Asian Journal of Bio Science, **Vol. 3 No. 2 : 340-345 (October, 2008)**

Marine phytochemicals as a source of pharmacological interest for drug resistant Methicillin Resistant *Staphylococcus aureus* (MRSA)

S. PRAKASH
Department of Biotechnology, Udaya School of Engineering, Ammandivillai P.O., KANYAKUMARI (T.N.) INDIA

(Accepted : September, 2008)

Antimicrobial drug resistance occurs in hospitals worldwide. One of the globally important micro-organisms is Methicillin Resistant *Staphylococcus aureus* (MRSA) which now causes more than 40% of all *S. aureus* bacteremias. Hence scientists are searching for natural compounds to control MRSA. Many marine organisms are known to produce bioactive compounds, but historically man has derived relatively few pharmaceutical agents from marine species. It is certainly true that most of the pharmacologically active products have been isolated from the sea especially from microalgae. In the present study a survey was made in the Nagercoil government hospital for studying bed sore infectious *Staphylococcus aureus*. From the samples collected, 20% were *Staphylococcus* spp. and 80% were other bacterial species. Of the 20% of *Staphylococcus aureus*, 33.4% Methicillin Resistant *Staphylococcus aureus* (MRSA). Different solvent extracts of four marine micro algae were screened against MRSA pathogens. The extraction of antimicrobials from marine micro algae reveals that n-Butanol gave maximum extraction. Among the total Four microalgal extracts used against the bed sore MRSA pathogens *Isochrysis galbana* extract showed highest percentage inhibition (52%) when compared to other extracts. The present study would therefore seems particularly worthy for further investigations of valuable compounds from marine microalgae.

Key words : Drug resistant, *Staphylococcus aureus*, Methicillin.

INTRODUCTION

Staphylococcus aureus, often referred to simply as 'staph', is a bacteria commonly found on the skin of healthy people. Occasionally, staph can get in to the body and cause infection. This infection can be minor (such as pimples, boils and other skin conditions) or serious (such as blood infections or pneumonia). Methicillin is an antibiotic commonly used to treat staph infections. Although methicillin is very effective in treating most staph infections, some staph bacteria have developed resistance to methicillin and can no longer be killed by this antibiotic. These resistant bacteria are called Methicillin Resistant *Staphylococcus aureus* or MRSA. Methicillin Resistant *Staphylococcus aureus* (MRSA) strains were first described in England in 1961, shortly after methicillin became available for clinical use. They have subsequently spread through out the world and are an important cause of nosocomial infections in many geographic areas, including the United States. Data from the National Nosocomial Infection Surveillance System reveal MRSA accounts for up to 40% of nosocomial *S. aureus* infections in large hospitals and 25% to 30% of such infections in smaller hospitals. MRSA is of special concern because it is resistant not only to methicillin, oxacillin, and nafcillin but also to all other β-lactams, including cephalosporins, imipenem and meropenem, and aztreonam. Most strains of MRSA are multidrug resistant.Resistance to erythromycin and clindamycin are very common and many strains are resistant to gentamycin, tobramycin and ciprofloxacin. In some geographic areas, resistance to co-trimazole and rifampin are also common. Many strains are susceptible to minocycline. Recently, strains resistant to methicillin and oxacillin but susceptible to many non-b-lactum agents such as clindamycin and gentamycin. Hence scientists are searching for natural compounds to control MRSA. Many marine organisms are known to produce bioactive compounds, but historically man has derived relatively few pharmaceutical agents from marine species (Rinehart and Shied, 1989). It is certainly true that most of the pharmacologically active products have been isolated from the sea especially from algae. The constant need to find new antibiotics is largely the result of an increased resistance in pathogenic bacteria caused by the continued use of these drugs. In recent years, several reports on antibacterial and antifungal activities of marine plants have been published (Khaleefa *et al*., 1975; Caccamesa *et al*., 1980; Reichelt and Borowitzk,1984). Antibacterial activity in connection with phytoplankton was first observed by Sieburth (1960) and Aubert *et al.* (1968) against some soil bacteria. Since then a number of screening programs led to the discovery of antibacterial

properties in extracts from phytoplanktonic organism of several families Diatoms, Dinoflagellates, Cyanophyta, Crysophyta (Burkholder *et al*., 1960; Aubert *et al*., 1968; Aubert *et al*., 1979; Gauthier, 1980; Reichelt and Borowitsk, 1984; Visco *et al*., 1987; Pesando, 1990).

