In vitro Assessment of Antimicrobial Properties in Different Concentration Crude Extracts of Ascidian *Didemnum granulatum* Tokioka, 1954 Against Isolated Human, Fish Pathogenic and Biofilm Microorganisms

N. Sri Kumaran[1-2]*, S. Bragadeeswaran[2] and V. K. Meenakshi[3]

[1]Department of Marine Biotechnology, AMET University, Kanathur, Chennai - 603112, India.
[2]Faculty of Marine Science, Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai – 608 502, India.
[3]Department of Zoology, A. P. C. Mahalakshmi College for Women, Tuticorin - 628 016, India.

doi: http://dx.doi.org/10.13005/bbra/1421

(Received: 15 August 2014; accepted: 10 October 2014)

The present study was carried for evaluate the antimicrobial properties of different solvent extracts of ascidian *Didemnum granulatum* against human, fish pathogenic and biofilm microbes. In this study anti microbial activities were carried out by standard disc diffusion method. In this experiment 50 human, fish bacterial, fungal pathogens and biofilm microbes were isolated and assayed against 7 different solvents such as methanol, acetone, ethanol, n-butanol, chloroform, ethyl acetate and dichloromethane. Each solvent assayed at different concentrations 25, 50, 75 and 100 mgmL^{-1}. The ascidian extracts exhibits profound antibacterial activity against human, fish pathogens and biofilm microbes. The higher concentration of solvents showed higher inhibition of bacterial pathogens but the fungal pathogens show resistant to tested solvents. The ascidian *D. granulatum* could be an ideal candidate for antimicrobial lead molecule development against microbial pathogens.

Key words: Ascidian extracts, Human pathogen, Fish pathogen, Biofilm micoorganisms.

Marine organisms have been attracting attention as potential sources of bioactive compounds with pharmaceutical interest. Several studies have reported the discovery of new bioactive compounds from marine organisms, focusing mainly on chemistry of secondary metabolites, which include now more than 15, 000 structurally diverse bioactive compounds isolated during the last 30 years (Salomon *et al.*, 2004). A large proportion of natural compounds have been extracted from marine invertebrates, especially sponges, ascidians, bryozoans and molluscs and some of them are currently in clinical trials (Proksch *et al.*, 2002). Tunicates have been reported to be rich sources of biologically active compounds and ranked third for their overall activities, next to sponges and bryozoans (Davis and Bremner, 1999). Recent chemical and biological investigations have revealed the importance of tunicates secondary metabolites in providing a chemical basis for survival of the adults and their larvae in predator-rich habitats. Saclike filter feeder ascidians have been reported to be an important source in drug discovery. Tetrahydroisoquinolone alkaloid

* To whom all correspondence should be
Mob.: +91 9791871889;
E-mail: s.kumaran08@gmail.com

'Ecteinascidin 743' from *Ecteinascidia turbinata*, cyclic depsipeptides 'Dehydrodidemnin B' and 'Didemnin B' from *Trididemnum solidum*, cyclic peptide 'Vitilevuamide' from *Didemnin cuculiferum* and 'Diazonamide' from *Diazona angulata* are a few tunicate compounds in anticancer preclinical or clinical trials (Jain *et al.,* 2008).

Infectious diseases are the major cause of morbidity and mortality worldwide (WHO, 2004). Synthetic drugs are not only expensive and inadequate for the treatment of diseases but are also often with adulterations and side effects. Therefore, there is a need to search for new infection-combating strategies to control microbial infections (Sieradzki *et al.*, 1999). The case of living marine surfaces the colonization process can additionally be affected by organic metabolites produced by the host organism. Most of the ascidians are known to produce bioactive metabolites which prevent bio-fouling and this can be considered as a kind of autogenic protection. These metabolites may affect bacteria in a number of ways, ranging from the induction of chemotactic responses to the inhibition of bacterial growth or cell death. Since they accumulate chemical defenses, ascidians have been screened in a variety of pharmacological bioassays. Biological activities which have been frequently observed in ascidian crude extracts include antibiosis against both human microbial pathogens and marine microorganisms (Mayer *et al.,* 2007). Such potential ascidians need to be explored for the pharmaceutical purpose. Hence a broad based screening of ascidians for bioactive compound is necessary. Through this study we plan to evaluate the anti microbial properties from biofoulants ascidians *Didemnum granulatum* against isolated human, fish pathogenic and biofilm micro organisms.

MATERIAL AND METHODS

Specimen Collection and Identification

Ascidians were collected as common and persistent biofoulants from the rocks of Tuticorin Coast (Lat. 8° 47' 20" and Long. 78° 09' 70"), India by SCUBA diving at the depth ranging from 1 to 3m between September, 2010. The samples were thoroughly washed with treated sea water and removed sand, mutt and overgrowing organisms at the site collection and transported to laboratory and collected specimens were identified by Dr. V. K. Meenakshi, Associate Professor, Department of Zoology, A. P. C. Mahalaxmi College for women, Tuticorin - 628002. A Voucher specimen No: AS 2235. The collected specimens were immediately shade dried.

Extraction

The extraction method was followed by Chellaram *et al.,* (2004). The freshly collected ascidian was weighed 15 gms in dry, each 15 gms were soaked in methanol, acetone, ethanol, n-butanol, chloroform, ethyl acetate and dichloromethane maintained for few days. The extracts were filtered through Whatman® No.1. Filter paper. The filtered solvents were concentrated by using rotary evaporator (VC100A Lark Rotavapor® at 30°C) with reduced the pressure to give a dark reddish gummy mass. The resultant residues were stored at 4°C for further analysis.

Test microorganisms and microbial culture

Human Bacterial and Fungal pathogens

The following human pathogens were used for test the antimicrobial assay, bacterial pathogens such as *E. coli*, *K. oxytoca*, *K. pneumoniae, P. mirabilis, S. paratyphi*, *S. typhi*, *Staphylococcus aureus*, *Enterococcus faecalis, V. cholerae* and *V. parahaemolyticus* the fungal pathogens such as *A. alternaria*, *A. flavus*, *A. niger*, *C. albicans*, *C. tropicalis*, *Mucor* sp., *Penicillium* sp., *Rhizopus* sp., *T. mentagarophytes* and *T. rubrum*. These microbes were obtained from Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalainagar. These bacterial and fungal strains were maintained on nutrient agar and fungal agar slants at 4°C respectively.

