Int J Pharm Bio Sci 2013 Apr; 4(2): (P) 876 - 888

Research Article **Pharmaceutics**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

STUDIES ON ANTIMICROBIAL COMPOUNDS FROM SELECTED MARINE PHYTOPLANKTONS

SELVENDRAN.M.

Centre for Marine Science and Technology Manonmanium Sundarnar University Rajakkamangalam-629 502.Tamil Nadu. India

ABSTRACT

The antimicrobial compounds were extracted from two marine microalgae namely *Nanochloropsis oculata* and *Chaetoceros calcitrans* using different organic solvents. The efficiency of those extracts on aquatic pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa, Vibrio harveyi*, *V. parahaemolyticus and Aeromonas hydrophila.* Among the extracts obtained from microalgae, chloroform + methanol (2:1) extract of *N. oculata* and butanolic extract of *C. calcitrans* had the maximum inhibiting activity against all tested aquatic pathogens. Most effective crude extracts were again purified by column chromatography and inhibitory activity of the eluted fractions tested against same pathogens. Active fraction was analyzed through FTIR for knowing its functional group. Column purified extracts of antimicrobials obtained from *N. oculata* was found to be light sensitive compound which was activated at an intensity of 200 lux for 18 h. Analysis on mode of activity showed inhibition of cell wall formation in the pathogen. Identified active functional groups were carboxyl and nitro group in *N. oculata* and nucleoside compound in *C. calcitrans*.

KEYWORDS:**Active compounds, functional group, *Nannochloropsis oculata*, *Chaetoceros calcitrans*. mode of activity.**

SELVENDRAN.M.
Centre for Marine Science and Technology Manonmanium Sundarnar University Rajakkamangalam-629 502.Tamil Nadu. India

INTRODUCTION

Marine organisms are rich sources 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 being developed as a new pharmaceutical product. [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 aquatic organisms. [3] Development of multidrug resistant bacteria due to uncontrolled usage of chemotherapeutic agents is a serious issue posing danger to aquatic animals and human health . 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 one of the greatest mysteries in the world of bio toxicology deserving significance as material for developing useful drugs . [4, 5] The bactericidal effects of cellular extract of *Asterionella japonica* on eight resistant pathogenic *Staphylococci* has been reported [6] The studies on active fractions of *Chaetoceros lauderi* showed an acid polysaccharide with a high molecular weight, which might include uronic group. [7] An antibiotic substance which proved to be a peptide was isolated from diatom (*Fragilaria pinnata*) and Chrysophyte (*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 (Reference). Another active fraction isolated from the cellular extract of *Asterionella japonica*[9] 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 C_{20} fatty acid with 5 malonic double bonds in position 5, 11, 14 and 17 [10]. Active fraction from *Chaetoceros lauderi* and *Skeletonema costalum* was also reported to be a fatty acid. [11, 12] Aquatic extracts of *Asterionella notata* [6]. inhibited growth of several bacteria and of some fungi such as *Candida albicans*, *Penicillum* and *Aspergillus* sp. [12]

Many compounds responsible for antimicrobial activity were identified and evaluated only since few decades owing to 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. Rampant use of innumerable drugs, antibiotics and chemicals 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 antimicrobial potential of two marine microalgae *Nanochloropsis oculata* and *Chaetoceros calcitrans* against aquatic pathogens and to characterize the active compounds obtained from them.

MATERIALS AND METHODS

(i) Collection of Marine Microalgae

The microalgae *N. oculata* and *C. calcitrans* were collected from Central Marine Fisheries Research Institute (CMFRI) at Tuticorin, Tamilnadu, India.

(ii) Culture of Marine Microalgae

The autoclaved seawater after cooling was transferred to clean (ml) conical flasks and required nutrients (media??) were added. About 20 % of inoculum in growth phase of culture was inoculated in to the culture flasks and was cultured at 28ºC under 1000 lux light intensity.

(iii) Separation of Algal Cells

When algal cultures reached exponential growth phase, the biomass was harvested by batch centrifugation at 8000 rpm for 10 min and bottom algal pellets were collected.

