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Isolation and partial characterization of the active metabolite of ascidian, *Polyclinum madrasensis* from the Palk Bay Region, Southeast coast of India

Kathirvel Iyappan and Gnanakkan Ananthan*

Tunicate Biology Laboratory, Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai - 608 502, TN, India.

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Ascidians are rich source of bioactive agents which could be used for novel antimicrobial drugs. The present investigation inspects the antibacterial potential of ascidian, *Polyclinum madrasensis* collected from Mandapam, the Palk Bay region, Southeast coast of India. The crude extracts were tested for inhibition of bacterial growth against human pathogens. Antibacterial assay was carried out by agar well diffusion method. The maximum inhibition zone (12.0±0.5 mm) was observed against the *Staphylococcus aureus* in crude methanol extract. The consequent zone of 6.5±0.1 mm was observed against *S. aureus in* ethanol extract and minimum inhibition zone (3.2±0.5 mm) was noticed with *Pseudomonas aeruginosa*. Molecular weight of tissue protein was determined through sodium dodecyl sulphate polyacryalamide gel electrophoresis (SDS-PAGE) and active metabolites were characterized by Fourier Transform Infrared spectroscopy (FTIR) analysis. The protein bands were at 36.5, 20.5, and 10.5 kDa, in SDS-PAGE and O-H stretch carboxylic acid compounds identified at the peak 3533.59 cm^{-1}. It could be concluded from the present study that crude extract of the ascidian, *P. madrasensis* has potential antimicrobial effect against human pathogens.

Key wards: *Polyclinum madrasensis,* bioactive compounds, Fourier Transform Infrared spectroscopy (FT-IR), sodium dodecyl sulphate polyacryalamide gel electrophoresis (SDS-PAGE).

INTRODUCTION

It is a real fact that the importance of marine organisms as a source of new substances is growing. With marine species comprising approximately a half of the total global biodiversity, the sea offers an enormous resource for novel compounds (Blunt et al., 2011). A very different kind of substances have been obtained from marine organisms among other reasons because they are living in a very exigent, competitive and aggressive surrounding and are very different in many aspects from the terrestrial environment, a situation that demands the production of quite specific and potent active molecules (De Vries, 1995). Peptidic compounds analyzed are obtained from very different marine organisms exhibiting different chemical structures and displaying a large variety of

*Corresponding author. E-mail: casananthan@yahoo.co.in. Tel: +91 4144-243223. Fax: +91 4144-243555.

pharmacological effects on specific targets (Aneiros and Garateix, 2004). In marine invertebrates so far, approximately 7,000 marine natural products have been reported (Venkataraman and Wafer, 2005). Ascidians have attracted attention as a source of antimicrobial proteins because they are marine sessile, filter feeding invertebrate which hold a phylogenetically strategic position close to the origin of the vertebrate line. The group of ascidians is one of the most compelling sources of metabolites both of chemical and biomedical interest in the marine environment (Blunt et al., 2011). Antitumor, antiviral and immunosuppressive active compounds are primarily isolated from tunicates (Schmitz et al., 1993).

Bacterial infection cause high rate of mortality in human population (Kandhasamy and Arunachalam, 2008). During the last few decades, the number of upcoming infectious disease is increasing in the developing countries (Murugan and Mohan, 2011). *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*, are well known to be causative agents for boils skin infections, abscesses, dysentery and diarrhoea (Levin, 1987; Ananthanarayanan and Paniker, 2008). Presently, various ascidians such as *Botryllus* sp., and *Didemnum* sp. have been reported for producing anti-cancer drugs (Azumi et al., 1990). Halocyamine A, an antimicrobial substance was isolated from haemocytes of the solitary ascidians *Halocynthia roretzi* (Azumi et al., 1990). Ascidian-based products may be a new boon to the development of antibiotics effective against antibiotic resistant strain. Then, attention has focused on ascidians because of their biologically active metabolites and the chemistry of ascidians has become one of the most active fields of marine natural products; it has been amply demonstrated that these sea creatures are prolific producers of unusual structures with significant bioactivities (Faulkner, 2002; Blunt, 2010).