Fish Bacterial and Fungal pathogens

Following fish pathogens were used for antimicrobial activity, the bacterial pathogens such as *Aeromonas hydrophila*, *Aeromonas* sp., *Klebsiella* sp., *Micrococcus* sp., *P. mirabilis*, *Proteus* sp1., *Streptococcus* sp., *V. cholerae*, *V. parahaemolyticus* and *Vibrio* sp1. the fungal pathogens *A. flaves*, *A. fumigatus*, *A. niger*, *Aspergillus* sp1., *Aspergillus* sp2., *Fusarium* sp., *Ichthyophonus* sp., *Microsporum* sp., *Rhizopus* sp. and *Rhizopus* sp1. These fish pathogens were isolated from infected fishes from Cuddalore Government fish hatchery during April and May

2010. These pathogens were identified based on the morphological, cultural and biochemical characteristics following Bergey's manual of Determinative Bacteriology (Holt, 1994) and manual of Clinical Microbiology (Mahony *et al.*, 1999). These Fish pathogenic bacterial and fungal strains were maintained on Zobell marine agar and fungal agar slants at 4°C.

Fouling organisms

The reference microbes were used to test antimicrobial assay, *Bacillus* sp., *Klebsiella* sp., *Micrococcus luteus, Micrococcus* sp., *Micrococcus* sp.1, *Proteus* sp., *Pseudomonas* sp., *S. aureus, Streptococcus* sp1., and *Streptococcus* sp2. These fouling bacteria were isolated from the biofilm formed over aluminium, fiber glass and wood panels by pour plate method of Wahl, (1995). The isolated strains were identified based on the morphological, cultural and biochemical characteristics following Bergey's manual of Determinative Bacteriology (Holt, 1994) and manual of Clinical Microbiology (Mahony *et al.*, 1999). These biofilm bacterial strains were maintained on Zobell marine agar slants at 4°C.

Antibacterial Activity

Antibacterial activity was carried out by using standard disc diffusion method by Laouer *et al.*, (2009). The test cultures were swabbed on top of the solidified media and allowed to dry for 10 mins. The human bacteria were maintained on nutrient agar plates, fouling and fish pathogens maintained on Zobell marine agar plates. Collected extracts were tested at different concentration such as 25, 50, 75 and 100 mg /mL and each extracts (30 µL) were applied on to 6 mm sterile discs and allowed to dry at room temperature. The extract loaded discs were placed on agar plates seeded with isolated microorganisms and incubated at 37°C for 24 hrs. The susceptibility of the test organisms were determined by radius of the zones inhibition around each disc. The tetracycline discs (30 mg $disc^{-1}$) were used as a positive control and solvents discs were used as a negative control. All the extracts were tested with triplicate at a concentration of 30 mg $disc^{-1}$.

Antifungal activity

Antifungal activity was carried out by using the standard disc diffusion method by National Committee for Clinical Laboratory Standards, (2006). Collected extracts were tested at different concentration such like 25, 50, 75 and 100 mg /mL and each extract (30 µL) were applied on to the 6 mm sterile discs and allowed to dry at room temperature. The extracts loaded discs placed on agar plates seeded with fungal pathogens and incubated at 37°C for 24 hrs. Zones of growth inhibition were measured in millimeters after incubation. The tetracycline discs (30 mg $disc^{-1}$) were used as a positive control and solvents discs were used as a negative control. All the extracts were tested triplicate at a concentration of 30 $mgdisc^{-1}$.

Statical Analysis

The results were expressed as mean ± SD of three independent values.

RESULTS

The ascidian, *D. granulatum* (725 gms. in wet wt.) was collected from Tuticorin fishing harbor. The ascidian species was identified by following standard literature of Rocha and Bonnet, 2009, Kott, 2001 and Tokioka, 1954. Different solvents of *D. granulatum* were concentrated under reduced pressure to give a dark reddish gummy mass of 4.78 to 2.65 gms. In this experiment different concentrations (25, 50, 75 and 100 µg mL^{-1}) of the crude extracts were assayed against isolated human, fish pathogens and bio film microorganisms by using the disc diffusion method. The high concentration extracts of *D. granulatum* showed high susceptibility to isolated micro organisms. In antibacterial assay following human pathogenic bacteria shows (Table. 1) high susceptibility against different solvent extracts such as *V. parahaemolyticus* showed high sensitivity (9.08±0.15 mm), (11.11±0.2 mm) and (10.08±0.15 mm) against methanolic, ethanol and ethyl acetate extracts (100 mg/mL), *V. cholerae* express high zone inhibition **(**9.08±0.12 mm) against acetone extract (100 mg/mL), *V. cholerae* shows high sensitivity (10.1±0.3 mm) against n-butanol extract (100 mg/mL), *K. pneumoniae* showed high sensitivity **(**12.05±0.1 mm) against chloroform extract (100 mg/mL) and *S. paratyphi* exhibit zone inhibition (7.18±0.25 mm) against dichloromethane extract. The antifungal activity of *D. granulatum* against human fungal pathogens shows in Table. 2. Following pathogens shows high sensitivity against solvent extracts, *Mucor* sp. exhibit high

Table 1.Antimicrobial activity of *D. granulatum* against human bacterial pathogens

