STUDIES ON ANTIMICROBIAL COMPOUNDS FROM SELECTED MARINE PHYTOPLANKTONS

Selvendran. M.[1], and Michael Babu. M

Centre for Marine Science and Technology, Manonmanium Sundarnar University
Rajakkamangalam - 629 502, Tamil Nadu. India
Email: [1]selvendran008@gmail.com

ABSTRACT

Two marine microalgae named *Nanochloropsis occulata* and *Chaetoceros calcitrans* were selected for the study. The crude extracts of the antimicrobial compounds were taken by using acetone, butanol, hexane and chloroform: methanol, under percolation method. The crude extracts were tested on aquatic pathogens such as *Staphylococcus aureus, Pseudomonas aeruginosa, Vibrio harveyi, Vibrio parahaemolyticus* and *Aeromonas hydrophila*. Among the four extract obtained using the said solvents, the chloroform: methanol resulted maximum inhibitory activity in the extract obtained from both the microalgae. Among the four extracts, two crude extracts were again purified in column chromatography and got different fraction of compounds. The fractionationed compounds were tested on aquatic pathogen, among those, some fraction ad maximum inhibitory activity. The active fractions were analyzed for its compound identification through FTIR technique. The active compounds were nitrogenous,carbonyl and alcohol group. The compounds were exposed to light (1000 lux) to increase the activity.

Key words: Antimicrobials, Aquatic pathogens, Active compounds, Functional group, Light sensitive compounds.

I. INTRODUCTION

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).

Marine organisms are a rich source of structurally novel and biologically active metabolites. 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.

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 biotoxicology (Hikmet Kartircioglu et al., 2004) (13).

Many microalgae have been recognised as potential source of antibacterial and antifungal substances (Water and Mahesh, 2000). Antibacterial effects have been noticed in all the algal classes, which is the major component of the phytoplankton. Marine algae are also a source of antifungal substances and have also been found to be effective against the fungi *Trichophyton entagrophytes, Trichophyton tubrum* (Mauter *et al.,* 1953), *Candida albicans* (Olesen et al., 1964) and a number of other fungal organisms (welch, 1962).

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 8 stocks of resistant pathogenic *Staphylococci* from the Roger Bellon Laboratory. The antibacterial activity of 11 cellular extracts of phytoplankton on 21 anaerobic germs from the Institute Pasteur in Paris those were resistant to usual antibiotics babu et al., 2001)[(5)].

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 [16]. An antibiotic substace which proved to be a peptide from a Diatom (*Fragillaria prinata*) and from a Chrysophycea (*Stichochysis immobilis*) was also isolated [7]. The cellular extract from *Asterionella japonica* has an active fractions of the extract which has a low molecular weight and contains ribosome could be a nucleoside[4]. Another active fraction has been isolated from the cellular extract of *Asterionella japonica*.

This was found to have remarkable bactericidal properties after exposure to light, and was especially active on *Sarcina lutea,* also on *Staphylococcus aureus* 209 P, *Clostridium tetani, Clostridium botulinum, Clostridium septicum*, and *Clostridium histolyticum*. It could be identified as a fatty acid whose structure was studied by ultra-violet and infra-red spectrophotometry, RNN and mass spectrography. The results of these observations indicate that it would appear to be a C_{20} fatty acid with 5 malonic double bonds in position 5, 11, 14 and 17 Burkholder et al 1960[8]. Similarly another active fraction of *Chaetoceros lauderi* was found to be fatty acid with the same bactericidal properties after exposure to light (Gauthier et al.,[10]. The active fraction of *Skeletonema costalum* to be a fatty acid was also found[1].

The antimicrobial activity of a marine Diatom *Asterionella notata* has been studied by Gauthier. The author reported that an aqueous extract from this Diatom inhibited the growth of several bacteria and of some fungi as *Candida albicans*, *Penicillum* and *Aspergillus sp*[10]. Many compounds responsible for antimicrobial activity was identified and evaluated their optimum concentration and effects were only estimated in the post 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 Glombitza et al.,[11].

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. 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 (Babu et al.,[5]. 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 aquaculture fields. 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 the compounds from microalgae were partially characterized.

II. MATERIALS AND METHODS

Collection of marine microalgae:

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

Culture of marine microalgae:

The autoclaved or heated sea water after cooling and poured in to clean conical flasks and required nutrients are added Macro, micro nutrients and vitamins (Waln's media) and about 20% of the inoculums in the growing phase culture of microalgae was transferred to the culture flasks, and then incubated at 28 c + 2 c under 1000 lux light illuminated room.

