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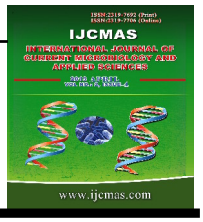

Original Research Article

Isolation, Characterization and phylogeny of Sponge - associated bacteria with Antimicrobial and Immunomodulatory potential

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Keywords

Sponge-associated bacteria, Phylogeny; Bioactive metabolites; Clinical, Fish-borne pathogens; Antimicrobials; Immuno-modulators.

A B S T R A C T

Sponge associated bacterial strains represent a rich source of bioactive metabolites. The aim of the present investigation was to isolate and characterize bacteria with antimicrobial and immunomodulatory activity from the Indian marine sponge. A total of 10 marine bacterial strains were isolated from the marine sponge *Callyspongia diffusa* (CSD 1- CSD 10) *that was collected from the Gulf of Mannar province*, Tuticorin. Of the ten bacterial strains, CSD 5 showed a remarkable antagonistic activity against clinical bacterial pathogens. Based on the Biochemical, morphological and 16 S rRNA the strain CSD 5 was identified as *Virgibacillus sp*. The bioactive metabolites produced by the strain *Virgibacillus sp.* was tested for its antimicrobial and immunomodulatory activity. The metabolites produced by the strain *Virgibacillus sp.* displayed a significant antibacterial activity against several clinical and fish-borne pathogens. Profound antimicrobial activity was found against the clinical strain *Pseudomonas aeruginosa* and the fish-borne pathogens viz., *Plesiomonas shigelloides*, *Vibririo mimicus* and *Vibrio alginolyticus* and *Aeromonas hydrophila* The antifungal activity was found to be significant against the pathogenic skin infection causing fungi *Tricoderma sp.* The immuno stimulative effect of the metabolite on murine model revealed its potential in enhancing both cell mediated and humoral immune response significantly. These findings suggest that the sponge associated bacterial strain *Virgibacillus sp*. may contribute the search for novel antibiotics to overcome the infections caused by the newly developing multi drug resistant bacterial strains and also for the production of potential immunomodulators. The biopotential of the compound produced by this sponge associated bacterial strain can be bioprospected to produce bioactive compounds with potential antimicrobial and immunostimulating activity which would impact in the production of novel antibiotics and potential immunomodulators in medical microbiology field.

Introduction

The world's oceans cover more than 70% of the earth's surface and represent an enormous resource for the discovery of chemotherapeutic agents. Given the diversity of marine organisms and habitats, marine natural products

encompass a wide variety of chemical classes such as terpenes, polyketides, acetogenins, peptides and alkaloids of varying structures representing biosynthetic schemes of stunning variety (Wright, 1998).Over the past 30–40 years marine organisms have been the focus of a worldwide effort for the discovery of novel natural products. A small number of marine plants, animals and microbes have already yielded more than 12,000 novel chemicals with hundreds of new compounds still being discovered every year (Donia and Hamann, 2003).

To date majority of these chemicals have been identified from marine invertebrates of which sponges predominate (Lie and Zhou, 2002).Sponges are known as prolific sources of bioactive compounds that could be used to treat various human diseases (Faulkner, 2002).They were determined as potential source of novel antimicrobial agents (Selvin and Lipton, 2004). Although many bioactive compounds have been discovered in sponges only a few of these compounds have been commercialized (Faulkner, 2000). A serious hurdle to the ultimate development of most marine natural products that are currently undergoing clinical trials or that are in the pre clinical trial evaluation is the problem of supply. Therefore, the research has been focused to disclose the mechanism of secondary metabolites synthesis (Abrell, 1997).Since marine organisms live in a significantly different environment from those of the terrestrial organisms, it is reasonable to expect that their metabolite will differ considerably. The presence of large amounts of microorganisms within the mesophyl of many demo sponges has been well documented (Imhorf and Stohr, 2003; Hentchel *et al.,* 2002).Bacteria can contribute up to 40% of the sponge biomass and are probably permanently associated with the host sponge unless they are disturbed by the external factors (Friedrich *et al*., 2001; Webster and Hill, 2001; Thoms *et al*., 2003). Many promising bioactive compounds including antimicrobial, anticancer, immuno-suppressive agents and enzymes are being discovered in marine actinomycetes (Lam, 2006).Microbial metabolite from the Bermudian marine sponge associated bacteria *Alteromonas* has been developed with anti-HIV potential as reverse transcriptase inhibitor (Stierile *et al*., 2002). The Indian coastline measures about 8129 km (Nair, 2003) which is distributed among nine coastal states and four Union Territories. The Gulf of Mannar, in South East coast of India, alone has 295 species of sponges, 180 species of marine algae and seaweeds, 190 species of gastropods, etc.(Ramadhas *et al*.,1999). Several reports have appeared on the characterization of the antimicrobial activity of marine macro organisms collected off the Indian coastline (Ely *et al*., 2004). Studies involving the screening, isolation and characterization of bioactive compounds from marine bacteria are yet to be undertaken on a systematic scale. In this present study the antimicrobial activity of the bioactive metabolites from the sponge -associated marine bacteria *Virgibacillus sp.* was evaluated for its antimicrobial activity against the clinical and the fish borne pathogens and the immunomodulatory potential of the secondary metabolites also investigated in Wistar rats *(Rattus norvegicus*).