Concentration of Extracts (µg ml^{-1})		Human Bacterial Pathogens									
		Staphylococcus aureus	*Enterococcus faecalis*	*E. coli*	*S. typhi*	*S. paratyphi*	*K. oxytoca*	*V. cholerae*	*V. parahaemolyticus*	*K. pneumoniae*	*P. mirabilis*
Methanol	25	5.1±0.02	4.11±0.1	3.01±0.33	5.10±0.2	4.11±0.16	6.01±0.2	4.01±0.1	5.08±0.18	2.05±0.24	4.15±0.6
	50	6.02±0.2	5.1±0.01	4.3±0.1	6.04±0.22	5.01±0.19	6.07±0.17	6.02±0.2	6.1±0.12	3.01±0.1	5.04±0.1
	75	8.1±0.1	6.08±0.18	6.01±0.04	6.12±0.3	6.01±0.41	6.16±0.3	7.05±0.15	6.12±0.11	6.4±0.1	6.08±0.18
	100	10.2±0.13	9.04±0.18	9.05±0.1	9.02±0.2	7.07±0.14	9.04±0.1	9.04±0.18	9.08±0.15	7.02±0.1	7.01±0.11
Acetone	25	-	2.03±0.16	2.11±0.16	2.03±0.16	2.23±0.33	3.12±0.18	2.11±0.25	3.13±0.5	3.01±0.15	5.06±0.15
	50	2.33±0.22	3.12±0.18	3.01±0.2	3.12±0.18	3.41±0.15	4.01±0.11	4.12±0.17	5.04±0.14	5.06±0.1	6.01±0.04
	75	3.41±0.15	4.01±0.22	5.01±0.3	5.12±0.11	4.11±0.11	5.04±0.11	6.04±0.15	6.07±0.17	6.04±0.14	7.03±0.11
	100	6.33±0.22	6.05±0.11	7.01±0.2	8.18±0.12	5.28±0.13	7.01±0.12	9.08±0.12	8.15±0.8	8.01±0.1	8.01±0.1
Ethanol	25	2.01±0.18	2.04±0.1	3.13±0.5	-	2.15±0.2	4.15±0.5	4.11±0.1	4.12±0.1	3.6±0.3	3.01±0.33
	50	2.05±0.01	3.08±0.11	2.15±0.11	2.03±0.11	4.3±0.1	5.1±0.01	6.01±0.04	5.06±.05	4.11±0.9	5.01±0.4
	75	4.05±0.55	4.06±0.01	5.06±.05	4.05±0.5	5.06±.05	6.08±0.18	7.06±0.11	8.02±0.3	6.02±0.4	6.01±0.41
	100	6.01±0.41	6.02±0.05	6.07±0.17	5.3±0.12	8.05±0.1	9.1±0.11	9.04±0.18	11.11±0.2	7.03±0.15	9.03±0.1
n-butanol	25	4.01±0.18	2.03±0.11	3.11±0.2	4.11±0.16	3.01±0.1	2.04±0.1	6.12±0.1	6.01±0.1	6.4±0.18	5.05±011
	50	5.01±0.19	3.04±0.1	5.08±0.18	5.01±0.19	5.04±0.14	3.05±0.18	6.08±0.18	7.06±0.11	8.02±0.03	6.02±0.15
	75	6.01±0.5	5.09±0.17	6.06±0.3	6.04±0.14	6.07±0.17	6.01±0.1	7.06±0.11	8.01±0.1	9.01±0.01	7.08±0.18
	100	6.07±0.17	6.04±0.22	9.08±0.15	9.04±0.18	7.05±0.15	8.01±0.1	10.1±0.3	5.2±0.22	12.1±0.1	8.02±0.3
Chloroform	25	2.11±0.25	2.12±0.11	3.19±0.25	-	1.04±0.13	1.01±0.18	2.06±0.16	3.02±0.15	3.04±0.12	-
	50	3.04±0.05	3.01±0.15	6.2±0.2	2.01±0.2	3.01±0.23	3.01±0.1	3.05±0.2	6.08±0.18	5.01±0.15	2.04±0.1
	75	3.11±0.2	6.02±0.4	5.06±.05	4.11±0.9	3.11±0.41	5.05±0.5	3.04±0.5	8.02±0.2	7.01±0.11	2.03±0.5
	100	5.01±0.4	7.06±0.11	8.01±0.1	8.02±0.3	6.01±0.2	6.04±0.14	7.03±0.15	9.02±0.12	12.05±0.1	5.03±0.2
Ethyl acetate	25	-	3.02±0.15	-	3.01±0.15	-	2.03±0.1	1.01±0.45	3.23±0.14	2.11±0.1	2.4±0.11
	50	-	2.08±0.14	1.06±0.02	3.11±0.3	2.07±0.19	3.01±0.15	1.02±0.14	5.06±0.15	3.11±0.25	3.02±0.15
	75	2.08±0.16	5.14±0.15	2.02±0.14	3.05±0.1	2.8±0.25	4.01±0.11	2.01±0.13	6.07±0.17	4.01±0.18	4.11±0.16
	100	3.22±0.25	6.05±0.11	7.01±0.11	9.01±0.1	4.1±0.1	4.12±0.17	4.05±0.5	10.08±0.15	5.3±0.12	6.04±0.14
Dichloromethane	25	3.05±0.16	3.01±0.23	3.05±0.2	4.07±0.21	2.04±0.14	4.15±0.11	2.01±0.15	2.02±0.2	2.01±0.1	2.01±0.14
	50	3.05±0.11	3.02±0.11	4.08±0.16	2.01±0.15	5.06±0.13	6.01±0.14	3.01±0.18	2.08±0.1	3.08±0.11	2.05±0.25
	75	3.02±0.2	5.01±0.19	6.07±0.17	5.08±0.18	6.01±0.5	6.04±0.14	5.04±0.14	2.05±0.18	2.04±0.1	4.08±0.15
	100	4.05±0.55	7.01±0.25	7.02±0.24	6.11±0.36	7.18±0.25	6.1±0.22	5.11±0.11	5.08±0.8	4.04±0.1	5.09±0.17
Positive Control		10.02±0.14	13.01±0.01	12.03±0.11	11.15±0.1	10.11±0.15	14.02±0.11	12.11±0.14	9.02±0.19	12.02±0.01	11.03±0.15
Negative Control		0	0	0	0	0	0	0	0	0	0

(3 replicates; mean ± SD (standard error); inhibition zones (diameter in mm))

Table 2. Antimicrobial activity of *D. granulatum* against human fungal pathogens