Separation of algal cells:

The biomass of algal cells was reached in exponential growth phase the biomass was recovered from culture by batch centrifugation at 8000 rpm for 10 min. The resulted algal pellets were collected.

Extraction of bioactive compounds:

0.5 gm algal cells were mixed with 5 ml solvent. The solvents used in this experiment were n-butanol, n-hexane, Acetone, methanol, and chloroform: Methanol (2:1). The solvent extract was centrifuged at 10,000 rpm for 15 min to remove cellular materials. The supernatant was collected and kept at 4°C.

Collection of aquatic pathogenic strains:

Test organisms (*Vibrio harveyi, V. Parahaemolyticus, Aeromonas hydrophilla* were collected from Microbiology unit, our Laboratory of centre for marine science, Manonmanian sundaranar University, Nagercoil, Tamil Nadu and India. the other two strains *Staphylococcus aureus,* (ATCC9144) *Pseudomonas aeruginosa* (ATCC25619) purchased from chandiagar microbiology lab.

Antibacterial Assay:

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

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 was used as a solvents like ethyl acetate and hexane. The crude extracts which was dried to remove solvent was dissolved in chloroform: methanol and 1ml of suspension is placed at the top of the column. Eluted fractions were collected in separate vials at regular time intervals and stored at 4°C.

Secondary antibacterial screening for different active fractions by disc diffusion method (Kirby and Bauer Method, 1966)

Preparation Antimicrobial disc

After the column elution, the eluted compounds were loaded on to sterile paper disc

Antibacterial assay

The column chromatography active fractions were secondary screened by agar disc diffusion method (Kirby and Bauer, 1966). The swabs were prepared from various stock cultures of pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Vibrio harveyi*, *Vibrio paraheamolyticus*, *Aeromonas hydrophila* and were spreaded over the medium, the plates were allowed to dry for 10 mins. Then the prepared discs were placed over the agar medium. The bactericidal activity was observed in the form of inhibition of zone development which was determined by measuring the diameter of the zone.

Microbial activity relation with light intensity

This activity performed by disc diffusion method. The Muller Hinton agar medium was poured in sterilized plates. Then swabs were prepared from various stock cultured pathogens and were 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 dark light intensities like 200, 250 and 300. After incubation, the achieving of the extract was in the form of inhibition of zone development by measuring the diameter of the zone.

FTIR analysis

The extracts such as *Nanochloropsis occulata and chaetocerus calcitrans* were analyzed qualitatively for the active compounds by Fourier Transform Infra Red (FTIR) method described Kemp (1991).

III. RESULTS

Primary screening

The wet paste of selected algae were extracted with different organic solvents such as acetone, n-butanol, hexane and chloroform : methanol (2 : 1). The crude extract was tested against different aquatic pathogens.

Nanochloropsis occulata (no.)

Among the four extract tested on aquatic pathogens, the maximum activity was noticed in chloroform methanol (2 : 1) extract. This extract inhibited the *Streptococcus aureus* on the media to the level of 22.6 ± 1.26 mm. The same extract also showed relatively higher inhibition on *Aeromonas hydrophilla*. The second solvent that inhibited the growth of wide range of bacteria was hexane, here the maximum inhibition was 18.3 ±0.6 mm against *V. harveyi*. The n-butanol and acetone, though evolved a inhibitory response on different bacteria. But the level of inhibitory was comparatively lower than the chloroform and hexane. The inhibition resulted by the chloroform : methanol against *S. aureus, A. hydrophilla*

and hexane extract against *V. harveyi* were significantly influenced. Above the results are summarized in table 1

Chateoceros calcitrans

Among the four tested *Chaetoceros* extract obtained from using acetone, n-butanol, hexane and chloroform : methanol (2 : 1), the maximum inhibitory activity were observed in the n-butanol extract. In this extract, the highest inhibition zone was measured in *Aeromonas hydrophila* (24.3 ± 0.6) and followed by 19.3 ± 1.6, 19.3 ± 0.4, 17.6 ± 0.4 and 16.6 ± 0.9 resulted in *V. harveyi, V. parahaemolyticus, P. aeruginosa* and *S. aureus.* Next to n-butanol, the chloroform methanol and followed by hexane on controlling the pathogenic bacteria (Table 2).

