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Full length Research paper

Exploration of antibacterial effects on the crude extract of marine ascidian *Aplidium multiplicatum* against clinical isolates

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In vitro **antibacterial screening of a marine ascidian *Aplidium multiplicatum*, collected from Mandapam, Southeast coast of India, against selected clinical isolates of bacteria was conducted in this study. The crude ethyl acetate extract was more active, exhibiting a broad spectrum antibacterial activity than the crude methanol extract and petroleum ether extract against each of the bacterial strains tested. Maximum inhibition zone (17 mm) was observed against the gram negative *Pseudomonas aeruginosa* in crude ethyl acetate extract, followed by methanol extract showed activity (12.5 mm) and the petroleum ether extract of *A. multiplicatum* showed minimum inhibition zone (1 mm) against *Klebsiella oxytoca*. The range of MICs were high in ethyl acetate extract (1.80 mg/ml) and it was low in the petroleum ether (0.25 mg/ml). The range of MBCs was high in the ethyl acetate extract (2.05 mg/ml) and low concentration was observed in petroleum ether (0.55 mg/ml). Hence an attempt was made on the present study to investigate the potent antibacterials from marine ascidian.**

Key words: Antibacterials, ascidians, clinical isolates, MIC, MBC.

INTRODUCTION

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) . Although research on bioactive compounds from ascidians were recently initiated, it is significant that the first marine natural product didemnin B is entering in to human clinical trial, 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). Most of the ascidians are utilized as food in various countries and they are known to produce bioactive metabolites which prevent bio-fouling and this can be considered as a kind of autogenic protection (Bergquist et al., 1978). This mechanism has proved to be timely alternative natural medicine to human beings. Filter feeders often account for the greater part of the biomass and production of consumers in the intertidal and sublittoral communities of rocky shores (Velimirov et al., 1977; Field et al., 1980a). These rocky shore communities are principally formed by several species of mussels, sponges, holothurians and barnacles and the ascidian. Phytoplankton and organic particles in suspension apparently constitute the bulk of the food of many species of ascidians. In *Microcosmus sulcatus* the brachial sac has been found to contain organisms such as bacteria, diatoms and radiolarians, characteristics of water immediately above the substratum (Costa, 1969). When present in large numbers, their high filtration rate can have a dramatic effect on available plankton and suspended organic matter (Riisgard and Larsen, 2000).

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From tunicate (ascidians) *Tridemnum solidum*, the first marine organism or compound entered in human cancer clinical trial as a purified natural product (Carte, 1996). Already various ascidians such as *Botryllus* sp. and *Didemnum* sp. was proved 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). The bioactive substance which possesses potent anticancer activity Ecteinascidin- 743 was isolated from Caribbean tunicate *Ecteinascidia turbinata* (Russo et al., 2008).

Such potential ascidians need to be explored for the pharmaceutical purpose. The case of living marine surfaces the colonization process can additionally be affected by organic metabolites produced by the host organism. 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 (Bell and Mitchell, 1972; Sieburth and Conover, 1965). The role of secondary metabolites as a chemical defense against epibiosis has been discussed (Bakus et al., 1986; Davis et al., 1989; Paul, 1992). This study is part of a programme on screening for the antibacterial properties of natural product such as marine ascidians in order to find a novel antibacterial agent and peptide that can restrain the growth of bacteria in human body. Hence an attempt was made on the present study to investigate the potent antibacterials from marine ascidian.

MATERIALS AND METHODS

Collection and preparation of samples

The ascidian *Aplidium multiplicatum,* Sluiter (1909) (Chordata: Ascidiacea: Enterogona: Polyclinidae) were collected during the low tide of the intertidal area of Mandapam at Gulf of Mannar, Tamil Nadu, (Latitude 9°16'N and Longitude 79°8'E), Southeast coast of India, between 15 and 17 June, 2009. The collected samples were rinsed with sterile sea water to remove associated debris and salt. The samples were weighed (10 g) and preserved separately in methanol, ethyl acetate and petroleum ether (1:2) and brought to the laboratory. Samples were then soaked in the above mentioned solvents for 48 h, the extracts were then obtained from the soaked samples by grinding, using pestle and mortar and filtering through Whatman No.1 filter paper, the filtrate were centrifuged at 3000 rpm. The solvent was evaporated under reduced pressure using desiccator and the residue was weighed and dissolved in Dimethyl formamide (1 mg/ml) for using them for the antibacterial activity. All the pathogenic bacterial strains were obtained from Raja Muthiah Medical College, Annamalai, University.