In the arena of marine habitat the colonization process is affected by organic metabolites produced by the host organism. Such metabolites may affect bacteria in a number of ways, ranging from the induction of chemo tactic responses to the inhibition of bacterial growth or cell death (Bell and Mitchell, 1972). In this study, we evaluated the antimicrobial potential of the ascidian, *Polyclinum madrasensis* and partially characterized its biologically active metabolites through sodium dodecyl sulphate polyacryalamide gel electrophoresis (SDS-PAGE) and Fourier Transform Infrared spectroscopy (FT-IR).

MATERIALS AND METHODS

Collection

The ascidian, *P. madrasensis* was collected from Mandapam (Lat. 0957' N: Long79 11' E) Palk Bay region, Southeast coast of India. The sample was collected by hand-picking during low tide season and transported to the laboratory with safe condition. Molluscan shell, calcrete rock fragments attached to the foot of the animal was carefully removed. They were identified using key to identification of Indian ascidians (Meenakshi, 1997).

Preparation of ascidian extract

The fresh tissues of *P.madrasensis* were freezed at -20°C and used for the extraction of antimicrobial metabolites. Their soft bodies were removed by breaking the outer layer. The whole body tissues of the sample (50 g) were cut into small pieces and the tissue sample was used for extraction using methanol and ethanol. The extracts were cold steeped over night at -18°C and filtered with What man No. 1 filter paper. The filtrate was evaporated to dryness in rotary evaporator (Becerro, 1994; Wright, 1998). The dried crude extracts were used for antibacterial assay against human pathogens (*P. aeruginosa*, *E. coli*, *S. aureus*, *Salmonella typhi*, *Vibrio cholera*, *Vibrio parahaemolyticus*, *Klebsiella pneumoniae* and *Proteus mirabilis)*. All the pathogenic bacterial strains were obtained from the Department of Clinical Pathology, Raja Muthiah Medical College, Annamalai University, Tamil Nadu, India.

Antibacterial assay

The ascidian, *P. madrasensis* crude extracts were tested for inhibition of bacterial growth against human human pathogens. Antibacterial assaywas carried out by agar well diffusion technique (El-Masry et al., 2000). Human pathogens were inoculated in sterile nutrient broth and incubated at 37°C for 24 h. Pathogens were swabbed on the surface of the Muller Hinton agar plates and wells were punched out using a sterile cork borer (6 mm diameter). About 50 µl of the different solvent of ascidian, *P. madrasensis* extracts, were transferred in to each well. For each pathogen, controls were maintained where pure solvents were used instead of the ascidian extract.

The plates were incubated at 37°C for 24 h (Mtolera and Semesi, 1996). The results were obtained by measuring the inhibition zone diameter for each well and expressed in millimetre (Mohamed et al., 2010).

Muscle protein molecular weight determination by SDS-PAGE

Molecular weight of the muscle protein of *P. madrasensis* was determined using SDS-PAGE following the procedure by Sambrro et al. (2006). Glass plates were assembled and 20 ml of 15% resolving gel was prepared and poured immediately to the notch plate. It was over laid with butanol. After polymerization was completed, it was poured off and the top layer was washed with deionized water. Then it was over laid with 8 ml of stock gel. Approximately 1 ml of 1% SDS gel loading buffer and sample were taken and it was heated at 100°C for 3 min. Then, it was carefully fixed in electrophoresis apparatus. 15 µL of samples with different molecular weight markers (14.0 to 97.4 kDa) were loaded, respectively in the well, run in the gel and stained with coomassive brilliant blue.

Fourier transform infrared (FT-IR) spectral analysis

The lyophilized methanol extract of *P. madrasensis* (10 mg) were mixed with 100 mg of dried potassium bromide (KBr) and compressed to prepare as a salt disc. The disc was then read spectrophotometrically (Bio-Rad FTIR-40-model, USA). The frequencies of different components present in sample were analyzed.

Table 1. Antibacterial assay of ascidian, *Polyclinum madrasensis* against human pathogens.

