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Antagonistic effect of bacteria associated with ascidians from Thoothukudi coast

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The antagonistic properties of marine ascidians and the bacteria associated with them were investigated. Ascidians were collected from submerged structures along the coast of Thoothukudi and four species of solitary ascidians and four colonial ascidians were identified. A total of 590 bacterial isolates from the ascidians were characterized up to generic level. All the bacterial isolates were tested for antagonistic property against indicator bacteria and 71 isolates were recognized as potentially antagonistic, however, only 9 of them could effectively inhibit test organisms that included six human and one shrimp pathogenic bacteria. The inhibitory activity of the crude extracts of extracellular products (ECP) of selected antagonistic bacteria was weak against test organisms, but the ethyl acetate crude extracts of both cells and cell free supernatants of antagonistic bacteria showed strong inhibitory activity. Extracts from DS_{13}, DP_{41} and P_{19} showed a high degree of inhibition. Methanol crude extract of *Eudistoma viride* showed good inhibitory activity against *Vibrio cholerae* and *V. harveyi*.

[**Keywords:** Ascidian, antagonistic bacteria, human pathogen, shrimp pathogen, methanol extract]

Introduction

Natural products derived from marine microorganisms have increased tremendously in recent years due to the demand for compounds having potential pharmaceutical applications and economical value as cosmetics, drugs, fine chemicals and functional personal-care products. Bioactive compounds have been isolated from marine invertebrates such as sponges[1], ascidians[2], mollusks and bryozoans[3], marine microorganisms and fungi[4]. Ascidians, commonly called sea squirts are marine sessile filter feeding animals belonging to the Phylum Chordata and Class Ascidiacea. Ascidians are reported to rank third next to sponges and bryozoans as rich sources of biologically active compounds[5]. A number of bioactive compounds have been isolated from ascidians, exhibiting activities such as antiviral[6], cytotoxic[7,8], antibacterial[9,10] and enzyme inhibitory activities[11] and these compounds are derivatives of alkaloids and peptides[12]. Numerous natural products from marine invertebrates show similarities to marine microbial metabolites suggesting that microorganisms are involved in the synthesis of these products[13]. Ascidians harbour microorganisms whose secondary metabolites inhibit the settlement of potential competitors and predators of ascidians[14]. The symbiotic microorganisms from the ascidians are a rich source of new metabolites with a range of biological activity and practical applications[15]. These metabolites affect bacteria in a number of ways, ranging from the induction of chemotactic response to the inhibition of bacterial growth or cell death[16]. The bioactive compounds of ascidians may possibly be derived from the secondary metabolites of the associated bacteria, therefore studying the antibacterial activity of the bacteria from the ascidians and antibacterial activity of ascidian extract will widen our understanding as to whether antibacterial activity of ascidians is due to the associated bacteria. Against this background, a study was proposed to elucidate the antagonistic bacterial flora associated with ascidians and to understand if the antibacterial substances produced by the bacteria and the ascidians have any similarities between them.

Materials and Methods

Marine ascidians were collected from three sampling sites of Thoothukudi, namely Thoothukudi Fishing Harbour, V. O. Chidambaram port and Tharuvaikulam, Thoothukudi. Samples were collected by snorkel diving and from trawl catch and were placed in sterile polythene bags, brought to the laboratory within an hour of collection and processed for identification and bacteriological analysis. The collected specimens were narcotized with

menthol crystals and fixed in formalin (mixture of 40% formaldehyde and seawater in the ratio 1:10 (effective concentration of 4%)) for identification. They were identified according to standard keys[17]. Bacteria were isolated from surface and whole body homogenate of ascidians and identified to generic level following taxonomic schemes[18,19].

A total of 25 bacterial isolates were evaluated for their antibacterial activity against 18 antibiotics namely ampicillin (10µg), cefoxitin (30µg), chloramphenicol (30µg), ciprofloxacin (5µg), colistin (10µg), erythromycin (15µg), furazolidon (50µg), gentamycin (10µg), kanamycin (30µg), nalidixic Acid (30µg), norfloxacin (10µg), penicillin G (10units), rifampicin (5µg), streptomycin (10µg), sulphafurazole (300µg), tetracycline (30µg), trimethoprim (5µg) and vancomycin (30µg) (M/s Himedia, Mumbai). Antibiogram was done by agar disc diffusion assay on Muller Hinton Agar (MHA)[20]. Those bacteria showing larger zones of inhibition and sensitive to most antibiotics were selected as indicator organisms for screening of antagonistic marine bacteria.

The cross streak method was followed for assaying the inhibitory activity of marine bacteria[21]. Marine bacteria which showed inhibitory activity against the indicator organisms were considered potentially antagonistic. Selected marine isolates from the preliminary screening of bacteria were further tested against human and shrimp pathogenic bacteria by the same method. *Escherichia coli* (MTCC 443), *Staphylococcus aureus* (MTCC 96), *Salmonella paratyphi* (MTCC 735), *Salmonella typhi* (Clinical isolate), *Pseudomonas aeruginosa* (MTCC 27853), *Vibrio cholerae* (Clinical isolate) and *Vibrio harveyi* (Shrimp larval isolate) were the human and shrimp pathogenic bacteria used in this study.

