Journal of Biological Research-Thessaloniki **10**: 171 – 179, 2008
J. Biol. Res.-Thessalon. is available online at http://www.jbr.gr
Indexed in: WoS (Web of Science, ISI Thomson), CAS (Chemical Abstracts Service) and DOAJ (Directory of Open Access Journals)

Antibacterial activity of the marine ascidians *Phallusia nigra* and *Herdmania pallida* from the Tuticorin coast, India

HAJAMOHIDEEN ABDUL JAFFARALI[1*], MADASAMY TAMILSELVI[2] and VELAYUDHAM SIVAKUMAR[3]

[1] *Department of Biotechnology, Islamiah College, Vaniyambadi – 635 752, Tamilnadu, India*
[2] *Department of Zoology, V.V. Vanniyaperumal college for women, Virudhunagar – 626 001, Tamilnadu, India*
[3] *SPIC Research Centre, Department of Zoology, V.O. Chidambaram College, Tuticorin – 628 008, Tamilnadu, India*

Received: 29 February 2008 Accepted after revision: 18 July 2008

This study was carried out in order to screen the antibacterial activity of the bioactive compounds extracted from the test body (body covering) and the mantle body of the two solitary ascidians, *Phallusia nigra* and *Herdmania pallida,* collected from the Tuticorin harbour area. Antibacterial activity of *P. nigra* was significantly higher than that displayed by *H. pallida*. The former species showed maximum antibacterial activity and inhibition against all the bacteria tested. The bacterial growth inhibitory effect was found to be dependant on the solvents used for extraction as well as on the specific to the species extracts. Of the nine strains examined, Gram positive were the most susceptible after treatment with all fractions except fraction VI which showed activity against *Escherichia coli* and *Salmonella paratyphii* (Gram negative). Lipophilic (chloroform) extracts were highly active against the pathogens tested. The study was also extended to find out the chemical nature of the active compound. IR, ^{1}H NMR and ^{13}C NMR spectra of the fraction II provided the complete carbon skeleton of a tyrosine derivative. This tyrosine derivative could be probably responsible for the antibacterial activity and a future study involving pharmacology would pave a way for the invention of a new antibiotic drug from *P. nigra*.

Key words: antibiotic activity, ascidian, bioactive compound, ecology, simple ascidians.

INTRODUCTION

In Asian regions, various antimicrobials are used in fish and shrimp production, to control deadly infectious diseases caused by a variety of pathogenic bacteria. Also, chemotherapeutic agents like antibiotics, disinfectants and non-specific synthetic immune stimulants, are commonly employed for disease management in small scale culture systems (Palavesam *et al*., 2006). However, in the case of large scale operations, this is not advisable because of the cost effectiveness and the potential development of drug resistance against antibiotics, due to improper administration practices (Kruse & Soram, 1994). In this scenario, the ascidian-based antimicrobials are gaining paramount importance in remaining a vast untapped source for medicines with enormous therapeutic potential.

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 & Bremner, 1999). Although research on bioactive compounds from ascidians was recently initiated, it is significant that the first marine natural product entering human clinical trials, didemnin B, is an ascidian metabolite. Cytotoxicity of the ascidian metabolite is the most frequently listed agent against a variety of tumor cell lines, followed by antimicrobial, antiviral and anti-inflammatory activities (Davidson, 1993).

* Corresponding author: tel.: +91 944 3528704, fax: +91 417 4235876, e-mail: jaffar_asc@yahoo.com

Phallusia nigra and *Herdmania pallida* are the most abundant ascidians along the Tuticorin coast (Abdul Jaffar Ali & Sivakumar, 2007; Tamilselvi, 2008). A review of literature showed that as compared to *H. pallida, P. nigra* presents interesting chemical defense mechanisms, such as presence of haemolytic and cytotoxic substances (Costa *et al*., 1996), high amount of vanadium in the soft body parts, presence of guanidine based neurotoxins (Freitas *et al*., 1996) and low pH in the tunic (Hirose, 1999).

Though enough information about chemistry and ecological as well as developmental implications of marine natural products is available, little attention has been paid to the ascidian metabolites in India. Even though the Indian marine environment is unique for its rich diversity, to date, only a fraction of this diversity has judiciously been explored for novel bioactive compounds that could be developed as new drugs and/or agrochemicals. In India, a preliminary study was carried out on *Styela pigmentata* and *Pyura pallida* for their bioactive potentiality against *Artemia salina* and also against the growth of the microalgae *Dunaliella tertiolecta* and *Isochrysis galbana* (Avelin *et al*., 1991). The antibacterial activity of the compounds isolated from the colonial ascidian *Didemnum psammathodes* was also reported by Ramasamy & Murugan (2003).

In this context, the present investigation was carried out to analyze the bioactive compounds of *Phallusia nigra* and *Herdmania pallida* and also their possible antagonistic effects against several bacterial pathogens.