Concentration of Extracts (µg ml^{-1})		Human Bacterial Pathogens									
		A. alternata	*A. flavus*	*A. niger*	*C. albicans*	*C.tropicalis*	*Mucor sp.*	*Penicillium sp.*	*Rhizopus sp.*	*T. Mentagarophytes*	*T. Rubrum*
Methanol	25	3.02±0.1	4.11±0.17	4.04±0.8	3.01±0.14	3.01±0.15	4.08±0.04	3.01±0.6	3.04±0.6	3.01±0.6	1.01±0.11
	50	4.08±0.12	5.04±0.14	5.01±0.5	5.01±0.1	5.06±0.15	5.03±0.7	5.05±0.01	4.04±0.18	4.08±0.12	5.02±0.1
	75	6.01±0.1	6.01±0.14	6.01±0.1	6.15±0.01	7.01±0.2	7.02±0.1	7.21±0.01	6.03±0.1	6.02±0.1	7.01±0.4
	100	7.02±0.12	7.01±0.3	7.06±0.16	7.04±0.17	8.01±0.14	9.06±0.15	7.04±0.11	7.05±0.17	7.02±0.13	9.03±0.2
Acetone	25	-	2.01±0.17	3.01±0.05	4.08±0.17	1.01±0.18	3.04±0.16	1.01±0.17	2.04±0.15	2.04±0.16	2.01±0.17
	50	3.06±0.14	3.01±0.16	3.2±0.4	5.04±0.16	2.04±0.22	5.02±0.14	2.06±0.18	3.06±0.14	3.02±0.14	3.06±0.16
	75	5.05±0.14	4.06±0.1	6.01±0.14	5.04±0.13	4.06±0.15	7.06±0.18	4.08±0.16	6.04±0.13	6.01±0.16	4.05±0.1
	100	7.05±0.4	7.07±0.01	7.02±0.12	8.05±0.01	6.04±0.1	9.01±0.11	7.04±0.14	9.14±0.8	10.06±0.06	11.06±0.1
Ethanol	25	-	4.05±0.1	3.04±0.14	3.2±0.4	5.04±0.22	3.05±0.04	3.01±0.15	6.04±0.3	4.02±0.2	5.01±0.11
	50	3.04±0.16	5.08±0.1	5.05±0.18	4.1±0.3	7.05±0.4	5.08±0.01	4.03±0.14	8±0.01	4.12±0.11	6.01±0.05
	75	4.1±0.03	6.06±0.06	6.08±0.1	4.08±0.17	9.08±0.1	7.07±0.01	6.01±0.16	9.02±0.1	6.11±0.01	8.05±0.14
	100	7.01±0.11	8.05±0.04	7.06±0.16	6.1±0.18	11.08±0.17	9.05±0.1	7.02±0.2	11.16±0.01	10.1±0.11	9.04±0.11
n-butanol	25	2.04±0.22	5.02±0.13	4.04±0.1	4.04±0.1	5.04±0.11	5.01±0.19	4.01±0.02	4.12±0.12	4.07±0.15	4.07±0.15
	50	3.01±0.18	7.05±0.17	5.1±0.5	5.1±0.5	6.01±0.21	7.01±0.2	7.12±0.11	6.12±0.18	6.05±0.11	6.04±0.88
	75	6.01±0.16	8.01±0.14	5.04±0.14	6.02±0.15	8±0.01	9.03±.01	9.13±0.01	7.8±0.01	7.1±0.15	7.01±0.9
	100	8.04±0.14	9.06±0.15	7.04±0.17	7.04±0.11	12.01±0.1	13.02±0.12	12.14±0.1	9.1±0.12	11.05±0.16	10.04±0.1
Chloroform	25	2.04±0.16	2.04±0.4	3.04±0.4	4.09±0.14	1.23±0.15	4.01±0.58	2.12±0.17	2.02±0.11	2.01±0.16	1.18±0.14
	50	3.06±0.16	4.1±0.6	5.03±0.3	5.06±0.15	3±0.18	5.15±0.17	4.05±0.33	4.02±0.12	4.06±0.15	4.14±0.18
	75	4.03±0.14	6.01±0.1	8.02±0.1	9.05±0.16	5.04±0.26	7.01±0.15	6.06±0.55	5.14±0.15	5.01±0.11	7.15±0.36
	100	6.01±0.05	7.01±0.16	9.01±0.6	6.04±0.18	7.06±0.2	10.04±0.18	8.05±0.17	7.05±0.6	7.06±0.2	9.14±0.14
Ethyl acetate	25	-	6.05±0.8	3.01±0.16	4.19±0.36	3.14±0.18	2±0.04	3.11±0.13	4.18±0.15	5.12±0.11	6.01±0.1
	50	2.06±0.18	7.01±0.1	5.06±0.1	5.15±0.18	4.12±0.1	4.05±0.36	5.12±0.11	5.16±0.02	6.05±0.2	6.02±0.1
	75	3.2±0.4	7.05±0.11	7.03±0.3	6.15±0.17	6.13±0.14	5.1±0.51	7.13±0.16	6.11±0.13	8.01±0.3	7.01±0.4
	100	5.08±0.01	8.06±0.12	11.01±0.6	7.17±0.17	8.44±0.25	7.05±0.05	8.02±0.1	7.01±0.2	9.03±0.01	7.21±0.01
Dichloromethane	25	2.04±0.15	3.01±0.15	3.01±0.16	4.05±0.19	4.14±0.15	2.04±0.25	4.06±0.19	5.01±0.13	4.02±0.17	4.06±0.1
	50	4.08±0.17	4.03±0.14	4.06±0.1	6.04±0.17	6.05±0.16	5.06±0.33	6.05±0.14	6.07±0.04	5±0.04	4.08±0.17
	75	6.1±0.18	5.05±0.14	6.08±0.17	8.05±0.16	7.05±0.01	7.05±0.11	6.04±0.16	7.06±0.05	7.02±0.1	6.1±0.18
	100	9.04±0.8	6.1±0.18	8.1±0.03	9.06±0.17	8.05±0.18	8.05±0.1	9.05±0.16	8.04±0.01	9.02±0.1	9.05±0.3
Positive Control	12.13±0.12	12.1±0.1	11.25±0.1	11.01±0.11	10.13±0.1	11.12±0.11	11.02±0.12	12.13±0.01	10.01±0.12	11.21±0.2	
Negative Control	0	0	0	0	0	0	0	0	0	0	

(3 replicates; mean±SD(standard error); inhibition zones (diameter in mm))