Materials and Methods

Sponge Collection and Identification

Marine sponge was collected from the Gulf of Mannar region, Hares Island,

Tuticorin coast by scuba diving at 10-15m depth. The specimens were kept for 2 h in sterilized aged sea water to remove loosed associated bacterial flora present inside and outside the sponge. Environmental water representing the sponge habitat was taken prior to sponge sampling and filled up in sterilized 1L glass bottles. The habitat water was used for the isolation of the sponge associated bacterial flora on Zobell Marine Agar. Samples were surface cleaned with sterile aged sea water and surface sterilized with 70% alcohol to eliminate epiphytic microorganisms. Then the samples were kept in a sterile incubator oven for 1h at 40°C to dry the surface, and frozen (-20°C) in sterile sip-lap bags. Identification of sponge sample was carried out by Dr. P.A. Thomas, Retired Scientist, CMFRI, Kerala, India.

Isolation of Sponge-associated bacteria

A measured area of sponge tissue (1-cm^2 area) was excised using a sterile scalpel from the internal mesophyl area. The tissue was homogenized in sterile ASW using a tissue homogenizer. The homogenate was serially diluted and all dilutions were plated on Zobell marine agar 2216 (Himedia, Mumbai), using a dilution series of 1-6. The plates were incubated at room temperature (approx.27–30°C) for 7 days and isolation of bacteria with different colony characteristics was carried out from the third day onwards up to the seventh day. On Day 7 the colonies were counted for the calculation of colony forming units (CFU). The isolated colonies were repeatedly streaked to obtain pure cultures and stored in Zobell marine agar slants at 4°C for further usage.

Antagonistic Activity

Conventional cross streak method was employed to find out the potential strain with potential antagonistic activity (Egorov, 1965).

Bacterial Identification

Motility test, Catalase test, Oxidase test (Kovacs, 1956), Indole test, H2S production (Winn *et al*., 2006), Citrate utilization test, Urease test, Nitrate reduction test (Collee *et al*., 2006) and glucose and lactose fermentation profile (Hansen and Sorheim, 1991) were carried out for the potential strain CSD-5. The results were compared with the existing microbial taxonomy data of Bergey's manual of systemic Bacteriology in order to identify the bacterial isolates.

Polymerase Chain Reaction

Genomic DNA was isolated from the culture pellet of the strain *Virgibacillus* sp. (CSD-5). Using consensus primers1.5 kb 16S rRNA fragment was amplified using Taq DNA polymerase. PCR product was sequenced using the forward primer 5^1 - AGAGTTTGATC (AC) TGGCTCAG-3^1 and the reverse primer 5^1-AAG GAGGTG (AT) TCCA (AG) CC-3^1. Sequence data of the strain CSD-5 generated in this reaction was aligned with the 16S rRNA sequence of other closely related species of the Gene bank database and analyses for finding the closest homologues for the microbes. After the sequence alignment it was subjected to blast in NCBI database and then preceded with the alignment of the RDP database (GeNei; NCBI Gen Bank and RDP database).

Cultivation of Sponge associated Bacterium *Virgibacillus* sp. (CSD-5) for Secondary Metabolite Production

The potential bacterial strain *Virgibacillus* sp. (CSD-5) was mass cultured using 20 Litres (10X2L) of Zobell marine broth dissolved in sea water. The inoculums of the strain *Virgibacillus* sp. was introduced into the medium and kept for incubation for 72 h at room temperature on a rotary shaker. After the incubation time is over, the cells were separated from the culture medium by centrifuging at 12,000 rpm for 20 minutes. The cell free supernatant was extracted three times with ethyl acetate and the solvent layer was separated using the separating funnel. The ethyl acetate extract was condensed using a rotary vacuum evaporator under reduced pressure to obtain the crude metabolite of this strain. The crude metabolite obtained was 20 g.

Antimicrobial Activity

The clinical pathogens such as *Micrococcus luteus* (NCIM2673) , *E.coli* (NCIM 2065), *Proteus vulgaris* (NCIM 2027), *Enterobacter aerogenes* (NCIM 2340), *Pseudomonas* aeruginosa(NCIM 2036), *Klebsiella pneumoniae* (NCIM 2719), *Candida albicans* (NCIM 3471) *and Tricoderma sp* (NCIM 1059) were procured from NCIM, Chandigarh and the fish borne pathogens including *Plesiomonas shigelloides, Vibrio alginolyticus, Vibrio mimicus, Vibrio harveyi, Vibrio parahaemolyticus, Aeromonas hydrophila* were obtained from Suganthi Devadason Marine Research Institute, Tuticorin and preserved in nutrient agar slants and Potato dextrose agar slants respectively for the bacterial and fungal pathogens for few months.