MATERIALS AND METHODS

The material of the present study was comprised of the ascidians *Phallusia nigra* and *Herdmania pallida*. Both species are common to the Tuticorin coast of India and inhabit in rocks, cement blocks and rafts. Specimens of the two species (for the production of extracts) were collected in November and December 2001 from the Tuticorin harbour basin (8°48′N and 78°11′E) situated 1200 m away from the South Break Water (SBW) (Fig. 1). Each species was collected at a depth of 2-3 m from the hull of the barge and various cement blocks by SCUBA diving. The specimens of *Herdmania pallida* were consistently found in association with an epibiont species whereas the specimens of *Phallusia nigra* were free of any epibiont. The epibionts were removed from all samples prior to extraction.

Isolation of bacterial pathogens

Nine bacterial strains [*Bacillus subtilis*, (BS), *Staphylococcus aureus* (SA), *Entrerobacter aerogenes* (EA), *Escherichia coli* (EC), *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA), *Salmonella paratyphii* (SP), *Salmonella typhii* (ST) and *Vibrio cholerae* (VC)] were used. They were obtained from the Department of Biotechnology, Madurai Kamaraj University, Madurai, India. The bacterial stock cultures were maintained on nutrient agar (Hi-media, Mumbai) slants and were sub-cultured in the same agar.

Preparation of animal material

The test and mantle bodies of the study animals were used for the isolation of the compounds. The freshly collected samples were cleaned and washed with fresh seawater to remove impurities and associated plants and animals. The animals were dissected to separate the test from the mantle body and then dried under shade at room temperature. The dried material was ground and sieved in order to remove shell particles.

Preparation of crude extract

The ground animal material was individually soaked with methanol at a ratio of 1 g/20 ml for about five days. The extracts were air dried overnight at room temperature for complete evaporation of the solvent before subsequent extraction. The extracts were collected, filtered and concentrated using a rotary evaporator (Buchitype) under reduced pressure at 40°C. The residues were weighed and dissolved in distilled water to obtain different concentrations.

Bioassay

Different extracts were individually tested for their action as antibacterial agents on nine bacterial strains using the standard filter paper disc diffusion method (Avelin *et al.*, 1991). Sterilized discs were soaked with the prepared extracts of different concentrations and kept overnight at room temperature. The soaked discs were then aseptically dried to ensure evaporation of the solvents. The Muller-Hinton agar medium was prepared using sterilized distilled water. Swabs were prepared from various stock cultures of pathogens and spread over the agar surface. The plates were allowed to dry for 20 min. Dried antimicrobial discs (with impregnated herbal extracts) and control discs (without antimicrobials) were carefully dispensed

