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An invitro antimicrobial activity and Bioactivities of Protein Isolated from Marine Sponge – *Callyspongia sp*.

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The marine sponge *Callyspongia sp*, collected from Mandapam coast, Tamilnadu, was studied for antibacterial and antifungal activity of proteins. Sponge species were identified based on spicules morphology. Chloroform and aqueous extracts of *Callyspongia sp* yielded a total amount of 4.8g and 5.98g from 500g of sponge respectively. Crude protein obtained from marine sponge aqueous extract was 2.1 mg/ml and that of chloroform extract was 0.9 mg/ml. The antimicrobial activity of chloroform extract and aqueous extract showed clear inhibition zone against *Pseudomonas sp. Vibrio cholerae, Aspergillus niger* and *Candida albicans*. Both the extracts exhibited hemolytic activity which was estimated as 10.1ht/ml for chloroform extract and 8.6ht/ml for aqueous extract. The partial purification of protein was carried out using DEAE cellulose. On SDS-PAGE the crude protein yielded three well defined bands at 109.9, 28.2, 12.4 KDa respectively by both the extracts.

Key words: *Callyspongia sp.,* protein purification, *Candida albicans* chloroform extract, and *Aspergillus niger.*

Sponges are the simplest of the multicellular invertebrate animals. They are exclusively aquatic, most of the sponges found in the deepest oceans to the edge of the sea. Sponges play important roles in many marine habitats. They can survive in a variety of circumstances, like in places where there is no or abundant light and in cold or warm water. They are found in the shallow tropical reefs (Thompson, 1985). There are approximately 5,000 different species of sponges in the world, of which 150 occur in freshwater, but only about 17 are of commercial valuable. A total of 486 species of sponges have been identified in India (Thomas, 1998). In the Gulf of Mannar and Palk Bay a maximum of 275 species of sponges have been recorded. The distribution of sponges in other area as reported by Thomas (1998) and at Gulf of Kutch - 25 species; and Orissa coast - 54 species. The rapid development of the pharmaceutical market has brought about a bloom of information regarding various toxins native to the sponges. Recently various technology developed to produce novel products from marine sponges; these could contribute to human healthcare (e.g. bioactive compounds that can be used for new medicines), A variety of natural products from the marine sponges have been found to exhibit remarkable antitumor and anti-inflammatory activities Thomas (1998). In the present study an attempt has been made to analyse the symbiotic bacteria and bioactive proteins from the marine sponge, *Callyspongia sp*.

MATERIALS AND METHODS

The study area was located at Mandabam coast, Tamil Nadu and it is a sandy beach towards the North and as it proceeds Southwards, it extends into a rocky beach.

Specimens of *Callyspongia sp*. were collected at extreme low tide by hand picking. They were brought to the laboratory with seawater. Subsequently they were kept in low temperature and preserved in 10% neutralized formalin for further study. The experiments were conducted from December to March, 2011.

Extraction of spicules: The sponge tissue were boiled with concentrated HNO_3 to extrude the spicules that were then identified based on the identification characters given by Thomas (1998).

Extraction of crude toxin

Aqueous extraction: The aqueous extract of sponge was prepared by squeezing the sand – free specimens in triple distilled water. The resultant solution was filtered and dialyzed by using Sigma dialysis membrane-500 (Av Flat width-24.26 mm, Av. Diameter -14.3 mm and capacity approx-1.61ml/cm) against D-glucose to remove the excess water. The supernatant so obtained was lyophilized (Labcono Freeze Dry System) and stored at 4 ^{o}C in a refrigerator for further use as crude aqueous extract.

Chloroform extraction: Crude toxin was extracted following the method of Bakus *et al.,* (1981) with certain modifications. The sponge was dried in air for 2 days and after that 10 g sponge tissue was shocked with 200 ml of chloroform, covered and kept standing for 5 hours. The solvent was then removed after squeezing the sponge and filtered through Whatman No 1 filter paper. The solvent was evaporated at low pressure by using a Buchi Rotavapor R-200 at 45 ^{o}C in refrigerator for further use as crude chloroform extracts.

Antioxidant assay: DPPH is a useful reagent for investigating the free radical-scavenging activities of compounds. In the DPPH test, the extracts were able to reduce the stable radical DPPH to the yellow coloured diphenylpicrylhydrazine. The DPPH reaction of both the extracts produced a positive result which indicated the presence of antioxidant molecule. The method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH by the reaction.

Antimicrobial activity: Petri dishes with nutrient agar and Potato Dextrose Agar (PDA) were inoculated with two different species of bacteria and fungus. Sponge extracts were sterilized by passing each through a 0.22 m Millipore GV filter (Millipore, U.S.A). Round paper discs with a radius of 0.8 cm were dipped into each sponge extract of different concentration of 5mg/ml and 10mg/ml and placed in the center on inoculated petridishes. The bacterial and fungal colonies were allowed to grow overnight at 37^{o}C and 20^{o}C respectively, and then the inhibition zone around the disc was measured.