Agar Disc diffusion Method

About 250 ml of Muller-Hinton Agar was prepared and after solidification appropriate bacterial cultures were swabbed over the agar media in a proper manner. After swabbing the culture, the discs impregnated with the crude metabolite of *Virgibacillus sp.* (100 µg w/v dissolved in 1 ml of DMSO) with different volumes *viz.*, 25µl, 50µl, 75µl were placed over the media.Then incubation of the petriplates were carried out over night at 37^0 C for 24 h. The diameter of inhibition halos was measured in the next day to evaluate the antibacterial activity. Streptomycin discs were used as standard (Prabha *et al*., 2009).

Antifungal activity

About 180 ml of Potato Dextrose Agar was prepared and after solidification appropriate fungal cultures were swabbed over the agar media in a proper manner. After swabbing the culture, the discs impregnated with the crude metabolite of *Virgibacillus* sp. (100 µg w/v dissolved in 1 ml of DMSO) with different volumes *viz*., 25µl, 50µl, 75µl were placed over the media .Then incubation of the petriplates were carried out at room temperature for 24 h. The diameter of inhibition halos was measured in the next day to evaluate the antifungal activity. Fluconozole discs were used as standard (Prabha *et al*., 2009).

Immunological Studies

Healthy rats (*Rattus norvegicus*) of either sex were selected for the study. The animals were fed on a commercial diet (Saienterprisei Pellets, Chennai) and water ad libitum. They were acclimated to laboratory hygienic conditions for two

weeks before commencing the experiment. Permission of the Institutional animal ethical committee was obtained for all animal experimentation as per the approved protocol (KU/IAEC/B. Pharm/020/2010).

Administration of Test Extract

Pyrogallol induced immunosuppression was employed to study the immunomodulatory effect of the crude metabolites obtained from the sponge associated marine bacterium *Virgibacillus* sp. Animals were randomly divided into four groups, consisting of six animals each. Group I animals served as control and received equivalent volume of sodium CMC (0.1% w/v) as a vehicle. Group II animals were administered pyrogallol (100mg/kg/i.p.daily for seven days). Group III animals were administered pyrogallol daily for seven days with the same dose and the test extract, suspended in 0.1 %(w/v) sodium carboxy methyl cellulose(CMC) with 100mg/kg daily, p.o from day 8-22. Group IV animals administered with pyrogallol daily for seven days and vitamin E suspension(150mg/kg p.o) beside above treatment all the groups received sheep red blood cells (SRBC,0.5X109 cells/100 g,i.p) on day 7 and 13, as the antigenic material to sensitize them for immunological studies(Atul et al.,2011).

Humoral antibody response to SRBC

On 13^{th} and 20^{th} day, the blood samples were collected from the retro-orbital plexus and the rat serum was used to determine the haemaglutination titer. In order to collect the serum the blood samples were subjected to centrifugation at 8000 rpm for 20 minutes and equal volume of individual serum samples of each group was pooled. Two fold dilutions of pooled serum samples made in 25µl in normal saline in micro titration plates was added 25µl of 1% SRBC suspension in saline. After mixing, the plates were incubated at 37°C for 1hrs and examined for haemaglutination. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titer.

Cell Mediated immune response (T cell E- rosette assay)

Isolation of B and T cells

Blood was collected from treated and control Wister rats previously mentioned, using a heparin pretreated vials; B and T cell count in the blood were carried out by the following method. 5-10 ml of blood was collected and it was introduced into sterile conical flask/ beaker containing (4-5) sterile glass beads. It was then continuously swirled until no sounds were heard from the beads. This indicates that all the fibrins have adhered to the beads. This blood was considered as defibrinated blood. This defibrinated blood was taken and diluted with equal volume of physiological saline; 4 ml of the lymphosep solution was taken in a centrifuge tube and added with equal volume of diluted blood in physiological saline drop by drop by keeping the centrifuge tubes at the slanting position and then the tubes were centrifuged at 1600 rpm for 20 minutes. The interphase (containing lymphocytes) was removed using pipette. The cells were washed with 1ml of saline. The sample was again washed with 1ml of saline. After centrifugation the pellet was suspended in RPMI 1640 medium and stored in refrigerator at 4°C.

Re-suspended lymphocytes were loaded into the activated nylon wool column. Then the column was held vertically above on eppendorf tube, now hot saline (at 60^0C) was slowly dripped into column (600µl). The hot saline passing out of the column was collected in the sterile eppendorf tube, which contain T lymphocytes. After collection the cold saline (600µl) was added along the top of the column to separate the B lymphocytes.

