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ANTIBACTERIAL ACTIVITY OF ASCIDIAN *PHALLUSIA NIGRA* (SAVIGNY, 1816) AGAINST BIOFILM FORMING BACTERIA

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Abstract: Antibacterial activity of methanol and methanol extracts of *Phallusia nigra* at different was evaluated by well diffusion method against various biofilm forming bacteria. The crude methanol extract was more active and showed broad-spectrum of activity than the crude ethyl acetate extract against the tested microbes. The biofilm forming bacteria used were *Pseudomonas aeruginosa, Escheria coli, Staphylococcus aureus* and *Vibrio parahaemolyticus.* Maximum inhibition zone (16 mm) was observed against *Staphylococcus aureus* in crude methanol extract (1 mg mL^{-1} concentration) and the minimum inhibition zone (6 mm) was observed against *Escheria coli* in ethyl acetate extract (0.5 mg mL^{-1} concentration).The range of inhibition in the test extract was less than the standard antibiotic used in all the strains.

Keywords: Antibacterial activity, *Phallusia nigra*, well diffusion method, biofilm forming bacteria

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INTRODUCTION

Ascidians are marine invertebrates which ranks second with promising source of drugs (Azumi *et al*., 1990). 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. From tunicate, "*Tridemnum solidum"*, the first marine compound entered human cancer clinical trial as a purified natural product (Carte, 1996) various ascidians such as *Botryllus* sp. and *Didemnum* Sp. were 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). Such potential ascidians need to be explored for the pharmaceutical purpose. A number of bioactive compounds have also been isolated from ascidians, exhibiting activities such as antiviral (Rinehart *et al.,* 1984), cytotoxic (Moquin-Pattey and Guyot, 1989), antibacterial (Azumi *et al.,* 1990), and enzyme inhibitory activities (Sato *et al.,* 1998). These compounds are mainly comprised of various derivatives of alkaloids and peptides. There are few examples of marine derived compounds which have successfully reached the market as therapeutic drugs. The evolution of antibiotic-resistant pathogenic bacteria has stimulated the search for alternative antimicrobial agents from alternative sources including sources from the ocean. The powers of marine organisms have been realized for thousands of years and their potential as producers of pharmaceutical products have been reviewed (Baker, 2004). The major features that distinguish biofilm forming bacteria from their planktonic counterparts are their surface attachment ability, high population density, extracellular polymeric substances (EPS) slime and a wide range of physical, metabolic and chemical heterogeneities (Beer and Stoodley, 2006). In view of this, our aim of study is to determine the antibacterial activity of various extracts of ascidians, collected from Southeast coast of India.

MATERIALS AND METHODS

Collection and preparation of samples

The ascidian, *Phallusia nigra* was collected during the low tide of the intertidal area at Thoothukudi coast, Tamil Nadu, Southeast coast of India. The collected samples were rinsed with sterile seawater to remove associated debrises and salts. The samples were weighed (10 g) and preserved separately in methanol and ethyl acetate (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 was centrifuged at 3000 rpm. The solvent was evaporated under reduced pressure using desiccators and the residue was weighed and dissolved in distilled water to test antibacterial activity.

Isolation and screening of biofilm forming bacteria

Four colonies with visually distinguishable morphologies were randomly selected and isolated by directly streaking on Nutrient agar plates and incubated for another 12-18 hours. The isolated colonies were then re streaked onto nutrient agar plates to obtain pure cultures. The viability of the isolated cultures was checked in Lauria Bretani (LB) broth and those found to be viable were selected for biofilm formation. Primary biofilm screening was done using tube staining assay (Christensen *et al.,* 1982) as well as the microtitre plate biofilm assay (Mack *et al.,* 1994; O'Toole *et al.,* 1999).