With this view the present study was undertaken to better understand the epidemiology of Methicillin Resistant *Staphylococcus aureus* (MRSA) of Bed sore infections in Kanyakumari District and to screen the anti bacterial activity of marine microalgae, which can be used as an alternate source for the commonly used antibiotics.

Materials and Methods

The Bed sore samples were collected from male and female patients from the Government Hospital of Nagercoil, Kanyakumari District. A wound swab specimen was collected without the contamination using a sterile cotton wool swab, which was placed in a sterile tube for the collection of the specimen. The yellowish exudates were collected by using two swabs. The collected swabs were placed in the test tubes with a transport media. Of the two swabs, one was used for direct microscopic examination and the other for laboratory culture. The direct microscopic examination includes, gram's staining, and motility test. The second swab was used for the inoculation into the solid media. Cultivation, isolation and identification of specific pathogens from samples collected from the patients had done based on the standard procedures. For each dilution to be plated, aseptically transferred 1ml sample suspension to 3 plates of Baird-Parker agar, distributing 1ml of inoculum equitably to 3 plates (*e.g*., 0.5ml, 0.3ml and 0.2ml.). Spreaded innculum over surface of agar plate, using sterile bent glass streaking rod. Retain plates in upright position until inoculum is absorbed by agar (about 10 min on properly dried plates). If inoculum is not readily absorbed, placed plates upright in incubator for about 1hr. Inverted plates and incubated 45-48 hr at 35^0C. Selected plates containing 20-200 colonies, unless only plates at lower dilutions (>200 colonies) have colonies with typical appearance of *S. aureus*. Counted and recorded colonies. If several types of colonies were absorbed which appear to be *S. aureus* on selected plates, count number of colonies of each type and record counts separately.

Coagulase test is used to differentiate *Staphylococcus aureus* (positive) from coagulase-negative Staphylococci (negative). A drop of coagulase plasma was placed (preferably by rabbit plasma with EDTA) on a clean and dried glass slide.A drop of distilled water or saline was placed next to the drop of plasma as a control.Using loop or straight wire , or wooden stick, a portion of the isolated colony was emulsified and made a smooth suspension. The above was mixed well with an applicator stick. The slide was rocked gently for 5 to 10 seconds. Clumping indicates the positive result. Mannitol salt agar medium is recommended for the selective isolation of pathogenic Staphylococci, since most of the other bacteria are inhibited by the high salt concentration of the medium. Colonies of *Staphylococcus aureus* (pathogenic) are surrounded by a yellow halo, indicating mannitol fermentation. A confirmatory coagulation test can be performed with the yellow colonies, in order to separated the pathogenic form (*S. aureus* coagulase positive) from the non-pathogenic form (coagulase negative). Antibiotic Sensistivity test by Disk method is the simplest method to perform the susceptibility test. Disks of filter paper impregnated with antibiotic are placed on an agar plates that is heavily and uniformly inoculated (lawn) with an activily growing culture of the organism. The medium of choice in muller agar.The test organisms grow in a smooth lawn of confluent growth on the plate expect in a clear zone around the antibiotic disk ,which inhibits the growth of the organism and indicates the susceptibility of the organisms. Bacteria showing resistance to an antibiotic show such inhibition ,they grow up to edge of the disc. Cultures isolated from clinical material were obtained from the routine antibiotic-sensitivity-testing laboratory. Methicillin susceptibility was measured by the two fold serial dilution technique. An inoculum of 0.5 ml of a 10^{-3} dilution of an overnight broth culture was used. Test strains were incubated overnight, and minimal inhibitory concentration (MIC) determinates were carried out in Trypticase soy broth, Mueller Hinton broth, and brain heart infusion broth. The MIC was read as that dilution at which there was no visible growth after 24 hr of incubation at 37^oC. Subsequently, the minimal bactericidal concentration (MBC) was determined by sub culturing 0.05ml of broth from the clear tubes to blood agar plates and also to thioglycolate broth. The MBC was read after 48 hr of incubation at 37^oC. Algal inoculum were obtained from Central Marine Fisheries Research Institute(CMFRI) centers as Tuticorin are made use of in the study are *Chlorella salina, Nannochloropsis occulata, Dicarteria inorta, Chromulina freibergensis*. The culture were maintained by autoclaved or heated seawater after cooling was poured to the conical flasks and required nutrients are added. Walne's medium enriched with vitamins is the ideal one to maintain the stock cultures of all the phytoflagellates. About 10 per cent of the inoculum in the growing phase were transferred to the culture flasks and the same is placed in