E- Rosette assay

The separated T lymphocyte of about 0.2 ml was taken in an eppendrof tube was added with 0.2 ml of 1% SRBC and the mixture was centrifuged at 1600 rpm for 12 minutes. After centrifugation the sample were incubated in a refrigerator at 4^0c for 5 minutes. After cold incubation, the pellet in the eppendorf tube was resuspended by gentle flushing with a Pasteur pipette. Then a drop of the sample was taken in a clean dry slide was observed for the formation of rosettes at 10 X and 40X. The rosettes formed were photographed using a camera (Sujatha *et al.,* 2010)

Delayed type Hypersensitivity Reaction

The edema was induced in the right paw of rat by injecting SRBC ($0.025X\ 10^{9)}$ in the sub planar region on 20^{th} day. After 48 hrs the increase in paw volume was assessed on a Dial Caliper. The mean percentage increase in foot bad volume was considered as delayed type hypersensitivity and as an index of cell mediated immune response. The left hind paw injected with phosphate buffered saline serves as a control (Dhasarathan et al., 2010).

Results & Discussion

Sponge Identification

The sponge was identified as *Callyspongia diffusa*.

Isolation of microorganisms from sponges

Ten different colonies of bacteria were isolated from the sponge *Callyspongia diffusa* and were designated as CSD 1- CSD 10. The isolates were restreaked two times to avoid the contamination from other microbes and maintained in Zobell marine agar slants at 4^0C.

Antagonistic activity

Antagonistic activity of bacterial isolates (CSD 1- CSD 10) was tested against the clinical pathogens. The strain CSD 5 showed broad spectrum of antagonistic activity against five pathogenic microbes. Hence the strain CSD 5 was further selected for the production of bioactive metabolites (Table 1).

Identification of Microorganisms

Based on the morphology, biochemical characterization (Table 2) and 16S rRNA sequencing (Fig.1) results the potential strain CSD 5 was confirmed as *Virgibacillus* sp.

Antimicrobial activity

The crude metabolite of *Virgibacillus* sp. showed potential antibacterial activity against the clinical bacterial and fungal isolates and fish-borne pathogens. Maximum level of inhibitory activity was found against the fish-borne pathogens

such as *Plesiomonas shigelloides, Vibrio alginolyticus, Vibrio mimicus, Vibrio parahaemolyticus, Vibrio harveyi* and *Aeromonas hydrophila* and the clinical isolates such as *Pseudomonas aeruginosa and Klebsiella pneumoniae*. Moderate level of inhibitory activity was found against the clinical pathogens viz., *Proteus vulgaris, Pseudomonas aeruginosa* and *E.coli* (Fig. 2, 3) .The antifungal activity was found to be significant against the fungal strain *Tricoderma* sp. (Fig.4). The present study revealed that metabolites produced by *Virgibacillus* sp. displayed broad spectrum of antimicrobial activity against both clinical and fish- borne pathogens.

Haemaglutination antibody titer (HA)

According to the results, the log2 titer values for the first group (Control,

Table. 1 Evaluation of Antagonistic Activity of *Callyspongia diffusa* associated bacterial strains against the pathogenic microorganisms

Bacterial isolates	**Sponge *Callyspongia diffusa* associated bacterial isolates screened for its antagonistic activity against the clinical and fish borne pathogens.**									
	CSD 1	CSD 2	CSD 3	CSD 4	CSD 5	CSD 6	CSD 7	CSD 8	CSD 9	CSD10
E.coli	+	+	+	+	+	+	+	+	+	+
Staphylococcus aureus	-	+	_	_	+	_	+	+	-	_
Vibrio mimicus	+	_	-	-	+	+	-	-	_	+
Serratia marcescens	-	-	-	-	-	-	-	-	-	-
Vibrio alginolyticus	-	+	-	+	+	-	-	-	+	-
Vibrio cholera	-	-	+	+	_	-	-	+	-	+
Salmonella typhi	-	-	-	-	_	-	-	-	+	-
Aeromonas hydrophila	+	-	+	-	+	+	+	-	-	-

+: Positive ; - : Negative.