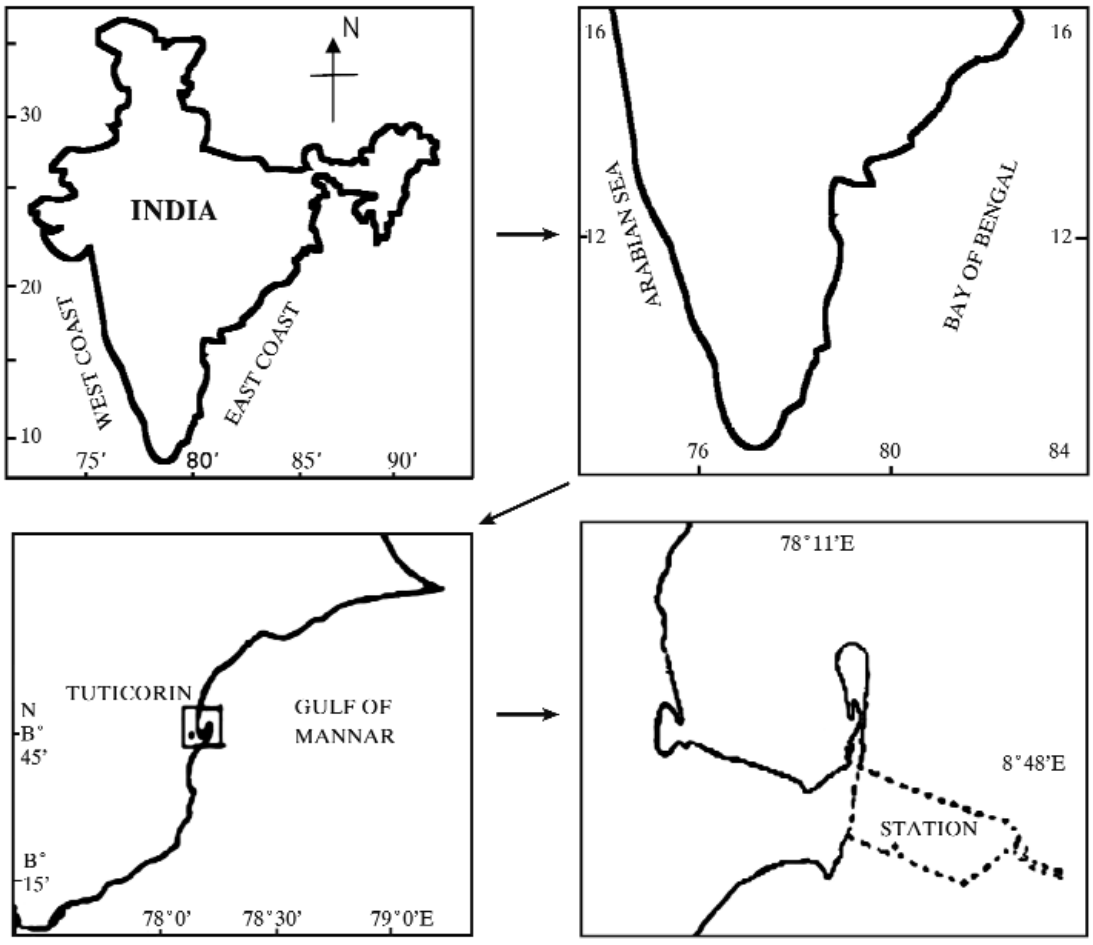


FIG. 1. Map showing the station where collections were made.

at uniform distances over the agar surface and were pressured for correct implantation. All plates were incubated at 35°C for 24 hrs. After incubation, plates were observed for inhibitory zone formation of the antimicrobial extracts on the microbial lawns.

Fractionation

The crude methanol extracts of the test and mantle bodies of *P. nigra* were fractionated by silica gel column chromatography with six different solvent systems. Elutions with hexane:chloroform (1:1), chloroform (100%), acetone (100%), benzene (100%), benzene:methanol (1:1) and methanol (100%) in the order of their polarity affording six fractions, namely fraction I, II, III, IV, V and VI. Certain amounts of extracts were taken and a concentration series of 0.1, 0.3 and 0.5 mg ml^{-1} was prepared.

Different fractions were tested against the nine bacterial strains using the standard filter paper disc diffusion method (Avelin *et al*., 1991).

The purified compounds were further characterized by ^{1}H NMR and ^{13}C NMR spectroscopy (FT NMR – BRUKER 300 MHz – Ultrashield) and IR spectroscopy (JASCO FT – IR – 410) (Source: Dr. Chellappa, School of Chemistry, Madurai Kamaraj University, Madurai, India). Several spectral values of the isolated metabolites were compared to published values to identify known metabolites.

Statistical analysis

The results are presented as means ± SD and are processed statistically by analysis of variance (ANOVA).

RESULTS

The antibacterial activity of the crude methanol extracts of test and mantle bodies of the two ascidian species was screened against different bacterial pathogens.

Table 1. Mean values of inhibition zones (diameter in mm) shown by the test and mantle bodies of the ascidians *P. nigra* and *H. pallida* (3 replicates, mean ± SD; BS: *Bacillus subtilis*, SA*: Staphylococcus aureus*, EA: *Entrerobacter aerogenes*, EC: *Escherichia coli*, KP: *Klebsiella pneumoniae*, PA: *Pseudomonas aeruginosa*, SP: *Salmonella paratyphii*, ST: *Salmonella typhii* and VC: *Vibrio cholerae*)

Bacterial strain		Ascidian species			
		P. nigra		*H. pallida*	
Code	Gram stain	Test	Mantle body	Test	Mantle body
BS	+	10.3 ± 0.8	6.3 ± 0.5	4.0 ± 0.3	2.2 ± 0.1
SA	+	12.3 ± 1.1	8.2 ± 0.8	4.4 ± 0.3	3.2 ± 0.3
EA	–	2.3 ± 0.2	0	0	0
EC	–	4.6 ± 0.4	3.1 ± 0.2	1.3 ± 0.1	0
KP	–	3.6 ± 0.2	0	1.0 ± 0.8	0
PA	–	2.3 ± 0.2	0	0	0
SP	–	9.6 ± 0.7	3.2 ± 0.3	2.3 ± 0.1	1.6 ± 0.14
ST	–	8.6 ± 0.9	3.2 ± 0.3	2.1 ± 0.2	1.0 ± 0.08
VC	–	4.0 ± 0.3	2.0 ± 0.1	1.0 ± 0.08	0
Average		6.4 ± 0.6	2.9 ± 0.3	1.8 ± 0.2	0.9 ± 0.1

The ascidian species tested displayed antimicrobial activity as shown in Table 1. Maximum antibacterial activity exhibited by 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). The corresponding zones of *H. pallida* were 4.4 ± 0.3 and 3.2 ± 0.3 mm respectively. Both species showed minimum activity against the Gram negative bacteria ($p > 0.05$).

The test of *P. nigra* exhibited a bactericidal effect, with the zone of inhibition ranging from 2.3 to 12.3 mm against all pathogens tested, as referred to the mantle body. The inhibitory effect of the mantle body of *H. pallida* on bacterial species was not remarkable; a zone of inhibition ranging from 1.0 to 3.2 mm in diameter was observed only in *S. aureus, S. paratyphii, B. subtilis* and *S. typhii.*

The overall means of the inhibition diameters featured marked differences between the test and mantle bodies of the ascidian species. Application of ANOVA between two species revealed that the antibacterial activity varied significantly ($p < 0.005$) and stronger antimicrobial activity was observed in *P. nigra* than in *H. pallida*.