front of the tube light (1000 lux). After 8-10 days, when the maximum exponential phase was reached, light is reduced for further growth. The time required for the maximum cell densities varies depending on the species. Almost all flagellates require 2 weeks for the completion of growth phase before entering into the decline phase. In the stationary phase, the flagellates can be kept for a period of 2 months in the stock culture room, under controlled condition of light and temperature. At that time of maximum exponential phase of growth the colour of the culture turns into dark brown and the cells are found as suspension without movement. The cells of flagellates emerge out for its further growth and multiplication. A minimum of 5 culture flasks were kept for each species as stock culture.Initially 250 ml conical flask having 200 ml media was used for culturing algae. All the glassware's used were first thoroughly washed with concentrated hydrochloric acid and then with tap water, the flask was rinsed with distilled water for minimum of five times. After drying, the glassware are kept in a hot air oven for an hour at 160^0C.

The filtered seawater was sterilized by autoclaving and after cooling to room temperature; 200 ml was poured into each conical flask. Prior to sterilization. Salinity and pH was checked by refractrometer and pH meter, respectively. The filtered sterilized seawater is enriched with required quantity of Walne's medium. Then 20 ml of the inoculums in the growing phase is transferred to the culture flask with 200 ml medium. Finally the culture flasks were placed in front of tube lights of 1000 lux. The temperature ranged from 28-33^0C. From the time of inoculation samples were taken and fixed with the help of formalin for the measurement of cell density. The initial count was calculated before inoculation. After inoculation the cell count was calculated for every 2 days and was tabulated. Cell density was determined with a Neubauer haemocytometer. Duplicates were also made and cell density recorded for alternative days. By following the above procedures, exponential phase of the algal cultures were determined. From this 100 ml culture will (condition inoculums are) taken as inoculums for culturing algae in 1 litre flask. The experiments were carried out in duplicates for each algal species. The 1 litre sterile conical flasks were filled with filtered sea water (salinity 21-35 ppt). The required nutrients for the growth of the algae were added. After thorough mixing of nutrients 100 ml of the inoculums was introduced. All cultures were maintained in room temperature (28-33^0C) and illuminated with 2 fluorescent lamps (1000 lux). A light dark regime of 12 h: 12 h was maintained. Shaking of the cultured flask thrice a day is essential for the proper growth. Inoculum density and initial cell count was taken immediately. Cell count was taken for alternative days and recorded.

Algal cells in exponential growth phase were recovered from culture by batch centrifugation at 3000 rpm for 10 minutes. The cells were repeatedly washed in sterile distilled water for three times by centrifugation at low speed. The resultant algal pellets were equally distributed in 5 tubes and stored in refrigerator till extraction process. The quantity of algal cells was weighed. 0.5 g of algal cells were mixed with 1 ml solvent. Five different solvent are used in this experiment. They were Acetone, Benzene, n-butanol, Isopropanol and sterile distilled water. By using these solvents algal cells are crushed in mortar and pestle. Finally crude extract were transferred to tubes and make upto 2 ml with the solvent. The solvent extract was centrifuged at 10000 rpm for 15 minutes to remove cellular materials. The supernatant was collected and kept at (low temperature 4^0C) refrigeration for further use. *Staphylococcus aureus* were subcultured in nutrient broth. About 15ml of sterile molten Muller Hilton was introduced aseptically into sterile Petri dishes and after solidification, 12 hr old nutrient broth culture of the test organism was spreaded uniformly on the surface of the agar plates with the help of a sterile cotton swab (lawn culture). Then the empty sterile paper disc were dipped in the respective extracts and air dried in the room temperature and placed on the agar medium in petridishes, pre spreaded with the bacterial pathogens using sterilized forceps. Then the plates were incubated at 37^oC for 24 hrs. The antibacterial activity of the marine micro algal extract was observed though zone of inhibition around the disc. Then this zone of inhibition was measured in mm and tabulated.