Well diffusion assay was followed for assaying of potential antibiotic producers against human and shrimp pathogenic bacteria[22]. In secondary screening, young cultures of antagonistic bacteria were inoculated in Seawater yeast extract peptone (SYEP) broth. The inoculated cultures were kept on a shaker at 80 rpm for 24 h at 30±2°C and used for well diffusion assay at a concentration of 10^6 – 10^7 cfu/ml. Wells of 5mm diameter were punched in Muller Hinton agar (MHA) plates seeded with test organisms. Alternatively stainless steel (SS) cylinders were placed over Muller Hinton agar plates for diffusion assay. 100µl of 24 h old broth culture of bacteria was added to the wells / cylinders and allowed for diffusion at 4°C for 30 min and the plates were incubated at 30±2°C for 24 h, subsequently inhibitory activity was detected as a zone of clearance around the wells. Diameter of the clearance zone was measured in millimeter. The cell free supernatant was prepared from the same culture after 48 h of growth in continuing agitation in a shaker at 80 rpm and centrifugation in a refrigerated centrifuge at 10,000 rpm for 15min at 5°C and supernatant collected. The supernatant was passed through 0.22 µm syringe filter and used for the assay as described above.

The extracellular products (ECP) were extracted from selected marine strains by ammonium sulphate precipitation[23]. The protein content of ECP was estimated[24]. The secondary metabolites of potential antibiotic producers were assayed against human and shrimp pathogenic bacteria by disc diffusion method. Sterilized Whatman No.1 filter paper discs (6mm) were impregnated with ECP of bacterial strains of known concentration of protein placed on Muller Hinton agar plates laid with uniform lawns of pathogenic bacteria. All plates were incubated at 4°C for 30 min for diffusion followed by incubation at 30±2°C for 24 h. The inhibition zone was measured in mm.

Extracellular enzymatic activities of antagonistic bacteria were tested by growing the culture on media with appropriate substrates. Isolates were examined for the production of lipase on lipid agar (Tributyrin agar), lecithinase on egg yolk agar, gelatinase on gelatin agar[25] and hemolysin on sheep and human blood agar[19].

Cell free culture extracts from marine antagonistic bacteria were tested for hemolytic activity by the method described by Sakurai *et al.*,[26], using human blood agar medium (2% washed human erythrocytes in TSAS medium). Clear β- hemolysis around the wells within 24 h was recorded as hemolytic. In addition to crude extracts of ECP, ethyl acetate extracts[27] of cultures as well as methanol extracts[28] of ascidians were also tested for hemolytic activity on blood agar plates by well diffusion assay. The zone of β- hemolysis was measured in mm.

Inhibitory activity of ethyl acetate crude extracts was tested against human and shrimp pathogenic bacteria by well diffusion assay or disc diffusion assay. Ethyl acetate extracts of harvested cells and cell free culture medium (spent medium) were prepared with slight modification of procedures described by Wratten *et al.,* [27]

Methanol extracts of ascidians was

prepared[28] and their protein content estimated[24]. The extracts of ascidians were individually tested for their antibacterial activity against pathogenic bacterial strains using the standard filter paper disc diffusion method[20]. Different concentrations of the prepared extracts were charged on the discs and aseptically dried to ensure evaporation of the solvents and were used for the assay on seeded plates and zone of inhibition recorded.

Minimum Inhibitory Concentration (MIC) and Minimum Cidal Concentration (MCC) of methanol extract of ascidians were determined against *Vibrio cholerae* and *V. harveyi*. The lowest concentration at which there was no growth of bacteria was taken as the MIC for ascidians extract. A loopful from the tubes of MIC test, where there was no growth was streaked on TSAS plates and observed for growth. The lowest concentration of ascidians extract that did not yield any colony growth on the medium after incubation was recorded as Minimum Cidal Concentration[29].

The methanol extract of one of the colonial ascidians that exhibited good inhibition against pathogenic bacteria was compared with commercial antibiotics against pathogenic bacterial strains. Discs impregnated with 10µg concentration of prepared extracts along with commercial antibiotic discs were placed on seeded Muller Hinton agar medium. The zone of inhibition was compared and recorded.

The isolates P_{19}, DP_{41} and DS_{13} were further confirmed by PCR using a universal primer[30]. Bacterial DNA was extracted and 16S rRNA gene was amplified[30, 31] and the PCR products were visualized using gel documentation system (Biorad, USA). PCR products were purified using HiYield PCR DNA extraction kit (RBC, USA) and sequenced in an automated DNA sequencer at *1st Base*, Malaysia. Sequences were checked for chimera and other sequence anomalies using pin tail programme and compared with the sequence available in NCBI – Gen bank using the BLAST tool and identified based on their homology.

Results

Four solitary and four colonial ascidians were collected from country crafts, submerged rocks and trawl net catch along Thoothukudi. Solitary ascidians were identified as *Cnemidocarpa aerolata*, *Herdmania momus, Microcosmus exasperatus* and *Phallusia nigra* and colonial ascidians as *Didemnum psammathodes*, *Diplosoma swamiensis*, *Eudistoma viride* and *Lissoclinum fragile*.

Indicator organisms were selected based on their sensitivity to antibiotics. 25 marine bacterial isolates from ascidians were subjected to antibiogram using 18 antibiotics. Based on their sensitivity pattern, 5 isolates that were highly susceptible to most antibiotics, belonging to *Arthrobacter* sp. (SA_9, SA_{10} and SA_{15}) and *Bacillus* sp. (SA_7 and SA_{25}) were chosen as indicator bacteria for screening antagonistic bacteria from ascidians. Five hundred and ninety bacterial isolates from ascidians were tested for their inhibitory activity against these five indicator organisms, of which 71 (12%) isolates belonging to both Gram positive and Gram negative bacteria showed antagonistic activity. The antagonistic bacterial isolates were identified as *Arthrobacter* sp. (12.6%), *Bacillus* sp. (78.9%), *Flavobacterium* sp. (2.8%) and *Vibrio* sp. (5.6%).