Human pathogens	Zone of Inhibition(mm)	
	Methanol extract	Ethanol extract
S. aureus	12.0±0.5	5.0±0.8
E. coli	8.2 ±0.5	5.0±0.5
P. aeruginosa	6.5±0.2	3.2±0.5
V. parahaemolyticus	6.5±0.8	5.0±0.1
V. cholera	5.5±0.6	3.4±0.8
K. pneumonia	5.5 ±0.5	3.3±0.5
P. mirabilis	5.5 ±0.8	3.4±0.2
S. typhi	4.3±0.7	3.3±0.5

Values are the mean of triplicates ± SD $p < 0.05$

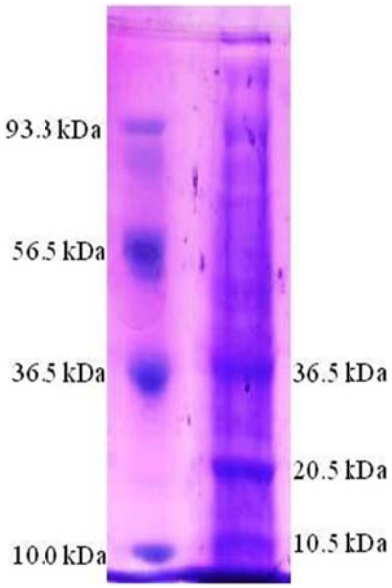

Figure 1. Molecular size of ascidian, *Polyclinum madrasensis* tissue protein determined by SDS. Lane 1: Standard protein molecular weight marker. Lane 2: Crude protein sample of ascidian, *Polyclinum madrasensis*.

RESULTS

Antibacterial activity

Two crude extracts of ascidian, P. madrasensis were screened against eight human bacterial pathogens for testing their antibacterial activities. The inhibition zones of methanol and ethanol extracts against the specific test organisms were shown in Table 1. The methanol extracts showed high antibacterial activity against *S. aureus* (12.0±0.5 mm), followed by *E. coli* (8.2 ±0.5 mm), *P. aeruginosa* (6.5±0.2 mm), *V. parahaemolyticus* (6.5±0.8 mm), *V. cholera*, (5.5±0.6 mm) *K. pneumonia* (5.5±0.8 mm) P. mirabilis (5.5 ±0.8 mm) and S. typhi (4.3±0.7 mm). The minimum inhibition zone (3.2±0.5 mm) was noticed with *P. aeruginosa* in ethanol extract. However, maximum zone of inhibition range between 5.0±0.1 - 5.0±0.8 mm against *E. coli*, *S. aureus* and *V. parahaemolyticus* in ethanol crude extract. Other indicated organisms were recorded as having meagre activity in ethanol extract.

Muscle protein molecular weight determination by SDS-PAGE

Crude protein sample of ascidian, *P. madrasensis* yielded three bands ranging from 25.0 to 110.0 kDa with well defined. The bands were at 36.5, 20.5 and 10.5 kDa, respectively. Ascidian, *P. madrasensis* sample was compared with the standard protein molecular weight marker (14.4 to 97.4 kDa) (Banglore Genei, India) (Figure 2).

FT- IR spectral analysis

The FTIR spectrum of the ascidian methanol extracts revealed characteristics of functional groups (Figure 1) such as O-H stretch carboxylic acid compounds, identified at the peak 3533.59 cm^{-1} and C-H stretching peak of alkanes group at 2954.95, 2922.16 and 2850.79 cm^{-1}, respectively. The –C (triple band) C–stretch peaks of alkenes were at 1641.42 and 1631.78 cm^{-1}. The peaks of 1458.18 and 1402.25 cm^{-1} indicate C-C stretch aromatic. Peak values were recorded at 1014.56 and 1083.99 cm^{-1}. Absorption indicated C-N stretch aliphatic amines.

DISCUSSION

Marine organisms have been recognized as a rich source of novel compounds that are of potential interest to mankind (Faulkner, 2000). They produce secondary metabolites and other compounds to repel and deter predators