Table 1. Primary antibacterial screening of different solvent extract of *Nannochloropsis 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 ± 0.4	12.3 ± 1.6	16.7 ± 1.2	16.3 ± 1.2
Hexane	17.0 ± 0.8	15.6 ± 0.4	18.3 ± 0.6	14.0 ± 0.0	16.3 ± 0.0
Chloroform ± Methanol (2 :1)	22.6 ± 1.26	16.0 ± 0.0	15.3 ± 0.6	17.3 ± 0.9	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 Paraheamolyticus*	*Aeromonas hydrophila*
Acetone	15.6 ± 0.4	15.0 ± 0.0	12.6 ± 0.4	15.6 ± 0.4	16.6 ± 0.6
n-butanol	16.6 ± 0.9	17.6 ± 0.4	19.3 ± 1.6	19.3 ± 0.4	24.3 ± 0.6
Hexane	17.0 ± 0.6	15.6 ± 0.4	15.3 ± 0.2	16.3 ± 0.6	18.0 ± 0.0
Chloroform ± Methanol (2 : 1)	16.6 ± 0.4	15.0 ± 0.8	19.6 ± 0.4	18.0 ± 0.4	17.6 ± 0.6

Table 3. 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. Paraheamolyticus*	*A. hydrophila*
Nanochloropsis occulata	Chloroform ± Methanol (2 : 1)	26.62 ± 1.7	19.4 ± 1.2	17.6 ± 0.9	21.6 ± 1.3	23.6 ± 1.4
Chaetoceros calcitrans	n-butanol	20.7 ± 0.8	22.6 ± 1.4	15.6 ± 1.5	24.7 ± 1.7	27.4 ± 0.83

Effect of secondary screened compound against aquatic pathogen

The crude extract that proved the maximum efficiency on controlling most of the tested pathogens were laded in silica column and eluted using ethyl acetate of different polarity of changing the percentage of EA.

The elution taken from *Nanochloropsis* extract obtained using chloroform methanol (2 : 1)

When different elution obtained from *Nanochloropsis* extract were tested on aquatic pathogens, EA40 elution resulted the maximum inhibitory activity against most of the pathogen tested than the other elution (Table 6)

Table 4. 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. Paraheamolyticus*	*A. hydrophila*
Nanochloropsis occulata	Chloroform ± Methanol (2 :1)	24.2 ± 1.3	17.1 ± 0.7	17.5 ± 0.6	19.2 ± 1.1	22.4 ± 1.3
Chaetoceros calcitrans	n-butanol	19.3 ± 1.2	21.5 ± 1.1	21.9 ± 0.8	22.7 ± 1.3	26.4 ± 1.6

Table 5. 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. Paraheamolyticus*	*A. hydrophila*
Nanochloropsis occulata	Chloroform ± Methanol (2 : 1)	25.2 ± 1.5	17.8 ± 0.6	18.4 ± 1.3	21.2 ± 1.4	22.8 ± 1.2
Chaetoceros calcitrans	n-butanol	20.4 ± 0.7	21.9 ± 0.6	22.3 ± 0.8	23.8 ± 0.7	25.9 ± 1.2

Table 6. 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. Paraheamolyticus*	*A. hydrophila*
EA20	14.3 ± 0.6	15.3 ± 0.6	12.3 ± 0.6	14.3 ± 1.6	13.0 ± 0.0
EA30	16.0 ± 0.0	14.2 ± 1.4	14.3 ± 0.4	18.7 ± 1.5	17.2 ± 1.4
EA40	17.6 ± 0.6	16.6 ± 0.6	22.3 ± 1.6	22.3 ± 0.6	26.3 ± 0.4
EA50	12.3 ± 0.8	17.6 ± 1.2	13.0 ± 0.0	15.3 ± 1.6	13.0 ± 0.4
EA60	15.3 ± 0.6	16.0 ± 0.6	12.3 ± 0.4	14.0 ± 0.0	14.6 ± 0.6

Table 7. 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. Paraheamolyticus*	*A. hydrophila*
EA20	12.3 ± 0.4	14.5 ± 1.2	13.3 ± 0.6	13.5 ± 1.1	16.3 ± 0.4
EA30	14.6 ± 0.4	15.0 ± 0.0	14.6 ± 0.4	15.2 ± 0.6	14.0 ± 1.4
EA40	13.6 ± 0.4	13.6 ± 0.4	14.4 ± 0.9	14.3 ± 0.4	15.3 ± 1.0
EA50	13.6 ± 0.4	13.6 ± 0.4	15.8 ± 1.3	14.3 ± 0.4	15.8 ± 0.7
EA60	21.6 ± 0.4	22.3 ± 0.4	25.6 ± 0.4	19.9 ± 0.9	17.3 ± 0.4
EA70	13.6 ± 0.4	14.6 ± 0.4	14.6 ± 0.4	15.7 ± 0.7	14.0 ± 0.7

The crude extract of *Chaetoceros calcitrans* obtained using n-butanol was loaded on silica column. The different elution obtained at different time intervals were tested on aquatic pathogens. Among the six tested elution, EA60, was selected as an effective elusion on controlling most of the pathogens (Table 7).