Antibacterial susceptibility assay

Ascidian crude extract was tested for inhibition of bacterial growth against human pathogenic bacteria. The clinical isolates were sub cultured from stock culture 24 h prior to the experiment in nutrient agar media and used for the study. All three extracts of *A. multiplicatum* were tested for antibacterial activity by disc diffusion method (Avelin et al., 1991). The bacterial strains such as *Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca, Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa, Salmonella paratyphi* and *Salmonella typhi* used for the antibacterial activity. All the bacterial strains were enriched in nutrient broth at 37°C for 18 - 24 h, after which they were streaked over Mueller Hinton agar surface using sterile cotton swabs. Then 20 l of the extract was pipetted on a 6 mm sterile paper disc, the solvent was allowed to evaporate and the disc was placed on the surface of the plate. The plates were incubated for 24 h at 37°C. Areas of inhibited bacterial growth were observed as clear halos (zones) around the disc.

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration was determined by the following procedure (Collins et al., 1995). Various concentrations of the methanolic crude extracts of *A. multiplicatum* ranging from 0.50 - 1.25 mg/ml were introduced into different test tubes, each tube was inoculated with an overnight culture of strains diluted to give a final concentration of 10^6 cells/ml. The tubes were incubated at 37°C for 24 h.

Minimum bactericidal concentration (MBC)

Minimum bactericidal concentration was experimented after the MIC in freshly prepared agar plates, followed by standard method of (Alade and Irobi, 1993). After culturing the test organisms separately in nutrient broth containing various concentrations of the active ingredients, the broth was inoculated onto freshly prepared agar plates to assay for the bactericidal effect. The culture was incubated at 37°C for 24 h. The lowest concentration of extract that does not yield any colony growth on the solid medium after the incubation period was regarded as minimum bactericidal concentration (MBC).

RESULTS AND DISCUSSION

In vitro antibacterial screening of a marine ascidian against selected clinical isolates were performed and the inhibition zones of the extract against the specific test organisms were given in (Table 1). Maximum inhibition zone (17 mm) was observed against the gram negative *P. aeruginosa* in crude ethyl acetate extract. The crude ethyl acetate extract of *A. multiplicatum* was more effective against Gram negative bacteria than Gram positive bacteria. This view is contrary with the findings of Abdul Jaffar Ali et al. (2008) who reported the maximum antibacterial activity exhibited by the Gram positive bacteria than in Gram negative bacteria of crude methanol extracts of the test and mantle bodies of *Phallusia nigra*. It is clearly evident that the antibacterial activity has been previously reported from extracts of some ascidian extracts caused growth inhibition in gram positive and negative bacteria,

Table 1. Antibacterial activity of the different crude extract of *Aplidium multiplicatum.*

Microorganisms	Inhibition zone (mm)		
	Methanol	Ethyl acetate	Petroleum ether
P. aeruginosa	12.5	17	10
E. coli	8	12	6
K.oxytoca	4	6.5	1
S. aureus	2	3	-
S. pneumoniae	3	8	6
K. pneumonia	-	6	-
S.paratyphi	-	2	-
S. typhi	7.5	10.5	2

-, No activity was observed.

Table 2. MIC and MBC of different crude extracts of *Aplidium multiplicatum.*

Microorganisms	Methanol extract		Ethyl acetate extract		Petroleum ether extract	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
P. aeruginosa	0.90	0.95	1.80	2.05	0.50	0.75
E. coli	0.85	0.95	1.05	1.15	0.25	0.55
K. oxytoca	0.70	1.10	0.90	1.25	0.90	0.85
S. aureus	0.85	0.90	0.85	1.10	-	-
S. pneumoniae	0.95	1.10	1.00	1.60	0.85	0.80
K. pneumonia	-	-	0.95	0.95	-	-
S. paratyphi	-	-	0.80	0.95	-	-
S. typhi	0.70	0.80	0.85	1.05	0.70	0.70

-, No activity.

indicating that these extracts do not selectively inhibit one group of microorganism (Thompson et al., 1985).

Minimum inhibition zone (1 mm) was observed against *K. oxytoca* in petroleum ether extract. Ethyl acetate extract, the range of inhibition of the clinical isolates of the bacteria varied from 2 - 17 mm with an average of 8.12 mm. Methanol extract, the range of inhibition of the clinical isolates of the bacteria varied from 2 - 12.5 mm with an average of 4.5 mm. This is in accordance with the findings of Mohamed and Ananthan (2009) who reported that the crude methanol extract of *Didemnum psammathodes*, the range of inhibition of the bacteria varied from 2 - 15 mm with an average of 4.3 mm. On the other hand Santhana and Murugan (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. Anand and Edward (2002) stated that ethyl acetate extract of ascidian exhibited strong antibacterial activity than methanol and petroleum ether of all the strain tested, ethyl acetate extract of *T. clinids*, the range varied between 1 and 8.5mm with an average of 3.18 mm.

