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Prospective study on antimicrobial protein of *Spirastrella inconstans* against Methicillin Resistant *Staphylococcus aureus* (MRSA)

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ABSTRACT

The bioactive compound have to be extracted from the marine sponge *Spirastrella inconstans* for treatment against Methicillin Resistant *Staphylococcus aureus* (MRSA), which were isolated from the chronic wound of fisherman community of east and west coastal villages of India. About 100 MRSA strains were isolated from the pus samples and confirmed using standard biochemical tests. Since those strains developed resistance to almost all type of antibiotics, alternative bioactive compounds are needed for the treatment. *Spirastrella inconstans* was selected in which the crude bioactive compound were extracted by solvent extraction method. The antimicrobial activity was performed using agar well cutting and paper disc diffusion methods, in which chloroform extract was the most active compared to that of other solvents, with a maximum of 30 mm diameter of zone of inhibition. That crude extract protein was estimated by using Lowry's method. The protein was precipitated using ammonium sulphate precipitation method and purified using dialysis. The purified protein was found to be of 3,000 Da molecular weight by SDS-PAGE, it can be used for the treatment of wound infection though the structural and molecular elucidations need to be done in the future.
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Keywords: MRSA, marine sponge, protein, antimicrobial.

INTRODUCTION

Staphylococcus aureus is a major pathogen causing large variety of infections worldwide and predominates in surgical wound infections with prevalence rate ranging from 4.6 to 54.4%. It causes superficial skin infections and life threatening diseases such as endocarditis, sepsis and soft tissue, urinary tract, respiratory tract, intestinal tract and bloodstream infections (Chakraborty et al., 2011).

S. aureus has developed resistance to most classes of antimicrobial agents. Penicillin was the first choice of antibiotics to treat staphylococcal infection. In 1994, by destroying the penicillin by penicillinase, *S. aureus* became resistant to penicillin. Methicillin, a semi synthetic penicillin was used to treat penicillin resistant *S. aureus* infection but resistance finally emerged in 1962. Following this, Methicillin Resistant *Staphylococcus aureus* (MRSA) emerged (Livermore, 2001; Cui et al., 2000).

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Since MRSA was first described in 1961, it has been regarded as a nosocomial pathogen that is not normally present in the community. However, this notion has changed greatly in the past 15 years, and CA-MRSA infections are now prevalent and widespread. Although MRSA infections acquired from the community were reported in Detroit, MI, USA, in 1982, all patients had predisposing risk factors for infection, such as previous hospital admission or intravenous drug abuse. The first genuine cases of CA-MRSA infection were reported among individuals from Kimberley, Western Australia, in the early 1990s (DeLeo et al., 2010).

Oceans are said to be the medicine chest of the future. Sponges and corals offer a rich source of unique and diverse natural products,most of which have potent pharmacological activities (Dovi Kelman et al., 2009). Antibiotics isolated from marine sponge were active against human pathogens or marine bacteria. The rich source of unique and diverse secondary metabolites from sponge has potent pharmacological activities, including anti-tumor, antifungal, anti-viral and anti-bacterial properties. The controlled diffusion of antibiotic agents in the living tissues of sponges may increase the efficiency and thereby provide a better defense against microbial infections (Dovi Kelman et al., 2001). Natural products were also found to play important biological and ecological roles for the producing organisms such as defense against predators, competition for space, prevention of fouling, roles in reproduction and antimicrobial activity. Antimicrobial activity has been extensively reported from various groups of marine organisms, such as sponges, bryozoans, ascidians, scleractinian corals, gorgonian octo-corals, and alcyonacean soft corals. Several antibiotics have been isolated, such as plakortin and manoalide from marine sponges (Dovi Kelman et al., 2009).

Despite the wealth of biologically active secondary metabolites isolated from marine sponges, the potential functions of these compounds in antimicrobial chemical defense have rarely been explored. The first report of antimicrobial activity of sponge extracts was done by Nigrelli et al. (1959).Since last two decades, research in to marine natural products has expanded rapidly. The oceans are a source of a large group of unique natural products, marine microorganisms whose immense genetic and biochemical diversity is only beginning, likely to become rich source of novel chemical entities for the discovery of more effective drugs. Among all marine organisms screened, marine sponges produce the largest number of structurally diverse natural products, and in fact represent the single best source of marine bioactive compounds, which acts as secondary metabolites (Radhika et al., 2007). The marine sponges are shown to exhibit anti-bacterial, anti-insecticidal, anti-viral, anti-plasmodial activities and anti-leishmanial activity (Lakshmi et al., 2009).