RESULTS AND DISCUSSION

For the present study a survey was made in the Government hospital, regarding the Methicillin Resistant *Staphylococcus aureus* infections among the hospital patients. The samples were collected from 17 male and 13 female patients. The proforma of the survey is given in Table 1. Immediately after collection the samples were subjected to Gram's staining for initial identification and serial dilutions and plating were done to isolate the aureus from bed sore samples. The suspected colonies were isolated and streaked on into a nutrient agar solvent for further tests. Isolation and identification of *S. aureus* was done on Baired Parker media and colony characteristics were displayed in the Table 3. The various

Table1 : Bed sore infection bacterial pathogens present in the sample collected

Sr. No	Samples	Organisms present
1.	S-1	NSA
2.	S-2	NSA
3.	S-3	SA
4.	S-4	NSA
5.	S-5	NSA
6.	S-6	NSA
7.	S-7	SA
8.	S-8	NSA
9.	S-9	SA
10.	S-10	NSA
11.	S-11	NSA
12.	S-12	NSA
13.	S-13	SA
14.	S-14	NSA
15.	S-15	NSA
16.	S-16	NSA
17.	S-17	NSA
18.	S-18	NSA
19.	S-19	NSA
20.	S-20	NSA
21.	S-21	SA
22.	S-22	NSA
23.	S-23	NSA
24.	S-24	SA
25.	S-25	NSA
26.	S-26	NSA
27.	S-27	NSA
28.	S-28	NSA
29.	S-29	NSA
30.	S-30	NSA

SA - *Staphylococcus aureus* is present
NSA - No *Staphylococcus aureus* is present

conventional metabolic biochemical tests were done to identify Staphylococcus aureus which were presented in the Table 2. Confirmation of *S. aureus* were carried out on Mannitol salt agar, cultural characteristics and growth patterns were given in Table 3. The percentage contribution of staphylococcal infections among the Bed sore infected patients were presented in the Fig. 1.

Both Methicillin Susceptible *Staphylococcus aureus* (MSSA) and MRSA has demonstrated by using antibiotic sensitivity test with commercially available antibiotics (Kirby-Bauer) method and Two fold serial dilution techniques(MIC and MBC). The isolated bed sore infected *Staphylococcus aureus* pathogens from collected samples were screened with methicillin, Ciprofloxacin, Erythromycin, Fusidic acid, Gentamycin, Mupirocin, Rifampin, Tetracycline, Trimethoprim

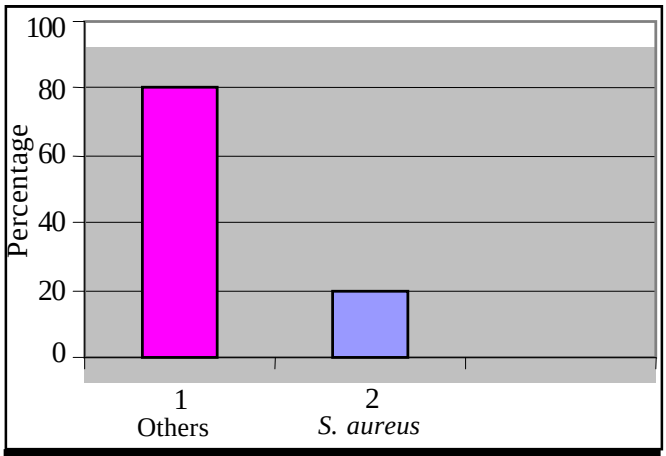

Fig. 1 : Percentage contribution of *Staphylococcus aureus* among patients

antibiotic discs. Inhibitation zone of diameter for each antibiotic against the *Staphylococcus aureus* are given in Table 6. Minimum Inhibiting Concentration and Minimum Bactericidal Concentration of selected Antibiotics were given in the Table 4.The isolated bacterial pathogens from all samples were tested against the bioactive compounds extracted from micro algae with various solvents. The Micro algae like *Nannochloropsis occulata,Chlorella salina, Chromulina freibergensis* and *Dicarteria inorta* were mass cultured using Walne's media. *Nannochloropsis occulata* on the initial day, the cells were $60x10^4$ cells/ml whereas it slightly increased to $640x10^4$ cells/ml on the 8th day and reached the exponential phase on 14th