(iv) Extraction of Bioactive Compounds

0.5 g algal concentrate was mixed with 5 ml solvent. 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 stored at 4ºC.

(v) Collection of Aquatic Pathogenic Strains

Test organisms *Vibrio harveyi*, *V. parahaemolyticus*, and *Aeromonas hydrophila* 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 into the plates. After solidification, swabs were prepared from various stock cultures of pathogens and spread over the medium. The plates were allowed to dry for 20 min and prepared antimicrobial extract loaded discs were placed over the Muller Hinton agar, using sterile forceps. After incubation, antimicrobial activity was observed in the form of inhibition zone development.

(vii) Phytochemical Analysis of N. oculata and C. calcitrans

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 few ml of filtrate, drops of Mayer's reagent were added to the side of the test tube. A creamy or white precipitate formation indicates the test is positive.

Test for Carbohydrates

Benedict's Test

To 0.5 ml of 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 (how much?) 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 side of test tube. 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 sample was boiled (time) 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 diluted ammonia solution, a portion of aqueous extract was added, followed by addition of concentrated sulphuric acid. Appearance of yellow colour indicated the presence of flavonoids.

Test for Terpenoids

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

Test for Phlobatannins

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

Determination of Total Phenols

The fat free algal sample was boiled with 50 ml of Ether for the extraction of phenolic compounds for 15 min. 5 ml of the extract was a pipette out into a 50 ml flask and 10 ml of distilled water was added .This 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 algal sample 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 mmonium hydroxide was added in drops to the extract until 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 crude extracts, silica gel was used as an 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 mix), 10 ml of suspension was taken and placed at the top of the column, and eluted 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)

Photometric analysis was made using spectrophotometer scanning (Make and Model No) and optical density of each fraction was determined with wavelength range of 350-750 nm to determine the maximum absorbance of eluted fractions.

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

Active fractions obtained from column chromatography were secondary screened by the agar disc diffusion method. The swabs were prepared from various stock cultures of pathogens namely, *S. aureus*, *P. aeruginosa*, *V. harveyi*, *V. paraheamolyticus, A. hydrophila* and were spread over the medium. The plates were allowed to dry for 10 min and prepared discs (with what) were placed over the agar medium. The bactericidal activity was observed in the form of inhibition zone development.

(Xi) Microbial Activity Relation with Light Intensity

This activity was determined by disc diffusion method. The Muller Hinton agar medium was poured in to sterilized plates. Then swabs were prepared from various stock cultured

pathogens and spread over the medium. Plates were allowed to dry and prepared discs were placed over agar using sterile forceps.and incubated at different light intensities 200, 400 and 800 lux. After incubation, the effect of light intensity on increasing the antimicrobial activity of light sensitive antimicrobial was determined by measuring inhibition zone developed.

(Xii) Study on Mode of Action

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

(Xiii) FT-IR Analysis

The column purified fraction obtained from *N. oculata* and *C. calcitrans* showing maximum antibacterial activity were analyzed qualitatively for the active compounds by Fourier Transform Infra Red (FT-IR) method described by Kemp,(REF NO)

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 oculata

Among the four extracts tested on aquatic pathogens, highest antibacterial activity was noticed with chloroform + methanol (2 : 1) extract. This extract inhibited growth of *S. aureus* forming an inhibition zone of 22 mm. Same extract also showed relatively higher inhibition on *A. hydrophila* with an inhibition zone of --- mm. The second solvent hexane extract (of which algae???), inhibited the growth of different bacteria, and expressed the maximum inhibition zone of 18 mm against *V. harveyi*. With n-butanol and acetone extract, the level of inhibition was comparatively lower than the chloroform + methanol and hexane. The effect of solvents on extracted compound activity was statistically significant ($P < 0.05$). 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. Highest inhibition zone by n-butanol extract was formed with *A. hydrophila* (24 mm) followed by 19 mm with *V. harveyi*, and 16 mm with *V. parahaemolyticus. C. calcitrans* extract obtained using n-butanol was found to be more effective in controlling pathogenic bacteria than extract obtained from *N. oculata*, obtained using same solvent. Effect of solvents on extraction of compounds and activity was studied, and was found statistically significant ($P < 0.05$). The results are presented in Table 2.