The objective of this study was to identify the drug from *Spirastrella inconstans* for the treatment of wound caused by MRSA among fisherman community.

MATERIALS AND METHODS

Isolation of MRSA

MRSA strains were isolated from the chronic wound caused due to fishing, or any other agents among fisherman community of east and west coastal villages of India. They were confirmed using standard routine biochemical tests (Mohankumar et al., 2012). Totally, 100 strains were isolated and their antimicrobial activity was evaluated using marine sponge, since they developed

resistance to almost all types of antibiotics (Baba et al., 2010).

Isolation of sponge

The sponge sample was collected from Gulf of mannar, Tamilnadu and the specimen were stored at -20 °C and transported by maintaining the temperature below 0 °C and identified as *Spirastrella inconstans*. The aqueous extract of sponge was prepared by squeezing the sand free specimens in distilled water. The resultant solution was filtered and dialyzed by using Sigma dialysis membrane 500 against D-glucose. The supernatant, the crude aqueous extract, was stored at 4 ºC.

Solvent extraction

10 mg of *Spirastrella inconstans* tissue was shocked with 200 ml of solvents, covered and kept standing for 5 hrs. The solvent was then removed after squeezing the sponge and filtered through Whatman No 1 filter paper then evaporated at 45 ºC according to the method of Gerald and Green Gerardo (1981).

Homogenized *Spirastrella inconstans* (20 mg) was taken with different solvents such as chloroform, dichloromethane, methanol, ethanol, petroleum ether and acetone. They were soaked, packed tightly and kept in dark condition for three days, though the biomolecules present in *Spirastrella inconstans* get mixed with solvent. The process was repeated using fresh solvent, until it is quite satisfied that the entire bioactive compound soluble in solvent has been extracted. The supernatant, which is yellowish when extracted for the first time, indicated the presence of solvent-soluble compounds while the supernatant, which is crystal clear when extracted for the third time, indicated that there are no more solvent-soluble compounds present in it (Zakaria et al., 2004). The solvent was evaporated to form layer. One ml of solvent was poured over the precipitate and the extract was stored in eppendorf for antimicrobial activity tests.

Antimicrobial activity of *Spirastrella inconstans* against MRSA

Paper disc diffusion method

A concentration of 500 µg $disc^{-1}$ of *Spirastrella inconstans* extracts was applied to each sterile paper discs (6 mm in diameter). The solvent was evaporated before they were placed on to agar plates that were previously seeded with MRSA. The diameter of the inhibition zones was measured in mm after incubation at 30 °C for 24 hrs. Solvent control discs without extract were prepared in the same manner and were never observed to inhibit bacterial growth (Thakur et al., 2003).

Well cutting method

Compared to Paper disc diffusion method, well cutting method showed efficient results so later was taken into account. MRSA turbidity was matched with Mac Farland standard and 100 µl of overnight culture was swabbed in Mueller Hinton Agar (MHA) plate, allowed it for 2-5 min then the wells were made using cork borer. 50 µl of the six different extracts were added in the order of single extract in center and the remaining extract around it, allowed it for 10 min, and kept at 4 °C for 30 min. After 24 hrs, the zones of diameter of inhibition were reported.

Quantitative estimation of protein by Lowry's method

Bovine serum albumin was pipetted in series of 0 µl (Blank), 5 µl, 10 µl, 20 µl, 30 µl, 40 µl, 50 µl, 60 µl, 75 µl, 100 µl and 150 µl. 0.2 ml of water added to blank; other tubes were also made to 0.2 ml. Then 1.0 ml of Solution D was added, after 10 min 0.3 ml of Solution E (Folin's phenol reagent) added, vortexed and incubated for 60 minute at room

temperature. A blank without protein was prepared. OD was measured at 660 nm. By standard graph, the amount or presence of protein was detected (Lowry et al., 1951).