Table 2 : Cultural characteristics on identified media

Sr. No.	Organisms	Blood Agar	Baird Parkar	Mannitol Salt Agar
S-1	*Staphylococcus aueus*	Milky white colonies with β- heamolysis	Black colour colonies	Golden yellow colour colonies
S-2	*Staphylococcus aueus*	Milky white colonies with β- heamolysis	Black colour colonies	Golden yellow colour colonies
S-3	*Staphylococcus aueus*	Milky white colonies with β- heamolysis	Black colour colonies	Golden yellow colour colonies
S-4	*Staphylococcus aueus*	Milky white colonies with β- heamolysis	Black colour colonies	Golden yellow colour colonies
S-5	*Staphylococcus aueus*	Milky white colonies with β- heamolysis	Black colour colonies	Golden yellow colour colonies
S-6	*Staphylococcus aueus*	Milky white colonies with β- heamolysis	Black colour colonies	Golden yellow colour colonies

Table 3 : Sensitivity pattern with commercially available antibiotics against *Staphylococcus aureus*

Chemotheropeutic agents	Symbol	Disc potency	Inhibition zone of diameter in m.m					
			S-3	S-7	S-9	S-13	S-21	S-24
Methicillin	M	10mcg	R	R	R	R	R	R
Ciproflaxin	Cf	10mcg	S	R	R	S	R	S
Clidamycin	Cd	10mcg	R	R	S	R	S	S
Erythromycin	E	10mcg	R	S	S	S	S	S
Fusidic acid	Fc	10mcg	R	S	R	S	R	S
Gentamycin	G	10mcg	S	S	R	S	R	R
Mupirocin	Mp	10mcg	R	R	S	R	S	R
Rifampin	R	15mcg	R	S	R	S	R	S
Tetracycline	T	10mcg	R	S	R	R	S	R
Trimethoprim	Tr	10mcg	S	R	S	R	R	S

Table 4 : Bactericidal Activity of Methicillin in Broth Media

Sample No.	Muller Hinton Broth		
	MIC [a]	MBC [b]	
		Thioglycolate Broth	Blood Agar plate
S-3	3.12	>100	6.25
S-7	3.12	>100	6.25
S-9	3.12	>100	6.25
S-13	3.12	>100	6.25
S-21	3.12	>100	6.25
S-24	3.12	>100	6.25

[a]Lowest concentration (µg/ml)of methicillin inhibited visible growth at 18 to 24 hrs
[b]Lowest Concentration (µg/ml) producing 99.9%kill after 48 hr of incubation at 37°C

day with the cell count of 880x10⁴ cells/ml (Table 5). *Chlorella salina* showed 36x10⁴/ml on the initial day whereas it was increased to 225x10⁴ cells/ml on the 8thday and then increased to the exponential phase of 500x10⁴ cells/ml on the 16th day (Table 5).

On the initital day, the *Chromulina freibergensis* was 5x10⁴ cells/ml and it was slightly increased to 80x10⁴ cells/ml on 8th day and reached 460x10⁴ cells/ml on the exponential phase (Table 5). On the initial day, the *Dicarteria inorta* was 40x10⁴ cells/ml and it was increased to 360x10⁴ cells/ml on the 8th day and it reached to the exponential phase of 540 x10⁴ cells/ml on the 16th day of culture(Table 5). On reaching the exponential phase, the algal cells were separated by centrifugation. The resultant pellets were crushed with 5 different solvents and centrifuges at 10000 rpm for 15 min. the supernatant was collected and loaded in the sterile paper disc. The crude extract of micro algae *Nannochloropsis occulata*

Table 5 : Growth rate of various marine micro algal for every two days

Days	Growth pattern of marine micro algae			
	C.salina cells per ml	*C. freibergensis* cells per ml	*D. inorta* cells per ml	*N. occulata* cells per ml
Initial	36 x10⁴	5 x10⁴	40 x10⁴	60 x10⁴
2nd day	55 x10⁴	15 x10⁴	80 x10⁴	130 x10⁴
4th day	80 x10⁴	45 x10⁴	170x10⁴	230 x10⁴
6th day	145 x10⁴	65 x10⁴	260 x10⁴	425 x10⁴
8th day	225 x10⁴	80 x10⁴	360 x10⁴	640 x10⁴
10th day	290x10⁴	95x10⁴	460x10⁴	805 x10⁴
12th day	480 x10⁴	120 x10⁴	500x10⁴	875 x10⁴
14th day	600 x10⁴	215 x10⁴	520 x10⁴	880 x10⁴
16th day	500x10⁴	460x10⁴	540x10⁴	890x10⁴