Table 3.Antimicrobial activity of D. granulatum against fish bacterial pathogens

Concentration of Extracts (μg ml^{-1})		Fish bacterial Pathogens									
		Aeromonas hydrophila	*Aeromonas sp.*	*Klebsiella sp.*	*Micrococ cus sp.*	*P. mirabilis*	*Proteus sp.1*	*Streptococcus sp.*	*V. cholerae*	*V. parahaemolyticus*	*Vibrio sp.1*
Methanol	25	4.01±0.12	4.08±0.1	2.01±0.1	3.01±0.1	4.01±0.1	2.02±0.2	3.01±0.1	4.03±0.1	3.11±0.2	3.03±.01
	50	5.02±0.2	6.01±0.2	4.01±0.12	4.02±0.4	6.02±0.1	2.3±0.01	4.01±0.1	7.01±0.2	4.1±0.2	5.02±0.12
	75	7.04±0.2	6.03±0.2	6.02±0.2	6.2±0.01	7.01±0.4	4.01±0.1	4.2±0.4	8.02±0.1	5.02±0.1	6.04±0.11
	100	9.05±0.1	8.01±0.2	7.04±0.2	8.1±0.1	8.21±0.01	7.01±0.2	6.2±0.01	10.15±0.01	6.02±0.1	7.01±0.21
Acetone	25	6.04±0.3	5.01±0.3	5.01±0.2	6.02±0.3	3.04±0.15	3.07±0.11	4.05±0.1	4.05±0.11	3.02±0.14	5.07±0.01
	50	6.02±0.1	6.01±0.1	6.01±0.1	7.04±0.12	3.01±0.1	5.06±0.1	5.05±0.4	6.02±0.17	6.01±0.47	6.06±0.06
	75	7.02±0.2	7.02±0.1	8.01±0.1	7.05±0.14	4.05±0.33	6.07±0.8	7.08±0.1	7.06±0.15	7.09±0.6	8.08±0.01
	100	8.02±0.01	9.01±0.4	9.03±0.2	8.01±0.1	5.06±0.3	8.1±0.16	9.04±0.8	9.01±0.16	8.02±0.5	9.04±0.22
Ethanol	25	3.01±0.16	5.05±0.14	6.01±0.2	2.11±0.14	3.01±0.15	3.05±0.04	4.04±0.1	4.05±0.33	4.12±0.05	4.08±0.1
	50	4.06±0.1	6.01±0.05	7.02±0.3	3.06±0.04	4.03±0.14	5.01±0.11	6.03±0.14	4.08±0.17	5.3±0.03	4.12±0.11
	75	5.08±0.17	7.2±0.4	8.04±0.12	5.04±0.15	6.01±0.16	5.05±0.1	7.05±0.11	5.08±0.1	7.12±0.1	5.02±0.12
	100	6.1±0.18	8.1±0.3	9.05±0.14	7.01±0.1	8.06±0.1	7.05±0.4	12.02±0.17	7.06±0.15	8.08±0.1	6.01±0.18
n-butanol	25	9.06±0.5	6.02±0.3	3.1±0.12	5.13±0.15	5.11±0.05	6.1±0.12	2.1±0.01	3.01±0.1	4.08±0.1	5.10±0.1
	50	10.1±0.1	7.10±0.1	5.01±0.11	6.11±0.05	6.11±0.15	7.01±0.15	3.12±0.04	6.1±0.14	5.12±0.11	6.04±0.11
	75	7.05±0.6	9.04±0.11	7.01±0.11	7.12±0.07	8.03±0.13	8.11±0.11	5.03±0.16	8.12±0.01	6.02±0.12	6.12±0.13
	100	11.04±0.5	9.12±0.13	10.01±0.05	8.02±0.01	9.05±0.11	10±0.01	5.11±0.05	9.01±0.01	9.01±0.18	8.12±0.05
Chloroform	25	2.11±0.1	5.3±0.03	6.4±0.15	2.33±0.22	3.12±0.1	3.18±0.11	4.12±0.11	3.12±0.01	3.12±0.1	3.12±0.1
	50	5.21±0.1	6.12±0.1	6.16±0.12	5.13±0.15	4.11±0.12	4.1±0.1	6.11±0.01	5.17±0.18	4.11±0.01	4.11±0.01
	75	6.1±0.12	7.08±0.1	6.01±0.1	6.11±0.15	6.11±0.15	3.11±0.01	6.01±0.02	7.01±0.11	6.12±0.1	6.12±0.1
	100	7.3±0.14	9.12±0.12	10.1±0.11	7.15±0.03	9.12±0.12	4.12±0.01	9.13±0.01	9.11±0.12	8.12±0.05	8.12±0.1
Ethyl acetate	25	-	2.11±0.16	1.33±0.22	-	2.11±0.2	2.41±0.15	-	2.3±0.14	2.13±0.12	3.02±0.12
	50	2.33±0.22	2.23±0.33	2.03±0.16	2.03±0.16	3.02±0.11	4.23±0.33	2.33±0.22	4.4±0.15	5.33±0.22	4.08±0.1
	75	2.41±0.15	3.15±0.15	3.12±0.18	2.11±0.2	4.03±0.33	6.12±0.11	4.12±0.1	5.16±0.12	6.41±0.15	5.12±0.11
	100	3.12±0.01	4.01±0.03	4.12±0.11	3.11±0.13	5.12±0.11	6.33±0.22	5.3±0.03	7.1±0.11	8.08±0.1	6.01±0.18
Dichloromethane	25	4.11±0.12	4.1±0.12	3.18±0.11	1.12±0.11	3.02±0.2	4.05±0.1	3.12±0.05	2.23±0.33	3.02±0.11	4.12±0.11
	50	4.12±0.11	4.02±0.4	5.04±0.2	2.13±0.16	4.01±0.12	4.1±0.1	5.01±0.05	3.15±0.15	5.02±.05	5.13±0.15
	75	6.01±0.05	6.2±0.01	7.02±0.2	318±0.15	6.01±0.1	6.01±0.1	6.02±0.1	5.02±0.1	6.01±0.2	6.11±0.05
	100	6.05±0.11	7.01±0.4	8±0.01	6.11±0.13	7.21±0.01	7.01±0.2	11.01±0.1	7.23±0.33	9.05±0.3	7.01±0.11
Positive Control		11.19±0.12	14.06±0.01	13.01±0.02	12.12±0.12	14.22±0.11	12.22±0.33	10.33±0.12	11.12±0.12	9.01±0.11	16.20±0.15
Negative Control		0	0	0	0	0	0	0	0	0	0

(3 replicates; mean ± SD (standard error); inhibition zones (diameter in mm))

