 2).

6. FT-IR Analysis for Chemical Nature of the Antimicrobial Active Principles

The active fractions were subjected to FT-IR spectroscopic analysis and the result is given in spectral graph (Table 9, 10). From the overall data the active fractions identified in *N. occulata* were carboxyl group and nitro group. In the fraction of *C. calcitrans* contained an active compound could be a nucleoside.

Table 1: Primary antibacterial screening of different solvent extract of *Nanochloropsis occulata* against the aquatic pathogens.

Solvents used	Zone of Inhibition in mm				
	S.aureus	***Pseudomonas aeruginosa***	***Vibrio harveyi***	***Vibrio Paraheamo -lyticus***	***Aeromonas hydrophila***
Acetone	14.3 ± 0.09	13.6 ± 0.40	14.3 ± 0.90	13.0 ± 2.10	15.0 ± 0.00
n-butanol	14.60 ± 0.9	13.6 ± 0.48	12.3 ± 1.06	16.7 ± 1.02	16.3 ± 1.26
Hexane	17.0 ± 0.08	15.6 ± 0.40	18.3 ± 0.06	14.0 ± 0.0	16.3 ± 0.0
Chloroform ± Methanol (2 :1)	22.6 ± 1.26	16.0 ± 0.0	15.3 ± 0.06	17.3 ± 0.96	20.6 ± 0.92

Table 2: Primary antibacterial screening of different solvent extract of *Chaetoceros calcitrans* against the aquatic pathogens.

Solvents used	Zone of Inhibition in mm				
	S.aureus	***Pseudomonas aeruginosa***	***Vibrio harveyi***	***Vibrio Paraheamo -lyticus***	***Aeromonas hydrophila***
Acetone	15.6 ± 0.47	15.0 ± 0.0	12.6 ± 0.48	15.6 ± 0.04	16.6 ± 0.66
n-butanol	16.6 ± 0.09	17.6 ± 0.46	19.3 ± 1.06	19.3 ± 0.48	24.3 ± 0.60
Hexane	17.0 ± 0.06	15.6 ± 0.43	15.3 ± 0.82	16.3 ± 0.06	18.0 ± 0.0
Chloroform ± Methanol (2 :1)	16.6 ± 0.40	15.0 ± 0.89	19.6 ± 0.40	18.0 ± 0.04	17.6 ± 0.86

Table 3: Phytochemical analysis of potent algal extract.

Test	*Nannochloropsis oculata*	*Chaetocerus calcitrans*
Mayers	-	-
Benedicts	-	-
Saponins	+	-
Ferric chloride	-	-
Tannins	-	-
Flavonoids	+	+
Terpenoids	+	+
Sterols	+	+
Phenols	+	+
Phlobatannin	-	-
Alkaloids	-	+

(+) Presence of compound

(-) Absence of compound

Table 4: Secondary screening of the different fractions of microalgal extract (*Nanochloropsis occulata*) against aquatic pathogens by disc diffusion method.

Types of elusion (polar and non polar) in %	Zone of Inhibition in mm				
	S. aureus	***P. aeruginosa***	***V. harveyi***	***V. Paraheamo-lyticus***	***A. hydrophila***
EA20	14.3 ± 0.86	15.3 ± 0.26	12.3 ± 0.36	14.3 ± 1.26	13.0 ± 0.0
EA30	16.0 ± 0.0	14.2 ± 1.34	14.3 ± 0.44	18.7 ± 1.05	17.2 ± 1.34
EA40	17.6 ± 0.36	16.6 ± 0.26	22.3 ± 1.26	22.3 ± 0.26	26.3 ± 0.46
EA50	12.3 ± 0.68	17.6 ± 1.22	13.0 ± 0.0	15.3 ± 1.26	13.0 ± 0.43
EA60	15.3 ± 0.26	16.0 ± 02.6	12.3 ± 0.24	14.0 ± 0.0	14.6 ± 0.26

Table 5: Secondary screening of the different fractions of microalgal extract (*Chaetoceros calcitrans*) against aquatic pathogens by disc diffusion method.