Table 6 : Sensitivity test with antimicrobials from marine micro algae against MRSA

Name of the marine Micro algae	Solvents used	Zone of inhibition in mm
		MRSA
Chorella salina	Acetone	Nil
	Benzene	Nil
	n-Butanol	Nil
	Iso propanol	Nil
	D/Water	Nil
Nannochloropsis occulata	Acetone	Nil
	Benzene	8
	n-Butanol	9
	Iso propanol	Nil
	D/Water	Nil
Dicarteria inorta	Acetone	Nil
	Benzene	Nil
	n-Butanol	7
	Iso propanol	Nil
	D/Water	Nil
Chromulina freibergensis	Acetone	Nil
	Benzene	Nil
	n-Butanol	Nil
	Iso propanol	Nil
	D/Water	Nil

gave maximum zone of inhibition of 9mm in diameter for n-butanol extract next to which benzene showed 8 mm (Table 6). The butanol extract of *Dicarteria inorta* showed maximum zone of inhibition by 7 mm against MRSA. The extracts of acetone, benzene and isopropanal has no antibacterial activity (Table 6). The crude extract of *Isochrysis galbana* showed activity in three solvents like acetone, benzene and n-butanol. The extract of n-butanol showed maximum zone of inhibition of 9 mm in diameter. (Table 6).The microalgae *Chromulina freibergensis* have no activity. Among the total 4 microalgal extracts used against the bed sore bacterial pathogens, *Isochrysis galbana* extract showed highest percentage of inhibition (52%) when compared to other extract.

REFERENCES

Aubert, M., Aubert, J. and Gauthier, M. (1968). Pouvoir autoepurateur del/eau de mer et substances antibiotiques produties par les organisms marine rev. *Intern. ocean.org.Med.*, **10** : 39-47.

Aubert,M., Aubert, J. and Gauthier, M. (1979). Antibiotic substances from marine flora : in : floppe, M.A. Lervign T., Tanaka, Ed In:*Marine Algae in pharmaceutical science*, pp 275-291.

Burkholder,P.R., Burkholder, L.M. and Almodovar, L.R. (1960). Antibacterial activity of some marine algae of Puerto Rico. *Bot. Mar.*, **2** : 149-156.

Caccamesa, S., and Azzolina, R., Furnari, G., Cormaci, M. and Grasso, S. (1980). Antimicrobial and antiviral activities of extracts from Mediterranean algae. *Bot. Mar.*, **23** : 285-288.

Caccamesa, S., and Azzolina, R., Furnari, G., Cormaci, M. and Grasso, S. (1981). Antimicrobial and antiviral activities of some marine algae from eastern Sicily. *Bot. Mar.*, **24** : 367.

Gauthier, M.J. (1980). Nate sur la friquence de la production d'antibioliques chezles algues Planetoniques. *Rev. Intern. Ocean. Org. Med.*, 41-44.

Khaleefa, A.F., Kharboush, A.M., Metwalli., A., Mohsam, A.F. and Serwai, A. (1975). Antibiotic fungicidal action from marine extracts of some weeds. *Bot. Mar.*, **118** : 163-165.

Pesando,D. (1990). Antibacterian and antifungal activities of marine algae. In : *Introduction to applied phycology*, pp3-26

Reichelt, J.L and Borowitzk (1984). Anibacterial activity from marine algae ;Results of a large scale screening program. *Hydrobiologia*., **116/117** : 158-170.

Sieburth, J.M. (1960). Acrylic acid an antibiotic principle in phaeocystis bloom in Antarctic waters. *J. Sci.*, **132** : 320-321.

Viso, A.C., Pesando, D. and Baby, C. (1987). Antibacterial and antifungal properties of some Marine diatoms in culture. *Bot. Mar.*, **30** : 41-45.