Antibiotic producing organisms that exhibited inhibitory activity against indicator organisms were tested for their ability to inhibit human pathogenic bacteria and shrimp pathogenic bacteria. As shown in Table 1, out of 71 antagonistic bacteria only 9 bacterial strains showed inhibitory activity against one or more of the test organisms. Of these 9 antagonistic bacteria that exhibited inhibitory activity against test organisms, 7 were identified as *Bacillus* sp. (AS_1, AS_3, AS_8, DP_{41}, DS_{26}, DS_{13} and DP_{11}) and one as A*rthrobacter* sp (DS_{32}) and one as *V. alginolyticus* (P_{19}). Of these, *B. firmus* (DP_{11}) and *Bacillus* sp. (DP_{41}) showed greater inhibitory activity against many test organisms upon cross streaking assay.

The crude extracts of extra cellular products (ECP) prepared by ammonium sulphate precipitation of cell free culture supernatant of 48 h old cultures of antagonistic bacteria (spent medium), although had low protein content exhibited inhibitory activity.

The inhibitory activity of ethyl acetate crude extract of cells and spent medium of six antagonistic bacteria (AS_8, AS_3, DP_{11}, DP_{41}, DS_{13}, P_{19}) was not similar against the tested pathogenic bacteria (Fig. 1). Crude extracts of cultures DS_{13}, DP_{41} and P_{19} (Table 2) were very effective in inhibiting all test organisms at both the concentrations used. Extracts of DP_{11} was effective against some organisms at 100µl concentration but not at 50µl concentrations. Extract of AS_3 was effective only against *S. typhi, P. aeruginosa* (MTCC 27853) and *V. cholerae*. Ethyl acetate crude extracts of the spent medium of all antagonistic bacteria could effectively inhibit all test organisms, however the zone of inhibition varied with different organisms (Fig. 2) (Table 3). The cell free supernatant showed least

activity against *S. paratyphi* (MTCC 735) and *V. cholerae* (Clinical isolate). Isolates P_{19}, DP_{41} and DS_{13} were further confirmed by PCR using a universal primer. Amplicons were sequenced and the anomaly-free partial sequences of 16S rRNA gene were obtained. BLAST search in the GenBank database revealed that three isolates, P_{19}, DP_{41} and DS_{13} showed 97-98 % similarity to the type strains of *V. alginolyticus*, *B. firmus* and *B. firmus* respectively. Based on the similarity of the 16S rRNA gene sequence to the available sequence in the NCBI database the isolates P_{19}, DP_{41} and DS_{13} were confirmed and deposited in NCBI GenBank as *Vibrio alginolyticus* (JQ24027), *Bacillus firmus* (JN993992) and *Bacillus firmus* (JN993991).

Table 1 - Antagonism of bacterial isolates from ascidians against human and shrimp pathogens by cross streaking method

S. No.	Source	Code	Genus	Inhibition against Pathogenic bacteria						
				T_1	T_2	T_3	T_4	T_5	T_6	T_7
1.	*Herdmania momus* (Epibionts)	AS_1	*Bacillus* sp.	-	-	-	+	-	-	-
2.	*Microcosmus exasperatus* (Epibionts)	AS_8	*Bacillus* sp.	+	-	-	-	-	-	+
3.	*Microcosmus exasperatus* (Epibionts)	AS_3	*Bacillus* sp.	+	-	-	-	-	-	+
4.	*Didemnum psammathodes*	DP_{11}	*Bacillus firmus*	+	-	-	+	-	+	+
5.	*Didemnum psammathodes*	DP_{41}	*Bacillus* sp.	-	-	-	+	-	+	+
6.	*Diplosoma swamiensis*	DS_{26}	*Bacillus* sp.	-	-	-	+	-	-	-
7.	*Diplosoma swamiensis*	DS_{13}	*Bacillus firmus*	-	-	-	-	-	+	+
8.	*Diplosoma swamiensis*	DS_{32}	*Arthrobacter* sp.	+	-	-	-	-	-	-
9.	*Microcosmus exasperatus*	P_{19}	*Vibrio alginolyticus*	-	-	-	+	-	+	-

+ Inhibition - No inhibition

T_1 – *Escherichia coli* (MTCC 443); T_2 – *Salmonella paratyphi* (MTCC 735); T_3 – *Salmonella typhi* (Clinical isolate); T_4 – *Pseudomonas aeruginosa* (MTCC 27853); T_5 – *Vibrio cholerae* (Clinical isolate); T_6 – *Vibrio harveyi* (Shrimp larval isolate); T_7 – *Staphylococcus aureus* (MTCC 96)

Table 2 - Inhibitory level of ethyl acetate crude extract of cells of antagonistic bacteria

Culture No.	Volume (µl)	Zone of inhibition against						
		T_1	T_2	T_3	T_4	T_5	T_6	T_7
AS_3	50	×	×	+ + + +	+	+ +	×	+ +
	100	×	×	+ + + +	+ + +	+ +	×	×
AS_8	50	×	×	+ + + +	+	×	×	×
	100	×	×	+ + + +	+	×	×	×
DS_{13}	50	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
	100	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + +
DP_{41}	50	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + +
	100	+ + + +	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
DP_{11}	50	×	×	+	×	×	×	×
	100	+ + +	×	+ + +	×	×	+ +	+ + + +
P_{19}	50	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + +
	100	+ + + +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
Control		×	×	×	×	×	×	×

× : No inhibition + : ≤ 10mm + + : 10-20mm + + + : 20-30mm + + + + : >30mm

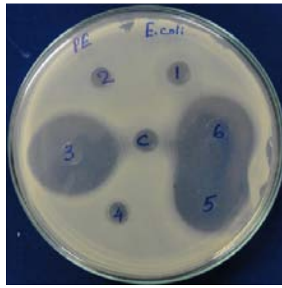
T_1 – *Escherichia coli* (MTCC 443); T_2 – *Salmonella paratyphi* (MTCC 735); T_3 – *Salmonella typhi* (Clinical isolate); T_4 – *Pseudomonas aeruginosa* (MTCC 27853); T_5 – *Vibrio cholerae* (Clinical isolate); T_6 – *Vibrio harveyi* (Shrimp larval isolate); T_7 – *Staphylococcus aureus* (MTCC 96)