Ammonium sulphate precipitation method for *Spirastrella inconstans* protein

5.0 mg body wall was homogenized 3 times with 100 ml of 50 mM Sodium acetate buffer at 4 °C. The homogenate was kept overnight at 4 °C. Centrifuged at 10,000 g for 20 min at 4 °C, then 20-80% saturation of a ammonium sulphate fraction of supernatant was prepared and precipitated protein was dissolved in PBSC (Bei-Wei Zhu et al., 2008).

Ammonium sulfate fractionation

Ammonium sulfate was added to fraction 1 at 20% saturation for 30 min. The precipitate was centrifuged at 10,000 rpm for 30 min (Fraction II) and to the supernatant ammonium sulfate was added at 40% saturation. Repeating centrifugation (Fraction III) was done. The supernatant was similarly treated with ammonium sulfate at 60 and 80% saturation and fractions IV and V obtained. All the precipitates (II-V) were dialyzed against the same buffer. All the concentrated fractions (II-V) were subjected to protein and enzyme activity for maximum activity. Enzyme activity is expressed as U/ml and one unit (U) of activity is defined as µ mols of free fatty acids liberated /min/ml by the enzyme solution under assay conditions. Ammonium sulfate added to precipitate the protein was calculated from the standard chart and represented in Table 1. The pellet obtained by precipitating the protein content was crude enzyme.

SDS-PAGE

One dimension SDS-PAGE was carried out following modified method of Laemmli, 1970. SDS-PAGE was run on vertical slab gel system. Proteins were electrophorised on 12% separating gel (0.75 mm thickness) overlaid with 5% stacking gel. A 10 % (w/v) stock solution of precipitated protein in deionized water was run in SDS-PAGE (Boobathy et al., 2009).

Purification of protein sample by dialysis

The dialysis bag was boiled with distilled water for half an hour, immersed in water for overnight. To the tighed dialysis bag 1.61 ml/cm sample was loaded, immersed in Sodium Phosphate buffer and dialyses were carried out for 24 hrs using Magnetic stirrer. The buffer solution was checked with Nesselers reagent for removal of Ammonia. 1.0 ml of purified enzyme, collected from 20%, 40%, 60% and 80% saturation levels were used for dialysis, to purify the impurities.

Table 1: Saturation Vs ammonium sulphate added to the supernatant.

S. No	% of saturation	supernatant obtained (ml)	Amount of ammonium sulfate added (gm)
1	Initial	43	-
2	20	43	4.859
3	40	43	5.203
4	60	43	5.590
5	80	45	6.020

RESULTS

Isolation of MRSA

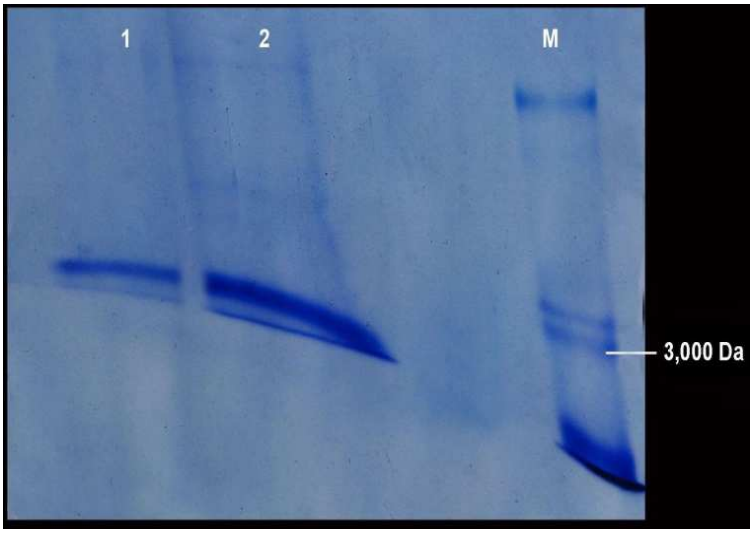
The isolated strains were confirmed as Methicillin Resistant *Staphylococcus aureus* (MRSA) by using standard biochemical characterization and by observing the positive growth in selective media for the growth of MRSA.