Since the more promising results were obtained from *P. nigra* against all bacterial pathogens studied, extracts of *P. nigra* were further fractionated to examine their inhibitory effects. The results of the antimicrobial activity of different fractions of the test and mantle bodies of *P. nigra* are shown in Table 2. The test body exhibited a more potent effect rather than the mantle body. Fraction II of the test body revealed a higher antibacterial activity indicated by a zone of inhibition ranging from 1.0 to 12.6 mm in diameter against all bacteria tested at the highest concentration (0.5 mg ml^{-1}) and this effect decreased at low concentrations. The zone of inhibition was narrow at a concentration of 0.3 mg ml^{-1} for the fractions I, III, IV, V and VI, whereas, no inhibitory effect was observed at 0.1 mg ml^{-1} except *B. subtilis* and *S. aeroginosa* by fraction I & III, *E. coli* by fractions IV& VI, *S. paratyphii* by fraction I and *Entrerobacter aerogenes* by fraction V.

Considering the magnitude of the inhibitory zone exhibited by the mantle body, a moderate effect was observed. Fraction II of the mantle body showed an inhibitory effect against all bacteria tested, except *E. coli, P. aeruginosa* and *S. paratyphii.* On the other hand, the mantle body did not show an inhibitory effect when fractionated with hexane and chloroform (1:1).

Gram positive bacteria were more susceptible to treatment with extracts of ascidian species than Gram negative bacteria. Of the nine strains examined *Staphylococcus aureus* was a susceptible bacterium after treatment with all fractions, but the most susceptible one to fraction II with an inhibitory zone of 12.6 mm at a concentration of 0.5 mg ml^{-1} followed by *B. subtilis* with a 10.9 mm inhibitory zone.

Table 2. Mean values of inhibition zones (diameter in mm) shown by different fractions at different concentrations of the test body of *P. nigra* (3 replicates, mean ± SD; BS: *Bacillus subtilis*, SA*: Staphylococcus aureus*, EA: *Entrerobacter aerogenes*, EC: *Escherichia coli*, KP: *Klebsiella pneumoniae*, PA: *Pseudomonas aeruginosa*, SP: *Salmonella paratyphii*, ST: *Salmonella typhii* and VC: *Vibrio cholerae*). Fraction I – hexane:chloroform (1:1), Fraction II – chloroform (100%), Fraction III – acetone (100%), Fraction IV – benzene (100%), Fraction V – benzene:methanol (1:1) and Fraction VI – methanol (100%)

Bacterial pathogens	Gram positive								Gram negative							
	BS		SA		EA		EC		KP		PA		SP		ST		VC	
FRACTION I																		
Concentrations (mg ml^{-1})	Test	Mantle body	Test	Mantle body	Test	Mantle body	Test	Mantle body	Test	Mantle body	Test	Mantle body	Test	Mantle body	Test	Mantle body	Test	Mantle body
0.1	1.5	0	1.0	0	0	0	0	0	0	0	0	0	1.1	0	0	0	0	0
0.3	2.0	0	2.1	0	0	0	1.3	0	0	0	1.3	0	2.4	0	0	0	1.5	0
0.5	4.0	0	3.3	0	0	0	4.2	0	0	0	2.6	0	3.6	0	2.3	0	3.6	0
FRACTION II																		
0.1	4	2.2	5.6	1.5	1.5	0	0	0	1.6	0	0	0	1.5	0	1.0	1.5	1.0	0
0.3	7.3	3.1	9.4	2.3	2.7	0	1.0	0	2.1	0	0	0	2.3	0	1.5	2.0	2.6	0
0.5	10.9	4.0	12.6	2.5	5.2	1.5	3.3	0	3.6	1.0	1.0	0	5.2	0	3.6	2.5	4.0	1.5
FRACTION III																		
0.1	1.7	0	3.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.3	4.1	1.5	6.4	0	0	0	1.0	0	0	0	0	0	1.7	0	0	0	1.6	0
0.5	6.3	2.0	8.4	0	2.0	0	2.0	0	0	0	0	0	4.6	0	1.0	0	2.3	0
FRACTION IV																		
0.1	0	1.0	0	0	0	1.0	1.6	0	0	0	0	0	0	0	0	0	0	0
0.3	2.1	1.0	1.6	0	0	1.0	2.5	0	0	0	0	0	0	0	0	0	0	0
0.5	4.5	1.5	4.6	0	1.5	1.5	4.5	0	0	0	0	0	2.4	0	0	0	0	0
FRACTION V																		
0.1	0	–	0	–	1.5	–	0	–	0	–	0	–	0	–	0	–	0	–
0.3	1.6	–	1.0	–	2.1	–	2.2	–	1.6	–	0	–	1.7	–	0	–	0	–
0.5	3.4	–	3.5	–	3.0	–	4.0	–	2.4	–	0	–	3.0	–	0	–	0	–
FRACTION VI																		
0.1	0	–	0	–	0	–	1.5	–	0	–	0	–	0	–	0	–	0	–
0.3	0	–	1.1	–	0	–	2.1	–	0	–	0	–	1.5	–	0	–	0	–
0.5	2.2	–	2.6	–	0	–	3.4	–	1.0	–	1.0	–	3.0	–	1.0	–	0	–

