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Antibacterial and Cytotoxic Activities of Ascidians *Polyclinum madrasensis* Sebestian, 1952 and *Phallusia nigra* Savigny, 1816 from Tuticorin Coast of India

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Abstract: Ascidians are involved with rich source of bioactive agents which could be used for novel antimicrobial drugs. Ascidians are belongs to phylum chordate and class Ascidiacea. In present study two ascidians *P. madrasensis* and *P. nigra* were collected from Tuticorin, Southeast coast of India. The antibacterial compounds were screened to evaluate antibacterial activity in and different solvent like crude, methanol, dichloromethane, 2-ethoxy ethanol, acetone, chloroform, n-butanol, ethyl acetate and diethyl ether. In antibacterial activity *S. aureus* shows most sensitive against *Phallusia nigra* n-butanol extracts (17 ± 0.124 mm). Except for the 2- ethoxy ethanol, n-butanol, ethyl acetate and diethyl ether extracts of *P. madrasensis* against *S. aureus, K. oxytoca, V. cholerae, E. coli* and *P. mirabilis*. The cytotoxic activity of *P. madrasensis* and *P. nigra* extracts have been tested at various concentrations and showed the results of highest cytotoxicity assay conducted, indicating the presence of cytotoxic compounds in these ascidians.

Key words: Ascidian · cytotoxic compounds · Antibacterial · Bioactive agents

INTRODUCTION

Marine invertebrates, especially ascidians are most prominent sources of new compounds with cytotoxic potential. Ascidians are marine sessile filter feeder animals that belong to the phylum chordate, class Ascidiacea and are known for their ability to concentrate vanadium and other metals found in marine water. Ascidians have attracted attention as a source of antimicrobial proteins. They are thus considered to contain antibacterial agents of relevance to either antifouling technology or clinical pharmacology and the tissues of broad spectrum screening for bactericidal, antiviral or cytotoxic activity [1]. The ability to recognise, neutralize and eliminate opportunistic or pathogenic microorganisms is an important determinant of an animal's fitness to survive and successfully colonise its habitat. Because invertebrates do not express specific immunoglobulins or T-type lymphocytes, the non-specific inflammatory cellular responses of the blood or body fluids have traditionally been considered to be of central importance in antimicrobial defense in the animals. Fewer and generally less extensive, investigations have been made of the antibacterial proteins of other invertebrate groups and although whole body homogenates of some marine invertebrates have been reported to contain a variety of antimicrobial compounds, bio chemical investigations of these proteins have been largely confined to ascidians, particularly Solitary species [2]. Hence, the present study was undertaken to investigate the anti bacterial and cytotoxicity effectors in crude extract of ascidians from Tuticorin coastal waters.

MATERIALS AND METHODS

Specimen Collection and Identification: Bulk samples of ascidians, *P. madrasensis* and *P. nigra* were collected as common and persistent biofoulants from the cement blocks, pilings and oyster cages of Tuticorin coast (Lat. 8° 47' 20" and Long. 78° 09' 70"), India by SCUBA diving at the depth ranging from 4 to 6 m between May and June, 2008. The samples were thoroughly washed with sea water and cleaned of sand, mutt and overgrowing organisms at the site collection and transported to laboratory and collected specimens were identified by the standard literature.

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Extraction: The extraction was followed by Malla Reddy *et al*. [3]. The freshly collected tunicates were soaked in methanol at the site of collection until workup. The initial methanol extract was decanted and the ascidian material was extracted with 1: 1 dichloromethane: methanol (3x0.5 L) at room temperature. The combined extract including initial methanol extract was filtered through Whatman®No.1. Filter paper and the solvents were concentrated by rotary evaporator (VC100A Lark Rotavapor® at 30°C) with reduced pressure to give predominantly an aqueous suspension and it was extracted into ethyl acetate (3x0.5 L) and was concentrated under reduced pressure to give a dark brown gummy mass.

Antibacterial Activity: Antibacterial activity was carried out by using standard disc diffusion method [4- 6]. The following microorganisms *P. aeruginosa, E. coli, S. typhi, S. paratyphi, K. oxytoca, K. pneumonia, S. aureus, V. cholerae, P. mirabilis* and *L. vulgaris* were used. The extracts were applied on to 6 mm sterile discs in aliquots of 30 µL of solvent, allowed to dry at room temperature and placed on agar plates seeded with microorganisms. The bacteria were maintained on nutrient agar plates and incubated at 37°C for 24hrs. Zones of growth inhibition were measured. All extracts were tested twice at a concentration of 30 mg $disc^{-1}$.