2. Phytochemical Analysis of Algal Extracts:

Qualitative analysis of crude phytochemical extracts of N. oculata, recorded the following phytochemical results

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 a positive reaction of colour change and indicated the presence of phytosterols. A dark green colour indicated presence of phenolic compound. A mixture of blue and green colour showed the presence of steroids. Appearance of yellow colour indicated presence of flavonoids. A reddish brown colour change indicated the presence of terpenoids.The qualitative analysis of crude extract from *Chatocerus calcitrans* recorded following results: A yellow precipitate was formed indicating presence of alkaloids in extract. Appearance of yellow colour indicated presence of flavonoids. A mixture of blue and green colour showed

presence of sterols. A dark green colour indicated presence of phenolic compounds. Other reactions viz. Mayer's, Benedict's, saponins, tannins, terpenoids, phlobatannins, were negative and indicated absence of the above compounds. The results are shown in Table 3.

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

The two microalgae, *N. oculata* and *C. calcitrans* showed considerable antibacterial activity against selected shrimp bacterial pathogens. The crude extracts were purified through column chromatography and 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 fractions were collected from *N. oculata* and among tested fractions, fraction no 3 (EA40) had maximum inhibitory activity, which controlled all tested pathogens. The inhibition zone size ranged from 16 to 26 mm. The 100 % of ethyl acetate fraction showed no effect on these pathogens. The minimum zone formation was found in fraction no 2 (EA20). The results are given in Table 4. Six fractions were collected from *C. calcitrans* and among these, EA60 was selected as an active fraction which controlled most of the pathogens. The results are shown in Table 5, and Fig 1. The values were statistically significant ($P<0.05$) with two way ANOVA.

4. Effect of Light Intensity on Antimicrobial Compounds

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

5. Mode of Action

The mode of action of active compounds on shrimp bacterial pathogens was studied. Fresh inoculum of pathogenic bacteria were treated with compound of different concentration for 2 to 3 h. After incubation, the bacterial suspension was observed for cell wall destruction. At 8 µg/ml concentration of *N. oculata* compound fraction damaged the cell wall resulting in pathogen forming irregular rod shape when compared with the negative control. (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 overall data the active fractions identified in *N. oculata* were carboxyl group and nitro group. The fraction of *C. calcitrans* contained an active compound, which could be a nucleoside.

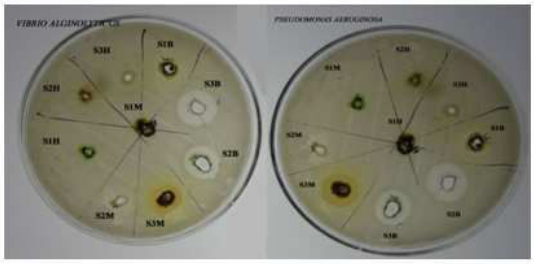

Figure 1
Antibacterial activity of microalgae (N.oculata) against aquatic pathogens .

Figure 2
Mode of activity of active compound (bactericidal effect) from N.oculata (explain the effect)

Table 1
Primary antibacterial screening of different solvent extract of Nannochloropsis oculata against tested aquatic pathogens

Solvents used	Zone of Inhibition in mm				
	Staphylococcus 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				
	Staphylococcus 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*	*Chaetoceros calcitrans*
Mayers	-	-
Benedicts	-	-
Saponins	+	-
Ferric chloride	-	-
Tannins	-	-
Flavonoids	+	+
Terpenoids	+	+
Sterols	+	+
Phenols	+	+
Phlobatannin	-	-
Alkaloids	-	+

(+) Presence of compound
(-) Absence of compound

Table 4
Secondary screening of different fractions of microalgal extract (Nanochloropsis oculata) against 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 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 oculata	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 butanolic extract of Nannochloropsis oculata through FTIR spectroscopic analysis.