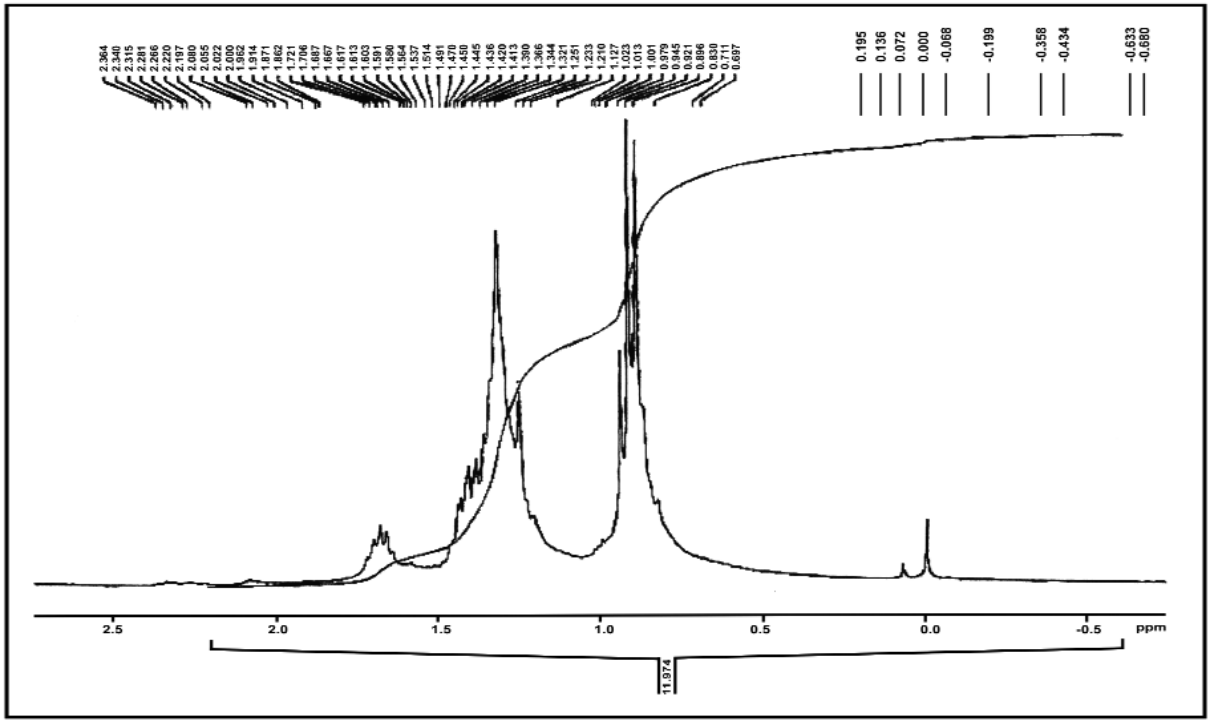


FIG. 2. ^{1}H NMR Spectra.