Table 3 - Inhibitory activity of ethyl acetate crude extracts of spent medium of antagonistic bacteria against pathogenic bacteria

Culture No.	Volume (µl)	Zone of inhibition against						
		T_1	T_2	T_3	T_4	T_5	T_6	T_7
AS_3	100	+ + + +	+ +	+ + + +	+ + + +	+ +	+ + + +	+ + + +
AS_8	100	+ + + +	+ +	+ + + +	+ + + +	+ +	+ + + +	+ + + +
DS_{13}	100	+ + + +	+ +	+ + + +	+ + + +	+ +	+ + + +	+ + + +
DP_{11}	100	+ + + +	+ +	+ + + +	+ + +	+ +	+ + + +	+ + + +
DP_{41}	100	+ + + +	+ +	+ + + +	+ + +	+ +	+ + + +	+ + + +
P_{19}	100	+ + + +	+ + +	+ + + +	+ + +	+ +	+ + + +	+ + + +
Control	100	×	×	×	×	×	×	×

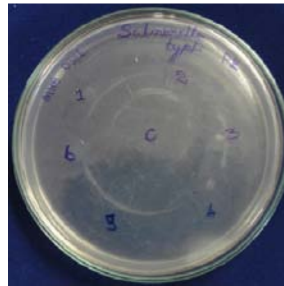
× : No inhibition + : ≤10mm + + : 10-20mm + + + : 20-30mm + + + + : >30mm

T_1 – *Escherichia coli* (MTCC 443); T_2 – *Salmonella paratyphi* (MTCC 735); T_3 – *Salmonella typhi* (Clinical isolate); T_4 – *Pseudomonas aeruginosa* (MTCC 27853); T_5 – *Vibrio cholerae* (Clinical isolate); T_6 – *Vibrio harveyi* (Shrimp larval isolate); T_7 – *Staphylococcus aureus* (MTCC 96)



T_1 – *Escherichia coli* (MTCC 443)

T_2 – *Salmonella paratyphi* (MTCC 735)



T_3 – *Salmonella typhi* (Clinical isolate)

T_4 – *Pseudomonas aeruginosa* (MTCC 27853)

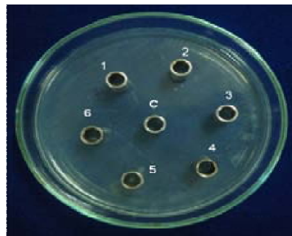
T_5 – *Vibrio cholerae* (Clinical isolate)

T_6 – *Vibrio harveyi* (Shrimp larval isolate)

T_7 – *Staphylococcus aureus* (MTCC 96)

1- AS_3
2- AS_8
3- DS_{13}
4- DP_{41}
5- DP_{11}
6- P_{19}
C- control

Fig. 1 - Inhibitory activity of ethyl acetate crude extract (50 µl) of cells of antagonistic bacteria against test organisms



T_1 – *Escherichia coli* (MTCC 443)

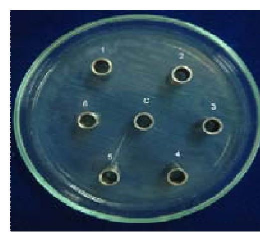
T_2 – *Salmonella paratyphi* (MTCC 735)

T_3 – *Salmonella typhi* (Clinical isolate)

T_4 – *Pseudomonas aeruginosa* (MTCC 27853)

T_5 – *Vibrio cholerae* (Clinical isolate)

T_6 – *Vibrio harveyi* (Shrimp larval isolate)



T_7 – *Staphylococcus aureus* (MTCC 96)

1- AS_3
2- AS_8
3- DS_{13}
4- DP_{41}
5- DP_{11}
6- P_{19}
C- control

Fig. 2 - Inhibitory activity of ethyl acetate extract (50 µl) of cell free supernatant of antagonistic bacteria against test organisms

All antagonistic bacteria were examined for their extracellular activities on suitable substrates. None of them produced hemolysin on human and sheep blood agar. All the cultures could hydrolyse gelatin. In addition to gelatinase, culture DS_{26} produced lipase and lecithinase, while cultures DS_{32} and P_{19} produced only lipase. Crude extracts of ECP of cultures did not exhibit hemolytic activity.

Antibacterial effect of methanol crude extracts from ascidians, *D. psammathodes*, *E. viride* (colonial ascidians), *H. momus* and *M. exasperatus* (solitary ascidians) was screened against human and shrimp pathogenic bacteria. Crude methanol extract from *D. psammathodes* exhibited moderate antibacterial activity against *P. aeruginosa*, with zone of inhibition level of 14mm, however, antibacterial activity was not observed against other pathogenic bacteria. Methanol extracts of *E. viride* showed strong antibacterial activity against *V. harveyi* and *V. cholerae* (28mm, 26mm) moderate activity against *E. coli* (MTCC 443), *S. aureus* (MTCC 96), *S. parayphi* (MTCC 735) and *S. typhi* (clinical isolate) (14mm) and lower activity against *P. aeruginosa* (MTCC 27853) (10mm) (Table. 4).

Antibacterial activity of methanol extract *H. momus* was moderate against *P. aerugenisa* (10mm) and *E.coli* (8mm) when tested at a concentrations of 50µg/disc. Extract of *M. exasperatus* showed inhibition against *S. paratyphi* (10mm) and *P. aeruginosa* (10mm). These ascidian extracts also exhibited β-hemolysis when charged with 20 µl volume on human blood agar.

The MIC and MCC values of methanol extracts of *E. viride* as shown by the protein concentration were 6.25 and 50 µg against *V. cholerae* and 3.125 and 12.5 µg against *V. harveyi* respectively.