HPLC: The following experiment illustrates the utility of high performance liquid chromatography (HPLC) as an analytical tool, (PLATE XII). In HPLC, narrow columns with internal diameters 2-80 mm are used. These columns are packed with particles having an average diameter of less than 50 microns (50 x 10^{-6}m). Such columns require high pressures

(1000-6000 psi) to maintain a convenient flow of the eluting solvent, usually in the range 0.1-10 mL/min, but occasionally higher. Resolution is considerably superior to that achieved with an ordinary column, in part because of the tight packing of the stationary phase, which reduces lateral diffusion, and because of the large surface area of the packing.
Compared with classical column chromatography, where the columns are gravity fed and a separation can take hours or even days, HPLC can offer analysis time of 5-30 min. Such times are comparable to that needed for GLC analyses. HPLC is especially suited for the analysis of compounds not readily assayed by GLC. For example, thermally labile compounds can be analysed by HPLC at ambient temperatures, and highly polar or nonvolatile compounds can be analyzed. Sample treatment is often minimal since aqueous solutions can be used in HPLC.

Protein estimation: Protein estimation was done as described by Lowry and Lopaz (1946), using Bovine Serum Albumin at the rate of 1mg/ml as the standard. Different concentrations of the standard ranging from 0.1 to 1mg/ml were taken and made up to 1 mg/ml. Then 5ml of alkaline copper reagent was added, mixed well and allowed to stand for 10 minutes at room temperature. Then 0.5ml of diluted Folins phenol reagent was added and mixed well. The mixture was incubated for 30 minutes at room temperature. The absorbance at 720nm was read spectrophotometrically. The protein concentrations of *Callyspongia sp*. extracts were estimated.

Partial purification of crude protein: Partial purification of the crude extract was carried out using DEAE Cellulose Anion Exchange chromatography according to the procedure of Stempein *et al.*, (1970).

Hemolytic assay: The micro hemolytic test was performed as described by Venkateshwaran (2001) in 96 well 'V' bottom micro titer plates. Different rows were selected for chick blood. Serial two fold dilutions of the crude toxin were made in 100ml of normal saline. This process was repeated upto the last well. Then 100: l of RBC was added to all the wells. Appropriate controls were included in the test. To the 1% RBC suspension 100: l normal saline was added, which served as negative control. The plate was gently shaken and then allowed to stand for two hours at room temperature and the results were recorded. Uniform red colour suspension in the wells was considered as positive hemolysis and a button formation in the bottom of these wells was considered as lack of hemolysis. Reciprocal of the highest dilution of the crude toxin showing pattern was taken as 1 Hemolytic Unit (HU).

SDS-PAGE: One dimension Sodium Dodecyl Sulphate (SDS) Polyacrylamide gel electrophoresis (PAGE) was carried out following the 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 was prepared in deionized water and stored in room temperature.

RESULTS AND DISCUSSION

The sponge identification was confirmed based on spicules morphology. Chloroform extract of marine sponge *Callyspongia sp*. yielded a total amount of 4.8g of crude extract from 500g of sponge. Similarly aqueous extract yielded a total amount of 5.98g of crude extract (Table1).

Both the extracts exhibited antioxidant property which was indicated by the appearance of yellow colour. The crude extract of aqueous and chloroform extracts at different concentration of 5mg/ml, 10mg/ml and 15mg/ml were tested against 2 species of bacteria *Pseudomonas* sp., *V. cholerae,* and 2 species of fungus, viz. *Aspergillusniger, Candida albicans*.. The results showed that the crude aqueous extract inhibit the growth of *V. cholerae* whereas in the chloroform extract a clear inhibition zone were observed only against *Pseudomonas* sp., The inhibition zone was measured and it was found to be 6mm for *Pseudomonas sp.* and 4mm for *V. cholerae* and the crude aqueous extracts inhibit the growth of *A. niger* whereas in the chloroform extract a clear inhibition zones were observed only against *C.albicans*. The inhibition zone was measured and it was found to be 6mm for *A. niger* and 6mm for *Candida albicans.* Burholder (1973) isolated two bromo compounds from *Verongi fistularies* and *V. vauliformis* that inhibited the growth of gram positive and gram negative bacteria. Bergquist and Bedford (1978) have suggested that the antibacterial agents produced by sponges may have a role in enhancing the efficiency with which sponge retain bacterial food and also reported that the activity was higher in temperate species than tropical species (87% as opposed to 58%) and the sponge extract more frequently inhibited the growth of marine bacteria.