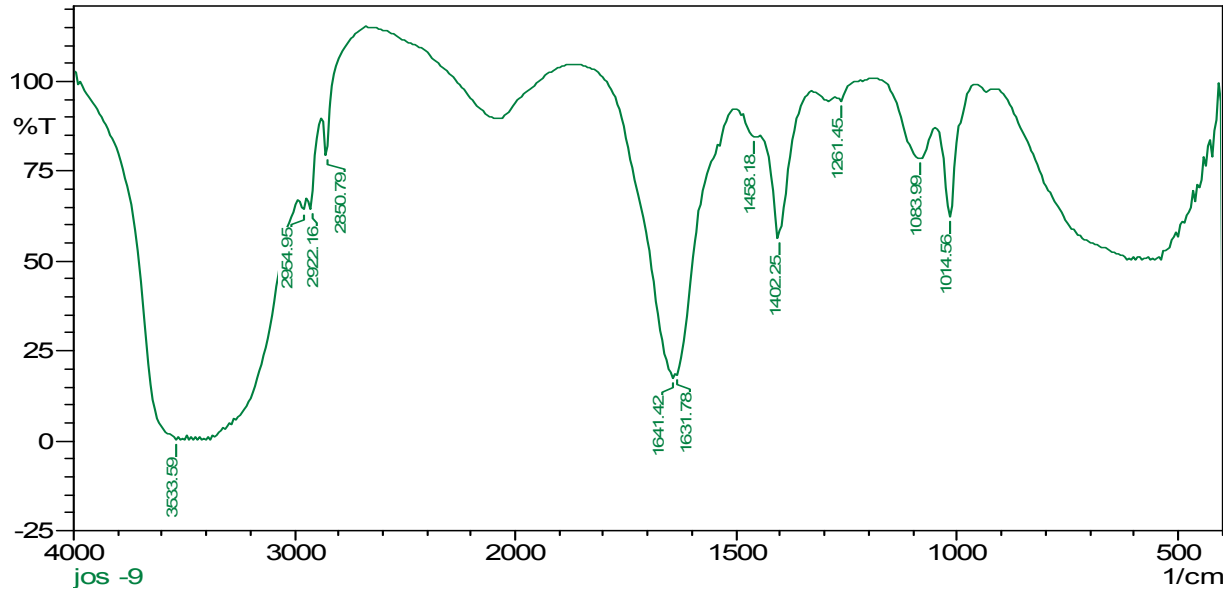

Figure 2. The FT-IR Spectra of lyophilized sample in *Polyclinum madrasensis*.

(Pawlik et al., 2002). Most of these pristine resources have not been explored for bio prospecting and microbial ecological studies. The ascidians are a potential chemical, ecological phenomenon, which provides sustainable source of supply for developing novel pharmaceutical leads. 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 (Thompson et al., 1985).

In the present study, a pronounced antimicrobial effective has been observed against some human pathogens. Both methanol and ethanol crude extract of *P. madrasensis* inhibit growth of human clinical pathogens. The maximum activity was observed against *S. aureus* (12.0±0.5 mm) and minimum activity was observed against *P. aeruginosa* (3.2±0.5 mm). Similarly, maximum activity was exposed by methanol extract in *S. aureus* and minimum activity was showed by ethanol extract in *P. aeruginosa* which was collected at Tuticorin coast (Bragadeeswaran et al., 2010). Selva Prabhu et al. (2012) have reported that the crude methanol extract of *P. madrasensis* of the maximum inhibition zone of against *E. coli* and the minimum of *P. aeroginosa* and ethanol extract produced *E. coli* and *P. aeruginosa*. It supports this work. In this present study, we used -18°C cold steep extract.

In the present investigation, the tissue extraction from *P. madrasensis* showed antimicrobial activity and it was subjected to SDS-PAGE in order to estimate the molecular weight. After electrophoresis, clear bands were detected in the gel which represented molecular weight of proteins ranging 11 to 97 kDa. Green et al. (2003) reported that the molecular weight of protein from hemolymph of the solitary ascidian, *Styela plicata* from Australian waters is 43 kDa. The endoderm specific alkaline phosphate proteins with molecular masses of 86 and 103 kDa were likewise reported by Kumano et al. (1996). The amount of carbohydrate, protein, lipid and minerals such as phosphorous and calcium contents in these ascidians were previously reported by Rajesh and Ali (2008) and Natarajan et al. (2011). FT-IR analysis reveals the presence of bioactive compound signals at different ranges. Previously, aliphatic chain was identified as the peak of 3394.43 and 2920.40 cm^{-1} in ethanol extract of *P. madrasensis* by Meenakshi et al. (2013). FT-IR spectroscopy is proved to be a reliable and sensitive method for detection of biomolecular composition (Komal Kumar and Devi Prasad, 2011). This study shows that the medicinal value of the ascidian *P. madrasensis* tissue may be due to high quality of bioactive metabolite.

The present study revealed that the species of *P. madrasensis* showed antibacterial activities against the human pathogens. So, they possess potential pharmacological action.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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