A comparison was made between the methanol crude extract of *E. viride* and commercial antibiotics of various groups against five pathogenic bacteria. At 10µg concentration, the methanol extract effectively inhibited *V. cholerae*, *V. harveyi* and *Salmonella paratyphi* (MTCC 735). The inhibition was less against *Staphylococcus aureus* (MTCC 96). As against *V. cholerae*, the effect of ascidians extract was similar or more than most antibiotics tested. The zone of inhibition of methanol extract at 10µg concentration was comparable to chloramphenicol and more than that of erythromycin, nalidixic acid, streptomycin and trimethoprim (Table 5). At the tested concentration, *E. coli* was not inhibited by the ascidian extract.

Table 4 - Inhibitory activity of methanol crude extracts of ascidians against human and shrimp pathogenic bacteria

S. No.	Source	Protein content (µg/ml)	Conc. /disc (µg/disc)	Zone of inhibition (diameter in mm)						
				T_1	T_2	T_3	T_4	T_5	T_6	T_7
1.	*Didemnum psammathodes*	380	25	-	-	-	14	-	-	-
2.	*Eudistoma viride*	190	20	14	14	14	10	26	28	14
3.	*Herdmania momus*	620	50	8	-	-	10	-	-	-
4.	*Microcomus exasperatus*	615	50	-	10	-	10	-	-	-

- No inhibition

T_1 – *Escherichia coli* (MTCC 443); T_2 – *Salmonella paratyphi* (MTCC 735); T_3 – *Salmonella typhi* (Clinical isolate); T_4 – *Pseudomonas aeruginosa* (MTCC 27853); T_5 – *Vibrio cholerae* (Clinical isolate); T_6 – *Vibrio harveyi* (Shrimp larval isolate); T_7 – *Staphylococcus aureus* (MTCC 96)

Table 5 - Comparison of inhibitory activity of methanol crude extract of *Eudistoma viride* and commercial antibiotics

S. No.	Ascidian extract / Antibiotics	Inhibition against human and shrimp pathogens (zone of inhibition in mm)				
		T_1	T_2	T_5	T_6	T_7
1.	*Eudistoma viride* (10µg)	R	14	22	16	10
2.	Ampicillin (10µg)	R	16(IM)	32(S)	R	R
3.	Chloramphenicol (30µg)	22(S)	20(S)	22(S)	20(S)	22(S)
4.	Erythromycin (15µg)	10(R)	10(R)	20(S)	14(IM)	12(R)
5.	Nalidixic acid (30µg)	16(IM)	18(IM)	16(IM)	14(IM)	18(IM)
6.	Streptomycin (10µg)	10(R)	10(R)	10(R)	R	10(R)
7.	Tetracycline (30µg)	R	16(IM)	26(S)	14(IM)	16(IM)
8.	Trimethoprim (5µg)	16(S)	16(S)	6(R)	R	14(IM)

R – Resistant; IM – Intermediate; S – Sensitive

T_1 – *Escherichia coli* (MTCC 443); T_2 – *Salmonella paratyphi* (MTCC 735); T_5 – *Vibrio cholerae* (Clinical isolate); T_6 – *Vibrio harveyi* (Shrimp larval isolate); T_7 – *Staphylococcus aureus* (MTCC 96)

Discussion

Ascidians are excellent sources of biologically active compounds and its metabolites posses antimicrobial, antiviral, anti-inflammatory and cytotoxic activities[9,32]. In the present study eight different ascidians samples were collected, characterized and harnessed as source of antibacterial substances and antagonistic bacteria.

For the isolation of marine antagonistic bacteria from ascidians, pigmented and non pigmented colonies were observed as it has been previously documented that antibiotic producing marine bacteria were often pigmented[33,34]. However in the study, the potential antibiotic producers were non pigmented. The population of non pigmented bacteria to the total bacterial isolates from marine samples was 87.1%. A few bacterial isolates with high antibiotic susceptibility were used as indicator bacteria for screening of marine bacterial isolates for antibiotic production. Indicator bacteria belonged to *Bacillus* and *Arthrobacter* sp. and one of them (SA_{25}) was sensitive to as many as 13 out of 18 antibiotics tested. These antibiotic sensitive bacterial isolates were used with the assumption that they would be highly sensitive to the inhibitory substances produced by antagonistic marine bacteria. Accordingly, out of the total of 590 bacterial isolates screened, 71 isolates were potentially antagonistic inhibiting the indicator bacteria. These antagonistic bacteria were mostly isolated from solitary ascidians (68%) and again most of the antagonistic bacteria belonged to *Bacillus* sp. However, in an earlier study, the majority of antagonistic bacteria isolated from marine samples including ascidians were Gram negative[34] and 37.35% of the antagonistic bacteria were pigmented. In this study, Gram negative bacterial isolates belonging to *Flavobacterium* and *Vibrio* also exhibited antagonistic activity against indicator bacteria but not against pathogenic bacteria except a very few. Out of the 71 potentially antagonistic bacterial isolates only 9 isolates had the ability to exhibit inhibitory activity against four test organisms that included *Escherichia coli* (MTCC 443), *P. aeruginosa* (MTCC 27853), *V. harveyi* (shrimp larval isolate) and *S. aureus* (MTCC 96). None of the isolates had the ability to inhibit *S. paratyphi* (MTCC 735) and *V. cholerae* by cross streaking assay. However, when live cultures were examined by well diffusion assay, four cultures could inhibit *Salmonella* sp. Many of the isolates from ascidians belonging to genus *Bacillus* were reported to possess inhibitory activity against Gram positive and Gram negative bacterial pathogens[12].