Effect of light intensity on antimicrobial compounds

In this experiment, the crude extract obtained from the *Nannochloropsis* and *Chaetoceros* using the two organic solvent proved as effective one on maximum control against the pathogen were selected. Among the three light intensity, 200, 400 and 800 lux tested, the increased light intensity affected to increase the antimicrobial activity on pathogen. The *Chaetoceros* extract obtained using n-butanol was found to be more effective in controlling the pathogenic bacteria than the extract obtained from *Nanochloropsis occulata* Table 3, 4 & 5 shows effect of light on bactericidal activity.

Spectroscopical Analysis for chemical nature of the antimicrobial active principles:

Three fractions were subjected for uv-visible and FTIR spectroscopical analysis and the FTIR result were as follows .

From the overall data the fractions of *Nanochloropsis occulata* contained the compounds seems to be a glycosidic aromatic amine and a linear carbon skeleton apart from the benzoid group. In the fraction of *Chaetoceros calcitrans* contained a compound could be a nucleoside.

IV. DISCUSSION

For a long time, a great number of medicines have been extracted from the sea plants. Many microalgae have been recognized as potential source of antibacterial and antifungal substances. Antibacterial effects have been noticed in all the algal classes, which is the major component of the phytoplankton. Marine algae are also a source of antifungl substances and have also been found to be effective against the fungi *Trichophyton mentagrophytes*, Trychophyton rubrum[14], *Candida albicans*[15] (Olesen et al.,), and a number of other fungal organisms[20]

The bad effects and biomagnification 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 animals' 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 out the active compounds which are involving in the control of human pathogenic bacteria such as *Staphylococcus aureus, Escherichia coli, Enterobacter aeroginosa, Pseudomonas aeroginosa* and *Proteus vulgaris.* The present study showed that the microalgal extracts obtained from *Tetraselmis suecica* claimed to be the best inhibitor of the growth of *Staphylococcus aureus* and *Proteus*

vulgaris. Similarly *Tetraselmis* showed inhibitory effect against soil bacteria[9].

They have a wide range of activity and or liable to be effective against pathogenic microorganisms, those are resistant to classic antibiotics (Aubert et al[2]). For example the work done by Si-Shen Li and Huai-Jen Tsai revealed that the medaca fish fed with the bovine lactoferricin (LFB) containing transgenic microalgae *Nannochloropsis occulata* would have bactericidal defence against *Vibrio parahaemolyticus* infection in its digestive tract[18]. (Si-Shen Li and Huai-Jen Tsai. 2008)

Chaetoceros launderi was tested against fungi resulted the significant activity was observed against all the dermatophytes (Gueho et al., 1979)[12]. In the present study, *Chaetoceros calcitrans* has proved to be the best controller of all the shrimp bacterial pathogens.

Several compounds have been isolated from marine source 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. *Skeletonema* is a good source for fatty acid derived antimicrobial substances[4]. In the present study the two fractions of *Tetraselmis suecica* contained the compounds which were seems to be a glycosidic aromatic amine and a linear carbon skeleton apart from the benzoid group. But Aubert et al in 1968, Persando and Gueho in 1977 had observed an unidentified molecule from the *Tetraselmis*, which showed inhibitory effect against soil bacteria.

In *Chaetoceros* sp. polysaccharides and fatty acids are the major candidate molecules for their antibacterial activity[4]. But in the present study the compound fraction from *Chaetoceros calcitrans* was predicted to be nucleoside derivative.

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 microbes. A commercially viable stategy will be worthful to establish this and high throughout drug screening system should be developed to develop a new class of drugs, which is from the natural source.

The active fraction of *Chaetoceros lauderi* was found to be a fatty acid with the same bactericidal properties after exposure to light (Gauthier, 1969). Acknan *et al* (1968) also found the active fraction of *Skeletonema costatum* to be a fatty acid and among the other fatty acid that have identified, acrylic acid was isolated by Sieburth (1960) from the *Chrysophycea phaeocystis poucheti*.

One active fraction has been isolated from the cellular extract of *Asterionella japonica*. This was found to have remarkable bactericidal properties after exposure to light, and was especially active on *Sarcina lutea*, also on *Staphylococcus aureus* 209P, *Clostridium tetani, Clostridium botuli* This fraction has be *num, Clostridium speticum, Clostridium histolyticum*. Pesando D. 1972. in the present investigation was the compounds exposure to the light the antimicrobial activity was decreased from the studies the compounds may be light sensitive compounds it expose to light it lose their antimicrobial property.

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