Antimicrobial activity of *Spirastrella inconstans* against MRSA

Among six solvents of *Spirastrella inconstans* (chloroform, dichloromethane, methanol, ethanol, petroleum ether and acetone) extract tested with the isolates showing more than 60% resistance to antibiotics, chloroform seems to be much effective and petroleum ether extract does not shows any activity. Optimum level of action was observed in case of other solvents extracts such as dichloromethane, methanol, ethanol and acetone. The strainsFM 4, FM 72, FM 84 and FM 88 showed maximum inhibition with chloroform extract, among which 30 mm diameter of zone of inhibition was observed with strain FM 72. MRSA strain FM 68 showed maximum inhibition with methanol extract of *Spirastrella inconstans*, the strain FM 22 showed maximum inhibition with ethanol extract compared to other MRSA strains (Table 2).

SDS-PAGE

The precipitates collected at 40% and 60% saturation levels were shown to content protein, which were quantified by Lowry's method and analyzed by SDS-PAGE. The bands obtained were compared with the low molecular weight marker, and revealed that the *Spirastrella inconstans* protein extract contains a 3,000 Da protein (Figure 1). Thus this study shows that this protein of *Spirastrella inconstans* has anti MRSA activity.



"1, 2": Protein precipitates by ammounium sulphate precipitation method;
"M": Low molecular weight marker.

Figure 1: Separation of crude protein in *Spirastrella inconstans* by SDS-PAGE.

Table 2: Antimicrobial activity of *Spirastrella inconstans* protein extracts against MRSA.

	Diameter of Zone of Inhibition (mm) of *Spirastrella inconstans* protein extracts						
S. No	**Strain No.**	**Chloroform**	**Dichloro methane**	**Methanol**	**Ethanol**	**Petroleum Ether**	**Acetone**
1	FM 4	24	16	16	16	10	-
2	FM 5	11	-	-	11	-	-
3	FM 6	10	11	-	-	-	11
4	FM 20	14	12	14	18	16	-
5	FM 21	10	-	8	10	-	-
6	FM 22	12	12	15	22	-	-
7	FM 23	11	11	10	16	-	-
8	FM 26	14	12	12	-	-	-
9	FM 27	10	-	11	8	-	-
10	FM 31	12	10	10	12	-	-
11	FM 32	12	11	16	10	15	-
12	FM 45	14	11	8	9	-	-
13	FM 47	14	9	13	11	-	-
14	FM 48	12	13	12	13	-	-
15	FM 49	20	18	14	18	15	-
16	FM 50	14	9	19	18	-	-
17	FM 51	14	14	10	11	-	-
18	FM 53	18	16	11	11	-	-
19	FM 54	14	10	11	10	-	-
20	FM 55	14	12	12	-	-	-
21	FM 56	13	-	-	11	-	-
22	FM 58	17	-	-	13	-	-
23	FM 60	14	18	10	11	-	-
24	FM 61	15	11	-	11	-	-
25	FM 62	15	10	14	11	-	-
26	FM 68	12	11	20	10	-	-
27	FM 69	15	10	10	17	-	-
28	FM 70	16	12	11	12	-	-
29	FM 71	15	15	10	-	-	-
30	FM 72	30	14	-	16	-	-
31	FM 73	12	-	11	13	-	11
32	FM 74	15	-	10	15	16	11
33	FM 76	13	14	10	15	-	-
34	FM 80	14	12	12	13	-	-
35	FM 81	10	-	16	15	-	-
36	FM 82	12	11	16	14	10	11
37	FM 83	15	11	10	15	10	12
38	FM 84	24	13	16	17	-	17
39	FM 85	17	-	-	15	-	-
40	FM 86	12	-	13	18	-	-
41	FM 88	23	8	15	18	11	14
42	FM 92	14	16	-	12	-	-
43	FM 93	11	-	-	13	-	-
44	FM 95	9	-	-	-	-	-

"FM": Fisherman wound MRSA strain; —: Absence of zone of inhibition.