Based on the inhibition of antagonistic bacteria against test organisms, six isolates were selected for extraction of antibacterial substances produced by them. Crude extracts of ECP of these antagonistic bacteria did show mild inhibitory activity against most test organisms. Even against T_2 (*S. paratyphi*, MTCC 75) which none of the antagonistic bacterial cells could inhibit on cross streak assay, the crude extracts of ECP could suppress the growth. However, the activity was very poor especially against T_3 (*S. typhi*, clinical isolate) T_4 (*P. aeruginosa,* MTCC 27853) and T_5 (*V. cholera*, clinical isolate) and the protein content was also very low. Therefore, it was assumed that the antibacterial substance may not be proteinaceous in nature. A few published reports have documented proteinaceous antibacterial substance from *Alteromonas*[35,36] while there are also reports of antibacterial substance being other than proteinaceous[33,34], as live cultures exhibited antibacterial activity but not crude extracts of ECP. The poor activity of ECP observed in the study may possibly be related to the disc diffusion assay as it was explained earlier[37] that if the compounds were cationic, they would be expected to adsorb to the surface of the disc and not diffuse into the medium.

Therefore, ethyl acetate crude extracts of both cells and culture free supernatant of antagonistic bacteria were tried which revealed excellent inhibitory activity. Extracts of cells of DS_{13} (*B. firmus*), P_{19} (*V. alginolyticus*) and DP_{41} (*B. firmus*) and extracts of culture free supernatants of all antagonistic bacteria exhibited inhibitory activity against all test organisms. These observations confirm that the antibacterial substance produced by the selected antagonistic bacteria may not be proteinaceous but of some other nature, which has not been deduced in this study. The efficiency with which ethyl acetate crude extracts of both cells and cell free supernants could inhibit pathogenic bacteria in this study was much higher than that observed with antagonistic *Alteromonas* sp.[34]. Further, the cytotoxic potential and other enzyme activities of these antagonistic bacteria when examined did not exhibit hemolytic activity, however all the cultures showed gelatinase activity, which is quite common with *Bacillus* sp. Thus some of these antagonistic bacteria could ideally be used as a source of antibacterial substance against human and shrimp pathogenic bacteria[35,37].

Ascidians have been reported to contain a wealth of interesting pharmacological substances including

antimicrobials[38]. The present investigation was aimed at understanding any possible relationship between the antibacterial substances produced by ascidians and the bacteria associated with them as several workers have documented similarities between the bioactive natural products from marine invertebrates and the metabolites of their associated microorganisms including bacteria[1,39,40]. In the present study, the antibacterial activity of the methanol crude extracts of four ascidians were evaluated and one of the four, *Eudistoma viride* exhibited inhibitory activity against all pathogenic test organisms with very strong activity against *Vibrio* sp. Other ascidians showed weak inhibitory effect against one or two test organisms. These results are in conformity with earlier studies who have found that the antibacterial activity was higher with the extract of *E. viride* than that of *D. psammathodes*[40]. Methanol extraction is the method of choice by earlier researchers and comparison of different polar and non-polar solvents for preparation of ascidian extracts revealed methanolic extract to have the highest cytotoxic activity and strong antibacterial activity[8]. Study on the antimicrobial activity of crude extracts of simple and colonial ascidians against eight bacterial pathogens showed that maximum inhibition zone (21mm) was observed from methanol extract against *Shigella boydii*[42]. However in another study, ethyl acetate extract was reported to have slightly higher inhibitory activity than ethanol extract of ascidans[43]. Although ascidian extracts have shown good bacterial activity, the active compounds in these ascidians were found at very low concentrations[8]. In this study, extract from *E. viride* could bring about higher level of inhibition at 20µg level than other ascidian extracts, thus proving to be an ideal source for harnessing antibacterial substances.

The MIC and MCC values of *E. viride* extract against *V. cholerae* observed in this study was very less when compared to the values obtained for ascidian *Aplidium multiplicatum* against other clinical isolates[43] and methanolic extract of ascidian, *Phallusia arabica* against human clinical isolates[44]. Studies have reported that methylene chloride extract of the ascidian *Ascidia sydneiensis* had MIC value of 1 µg/ml against *Streptococcus pneumonia*, *Shigella* sp., *Klebsiella pneumonia* and *Pseudomonas putida* and 10 µg/ml for *Staphylococcus epidermidis*[45]. Antibacterial activity of methanol extracts of *Polyclinum madrasensis* against clinical isolates showed the MIC and MCC to range between 0.70-0.95 mg/ml and 0.85-1.1 mg/ml respectively[46]. When evaluated against antibiotics of various groups, extracts of *E. viride* at 10µg level was comparable with tetracycline and erythromycin (macrolide) and better than ampicillin (betalactamine) streptomycin (aminoglycosidase) and trimethoprim. The effect of Nalidixic acid (quinolone) was higher against some test organisms while chloramphenicol was much more effective against all test organisms than that of the ascidian extract. Such a comparison of antibiotics with ascidian extract against several pathogenic organisms was reported previously[10] who suggested that ascidian extract may possibly be thought as an alternative for antibiotics in future to solve the problem of multi drug resistance in microorganisms. In general, the extract of *E. viride* could effectively control *Vibrio* sp. than other test organisms even at low concentration, suggesting that the antimicrobial substances are more effective against marine pathogenic bacteria which are naturally encountered in their normal habitats.

The methanol crude extracts of all four ascidians exhibited hemolytic activity on human blood agar suggesting that the mechanism of cytotoxicity is a result of membrane damage. Ascidian extracts exhibiting hemolytic activity over a variety of mammalian erythrocytes including human was well documented[8]. However, extracts of *E. viride* did not show hemolytic activity although cytotoxic effect was observed with tumour cell lines and on sea urchin embryos[47].

Conclusion

Ascidians have strong antibacterial compounds and thus could serve as a potential source for looking into newer alternative for antibiotics used in clinical treatments. Further, bacteria from marine sources are rich in bioactive compounds that could be harnessed for newer drugs for therapeutical applications.