Cytotoxicity Using Brine Shrimp Lethality Assay: To determine the toxicity effects on *Artemia salina* (brine shrimp) were determined Meyer *et al.* [7]. The extracts were dissolved in 0.01 ml of DMSO and incorporated into 5 ml of sea water (pH= 8.8 and Salinity = 28 ‰) containing ten *Artemia salina*. Each concentration (10, 20, 40, 60, 80 and 100 µgmL^{-1}) was tested thrice and a control DMSO was done each time. The vials were maintained under illumination. Survivors were counted after 24 hrs and the percentage of deaths at each dose and control (DMSO and saline water). The LC_{50} values of brine shrimp were obtained from counts using the prohibit analysis method described by Litchfield and Wilcoxon, [8].

RESULTS

Sample Collection and Identification: The ascidian, *P. madrasensis* Sebestian, 1952 (548 gms. in wet wt.) and *P. nigra* Savigny 1816 (680 gms. in wet wt.) were collected as common and persistent biofoulants from the cement blocks, pilings and oyster cages of Tuticorin coast. Collected specimens were identified by following the standard literature of Kott, [9] and Meenakshi, [10].

Extraction: 1: 1 dichloromethane: methanol (v/v) extracts of both *P. madrasensis* and *P. nigra* were concentrated under reduced pressure to give a dark brown gummy mass of 10.98 and 13.50 gms respectively. These crude extracts were further studied for biological activities.

Antibacterial Activity: Antibacterial activity of *P. madrasensis* and *P. nigra* radius of the zone of inhibition around the disc were represented (Table 1, 2). In the present study, *P. madrasensis* and *P. nigra* showed a promising source of antibacterial compound were screened to evaluate antibacterial activity in crude,

Table 1:Antibacterial activity of *Polyclinum madrasensis* against human pathogens

	Zone of Inhibition* (mm)									
Pathogens	C	M	DCM	E	A	CH	B	EA	DE	Control
Staphylococcus aureus	6 ± 0.081	7.2 ± 0.081	8 ± 0.081	0	4 ± 0.124	3 ± 0.081	7 ± 0.081	3 ± 0.081	3 ± 0.081	0
Salmonella typhi	7 ± 0.081	10 ± 0.081	13 ± 0.124	3.2 ± 0.163	5 ± 0.081	6.7 ± 0.205	6.5 ± 0.124	4.3 ± 0.081	3.5 ± 0.04	0
Salmonella paratyphi	8 ± 0.081	9 ± 0.286	11 ± 0.094	5.5 ± 0.047	3 ± 0.047	2 ± 0.081	2 ± 0.081	5 ± 0.081	3 ± 0.081	0
Klebsiella oxytoca	7 ± 0.081	6 ± 0.081	5 ± 0.081	0	6 ± 0.081	9 ± 0.286	12 ± 0.163	5 ± 0.081	7 ± 0.081	0
Klebsiella pneumonia	12 ± 0.163	15 ± 0.124	6 ± 0.081	10 ± 0.124	8 ± 0.081	4 ± 0.124	4 ± 0.124	4 ± 0.124	5 ± 0.124	0
Vibrio cholerae	6 ± 0.081	7 ± 0.081	6 ± 0.081	4 ± 0.124	4 ± 0.124	3 ± 0.081	4 ± 0.124	0	0	0
Pseudomonas aeruginosa	6 ± 0.081	7 ± 0.081	9 ± 0.081	8.5 ± 0.047	8 ± 0.081	3 ± 0.081	5 ± 0.124	7 ± 0.081	6 ± 0.081	0
E. coli	6.2 ± 0.081	7.2 ± 0.124	5 ± 0.124	5 ± 0.124	5 ± 0.124	3.5 ± 0.081	4 ± 0.124	4 ± 0.124	0	0
Proteus mirabilis	6 ± 0.081	9.6 ± 0.081	6.7 ± 0.081	7 ± 0.081	6.5 ± 0.124	4.5 ± 0.081	0	5 ± 0.124	8 ± 0.081	0
Lactobacillus vulgaris	8 ± 0.081	9 ± 0.081	5 ± 0.124	4 ± 0.124	4 ± 0.124	6 ± 0.081	5 ± 0.124	7 ± 0.081	8 ± 0.081	0

C= Crude; M= Methanol; DCM= Dichloromethane; E= 2- Ethoxy Ethanol; A= Acetone; CH= Chloroform; B= n-Butanol;

EA= Ethylacetate; DE= Diethyl ether.