Table. 2 Biochemical profile of the sponge *Callyspongia diffusa* associated Bacterium *Virgibacillus sp.*

Characteristic of isolates	***Virgibacillus* spp.**
Spore morphology	Spherical
Shape	25-48
Temperature range for growth(^{0} C)	
Growth in	+
0.5% NaCL	-
25% NaCL	+
Anaerobic Growth	-
Nitrate reduction	+
Hydrolysis of :	
Starch	+
Gelatin	+
Tween 20	+
Tween 80	+
Casein	+
H_2S Production	
Carbohydrate fermentation test:	
D-Glucose	+
D-Galactose	+
D-Fructose	+
D-Lactose	+
D- Mannitol	+
N acetyl glucosamine	+
Gram Staining	Gram positive , rod shape
Catalase test	Positive
Oxidase test	Positive
Citrate utilization test	Positive
Indole test	Positive
Methyl red test	Positive
Voges-Prascaurs test	Positive
Antibiotic Sensitivity test:	
Bacitracin	24 mm
Tetracycline	32mm
Offloxicilin	24mm
Streptomycin	29mm
Ciprofloxin	28mm
Neomycin	20mm
Amphicillin	13mm

Table.3 Evaluation of HA titer and E-Rosette formation in Wistar rats treated with metabolite of *Virgibacillus* spp.

GROUP	Dose level	Compound	DTH response (mm)	
			After 24 hrs.	After 48 hrs.
I	100 mg/kg (w/v)	0.1% CMC	8.10±0.12	8.78±0.03
II	100mg/kg (w/v)	Pyrogallol	8.10±0.12	8.42±0.06
III	100mg/kg (w/v)	Metabolite of *Virgibacillus* sp.	8.33±0.07	8.68±0.11
IV	150mg/kg (w/v)	Vitamin E suspension	9.80±0.34	9.87±0.36

DTH: Delayed Type Hypersensitivity and CMC: Carboxy Methyl Cellulose.

Table.4 Evaluation of Delayed Type Hypersensitivity response in Wistar rats treated with metabolite of *Virgibacillus* sp.

Group	Treatment	Ab (Log $^{2}_{2}$)	E –rosettes
I, Control	0.1% CMC	5.83±0.17	79.83±2.47
II	Pyrogallol (100mg/Kg)	4.33±0.21	38.17±0.65
III	Metabolite of *Virgibacillus* sp. (100mg/kg)	6.50±0.22	55.00±1.06
IV:	Vitamin E suspension(150mg/kg)	7.33±0.21	84.67±1.28

CMC: Carboxy Methyl Cellulose and Ab: Antibody.

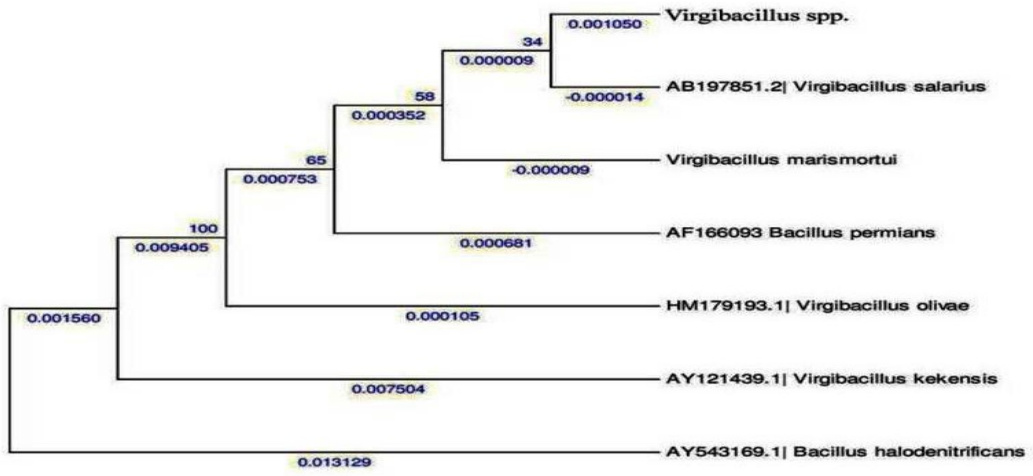
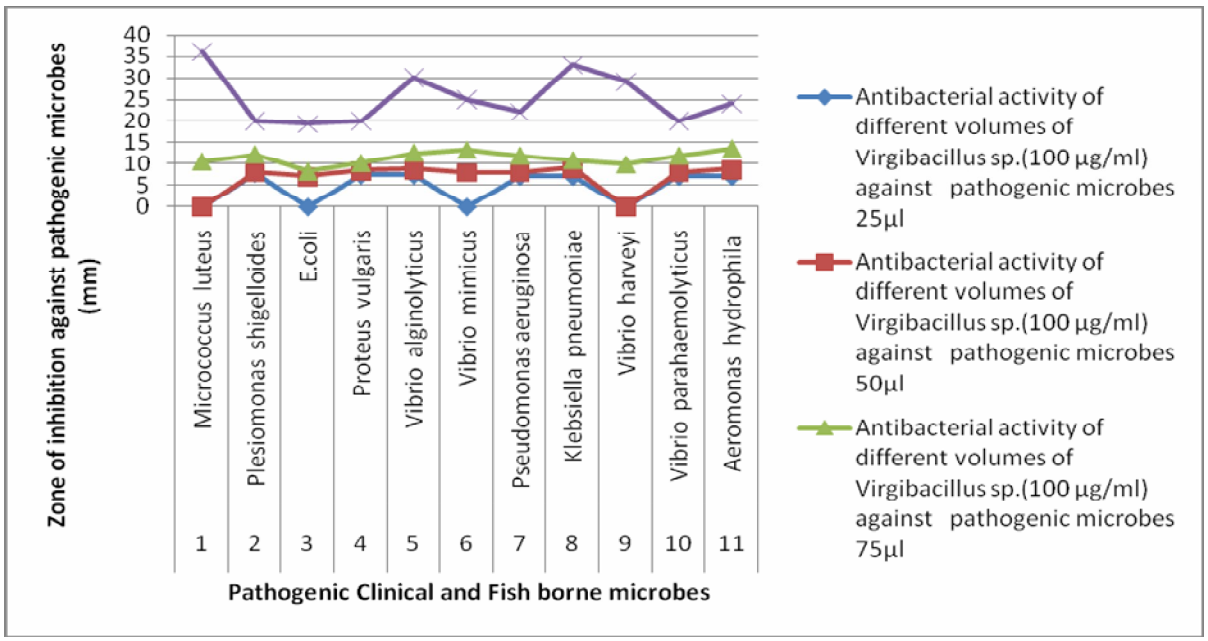