The protein content in crude extract of *Callyspongia sp*. was found to be 0.9 mg/ml in the case of chloroform extract and 2.1mg/ml in the case of aqueous extract (Table 2).
Similarly, 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 crude chloroform extract induced hemolysis on chicken erythrocytes. The hemolytic titer in methanolic extract was 11 ht/mg and its specific activity was estimated at 10.1 HU/mg of protein (Table: 5). The hemolytic titer of aqueous extract was found 8ht/mg and its hemolytic activity was 8.66 HU/mg of protein. Stempien (1970) reported that halitoxin showed better hemolytic activity isolated from *genus Haliclona.*

In the chloroform extract the retention time of 4.37min, the percentage intension was found to be 11 for aqueous extract and 25 for chloroform extract. In the aqueous extract the retention time of 6.75min, the percentage intension was found to be 32 for aqueous and 30 for chloroform extract.

SDS-PAGE analysis (Figure:1) in the present study revealed that medium sized proteins in the crude protein of both the extracts. Prominent bands indicated proteins of 109.9, 28.2 and 12.4 kDa to be common in the proteineous toxin have been reported from a number of marine species especially fishes, although non-proteineous material are also certainly involved originally thought to be induced by heat shock, the production of stress proteins are now shown to be produced by environmental stress, pathophysiological state, as also non-stressful conditions such as cell cycle, growth factors and development and the differentiation. This result assumes significance considering the presence of one band at 28.2 kDa in the present study, which might be a stress protein in the case of marine cat fishes (Barton, *et al*; 1991**).**

Table 1. Preparation of crude extract of *Callyspongia sp*.

S.No	Name of the solvent	Yield (in grams for 500 g of sample)
1.	Chloroform	4.8
2.	Aqueous	5.98

Table – 2. Protein estimation from aqueous and chloroform extracts of *Callyspongia sp.*

S.No	Type of extract	Concentration of protein in mg / ml
1.	Chloroform	0.942
2.	Aqueous	2.11

Table – 3 Antibacterial activity of *Callyspongia sp.*

S.N0	Bacterial culture	Chloroform extract(mm)		Aqueous extract (mm)	
		5mg/ml	10mg/ml	5mg/ml	10mg/ml
1.	***Pseudomonas* sp.**	**3±0.1**	**6±0.2**	**-**	**-**
2.	***Vibrio cholerae***	**-**	**-**	**-**	**4±0.2**

Table – 4. Antifungal activity of *Callyspongia sp.*

S.No	Fungal culture	Chloroform Extract (mm)		Aqueous Extract (mm)	
		5mg/ml	10mg/ml	4mg/ml	10mg/ml
1.	***Aspergillusniger***	**-**	**-**	**-**	**6±0.2**
2.	***Candida albicans***	**2±0.2**	**6±0.1**	**-**	**-**

Values mean ± SD, "-" No Inhibition

Table – 5. Hemolytic assay of *Callyspongia sp.*

S.No	Type of extract	Protein (mg)	Total Hemolysis upto Dilution	Hemolytic titre	Specific Hemolytic Activity (µg/ml)
1	**Chloroform**	0.9	11	12	10.1
2	**Aqueous**	2.1	8	9	8.6

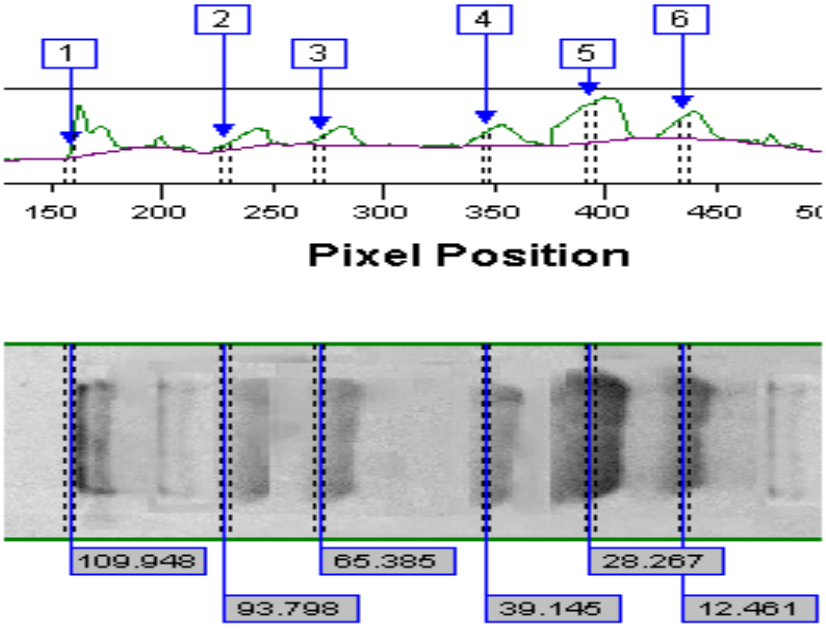

Figure:1 SDS-PAGE of *Callyspongia sp.*

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