*Zone in mm indicates the distance from the border of the disc to the edge of the clear zone.

[a] Solvent has a zone inhibition of 0 mm.

Table 2: Antibacterial activity of *Phallusia nigra* against human pathogens

	Zone of Inhibition* (mm)									
Pathogens	C	M	DCM	E	A	CH	B	EA	DE	Control
Staphylococcus aureus	7 ± 0.081	13 ± 0.124	6 ± 0.081	9.5 ± 0.081	5 ± 0.124	6 ± 0.081	17 ± 0.124	7.4 ± 0.124	6.3 ± 0.124	0
Salmonella typhi	13 ± 0.124	15 ± 0.124	12 ± 0.163	5.5 ± 0.047	6 ± 0.081	12.4 ± 0.09	7 ± 0.081	7.4 ± 0.124	12 ± 0.163	0
Salmonella paratyphi	12 ± 0.163	11 ± 0.09	9 ± 0.081	8 ± 0.081	2 ± 0.081	4 ± 0.124	7 ± 0.081	4 ± 0.124	8 ± 0.081	0
Klebsiella oxytoca	7 ± 0.081	6 ± 0.081	5.5 ± 0.047	6 ± 0.081	3 ± 0.081	3 ± 0.081	4 ± 0.124	12.1 ± 0.081	4 ± 0.124	0
Klebsiella pneumonia	8.5 ± 0.124	6.5 ± 0.124	5 ± 0.124	9 ± 0.081	6 ± 0.081	5 ± 0.124	3 ± 0.081	4 ± 0.124	7 ± 0.081	0
Vibrio cholerae	7 ± 0.081	12 ± 1.63	6.5 ± 0.124	7 ± 0.081	5 ± 0.124	8 ± 0.081	7 ± 0.081	7.5 ± 0.124	11 ± 0.124	0
Pseudomonas aeruginosa	8.5 ± 0.124	8 ± 0.081	6 ± 0.081	5 ± 0.124	7 ± 0.081	7.4 ± 0.124	8.5 ± 0.124	2 ± 0.081	2 ± 0.081	0
E. coli	7.5 ± 0.124	5.5 ± 0.047	4.5 ± 0.081	6 ± 0.081	5 ± 0.124	4 ± 0.124	3.5 ± 0.081	4 ± 0.124	7 ± 0.081	0
Proteus mirabilis	9 ± 0.081	10 ± 0.081	8.8 ± 0.081	7 ± 0.081	6.3 ± 0.124	4.5 ± 0.124	2 ± 0.081	7 ± 0.081	6.5 ± 0.124	0
Lactobacillus vulgaris	9 ± 0.081	9 ± 0.081	8 ± 0.081	6 ± 0.081	6 ± 0.081	4 ± 0.124	6 ± 0.081	5 ± 0.124	8 ± 0.081	0

C= Crude; M= Methanol; DCM= Dichloromethane; E= 2- Ethoxy Ethanol; A= Acetone; CH= Chloroform; B= n-Butanol;
EA= Ethylacetate; DE= Diethyl ether.
*Zone in mm indicates the distance from the border of the disc to the edge of the clear zone.
[a] Solvent has a zone inhibition of 0 mm.