References

1. Proksch, P., Edrada, R.A. & Ebel, R., Drugs from the sea - current status and microbiological implications, *Appl. Microbiol. Biotechnol*., 59(2002) 125-134.
2. Jang, W.S., Kim, H.K., Lee, K.Y., Kim, S.A., Han, Y.S. & Lee, I.H., Antifungal activity of synthetic peptide derived from Halocidin antimicrobial peptide from the tunicate, *Halocynthia aurantium*, *FEBS Lett*., 580(2006) 1490-1496.
3. Jimenez, J.T., Sturdikova, M. & Sturdik, E., Natural products of marine origin and their perspectives in the discovery of new anticancer drugs, *Acta. Chemica. Slovaca*., 2(2009) 63-74.

4. Austin, B., Novel pharmaceutical compounds from marine bacteria, *J. Appl. Bacteriol*., 67(1989) 461-470.
5. Davis A R & Bremner J B, Potential antifouling natural products from ascidian: A Review, in: *Bioactive Compounds from Marine Organisms*, edited by M. F. Thompson, R. Sarojini & R. Nagabhushanam, (Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi) 1999, pp. 259-310.
6. Rinehart, K.L., Gloer, J.B., Wilson, G.R., Hughes, R.G., Li, L.H., Renis, H.E. & Mc Govern, J.P., Antiviral and antitumor compound from tunicates, *Fed. Proc*., 42(1983) 87-90.
7. Ogi, T., Taira, J., Margiastuti, P. & Ueda, K., Cytotoxic metabolites from the Okinawan ascidian *Diplosoma virens*, *Molecules*., 13(2008) 595- 602.
8. Bragadeeswaran, S., Ganesan, K., Prabhu, K., Balasubramanian, T., Venkateswarlu, Y. & Meenakshi, V.K., Pharmacological properties of biofouling ascidian *Polyclinum madrasensis* Sebastian, 1952 from Tuticorin coast of India, *Adv. Environ. Biol*., 4(2010) 138-146.
9. Anand, T.P. & Edward, J.K.P., Antimicrobial activity in the tissue extracts of five species of cowries *Cypraea* spp. (Mollusca: Gastropoda) and an ascidian *Didemnum psammathodes* (Tunicata: Didemnidae), *Indian J. Mar. Sci*., 31(2002) 239-242.
10. Amutha, B.K., Meenakshi, V.K. & Senthamarai, S., Evaluation of antibacterial activity and antimitotic activities of biofouling marine ascidian extracts of Tuticorin coast, *Intl. J. Pharma. Sci*., 2(2010) 750-758.
11. Romanenko, L.A., Kalinovskaya, N.I. & Mikhailov, V.V., Taxonomic composition and biological activity of microorganisms associated with a marine ascidian *Halocynthia aurantium*, *Russian J. Mar. Biol*., 27(2001), 291-295.
12. Aneiros, A. & Garateix, A., Bioactive peptides from marine sources: pharmacological properties and isolation procedures, *J. Chromatogr. B,* 803(2004), 41-53.
13. Chelossi, E., Milanese, M., Milano, A., Pronzato, R. & Riccardi, G., Characterization and antimicrobial activity of epibiotic bacteria from *Petrosia ficiformis* (Porifera, Demospongiae), *J. Expt. Mar. Biol. Ecol*., 309(2004) 21-33.
14. Tait, E., Carman. M. & Sievert, S.M., Phylogenetic diversity of bacteria associated with ascidians in Eel pond (Woods Hole, Massachusetts, USA), *J. Expt. Mar. Biol. Ecol*., 342(2007) 138-146.
15. Fenical W & Jensen, P R, Marine microorganisms: A new biomedical resource. in. *Marine Biotechnology. Pharmaceutical and Bioactive Natural Products.* Edited by Attaway D H, Zaborsky O R (London Plenum Press, New York) 1993, pp. 419-459).
16. Paul, V.J. & Ritson-Williams, R., Marine chemical ecology, *Review Nat. Prod. Rep*., 25(2008) 662-695.
17. Meenakshi, V.K., *Marine biodiversity - Taxonomy of Indian ascidians. Final technical report submitted to the ministry of environment and forests*, New Delhi, 2003 pp. 1-103.
18. Le Chevallier, M.W., Seidler, R.J. & Evans, T.M., Enumeration and characterization of standard plate count bacteria in chlorinated and raw water supplies, *Appl. Environ. Microbiol*., 40(1980) 922-930.
19. Surendran, P.K., Nambiar, V.N., Lalitha, K.V., *Laboratory manual on microbiological examination of seafood*. Central Institute of Fisheries Technology. Cochin. India, 2003.
20. Bauer, A.W., Kirby, W.M.M., Sherris, J.C. & Turck, M., Antibiotic susceptibility testing by a standardized single disc method, *Am. J. Clin. Pathol*., 45(1966) 493-496.
21. Lemos, M.L., Dopazo, C.P., Toranzo, A.E., Barja, J.L., Competitive dominance of antibiotic producing marine bacteria in mixed cultures, *J. Appl. Bacteriol*., 51(1991) 228-232.
22. Tagg, J.R. & McGiven, A.R., Assay system for bacteriocins, *Appl. Microbiol*., 21(1971) 943-944.
23. Honda, T., Goshima, K., Takeda, Y., Sugino, Y. & Miwatani, T., Demonstration of the cardiotoxicity of the thermostable direct heamolysin (lethal toxin) produced by *Vibrio parahaemolyticus*, *Infect. Immun*., 13(1976) 163-171.
24. Lowry, O.H., Roseborough, H.J., Farr, A.L. & Randall, R.J., Protein measurement with the Folin - phenol reagent, *J. Biol. Chem*., 193(1951) 265-275.
25. West P A & Colwell R R, Identification and classification of Vibrionaceae - an overview, in: *Vibrios in the environment*, edited by R. R. Colwell, (John Willey and Son, New York) 1984, pp. 285-363.
26. Sakurai, J., Honda, T., Jinguji, Y., Arita, M. & Miwatani, T., Cytotoxic effect of thermostable direct haemolysin produced by *Vibrio parahaemolyticus* on FL cells, *Infect. Immun*., 13(1976) 876-883.
27. Wratten, S.J., Wolfe, M.S., Andersen, R.J. & Faulkner, D.J., Antibiotic metabolites from a marine Pseudomonad, *Antimicrob. Agents Chemother*., 11(1977) 411-414.
28. Ali, H.A.J., Tamilselvi, M. & Sivakumar, V., Antibacterial activity of the marine ascidians *Phallusia nigra* and *Herdmania pallida* from the Tuticorin coast, *Indian J. Biol. Res*., 10(2008) 171-179.
29. Alade, P.L. & Irobi, O.N., Antifungal activities of crude leaf extract of *Acalypha wilkesiana*, *J. Ethnopharmacol*., 39(1993) 171-174.
30. Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J., 16S Ribosomal DNA amplification for phylogenetic study, *J. Bacteriol*., 173(1991) 697-703.
31. Ausubel F M, Brenet R, Kingston R E, Moore D D, Seidman J G, Smith J A & Struhl K, *Short protocols in molecular biology* (John Wiley and Sons, New York) 1995, pp 836.
32. Davidson, B.S., Ascidians: Producers of aminoacid derived metabolites, *Chem. Rev*., 93(1993) 1771-1791.
33. Lemos, M.L., Toranzo, A.E. & Barja, J.L., Antibiotic activity of epiphytic bacteria isolated from intertidal seaweeds, *Microb. Ecol*., 11(1985) 149-163.
34. Jayanth, K., Jeyasekaran, G. & Shakila, R.J., Isolation of marine bacteria, antagonistic to human pathogens, *Indian J. Mar. Sci*., 31(2002) 39-44.
35. Barja, J.L., Lemos, M.L. & Toranzo, A.E., Purification and characterization of an antibacterial substance produced by a marine *Alteromonas* sp., *Antimicrob. Agents Chemother*., 33(1989) 1674-1679.
36. Mc Carthy, S.A., Johnson, R.M. & Kakimoto, D., Characterization of an antibiotic produced by *Alteromonas luteoviolacea* Gauthier, 1982, 85 isolated from Kinko bay. Japan, *J. Appl. Bacteriol*., 77(1994) 426-432.
37. Burgess, J.G., Jordan, E.M., Bregu, M., Spragg, A.M. & Boyd, K.G., Microbial antagonism: a neglected avenue of natural products research, *J. Biotechnol*., 70(1999) 27-32.
38. Rinehart, K.L., Shaw, P.D., Shield, L.S., Gloer, J.B., Harbour, G.C., Koker, M.E., Samian, D., Schwartz, R.E., Tymiak, A.A., Weller, D.L., Carter, G.T. & Munro, M.H.G., Marine natural products as sources of antiviral, antimicrobial and anti-neoplastic agents, *Pure Appl. Chem*., 53(2006) 795- 817.