Fig.1 **Phylogenetic tree of the strain CSD-5 associated with the marine sponge *Callyspongia diffusa*.**

Figure.3 Antibacterial activity of metabolite of *Virgibacillus* spp. against pathogenic clinical and fish borne microorganisms



0.1% CMC) of animals were five and for the groups treated with the immunosuppressive drug pyrogallol was four and for the III group of rats six, which was treated with the metabolites from the marine bacterium *Virgibacillus* sp. associated with the sponge *Callyspongia diffusa* was and for the IV group treated with vitamin E suspension was six. (Table. 3).

Delayed Type Hypersensitivity Reaction (DTH)/Footpad Thickness Test in Rats

The DTH response was low for the second group of rat that was treated with the immunosuppressive drug pyrogallol when compared with the control group after 24 and 48 hours from the SRBC treatment. There was an increase in the DTH response in the group which was treated with the metabolite of *Virgibacillus* sp. The result indicated that the bacterial metabolite had elevated DTH response which is an expression of cell mediated immunity. The enhancement in bacterial metabolite treated group more or less similar to vitamin E treated group. The DTH response was also similar for the fourth group which has been treated with Vitamin E suspension (Table 4).

Haematological parameters

In hematology profile, the WBC count was decreased in group II treated with the immunosuppressive drug pyrogallol when compared with the control group. But in the III group treated with bacterial metabolite, WBC count increased significantly higher than that of the group II rats. Moreover in the IV group also the WBC count was elevated significantly (Fig.4) higher than that of the II, III group but was lower than that of the controlled group. The neutrophil count was increased in both groups (III and IV) significantly. Of the two groups, the group IV animals were found with greater level of production of neutrophils than that of the

group I. Also group III animals were found with an equal level of increase in the neutrophil counts when compared with control group, I. The WBC count in pyrogallol treated (group, II) was less when compared with the mean WBC count of the group I control. In the group III, IV the mean level of WBC count was increased than that of the group II animals treated with the immunosuppressive drug, pyrogallol (Fig.5). The mean eosinophil count had increased only in group II treated with immunosuppressive drug pyrogallol and not found in the remaining groups. The significant increase in the mean lymphocytes value was observed in both III and IV groups treated with bacterial metabolite and vitamin E suspension respectively but the group II animals treated with pyrogallol showed a low level of lymphocyte value when compared with group I control (Fig.6).

E- Rosette assay

In group II animals, the mean number of E-Rosette production was comparatively less than the group I, control. Meanwhile in the group III and IV an increase in number of rosettes formation was observed. This indicated that the in the group II animals T- lymphocyte synthesis in the thymus and their proliferation was affected by the action of the immunosuppressive drug. But in the group III and IV the bioactive compounds had stimulated the proliferation, which resulted in the formation of a higher number of rosettes in the E-rosette assay (Table 3).

Figure.4 Antifungal activity of Metabolite of *Virgibacillus sp.* against clinical fungal pathogens