Table 4. Antimicrobial activity of *D. granulatum* against fish fungal pathogens

Concentration of Extracts (µg ml^{-1})		Fish bacterial Pathogens									
		A. flaves	*A. fumigatus*	*A. niger*	*Aspergillus* sp.1	*Aspergillus* sp.2	*Fusarium* sp.	*Ichthyophonus* sp.	*Microsporum* sp.	*Rhizopus* sp.	*Rhizopus* sp.1
Methanol	25	3.02±0.18	2.05±0.8	1.06±0.18	2.01±0.01	2.01±0.1	4.12±0. 1	1.01±0.22	1.02±0.12	3.11±0.41	2.02±0.1
	50	4.09±0.14	3.04±0.14	1.07±0.18	3.02±0.1	3.03±0.25	4.15±0.2	2.11±0.1	2.06±0.4	5.01±0.11	3.01±0.11
	75	5.07±0.15	4.01±0.55	2.03±0.19	3.07±0.18	5.12±0.11	6.16±0.3	3.23±0.14	3.04±0.1	6.05±0.5	4.11±0.12
	100	6.01±0.1	5.07±0.14	3.01±0.12	4.17±0.14	7.01±0.2	6.12±0.1	4.04±0.2	5.04±0.1	7.01±0.61	7.01±0.1
Acetone	25	2.01±0.07	2.01±0.01	-	-	1.2±0.1	3.15±0.5	1.01±0.45	1.03±0.2	2.03±0.5	1.01±0.18
	50	3.05±0.33	2.04±0.21	2.06±0.11	1.04±0.23	3.1±0.33	4.1±0.12	2.03±0.1	2.4±0.11	3.04±0.5	2.11±0.16
	75	4.12±0.6	3.07±0.18	2.08±0.14	2.08±0.1	4.2±0.3	6.4±0.1	3.01±0.33	3.02±0.12	5.03±0.2	3.02±0.15
	100	5.01±0.14	4.04±0.17	3.05±0.15	2.09±0.44	5.12±0.1	6.6±0.3	4.05±0.1	5.01±0.1	6.1±0.12	6.01±0.15
Ethanol	25	2.04±0.1	2.05±0.18	1.01±0.2	2.05±0.15	2.14±0.01	2.15±0.2	1.02±0.21	2.04±0.17	1.02±0.21	1.11±0.2
	50	3.02±0.18	2.08±0.15	3.02±0.017	3.01±0.12	3.15±0.02	4.16±0.3	3.05±0.22	4.05±0.19	2.01±0.5	3.01±0.23
	75	3.08±0.04	3.04±0.14	3.04±0.12	5.05±0.14	4.01±0.1	6.01±0.1	3.02±0.12	5.06±0.17	4.04±0.22	4.03±0.11
	100	4.01±0.55	3.08±0.5	5.04±0.17	6.01±0.16	5.03±0.25	7.1±0.05	5.01±0.1	6.05±0.18	6.01±0.5	4.01±0.1
n-butanol	25	3.07±0.01	2.06±0.11	2.07±0.1	2.07±0.19	2.08±0.16	3.2±0.1	3.01±0.22	2.04±0.17	2.06±0.4	3.02±0.12
	50	5.01±0.14	3.08±0.17	3.04±0.05	3.05±0.15	5.14±0.15	3.01±0.1	4.11±0.1	4.14±0.19	3.04±0.1	5.01±0.15
	75	6.04±0.17	3.11±0.3	4.07±0.16	5.04±0.11	6.07±0.18	4.11±0.2	5.01±0.1	4.11±0.17	5.01±0.1	5.1±0.1
	100	7.01±0.03	5.09±0.18	5.04±0.8	6.01±0.1	7.17±0.14	5.1±0.5	6.02±0.2	7.12±0.4	6.01±0.45	6.05±0.4
Chloroform	25	2.05±0.8	2.04±0.14	-	-	3.01±0.2	2.02±0.01	1.04±0.04	2.05±0.15	1.02±0.12	2.02±0.2
	50	3.07±0.01	3.04±0.1	1.06±0.02	1.01±0.03	4.02±0.1	3.02±0.3	3.02±0.19	3.01±0.33	2.4±0.11	3.05±011
	75	3.08±0.17	3.06±0.5	2.05±0.18	2.04±0.14	6.1±0.2	4.01±0.1	4.05±0.25	2.04±0.17	4.01±0.22	5.01±0.1
	100	5.07±0.15	5.07±0.14	2.04±0.14	3.04±0.12	7.02±0.1	5.02±0.2	5.01±0.16	4.05±0.19	4.08±0.15	6.2±0.2
Ethyl acetate	25	-	1.06±0.02	1.02±0.14	2.06±0.48	-	-	1.11±0.26	-	1.01±0.22	1.03±0.2
	50	1.04±0.6	2.08±0.14	2.08±0.16	3.01±0.13	1.01±0.1	2.1±0.1	2.03±0.14	-	2.11±0.1	4.05±0.1
	75	2.01±0.1	3.04±0.05	3.01±0.15	4.12±0.02	1.03±0.33	3.01±0.01	2.04±0.17	1.01±0.17	3.23±0.14	3.05±0.22
	100	2.14±0.11	5.14±0.15	4.09±0.14	6.2±0.11	2.1±0.3	4.1±0.3	4.14±0.19	2.01±0.16	4.04±0.1	5.04±0.1
Dichloromethane	25	2.07±0.17	1.04±0.6	2.01±0.1	2.04±0.16	2.01±0.2	2.01±0.1	1.01±0.15	2.03±0.1	2.01±0.1	1.04±0.2
	50	3.02±0.11	2.06±0.11	2.14±0.11	3.01±0.16	3.02±0.01	3.1±0.03	3.05±0.5	3.01±0.33	2.02±0.2	3.02±0.12
	75	4.05±0.14	3.04±0.23	3.02±0.11	3.05±0.14	5.01±0.2	5.02±0.22	4.01±0.18	4.15±0.11	4.07±0.21	5.01±0.1
	100	5.06±0.15	5.04±0.11	5.04±0.17	4.03±0.19	6.02±0.1	6.1±0.1	6.05±0.11	5.08±0.8	5.11±0.11	5.02±0.21
Positive Control		12.02±0.22	10.05±0.25	11.02±0.12	14.02±0.16	12.03±0.17	12.01±0.12	11.02±0.14	12.01±0.11	11.02±0.14	12.03±0.36
Negative Control		0	0	0	0	0	0	0	0	0	0

(3 replicates; mean ± SD (standard error); inhibition zones (diameter in mm))

Table 5. Antimicrobial activity of *D. granulatum* against Biofilm Microorganisms