Table 3: ytotoxicity of *P. madrasensis* extracts against *Artemia salina* larvae

S. No.	Extracts	LC $_{50}$ (µg m^{-1})
1.	Control (DMSO + Saline)	-
2.	Crude	92
3.	Methanol	97
4.	Dichloromethane	79
5.	2- Ethoxy Ethanol	78
6.	Acetone	54
7.	n-Butanol	74
8.	Ethylacetate	21
9.	Diethyl ether	43

Table 4:Cytotoxicity of *P. nigra* extracts against *Artemia salina* larvae

S. No.	Extracts	LC $_{50}$ (µg m^{-1})
1.	Control (DMSO + Saline)	-
2.	Crude	130
3.	Methanol	145
4.	Dichloromethane	67
5.	2- Ethoxy Ethanol	35
6.	Acetone	69
7.	n-Butanol	52
8.	Ethylacetate	64
9.	Diethyl ether	56

methanol, dichloromethane, 2-ethoxy ethanol, acetone, chloroform, n-butanol, ethyl acetate, diethyl ether extracts, because ascidians, have been already established as a group with higher percentage of bioactivity [11, 12]. It showed high and moderate antibacterial activity against 10 pathogens assayed. From the bacteria tested, *S.aureus* is the most sensitive against *P. nigra* n-butanol extracts (17 ± 0.124 mm). Except for the 2-ethoxy ethanol, n-butanol, ethyl acetate and diethyl ether extracts of *P. madrasensis* against *S. aureus, K. oxytoca, V. cholerae, E. coli* and *P. mirabilis* and all extracts are active against bacteria.

Cytotoxicity Using Brine Shrimp Lethality Assay: Both *P. madrasensis* and *P. nigra* extracts have been tested at 10, 20, 40, 60, 80 and 100 µgmL^{-1} and showed the results of highest cyto toxicity assay conducted, indicating the presence of cytotoxic compounds in these ascidians. The LC_{50} values are given in (Table 3. and 4). The different solvent system extracts of both *P. madrasensis* and *P. nigra* are showing cytotoxic properties against *Artemia salina* larvae.

DISCUSSION

The abundant evidence that ascidians have cytotoxic and antimicrobial activity [13] has generated interest from natural product chemists to identify the molecules responsible for these actions. Because most previous researchers have concentrated on extracting cytotoxic secondary metabolites from intact ascidians, that natural products are indeed viable source and resources for drug discovery and development [14]. They are thus considered to contain antibacterial agents of relevance to either antifouling technology or clinical pharmacology and the tissues of several solitary species have been subjected to broad spectrum screening for bactericidal, antiviral or cytotoxic activity [1]. Antibacterial activity has been previously reported from extracts of some ascidians.

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 [15]. In the present study, *P. madrasensis* and *P. nigra* showed a promising source of antibacterial compounds were screened to evaluate antibacterial activity in crude and solvents extract. It showed high and moderate antibacterial activity against 10 pathogens assayed, from the bacteria tested; *S. aureus* is the most sensitive against *P. nigra* n-butanol extracts (17 ± 0.124 mm). Except for the 2-ethoxy ethanol, n-butanol, ethyl acetate and diethyl ether extracts of *P. madrasensis* against *S. aureus, K. oxytoca, V. cholerae, E. coli* and *P. mirabilis,* all extracts are active against bacteria.

Some compounds exhibited mild antibacterial activity as well as toxicity towards to brine shrimps. Coproverdine is a cytotoxic alkaloid isolated by bioassay directed fractionation of a unidentifed ascidians collected at the three Kings Islands, New Zealand [16]. Cytotoxicity towards a variety of murine and human tumor cells lines was observed. Rubrolide-M recently isolated from a Spanish collection of the ascidians *Synoicum blochanni* [17] was synthesis using palladium catalyzed coupling methodology [18]. The compound and related congeners were found to exhibit cytotoxicity towards human tumor cell-lines. Sebastianines A and B isolated as biologically active pyridoacridine metabolites, which show cytotoxic activities towards colon cancer cells, have been extracted from a Brazilian collection of the ascidians *Cystodytes dellechiajei* [19]. Thai ascidian *Ecteinascidia thurstoni*, using a KCN- pretreatment isolation procedure, identified the Known two Alkaloids ecteinascidins and the two novel analogues ecteinascidins [20]. Such like many species of ascidians showed cytotoxicity. In the presence work *P. madrasensis* and *P. nigra* extracts have been tested at various concentrations and different solvent system, showed the highest cytotoxicity assay conducted, indicating the presence of cytotoxic compounds in these ascidians. The different solvent system extracts of both *P. madrasensis* and *P. nigra* are showing cytotoxic properties against *Artemia salina* larvae. Thus the current studies revealed the presence of potent antimicrobial compounds from ascidians of Tuticorin coast.

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