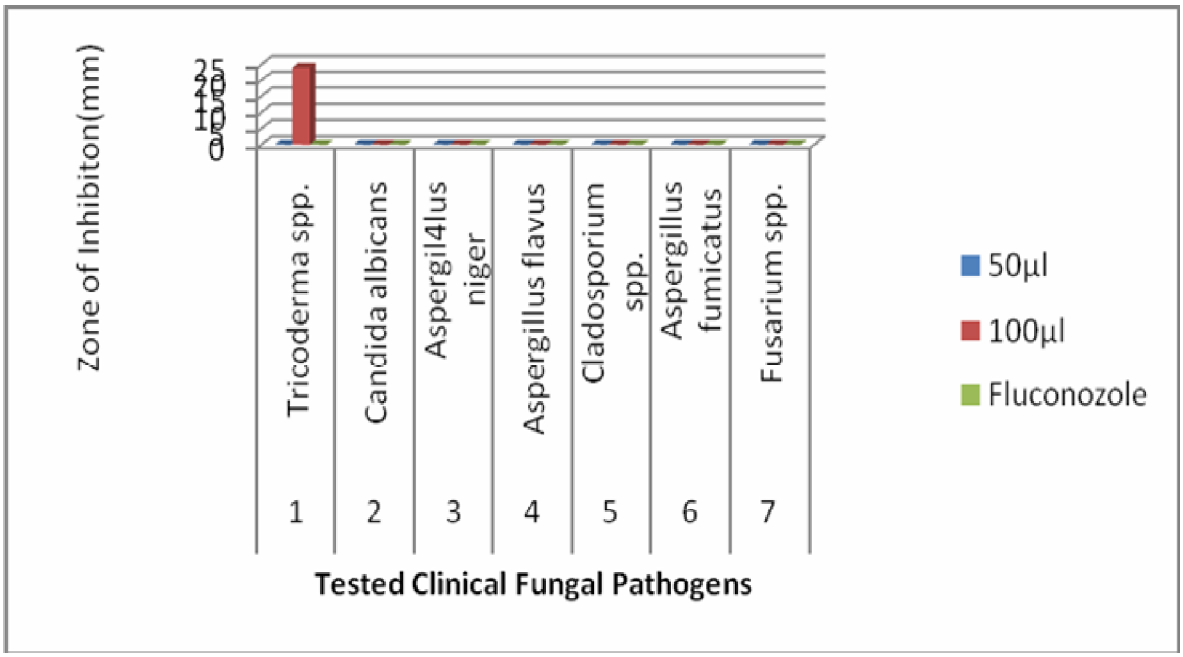


Figure.5 Evaluation of haematological indices of Wistar Rats treated with different types of compounds.

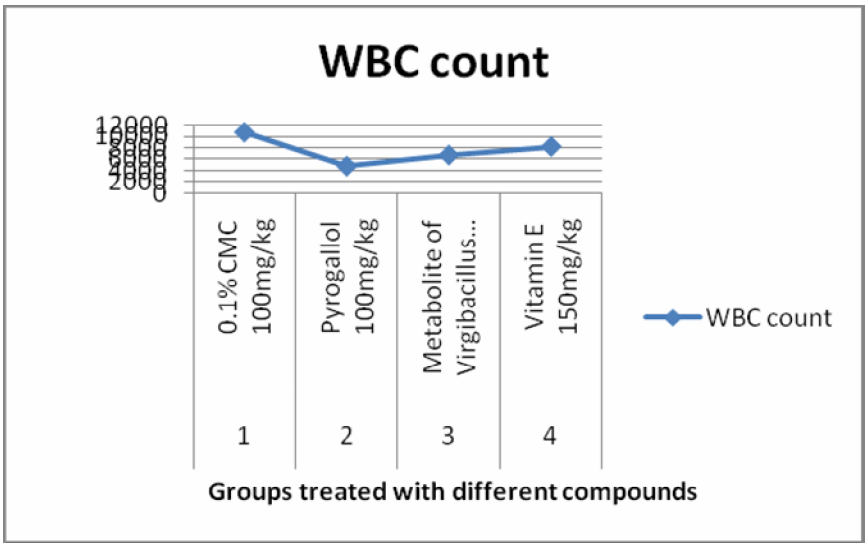
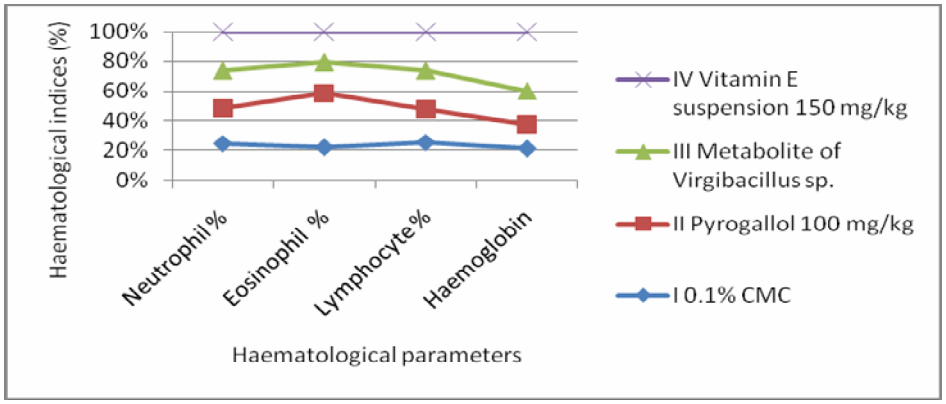