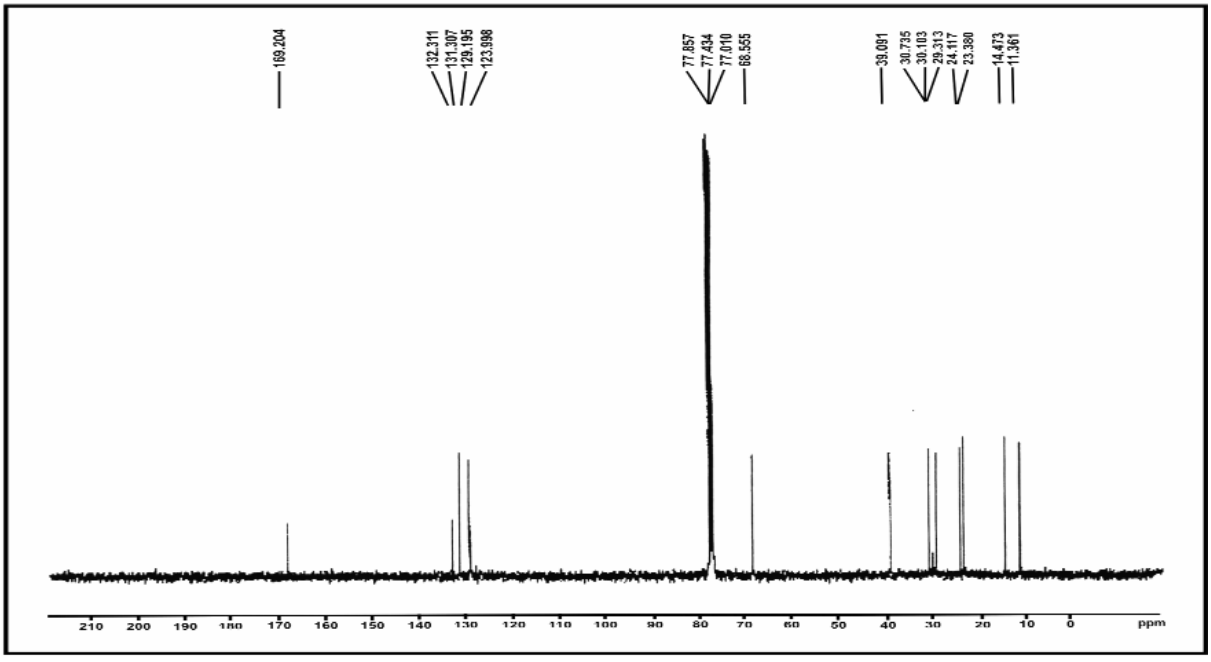
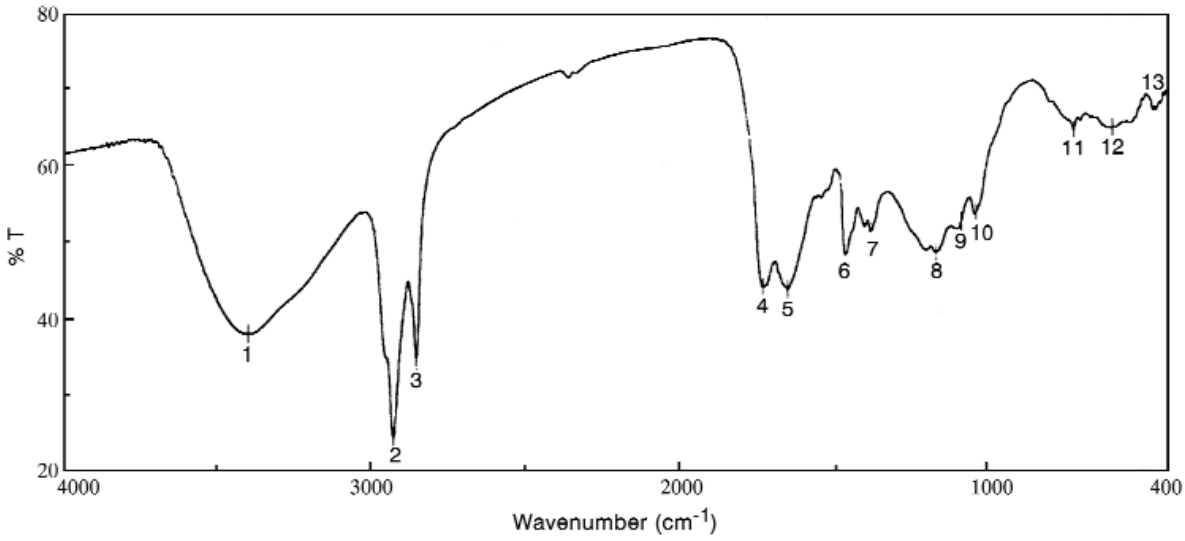


FIG. 3. ^{13}C NMR Spectrum.

As fraction II showed a more potent antibacterial activity than the rest of the fractions, it was further purified with TLC and characterized with IR, ^{1}H NMR and ^{13}C NMR spectra (Figs 2, 3, 4).

The ^{1}H NMR spectrum of the compound showed three major signals. The symmetrical quartlet pattern between 7.519 and 7.726 ppm corresponding to the para-substituted phenyl ring protons. The singlet at the chemical shift (δ) value of 7.266 ppm revealed the presence of phenolic OH protons. The apparent triplet at 4.27-4.16 ppm is the NH_2 protons. The COOH proton is off scale in the spectrum, since ^{1}H NMR



1: 3399.89, 38.0460 2: 2923.56, 24.8187 3: 2852.20, 34.9893 4: 1729.83, 44.5081
5: 1650.77, 44.2222 6: 1463.71, 48.7928 7: 1380.78, 51.8033 8: 1168.65, 49.1441
9: 1085.73, 53.1205 10: 1035.59, 54.3379 11: 721.25, 65.1174 12: 597.82, 65.3618
13: 458.98, 67.9681

FIG. 4. IR Spectrum.

was recorded only between 0 and 10 ppm.

^{13}C NMR confirms the presence of a C = O group (168.2 ppm) and a phenyl group (132.8-127.98 ppm for 4 carbons). The signal at 68.55 ppm indicates the presence of 1 carbon of the CH group. The triplet between 30.73 and 29.13 ppm corresponds to the carbon of the CH_2 group.