Concentration of Extracts (μg ml^{-1})		Biofilm Microorganisms									
		Bacillus sp.	*Klebsiella* sp.	*Micrococcus luteus*	*Micrococcus* sp.	*Micrococcu* s sp.1	*Proteus* sp.	*Pseudomonas* sp.	*S. aureus*	*Streptococcus* sp.1	*Streptococcus* sp.2
Methanol	25	-	2.14±0.16	1.04±0.17	1.04±0.18	4.07±0.15	2.06±0.14	1.01±0.11	3.08±0.1	1.01±0.11	1.02±0.22
	50	2.05±0.16	2.18±0.25	2.06±0.26	3.05±0.16	6±0.01	3.01±0.9	2±0.04	4.04±0.14	2.03±0.7	1.05±0.25
	75	3.05±0.18	4.08±0.16	4.05±0.25	4.14±0.15	7.1±0.15	5.04±0.1	3.04±0.6	5.05±0.11	3.04±0.6	2.02±0.12
	100	3.15±0.36	5.12±0.18	5.05±0.14	5.05±0.16	10.05±0.16	6.05±0.11	5.03±0.7	6.05±0.18	5.01±0.14	3.02±0.16
Acetone	25	-	1.14±0.14	1.15±0.15	1.25±0.2e6	1.14±0.14	1.14±0.18	3.01±0.14	2.04±0.14	3.01±0.15	-
	50	2.18±0.36	2.04±0.17	3±0.18	3.04±0.23	3.05±0.5	4.12±0.1	4.04±0.8	3.06±0.12	3.01±0.5	1.01±0.12
	75	2.17±0.77	4.08±0.16	4.01±0.02	6.05±0.11	4.14±0.18	5.44±0.25	4.01±0.6	4.01±0.16	4.01±0.1	1.02±0.14
	100	3.15±0.11	6.04±0.17	5.01±0.2	7.15±0.36	6.04±0.17	6.15±0.17	6.11±0.17	6.02±0.15	5.05±0.01	1.01±0.11
Ethanol	25	-	2.18±0.25	2.04±0.17	2.04±0.17	2.14±0.14	2.06±0.15	3.01±0.1	2.04±0.8	3.06±0.15	2.02±0.14
	50	2.18±0.36	4.17±0.4	4.14±0.19	4.05±0.19	3.15±0.18	4.04±0.88	4.07±0.3	3.01±0.6	4.08±0.12	3.03±0.36
	75	3.17±0.14	5.01±0.16	4.11±0.17	5.06±0.17	7.17±0.17	6.07±0.15	5.06±0.1	4.11±0.17	5.04±0.18	3.02±0.22
	100	4.15±0.11	6.06±0.5	7.12±0.4	6.05±0.18	8.19±0.36	6.05±0.16	7.05±0.2	7.06±0.15	7.04±0.14	4.05±0.25
n-butanol	25	1.12±0.22	1.01±0.15	1.04±0.04	1.01±0.15	3.04±0.23	2.01±0.15	1.01±0.14	3.01±0.15	1.01±0.14	3.02±0.12
	50	1.18±0.17	2.14±0.16	3.02±0.19	3.05±0.5	4.05±0.14	4.01±0.5	3.01±0.2	4.02±0.1	2.01±0.2	4.02±0.16
	75	2.17±0.77	3.02±0.19	4.05±0.25	4.01±0.18	7.15±0.36	5.01±0.1	4.04±0.6	5.01±0.6	3.040±0.6	5.03±0.17
	100	2.16±0.11	4.01±0.15	5.01±0.16	6.05±0.11	8.06±0.26	7.05±0.01	5.08±0.7	7.08±0.04	5.08±0.7	6.11±0.17
Chloroform	25	-	1.05±0.6	-	1.01±0.17	4.17±0.4	3.06±0.15	1.24±0.16	2.04±0.14	1.01±0.1	1.04±0.14
	50	1.1±0.19	1.01±0.16	1.1±0.19	2.01±0.16	1.15±0.15	4.08±0.12	2.01±0.12	3.24±0.16	3.07±0.3	3.05±0.14
	75	1.11±0.26	1.01±0.17	1.11±0.26	2.05±0.15	1.25±0.26	5.04±0.18	3.03±0.12	4.01±0.12	4.06±0.1	4.14±0.18
	100	2.03±0.14	3.05±0.18	2.03±0.14	3.01±0.33	1.14±0.14	7.04±0.14	5.01±0.14	5.03±0.12	5.05±0.2	5.05±0.12
Ethyl acetate	25	-	2.1±0.51	-	1.04±0.16	-	-	1.01±0.1	-	-	-
	50	-	2.04±0.25	-	1.04±0.14	1.04±0.14	1.01±0.11	3.09±0.16	1.01±0.1	1.01±0.11	1.18±0.25
	75	2.05±0.16	3.05±0.36	2.01±0.15	2.05±0.11	2.04±0.16	3±0.1	3.04±0.3	1.09±0.16	2.01±0.14	2.08±0.16
	100	3.06±0.15	4.05±0.05	5.06±0.33	3.14±0.14	3.06±0.26	4.08±0.04	5.02±0.12	2.04±0.3	3.04±0.6	3.12±0.18
Dichloromethane	25	-	1.05±0.36	1.05±0.1	1.2±0.15	2.05±0.13	1.02±0.1	3.01±0.18	1.4±0.12	1.04±0.8	1.14±0.14
	50	2.16±0.11	2.1±0.51	1.12±0.22	2.05±0.1	5.03±0.14	2.01±0.6	5.05±0.1	3.01±0.1	2.01±0.6	2.04±0.17
	75	3.04±0.17	3.05±0.05	2.06±0.15	4.05±0.33	6.06±0.55	3.01±0.15	7.02±0.08	4.01±0.14	3.11±0.17	3.08±0.16
	100	5.12±0.18	4.04±0.25	2.09±0.04	5.04±0.26	7.05±0.6	4.04±0.14	8.01±0.1	5.02±0.08	4.06±0.15	4.04±0.17
Positive Control		12.02±0.22	10.05±0.25	11.02±0.12	14.02±0.16	12.03±0.17	12.01±0.12	11.02±0.14	12.01±0.11	11.02±0.14	12.03±0.36
Negative Control	0	0	0	0	0	0	0	0	0	0	

(3 replicates; mean ± SD (standard error); inhibition zones (diameter in mm))

sensitivity (9.06±0.15 mm), (13.02±0.12 mm) and (10.04±0.18 mm) against methanolic, n-butanol and Chloroform extracts (100 mg/mL), *T. rubrum* showed high sensitivity (11.06±0.1 mm) against acetone extract (100 mg/mL), *Rhizopus* sp. express high zone inhibition (11.16±0.01mm) against ethanol extract (100 mg/mL), *A.niger* exhibit high sensitivity (11.01±0.6 mm) against ethyl acetate extract (100 mg/mL) and finally *C. albicans* shows high sensitivity (9.06±0.17 mm) to dichloromethane extract (100 mg/mL).