Figure.6 Haematological indices of Albino Wistar Rats treated with different types of compound



As the antimicrobial activity of bioactive metabolites was found to be effective against the fish-borne pathogens, it's expected that the potential compounds produced by this sponge residing microorganism may protect fishes living in association with sponge deposits by repelling out the pathogens. Bacteria of the genus *Vibrio* found abundantly in marine environments, often lead to microbial fouling and are responsible for coral and shellfish diseases (Israely *et al*., 2001). As *Virgibacillus sp.* has high activity against *Vibrio* sp., it can help to overcome the outbreak caused by these microorganisms in aquaculture industry. *Aeromonas hydrophila* are pathogens of fishes, crustaceans and other culturable organisms (Starliper and Morrision, 2000). As the metabolite from *Virgibacillus* sp. had shown a significant inhibitory effect against these organisms, it may contribute in aquaculture as an effective antibiotic where *Aeromonas hydrophila* causes

major outbreaks. The metabolite showed significant antifungal activity against the skin infection causing fungal isolate *Tricoderma* sp. Hence, it's possible to develop an ointment to prevent the skin infections caused by this fungal pathogen. The increased antibody titre value was found in group III rats treated with metabolite of *Virgibacillus* sp.

The fourth group rats treated with vitamin E solution also showed increased level of antibody titre valus. This result indicated that the bioactive compound and the vitamin E suspension showed its impact on the proliferation of B- lymphocytes in the murine immune system. The DTH response in group III rats treated with metabolite of *Virgibacillus* sp. is more or less similar to vitamin E treated group. This indicates the compound has its own potential activity in the proliferation of various types of T cells towards increasing the cell mediated immune response which was comparable to group IV rats treated with Vitamin E suspension. The immunomodulatory potential was due to the potential compound which present in the crude bacterial metabolite. Delayed type hypersensitivity requires the specific recognition of given antigen by activated T cells and release of cytokines. DTH is a part of the process of graft rejection, tumor immunity and most important immunity to many intracellular microorganisms. It can also be due to activation of complement, release of reactive oxygen or nitrogen sp. by activated phagocytes and proinflammatory cytokines.

The bacteria of the order Spingomonadales are yellow pigmented, Gram-negative, rod shaped bacteria that contain glycoshingolipids in their cell envelope (Yabuuchi *et al*., 1990). Glycosphingolipids are a class of compounds that had been shown to be potent stimulators of natural killer T cells (Kinjo *et al*.,2005; Mattner *et al*.,2005) Glycoshingolipids isolated from *Sphingomonas* sp. showed stimulatory effect on the natural killer T cells (Sriram *et al*., 2005) (Table 4).

The increased level of WBC count in group III may be due to enhanced haemopoietic activity due to the potential of bioactive metabolites. This had stimulated the proliferation of B cell clones to produce excess antibody when it was challenged with SRBC as an antigen. This indicated that the group II animals had undergone severe allergetic reactions caused by pyrogallol, an immunosuppressive drug. The elevation of B- lymphocytes was found in group III and group IV rats. The increase in number of B-lymphocytes count might have increased humoral immune response as evidenced in the hemaglutination titer of the immunized rats. Lymphocytes are responsible for the primary recognition of antigen and also function as immunologically specific effector cells. Lymphocytes produce cell surface molecules that serve as receptor sites (paratopes) for reaction with antigen and in turn produce antibodies (Didler and So,1998).The hemoglobin level in group II was decreased than that of the group I, control and in the group III and group IV it was increased . This result showed that the immunosuppressive drug pyrogallol has its damaging effects in the heme group of haemoglobin in the blood treated rats and in turn affects transport of oxygen in the blood vessels which led to breathing problem. The number of E-rosettes formation in group III rats were almost equal to the normal group I control rats. This is expected may be due to the presence of CD^4 receptors on the surface

of T-lymphocytes which is having the potential to make clumps with the SRBC, a cellular antigen.

In the present investigation, the sponge Callyspongia diffusa associated marine bacteria *Virgibacillus sp.* was found with potential antimicrobial and immunostimulatory potential. The antimicrobial activity of the bioactive metabolite produced by this strain showed effective activity in inhibiting the clinical as well as the fish-borne pathogens, it The crude metabolite of this bacterium had been found to stimulate both humoral as well as the cell mediated immune response. The sponge and bacterial association in the marine environment is being the major focus of the many marine researchers because the compounds produced by the associated microbes were found with potential pharmacological activity. Moreover the associated microbes on the sponges may provide some defensive mechanisms to depend it in the odd environment. From this research it is concluded that isolation and characterization of the exact bioactive compounds present in the crude metabolite of *Virgibacillus* sp. will help to develop a novel antimicrobials that will be useful in medical field and aquaculture industry as well.

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