DISCUSSION

The crude methanol extracts of the test and mantle bodies of *P. nigra* and *H. pallida* examined in the present study indicated that both species possess substance(s) that inhibit the growth and multiplication of pathogenic bacteria at a varying spectrum. Chemical antibacterial defense has been suggested as one of an array of defenses potentially available to sessile invertebrates (Wahl & Banaigs, 1991).

Our results showed a significant difference in the antimicrobial activity of *P. nigra* compared to *H. pallida*. This could be justified by the observation that bacteria and other macrofoulers were present on the surface of the latter species, while *P. nigra* featured a clear axenic surface. This is in agreement with the results by Meenakshi (1997) who reported that among 47 species studied along the gulf of Mannar, *P. nigra* and *P. arabica* were free of any epibionts on their surface throughout the year.

Our results also showed that contrary to the mantle body, test body extracts exhibited varying levels of antimicrobial activity against the examined bacterial strains. This could be attributed to the fact that the test body might contain secondary metabolites which inhibit the growth of bacteria. This view is consistent with the findings of Abdul Jaffar Ali (2004), who reported that the test body of *P. nigra* harboured smaller number of total heterotrophic bacteria compared to that of the surrounding water medium.

Stoecker (1978) reported that tunic acidity is significantly associated with lack of epibionts. Parry (1984) claimed that the defensive ability of tunic acid is doubtful, because the undamaged tunic is neutral and the acid released by the damaged tunic becomes rapidly neutralized in the seawater. Hirose *et al.* (2001) and Stoecker (1978) considered that neutralization of the tunic acid by the seawater would not occur rapidly and tunic acid could be an effective agent in *P. nigra* by protecting against predation, fouling and infections. Shobu and Pawlik (2007) reported that crude organic extracts of whole tunic and internal tissues of *Phallusia nigra* contained vanadium metabolites (225 and 750 ppm dry mass, respectively) and were palatable to bluehead wrasse, *Thalassoma bifasciatum*. Crude extracts also exhibited no antimicrobial effects against a panel of four marine bacteria

known to be pathogens of marine invertebrates (*Vibrio parahaemolyticus*, *Vibrio harveyi*, *Leucothrix mucor* and *Deleya marina*).

In the present study, NMR spectra of the compound isolated from the test body provided a complete carbon skeleton of a tyrosine derivative which might be responsible for the potent antibacterial activity. This is in agreement with Rinehart (1990), who reported that a tyrosine-derived compound (called ecteinascidine) from *Ecteinascidia* sp. showed *in vitro* and *in vivo* antitumor activity. Another tyrosine-derived compound (etzionin) from an unidentified Red Sea tunicate, exhibited antifungal activity against *Candida albicans* with a MIC of 3 μg ml^{-1} as well as against *Aspergillus nidulans* and the Gram positive bacterium *Bacillus subtiles* (Lindquist & Fenical, 1990). Davidson (1993) also observed that the lissoclinotoxin and a tyrosine – phenylalanine derivatives exhibit antifungal and antibacterial activities, towards both Gram positive and Gram negative bacteria with MIC of 0.6 to 0.1 mg ml^{-1}, as well as against the pathogenic fungi *Candida albicans* and *Trychophyton mentagrophytes*. Kang & Fenical (1997) isolated four aromatic alkaloids, ningalins A-D, three of which possess new carbon skeletons and one is tyrosine derived, from an undescribed ascidian of the genus *Didemnum* collected in Western Australia near Nigalooo Reef. Components of ecteinascidins 722 and 736 exhibit tryptophan and tyrosine biosynthetic origins, a feature that is not common in alkaloids (Sakai *et al*, 1992). The detection of such compounds offers scope for the potential occurrence of a range of new structural types of mixed amino acid origin.

Of the nine bacterial strains tested, *Staphylococcus aureus* was the most susceptible to fraction II and the difference in response may be due to the species specific characters. In general, ascidians are known to contain a variety of novel and highly bioactive compounds which have been hypothesized to function in chemical defense. Earlier studies indicated that secondary metabolites particularly the tyrosine-derived metabolites showed antiviral, antifungal, antibacterial and antitumour activities. Hence, the present study indicated that the tyrosine derivative from the test body of *P. nigra* showed antibacterial activity which could be used in pharmacological research.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Edwin, Head of the Department of Zoology, V.O.C. College, Tuticorin and the first author expresses his gratitude to Dr. Chellappa, Professor, Department of Inorganic Chemistry, School of Chemistry, Madurai Kamaraj University, Madurai, for his immense help in acquiring NMR and IR spectra and Dr. Easwaran, Lecturer, Department of Chemistry, V.O. Chidambaram College, Tuticorin for the interpretation of the spectra. We acknowledge C. Khaiser Ahmed Sahib, Secretary and Dr. Major Syed Shahabudeen, Principal, Islamiah College, Vaniyambadi for their great enthusiasm and wise advice.