DISCUSSION

Antibacterial activity of the crude *Spirastrella inconstans* protein extract showed zones of inhibition in the range of 8-30 mm against *S. aureus*. But in antibacterial activity of the crude induced hemolymph of *Scylla serrata* against *S. aureus* by disc diffusion technique was determined as 11 mm. The antibacterial activity of the nitrogen heterocyclic sponge constituent cribrostatin 6 was examined, which was bacteriostatic for a variety of gram-positive species. Infections caused by antibiotic-resistant gram-positive bacteria are a major cause of morbidity and mortality. Resistance includes Vancomycin resistance in enterococci, Penicillin resistance in streptococci and Methicillin resistance in staphylococci according to Pettit et al. (2004).

All the protein fractions were subjected to antimicrobial sensitivity testing against those MRSA that were sensitive to the crude *Spirastrella inconstans* protein previously. The fractions F4 and F79 were found to show activity against MRSA that have already shown sensitivity to the crude protein, whereas other fractions, apart from the above two i.e, F1 to F300 did not show any activity in our report. All the protein fractions were subjected to antimicrobial sensitivity testing against those bacteria that have activity to the crude induced hemolymph. The fractions F1 to F9 were found to show an activity against them, whereas other fractions (F10 to F17) did not show any activity (Hoq et al., 2003) (this reference seems not at the correct position, no need to cite references for a work done by the author himself!).

The antibacterial activity of our sponge protein Glutamic acid was against gram positive bacteria *Staphylococcus aureus.* But Schroder et al. (2003) suggested that the antibacterial activity of sponge protein LEC_SUBDO was against the Gram-negative bacteria *E. coli* and the Gram-positive bacteria *S. aureus.* The lectin was added to the bacteria and incubated. The antibacterial activity was determined in colony-forming units and expressed in percent inhibition. The Inhibitory effect of the lectin on the Gram-positive bacteria *S. aureus* was comparatively low. At a concentration of 300 µg/ml natural lectin, an inhibition of only 15% was appeared; for the recombinant protein, the activity rather lower of 8%, remains as the contrast report in this study.

MRSA of our study develops multidrug resistance against other marine organism due to its thick wall and negative charge. The ability of *Staphylococcus aureus* to adhere to the extracellular matrix and plasma proteins deposited on biomaterials is the significant factor in the pathogenesis. Rajagopal et al. (2008), reported that *Clathria* sp. showed more antimicrobial activity than the other sponges against *Staphylococcus* sp. Dovi Kelman et al. (2009) reported the eight out of eleven (73%) of the sponge species which inhibited at least one bacterial isolate. Among them, *Amphimedon chloros* exhibited the highest antimicrobial activity. *A. chloros* associated bacteria, resistant to them as well as to polymixin B, lead us to suggest that the amphitoxins and halitoxins mimic the mode of action of this class of antibiotics. Furthermore, it leads us to hypothesize that these resistant bacteria probably have special membrane properties that interfere with the antibiotic action. It will be interesting to test the charge on the surface of these microbes and investigate the ability of positively charged antibiotics to bind to these membranes.

The crude Chloroform extract, in our report of *S. inconstans* showed moderate activity against MRSA compared to purified extract. Isnansetyo et al. (2009) reported the MeOH extract of *Geodia* sp. exhibited potent antibacterial activity against oxy-tetracycline resistant *V. harveyi* with MIC 31.25 µg mL-1. The extract showed bacteriostatic activity at low concentration and bactericidal activity at the concentration of 4XMIC. This extract might be applied to control the disease caused by the bacterium as the extract did not show any toxic effect up to 125 µg mL-1 (4XMIC).

A single well defined band showing the molecular weight of 3,000 Da was obtained using the fractions of 4 and 79 of marine sponge mainly by chloroform extract in our research. Boobathy et al. (2009) obtained the crude protein content of 1.62 mg/mL in methanolic crude extract and 1.43 mg/mL in the aqueous extract of marine sponge *Callyspongia diffusa*. The SDS-PAGE on gel, crude protein toxins yielded 6 bands in the chloroform extract and 5 bands in the aqueous extract of *S. fibulatus*, ranging from 14.4 to 116 kDa molecular weight with 5 well-defined bands of 28.5, 35.4, 45.0, 59.5, 72.3 kDa in both the extracts. The presence of 3 protein bands viz, 19.5, 39.0 and 66.2 kDa were already reported in *C. diffusa*.

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