Types of elusion (polar and non polar) in %	Zone of Inhibition in mm				
	S. aureus	***P. aeruginosa***	***V. harveyi***	***V. Paraheamo-lyticus***	***A. hydrophila***
EA20	12.3 ± 0.40	14.5 ± 1.2	13.3 ± 0.46	13.5 ± 1.31	16.3 ± 0.64
EA30	14.6 ± 0.24	15.0 ± 0.0	14.6 ± 0.24	15.2 ± 0.6	14.0 ± 1.24
EA40	13.6 ± 0.46	13.6 ± 0.04	14.4 ± 0.09	14.3 ± 0.46	15.3 ± 1.90
EA50	13.6 ± 0.43	13.6 ± 0.14	15.8 ± 1.36	14.3 ± 0.24	15.8 ± 0.27
EA60	21.6 ± 0.40	22.3 ± 0.14	25.6 ± 0.44	19.9 ± 0.19	17.3 ± 0.54
EA70	13.6 ± 0.04	14.6 ± 0.04	14.6 ± 0.43	15.7 ± 0.37	14.0 ± 0.37

Table 6: Light induced antimicrobial activity of column purified compound on controlling the pathogenic bacteria (200 lux).

Name of the microalgae	Solvents used	Zone of Inhibition in mm				
		S. aureus	***P. aeruginosa***	***V. harveyi***	***V. Paraheamo -lyticus***	***A. hydrophila***
Nanochloropsis occulata	Chloroform ± Methanol (2 :1)	24.2 ± 1.03	17.1 ± 0.78	17.5 ± 0.68	19.2 ± 1.18	22.4 ± 1.33
Chaetoceros calcitrans	n-butanol	19.3 ± 1.02	21.5 ± 1.19	21.9 ± 0.86	22.7 ± 1.23	26.4 ± 1.86

Table 7: Light induced antimicrobial activity of column purified compound on controlling the pathogenic bacteria (400 lux).

Name of the microalgae	Solvents used	Zone of Inhibition in mm				
		S. aureus	***P. aeruginosa***	***V. harveyi***	***V. Paraheamo -lyticus***	***A. hydrophila***
Nanochloropsis occulata	Chloroform ± Methanol (2 :1)	25.2 ± 1.56	17.8 ± 0.60	18.4 ± 1.38	21.2 ± 1.48	22.8 ± 1.29
Chaetoceros calcitrans	n-butanol	20.4 ± 0.78	21.9 ± 0.68	22.3 ± 0.83	23.8 ± 0.37	25.9 ± 1.26

Table 8: Effect of light active column purified antimicrobial compound on different pathogenic bacteria (800 lux).

Name of the microalgae	Solvents used	Zone of Inhibition in mm				
		S. aureus	***P. aeruginosa***	***V. harveyi***	***V. Paraheamo -lyticus***	***A. hydrophila***
Nanochloropsis occulata	Chloroform ± Methanol (2 :1)	26.62 ± 1.79	19.4 ± 1.26	17.6 ± 0.96	21.6 ± 1.03	23.6 ± 1.04
Chaetoceros calcitrans	n-butanol	20.7 ± 0.80	22.6 ± 1.42	15.6 ± 1.52	24.7 ± 1.07	27.4 ± 0.83

Table 9: Molecular stretches of active principles Characterized from the butanolic extract of *Nannochloropsis oculata* through FTIR spectroscopic analysis.

1	367.45	C – L
2	387.7	C – L
3	737.8	O -
4	900.79	RCH = CH_2
5	952.87	R_3N - O^+
6	990.48	RC_4 = CH_2
7	1042.56	C – N
8	1073.42	C – O
9	1112.96	C – F
10	1215.19	C – N
11	1250.88	C – O
12	1338.64	R_2S (= O) O
13	1379.15	RNO_2

14	1462.09	R - S(=O_2) - OR
15	1654.98	RO – N = O
16	2871.14	C_{SP}3 – N
17	2932.83	C_{SP} 3 – 4
18	2960.83	C_{SP} 3 – 4
19	3335.03	H bonded

Table-10: Molecular stretches of active principles Characterized from *Chaetocerus calcitrans* through FTIR spectroscopic analysis.

S.No	peak	Functional group
1	649.97	C-Br, C=C-H
2	669.25	C-Br, C=C-H
3	1022.2	C-F,C-O
4	1045.35	C-F,C-O,C-N,R_2SO
5	1083.92	C-F, C-O,C-N
6	1213.14	C-N,C-O,C-F
7	1348.15	C-F,C-C,$R_2S(=O)$,O.R-S(=$O)_2$-OR',$RONO_2$
8	1379.01	C-C,R-S(=$O)_2$-OR',RNO_2
9	1450.37	RNO_2,C-C
10	1641.31	Ro-N=o
11	2360.71	$RNH_3^+R_2NH^+R_3NH^+$:R_2C=N=N:RN=C=CO
12	2977.89	$Csp^{3\text{-}H}$