REFERENCES

Abdul Jaffar Ali H, 2004. Comparative study on the ecology of *Phallusia nigra* Savigny, 1818 from Tuticorin (South east coast) and Vizhinjam (South west coast). Ph. D. Thesis, Manonmaniam Sundaranar University, India.

Abdul Jaffar Ali H, Sivakumar V, 2007. Occurrence and distribution of ascidians in Vizhinjam Bay (south west coast of India). *Journal of experimental marine biology and ecology*, 342: 189-190.

Avelin MS, Vitalina M, Nagabhushanam K, 1991. Extracts of ascidians *Styela pigmentata* and *Pyura pallida.* In: Thompson MF, Sarojini R & Nagabhushanam R, eds. *Biological potency in bioactive compounds from marine organisms*. Oxford & IBH Publishing Co. Pvt. Ltd, New Delhi: 241-248.

Costa LV, Malpezzi ELA, Matsui DH, Machado-Santelli GM, Freitas JC, 1996. Cytotoxic activity of a methanol extract of *Phallusia nigra* (Tunicata, Ascidiacea). *Brazilian journal of medical and biological research*, 29: 367-373.

Davidson BS, 1993. Ascidians: producers of amino acid derived metabolites. *Chemical reviews*, 93: 1771-1791.

Davis AR, Bremner JB, 1999. Potential antifouling natural products from ascidian: A review. In: Thompson MF, Sarojini R, Nagabhushanam R, eds. *Bioactive compounds from marine organisms*. Oxford & IBH Publishing Co. Pvt. Ltd, New Delhi: 259-310.

Freitas JC, Ogata T, Veit CH, Kodama M, 1996. Occurrence of tetrodotoxin and paralytic shellfish toxins in *Phallusia nigra* (Tunicata, Ascidiacea) from the Brazilian coast. *Journal of venomous animals and toxins*, 2: 28-38.

Hirose E, 1999. Pigmentation and acid storage in the tunic: protective functions of the tunic cells in the tropical ascidian *Phallusia nigra*. *Invertebrate biology*, 118: 414-422.

Hirose E, Yamashiro H, Mori Y, 2001. Properties of tunic acid in the ascidian *Phallusia nigra* (Ascidiidae, Phlebobranchia). *Zoological science*, 18: 309-314.

Kang H, Fenical W, 1997. Ningalins A-D: Novel aromatic alkaloids from a Western Australian ascidian of the genus *Didemnum*. *The journal of organic chemistry*, 62: 3254-3262.

Kruse H, Sørum H, 1994. Transfer of multiple drug resistance plasmids between bacteria of diverse origin in natural microenvironments. *Applied and environmental microbiology*, 60: 4015-4021.

Lindquist N, Fenical W, 1990. Polycarpamines A-E, antifungal disulfides from the marine ascidian *Polycarpa auzata*. *Tetrahedron letters,* 31: 2389-2392.

Meenakshi VK, 1997. Biology of few chosen ascidians. Ph. D Thesis, Manonmaniam Sundaranar University, India.

Palavesam A, Sheeja L, Immanuel G, 2006. Antimicrobial properties of medicinal herbal extracts against pathogenic bacteria isolated from the infected grouper *Epinephelus tauvina. Journal of biological research-Thessaloniki*, 6: 167-176.

Parry DL, 1984. Chemical properties of the test of ascidians in relation to predation. *Marine ecology progress series*, 17: 279-282.

Ramasamy MS, Murugan A, 2003. Chemical defense in ascidians *Eudistoma viride* and *Didemnum psammathodes* in Tuticorin, south east coast of India: Bacterial epibiosis and fouling deterrent activity. *Indian journal of marine science*, 32: 337-339.

Rinehart KL, 1990. Antitumor, antiviral and immunosuppressive compounds form marine tunicates. In: Thompson MF, Sarojini R, Nagabhushanam R, eds. *Marine Biodeterioration – bioactive compounds from marine organisms*. Oxford & IBH Publishing Co. Pvt. Ltd, New Delhi: 301-307.

Sakai R, Rinehart KL, Guan Y, Wang AH, 1992. Additional antitumour ecteinascidins from a Caribbean tunicate: crystal structures and activities *in vivo*. *Proceedings of the national academy of sciences USA*, 89: 11456-11460.

Shobu O, Pawlik J, 2007. The role of vanadium in the chemical defense of solitary tunicate, *Phallusia nigra*. *Journal of chemical ecology*, 33: 643-654.

Stoecker D, 1980. Chemical defenses against predators. *Ecology*, 61: 1327-1334.

Tamilselvi M, 2008. Ecological studies on ascidians of Tuticorin coast. Ph. D. Thesis, Manonmaniam Sundaranar University, India.

Wahl M, Banaigs B, 1991. Marine epibiosis. III. Possible antifouling defense adaptations in *Polysyncraton lacazei* (Giard) (Didemnidae, Ascidiacea). *Journal of experimental marine biology and ecology*, 145: 49-63.