The crude ethyl acetate extract showed maximum activity against *P. aeruginosa*, followed by *E. coli, S. typhi, S. pneumoniae, K. oxytoca, K. pneumoniae* and the minimum activity was noticed against *S. aureus* and *S. paratyphi* .The crude methanol extract showed maximum activity against *P. aeruginosa,* followed, by *E. coli, S. typhi, K. oxytoca* and the minimum activity was noticed against *S. pneumoniae* and *S. aureus.* There is no activity against *K. pneumoniae* and *S. paratyphi.* The crude Petroleum ether extract showed maximum activity against *P. aeruginosa* followed by, *E. coli* and *S. pneumoniae* and the minimum activity was noticed against *S. typhi* and *K. oxytoca.* There is no activity against *S. aureus, K. pneumoniae* and *S. paratyphi.*

The minimum inhibitory concentrations (MICs) and minimum bactericidal concentration (MBCs) of the crude extracts were shown in (Table 2). The range of MIC varied between 0.50 - 1.80 mg/ml against all the bacterial strains used in this study and MBC ranges between 0.55 - 2.05 mg/ml against all the bacterial strains. Ascidians

are already reported for rich nitrogenous source with a wide range of biological activities (Biard et al., 1994). The range of MICs highest in ethyl acetate extract (1.80 mg/ml) followed by methanol extract (0.95 mg/ml) and petroleum ether extract (0.90 mg/ml). Range of MBCs was high in ethyl acetate extract (2.05 mg/ml) followed by, methanol extract (1.10 mg/ml) and petroleum ether (0.85 mg/ml). The range of MICs was low in the ethyl acetate extract (0.80 mg/ml) followed by, methanol (0.70 mg/ml) and petroleum ether extract (0.25 mg/ml) . The range of MBCs was low in ethyl acetate extract (0.95 mg/ml) followed by methanol (0.80 mg/ml) and petroleum ether extract (0.55 mg/ml). On the other hand (Natarajan et al*.,* 2010) reported that the minimum inhibitory concentrations (MICs) and minimum bactericidal concentration (MBC) of the methanolic extract of *Polyclinum madrasensis*, MIC varied between 0.70 - 0.95 mg/ml and MBC ranges between 0.85 - 1.10 mg/ml against all the bacterial strain tested.

Tsukamoto et al. (1994) have been reported that methanol extracts of the hepatopancreas of *H. roretzi* have antibacterial and antifungal properties towards *Vibrio alginolyticus* and *Mortierella ramanniana*, respectively. Meenakshi (2002) revealed that the preliminary screening of nine species of ascidian indicated the presence of antibacterial activity of the three different solvent was tested (methanol, methylene chloride and hexane), methylene chloride extracts showed maximum activity followed by methanol and hexane. Methanol and methylene chlorine extracts of *Aplidium indicum* were active against all pathogens including hospital isolates. *S. pneumonia, Corynebacterium diphtheria, Vibrio cholerae, S. typhi, Pseudomonas putida* and *Enterococcus faecalis* showed sensitivity to methylene chloride extracts of all the ascidians studied. Abourriche et al. (2003) evaluated the antibacterial activity against *Agrobacterium tumifaciens, E. coli, P. aeruginosae* and *S. aureus* from the extracts of Morocco Atlantic sea ascidian, *Cynthia savignyi*. It showed activity, except the dichloromethane extract, all extracts were active against bacteria. *A. tumifaciens* was most sensitive. Activity of hexane and diethyl ether extracts of this ascidian against *A. tumifaciens* was slightly less, but higher than the activity of *Lissoclinum fragile* extracts. Tunicates have the potential to yield novel compounds of ecological, chemical and also biomedical interest (Paul et al., 2008). In particular, the cosmopolitan genus *Aplidium* is renowned for the variability of its metabolites. A large variety of alkaloids have been isolated from this group, such as piperidins, tetracyclic alkaloids and indoles, which display potent bioactivities (Zubia et al., 2005). However, even though a wide range of natural products has been isolated from tunicates, little is known about the ecological roles of most of these metabolites and their allocation within ascidian tissues (Paul et al., 2008; Pisut and Pawlik, 2002; Avila et al., 2008). Most of these compounds have been reported to possess cytotoxicity. Among them, dehydrodidemnin B (DDB or aplidine) from *Aplidium albicans* is one of the most renowned ascidian natural products as it made antitumor phase II clinical trials. (Depenbrock et al., 1998; Urdiales et al., 1996; Cardenas et al., 2001). The discovery of new and/or bioactive natural products from Australian ascidians, two new tyrosine derivatives, botryllamides K (1) and L (2), together with six known metabolites, were isolated from *Aplidium altarium.* Compounds were evaluated for their cytotoxicity towards the tumour cell lines, MCF-7 (breast), H460 (lung) and SF268 (central nervous system). This is the first reported chemistry from *A. altarium*. (Davis et al., 1999, 2002; McKay et al., 2005), report the isolation, structural elucidation and cytotoxicity of compounds.

Conclusion

We deduce that, the continuing and overwhelming contribution of ascidians metabolites to the development of new pharmaceuticals are clearly evident and need to be explored. After taking into consideration the immense side effects of synthetic drugs, great attention has to be paid for the discovery of novel drugs from marine natural products.

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