39. Thiel, V. & Imhoff, J.F., Phylogenetic identification of bacteria with antimicrobial activities isolated from Mediterranean sponges, *Biomol. Eng*., 20(2003) 421-423.
40. Radjasa, O.K., Sabdono, A., Junaidi. & Zocchi, E., Richness of secondary metabolite producing marine bacteria associated with sponge *Haliclona* sp. *Intl. J. Pharmacol*., 3(2007) 275-279.
41. Ramasamy. M.S. & Murugan, A., Chemical defense in ascidians *Eudistoma viride* and *Didemnum psammathodes* in Tuticorin, Southeast coast of India: Bacterial epibiosis and fouling deterrent activity, *Indian J. Mar. Sci*., 32(2003) 337-339.
42. Karthikeyan, M.M., Ananthan, G. & Balasubramanian, T., Antimicrobial activity of crude extracts of some ascidians (Urochordata: Ascidiacea), from Palk Strait, (Southeast Coast of India), *World J.Fish Mar. Sci*., 1(2009) 262-267.
43. Sivaperumal, P., Ananthan, G. & Hussain, S.M., Exploration of antibacterial effects on the crude extract of marine ascidian *Aplidium multiplicatum* against clinical isolates, *Intl. J. Med. Med. Sci*., 2(2010) 382-386.
44. Prabhu, A.S, Ananthan, G., Hussain, S.M. & Balasubramanian, T., Antibacterial activity of ascidian *Phallusia arabica* against human clinical isolates. *J. Appl. Pharma. Sci*., 1(2011) 143-145.
45. Meenakshi V.K., Senthamarai, S., Paripoorana Selvi, M., Gomathy, S., Shanmuga Priya D. & Chamundeswari, K.P., Antibacterial activity of simple ascidian *Ascidia sydneiensis* (Family: Ascidiidae) against human pathogens, *J. Microbiol. Biotech. Res*., 2(2012) 894-899.
46. Natarajan, K., Sathish, R., Regupathi, T. & Riyaz, A., Antibacterial activity of crude extracts of marine invertebrate *Polyclinum madrasensis* Sebastian, *Indian J.Sci.Technol*., 3(2010) 303-304.
47. Jimenez, P.C., Fortier, S.C., Lotufo, T.M.C., Pessoa, C., Moraes, M.E.A., de Moraes, M.O. & Costa-Lotufo, L.V., Biological activity in extracts of ascidians (Tunicata: Ascidiacea) from the northeastern Brazilian coast, *J. Expt. Mar. Biol. Ecol*., 287(2003) 93-101.