#	What	what
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-H}

DISCUSSION

Evolving drug resistance in medical pathogens can be overcome through new citations about active principles from microalgae. A commercially viable strategy will be worth to establish this and high throughout the drug screening system should be developed to develop a new class of drugs [14] . Even though the antibacterial activity of microalgae has frequently been observed, these substances have only rarely been used for pharmaceutical purposes. The present study was undertaken with two microalgae to find the active compounds involved in the control of aquatic bacterial pathogens such as *S. aureus, V. parahaemolyticus, P. aeruginosa* and *A. hydrophila*. The present study showed that microalgal extracts obtained from *N. oculata and C. calcitrans* inhibited growth of *S. aureus* and *V. harveyi and A. hydrophila*. It was reported[15] that marine microalgae *Tetraselmis* showed inhibitory effect against soil bacteria. The microalgae *N. oculata* had bactericidal activity against *V. parahaemolyticus*[27.] Studies concerning the effectiveness of extraction method highlights that methanol extraction yields the higher antimicrobial activity than n-hexane and ethyl acetate (ref) It was reported[17, 23] that chloroform is better than methanol and benzene in yielding higher antimicrobial activity. However, in this study, chloroform methanol extract of both algal species. had the maximum inhibitory activity. From our findings and earlier reports [23] it is evident that difference of activity based on solvent is due to property of the compound to dissolve variously in different organic solvent.. The solvent oriented inhibition activity may be due to the different compounds extracted in different solvents. Some active compounds which have antimicrobial activity of some bacteria might have extracted with some selective solvents only. This may be the reason that the extract obtained from different solvent showed different activity for different pathogens[19.] (Repetition, take reference 19 to earlier para) Our results also confirm that the inhibitory activity of the compound found in microalgae varies with organic solvent used. The extract of *N. oculata* 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 a minimum level of activity. The observation that antibacterial activity depends upon the solvent medium used for extraction has been previously reported[20.] An earlier study conducted by Moreau [21] revealed antimicrobial activity of *C. launderi* tested against some dermatophytic fungi. A significant activity was observed by them against all studied dermatophytes[21]. In the present study also *C. calcitrans* has proven to be the best preventer of all the shrimp bacterial pathogens (Several compounds

have been isolated from marine sources and their structure and functions have been clearly defined. The major antibacterial activity was exhibited by compounds like fatty acids, terpenes, carbonyl, chromophenol, nucleosides and glycosidic compounds. Microalgae *Skeletonema* is a good source of fatty acid derived antimicrobial substances[9.] In the present study, 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 (you have not used Tetraselmis suecica????). 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 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 shrimp bacterial pathogens. In *Chaetoceros* sp. polysaccharides and fatty acids are the major candidate molecules contributing to antibacterial activity[9]. But in the present study, active compound fraction from *C. calcitrans* was predicted to be nucleoside derivative, and active compound fractions from *N. occulata* were carboxyl group and nitro group. The active fraction of *C. lauderi* was found to be a fatty acid with the same bactericidal properties after exposure to light[9, 11] was also found the active fraction of *Skeletonema costatum*. Acrylic acid was isolated as active fraction from the Chrysophyte *Phaeocystis poucheti* [27] One active fraction isolated from the cellular extract of *Asterionella japonica*[7.] has been found to have remarkable bactericidal properties after exposure to light, and was especially active on *Sarcina lutea*, also on *S. aureus* 209. In the present investigation, when the compounds were exposed to light, the antimicrobial activity increased. A study has 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 the same to be 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 compounds may responsible for the antibacterial activity (too much repetition related to Skeleto, please mention only once in the appropriate place) There is an urgent need for development of new antibacterial agents which can be made inexpensively and are safe for long-term applications in mammals and aquatic animals. The present study reports extracts from marine algae *N. oculata*. with antibacterial activity , especially active against *Vibrio* sp. The antibacterial extracts of *N. oculata* are believed to occur either naturally or produced by them in response to the presence of bacterium and other algae that compete against them for living space.[28] (why no mention of compounds from C. calcitrans???)

CONCLUSION

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. (better to rewrite it – mentioning your results in brief and that that there is no risk of bacterial resistance development with microalgal extracts and also stressing furture studies needed in relation to your results)

ACKNOWLEDGEMENT

The corresponding author Dr. M. Michael Babu, greatly acknowledges University Grants Commission, India for its research fund to carry out this research work through the fund sanctioned to him No F.2-272/2009.

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