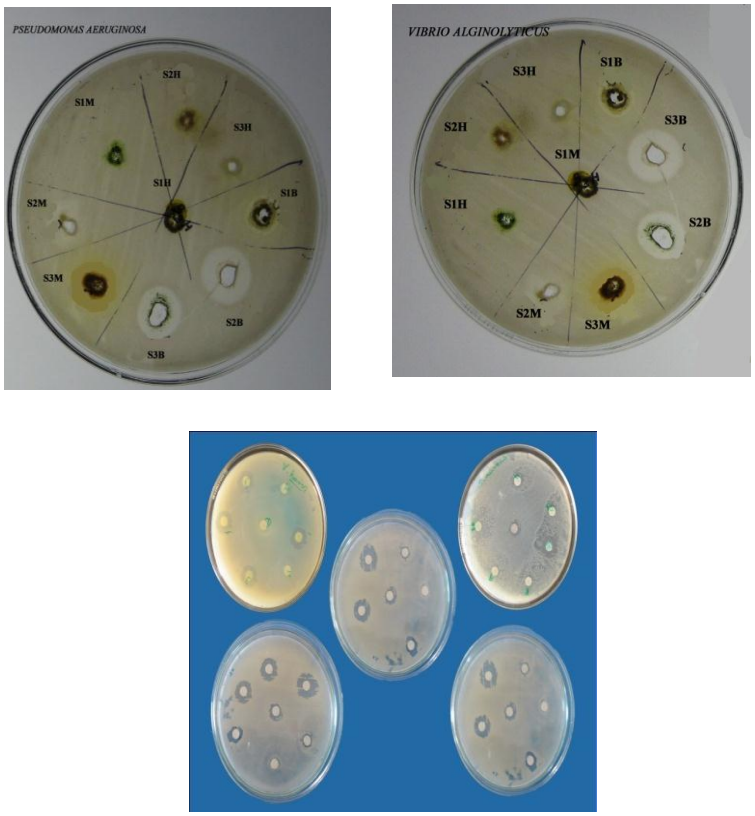

Fig. 1: Antibacterial activity of microalgae (*N.occulata*) against aquatic pathogens.

Fig. 2: TLC analysis of Nanochloropsis extracts.

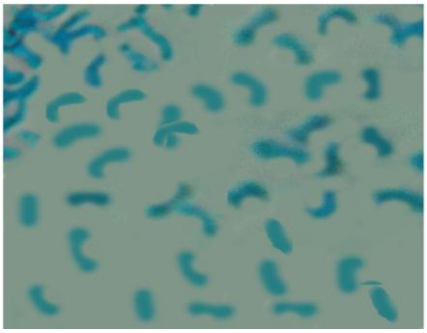

Fig. 3: Mode of activity of active compound (bactericidal effect) from *N.occulata*.

DISCUSSION

Since marine microalgae seems to be a potential source for both antibacterial as well as other bioactive compounds, further explorations towards this venture is needed. Evolving drug resistance in medical pathogens can be overcome through new citations about active principles from these micro organisms. A commercially viable strategy will be worth to establish this and high throughout drug screening system should be developed to develop a new class of drugs.[14] The bad effects and biomagnifications of antibiotics have been realized. Due to the presence of plasmids in bacteria, they will became resistant to the classic antibiotics which are being used for the control of human and animal ailments. Marine microphytoplanktons which have been proved to be act against bacteria present in water and animal's body.

Even though the antibacterial activity has frequently been observed, these substances have only rarely been used for pharmaceutical purposes. The present study was undertaken to find the active compounds which are involving in the control of aquatic pathogenic bacteria such as *S. aureus, V. parahaemolyticus, P. aeroginosa* and *A. hydrophilla*. The present study showed that the microalgal extracts obtained from *N. occulata and C. calcitrans* claimed to

be the best inhibitor of the growth of *S. aurius* and *V. harveyi and A. hydrophilla*. It was reported[15] that the marine microalgae *Tetraselmis* showed inhibitory effect against soil bacteria. The microalgae *N. occulata* had bactericidal activity against *V. parahaemolyticus*.[27]

The studies concerning the effectiveness of extraction methods highlights that methanol extraction yields higher antimicrobial activity than n-hexane and ethyl acetate and it was reported[17,23] that chloroform is better than methanol and benzene in yielding higher antimicrobial activity. But in the present study the chloroform methanol extract of *Nanaochloropsis* sp. and *Chaetocerus* sp. had the maximum inhibitory activity. From our findings and the earlier study[23], the difference of activity based on the solvent is due to the property of the compound to dissolve variously in different organic solvent. According to our results, the chloroform and methanol showed the maximum antimicrobial activity. From our result it is evidenced that the inhibiting activity of the compound found in the microalgae varies with the organic solvent. The extract of *N. occulata* obtained from chloroform + methanol and hexane showed maximum inhibitory activity against all the five shrimp bacterial pathogens tested. The other solvents such as acetone and butanol had minimum level of activity.