The antibacterial activity the fish pathogenic bacteria tested shows in Table. 3. The *V. cholerae* exhibit high sensitivity (10.15±0.01 mm) against methanolic extract (100 mg/mL)**,** *Vibrio* sp.1 express high zone inhibition **(**9.04±0.22 mm) against acetone extract (100 mg/mL), *Streptococcus* sp. showed high sensitivity (12.02±0.17 mm), (9.13±0.01) and (11.01±0.1mm) against ethanol, chloroform and dichloromethane extracts (100 mg/mL), *Aeromonas hydrophila* (11.04±0.5 mm) exhibit high zone inhibition against n-butanol extract (100 mg/mL) and *V. parahaemolyticus* showed high sensitivity (8.08±0.1 mm) against ethyl acetate extract (100 mg/mL). Antifungal activity of *D. granulatum* against fish fungal pathogens shows in Table. 4. The *Rhizopus* sp. exhibit high sensitivity (7.01±0.61 mm) against methanolic extract (100 mg/mL), *Fusarium* sp. showed high zone inhibition (6.6±0.3 mm), (7.1±0.05 mm) and (6.1±0.1 mm) against acetone, ethanol and dichloromethane extracts (100 mg/mL), *Aspergillus* sp.2 exhibit zone inhibition (7.17±0.14 mm) and (7.02±0.1mm) against n-butanol and chloroform extracts (100 mg/mL) and finally *Aspergillus* sp.2 showed high sensitivity (6.2±0.11 mm) against ethyl acetate (100 mg/mL).

The antimicrobial activity of *D. granulatum* extracts against biofilm microorganisms showed in Table 5. *Micrococcus* sp.1 shows high sensitivity (10.05±0.16 mm), (8.19±0.36 mm) and (8.06±0.26 mm) against methanol, ethanol and n-butanol extracts (100 mg/mL), *Proteus* sp. showed exhibit zone inhibition (6.15±0.17 mm) and (7.04±0.14 mm) against acetone and chloroform extracts (100 mg/mL), *Pseudomonas* sp. express high inhibition zone **(**5.02±0.12 mm) and (8.01±0.1 mm) against ethyl acetate and dichloromethane (100 mg/mL).

DISCUSSION

Antimicrobial peptides have recently become the focus of considerable interest as a candidate for a new type of antibiotic, due primarily to their potency against pathogenic microbes that are resistant to conventional antibiotics, as well as their broad-spectrum activity (Bulet et al., 2004). The search for such antimicrobial peptides continues, in the hopes of locating an effective candidate for the development of a new type of antibiotic (Ngai *et al.*, 2005; Thevissen *et al.*, 2005). In the present investigation a promised antimicrobial activity has been observed in different concentration (25, 50, 75 and 100 mg/mL) and different solvents (methanol, acetone, ethanol, n-butanol, chloroform, ethyl acetate and dichloromethane) of the crude samples against isolated bacterial and fungal strains. Antimicrobial properties of *D. granulatum* extracts were measured by the radius of the zone of inhibition around the discs. In the present study the ascidian extracts were showed positive source of antimicrobial compounds towards isolated microbes.

These findings are consistent with previous studies on ascidians. Ascidians are already reported for rich nitrogenous source with a wide range of biological activities (Biard *et al.*, 1994). The present investigation shows the broad spectrum antibacterial activity of ascidian and this may be due to the nitrogenous bioactive principles. Murugan and Santhana Ramasamy (2003) has reported that the crude methanol extract of *D. psammathodes*, the range of inhibition of the bacteria varied from 6 and 10 mm with an average of 7.1 mm. Prem Anand and Patterson Edward (2002) reported that in *D. psammathodes* the highest activity was seen against *P. mirabilis* (7 mm), *Shigella flexneri* (8 mm) and *Salmonella typhi* (6 mm).Abdul Jaffar Ali *et al.* (2008) reported the maximum antibacterial activity of the crude methanol extracts of the test and mantle bodies of *P. nigra* against the Gram positive *Staphylococcus aureus* (inhibitory zones of 12.3±0.8 and 8.2±0.8 mm in diameter, respectively).Activity of hexane and ether extracts of this tunicate against *A. tumefaciens* is slightly less than tetracycline activity, but higher than activity of *Lissoclinum fragile* extracts (Badre *et al.*, 1994). On the other hand, antibacterial activity of *C. savignyi* extracts against *E. coli*, and *S. aureus* is less than the activity of *L. perforatum* extracts (Litandon and Guyot, 1991). Antibacterial activity has previously been detected in methanol/dichloromethane extracts of the ascidians *H. pyriformis* and a mixture of two *Styela* species where one of the species was *S. rustica* (Lippert *et al.*, 2003). Methanolic extract of *P. madrasensis* demonstrated high degree of activity against all tested bacterial isolates whereas hexane extract showed good activity against gram-negative pathogens and moderate against gram-positive pathogens (Natarajan *et al.*, 2010). Ronald, (1997) has reported that the fungi are more resistant than the bacterial strains to the tested compound, this could be leads to the nature of fungal cell wall made up of chitin, the hard cover of the exoskeletons of the arthropods are also made up of chitin, which is relatively resistant, including microbial decomposition. Antifungal activity has been reported for *C. intestinalis* in the form of tribromophenol, which is a known fungicide (Kotterman *et al.*, 2003). Overall, ascidian extracts caused growth inhibition in gram positive and gram negative bacteria, indicating that these extracts do not selectively inhibit one group of microorganism. Because some of antimicrobial peptides physically attack the microbial membranes, thereby killing even microbes that are equipped with antibiotic-resistant mechanisms, they have been the focus of increasing interest, and are being heralded as excellent candidates in the search for new types of antibiotics (Janga *et al.*, 2006). These results indicate that ascidians exhibits amazing activity against microbes. Therefore the current studies exposed the presence of potent antimicrobial compounds from ascidians of Tuticorin coast. Hence further purification may lead to the discovery of novel antimicrobial compounds.

CONCLUSION

Screening tactics followed by ecological knowledge of marine organisms are being increasing deployed in the investigation of novel bioactive compounds. Our preliminary result reveals that many of the marine organisms produce more or less structurally diverse secondary metabolites which could be of pharmaceutical interest. The ascidian *D. granulatum* seems to be a promising source of antimicrobial compounds.

ACKNOWLEDGMENTS

Authors are thankful to the Dean & Director, Center of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai, Tamil nadu, India for Facilities provided.

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