The solvent oriented inhibition activity may be due to the different compounds extracted in different solvents. Some active compounds which have antimicrobial activity on some bacteria might have extracted in some selective solvents only. This may be the reason that the extract obtained from different solvent showed different activity for different pathogens.[19] The same observation was already reported by the earlier workers.[20] The antibacterial activity was depends upon the solvent medium used for extraction.[20] An earlier study conducted by Moreau[21] revealed an observation of antimicrobial activity obtained from *C. launderi* tested against some dermatophytic fungi. A significant activity was observed by them against all the studied dermatophytes.[21] In the present study also *C. calcitrans* has proved to be the best preventer of all the shrimp bacterial pathogens. Several compounds have been isolated from marine sources and their structure and function have been clearly defined. The major antibacterial activity was exhibited by compounds like fatty acids, terpenes, carbonyl, chromophenol, nucleosides and glycosidic compounds. The *Skeletonema* is a good source for fatty acid derived antimicrobial substances[9]. In the present study, the two fractions of *T. suecica* contained the compounds which were seems to be a glycosidic aromatic amine and a linear carbon skeleton apart from the benzoid group. But, the previous

workers[7, 9, 21] observed an unidentified molecule from the *Tetraselmis*, which showed inhibitory effect against soil bacteria. Some of the phytochemical compounds e.g. glycoside, saponin, tannin, flavonoids, terpenoid, alkaloids have variously been reported to have antimicrobial activity.[22, 26] In the present study, the phytochemical compounds of selected marine micro algae *N. oculata* and *C. calcitrans* contained alkaloids, phytosterols, phenolic compounds, flavonoids and terpenoids. These compounds might have shown inhibitory activity against the shrimp bacterial pathogens.

In *Chaetoceros* sp. polysaccharides and fatty acids are the major candidate molecules for their antibacterial activity.[9] But in the present study, the active compound fraction from *C. calcitrans* was predicted to be nucleoside derivative, and the active compound fractions from *N. occulata* were carboxyl group and nitro group. It is responsible for their antimicrobial activity. The active fraction of *C. lauderi* was found to be a fatty acid with the same bactericidal properties after exposure to light[9, 11] also found the active fraction of *Skeletonema costatum* to be a fatty acid and among the other fatty acid that have identified. Earlier acrylic acid was isolated from the *Chrysophycea phaeocystis poucheti*[27] One active fraction has been isolated from the cellular extract of *Asterionella japonica*.[7] This was found to have remarkable bactericidal properties after exposure to light, and was especially active on *Sarcina lutea*, also on *Staphylococcus aureus* 209. In the present investigation, when the compounds was exposed to the light the antimicrobial activity increased. From this study the compounds may be light sensitive compounds. Earlier researcher[9] extracted one low molecular weight compound which is sensitivity to light and it was identified as nucleosides[9] Borowitzka[11] have studied the light sensitive antimicrobial compound from *C. laudrii*, and they found that the light sensitive antibacterial compound present in the diatoms were fatty acid compounds. The same fatty acid compounds also excreted the antibacterial activity in another diatom *S. costatum*. This compound was also identified as fatty acid and terpenes[10] and those compound may responsible for the antibacterial activity.

Additionally, there is a need for the development of new antibacterially effective agents which can be made inexpensively and are safe for applications in mammals and aquatic animals. The present invention provides antibacterial agents which are based on the extracts of the marine algae *N. occulata*. Fractions and isolates of *N. occulata* extracts have been shown to provide antibacterial activity which was especially active against *Vibrio* sp. The antibacterially effective extracts of the *N. occulata* are believed to occur either naturally in

the unicellular algae or produced by the algae in response to the presence of bacterium and other algae that compete against *N. occulata* for living space.[28]

CONCLUSIONS

The commercial antibiotics are highly effective to kill the bacterial pathogens involved in common infection produced by bacteria. The solvent extracts of two different marine microalgae used in the present study showed significant bactericidal action. The interesting information is that the product obtained is in the form of naturally good one for health and also fails to cause side effects. From these preliminary investigations, it was concluded that the product obtained from marine natural resources contain considerably useful bioactive compounds.

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