Antibacterial assay

Antibacterial activity was determined against four biofilm forming bacteria such as *Pseudomonas aeruginosa, Escheria coli, Staphylococcus aureus* and *Vibrio parahaemolyticus* using the well diffusion method (Tagg et al 1971). Agar plates were surface swabbed with overnight broth culture of the tested microorganisms. Then wells were made on the swabbed surface with help of well cutter. Then the crude extract were loaded at different concentrations on the wells and plates were incubated at 37°Cfor 24h

RESULTS AND DISCUSSION

The results of antibacterial activity of the crude methanol and ethyl acetate extract of *P. nigra* against various biofilm forming bacteria is given in Table. 1 and Plate.1. Methanol extract at 1mg/ml concentration produced a maximum zone of 16 mm against *S. aureus* and the minimum zone of 10mm against E. coli and P. aeruginosa. The ethyl acetate extract produced a maximum zone of 14 mm and minimum of 8 mm against *S. aureus* and *E .coli* respectively. Both extracts in two different concentrations showed minimum activity against *E. coli* whereas minimum activity was observed in the same strain at a concentration of 0.5mg/ml. Both extracts showed a broad spectrum of antibacterial activity against *S. aureus* followed by *V. parahaemolyticus* and *E. coli* in all the two concentrations. Antibacterial activity of ascidians extracts increased with increasing concentrations.

The results of the present study showed considerable antimicrobial activity. This proves that ascidians contain antibacterial agents of relevance to either antifouling technology or clinical pharmacology. Antibacterial activity of the Crude extract of ascidian shows inhibitory activity against almost all the strains tested here. However, methanolic extract of *P. nigra* shows

prominent antibacterial activity Against all the bacterial strains.

In this study the antibacterial activity against the biofilm forming bacteria were also performed and it was evident that the gram negative strains were more resistant. On the other hand Ananthan *et al.* (2011) reported the maximum antibacterial activity of the crude methanol extracts of the test and mantle bodies of *P. nigra* against the isolated urinary tract *S. aureus*. Martinez and Baquero (2002) stated that some of these bacteria were developed multi drug resistance to these antibiotics. Recent study in USA, Dowzicky and Park (2008) reported that UTI bacterial pathogens have exhibited decreased susceptibility rates to tigecycline over the years. Present results showed moderate antibacterial activity against the biofilm forming bacterial culture by the test body of *P. nigra*. This could be attributed to the fact that the test body might contain antifouling aspect which inhibits the growth of biofilm forming bacteria. This view is consistent with the findings of Paul *et al.* (2008) reported that the tunicates have the potential to yield novel compounds of ecological, chemical and also antifouling compounds. Based on the observed zone of inhibition, present study revealed that *P. nigra* extract are more effective than other marine invertebrates similar to sponges and bryozoans. A number of drugs have been purified and characterized with a view to developing novel pharmacological or commercially useful products (Rodrigues *et al*. 2004). In some ascidians peptides with antibiotic properties have been shown to have other biological effects for example protection against predation, digestion or prevention of surface epibiosis. There is a great scope for finding further novel antimicrobial proteins in ascidian group and analysis of their biochemical and phylogenetic relationship to other biologically active peptides will be of great invention, An understanding of their induction and modulation in vivo and an assessment of the ways they exert their biological effects is needed on susceptible micro-organisms. It could be concluded that, the continuing and overwhelming contribution of ascidians extracts to the development of antifouling potential compounds are clearly evident and need to be explored.

CONCLUSION

The methanolic extract has significant inhibitory effect against the tested biofilm forming bacteria and thus *P. nigra* needs further attention in this field. The fractions and extracts prepared were complex and in most cases probably contained a multitude of antifouling compounds. The most potent fractions displayed antibacterial activity at concentrations as low as 0.5 mg/ml. Extracts from the ascidian *P. nigra* were especially efficacious, showing full inhibition of microbial and fungal growth at the lowest concentration tested, making this species a promising candidate for further studies.

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TABLE. 1 The antibacterial zone formation of *P. nigra* against biofilm forming bacteria

Sl. no	Pathogens	Methanol (mm)		Ethyl acetate (mm)	
		0.5mg/ml	1mg/ml	0.5mg/ml	1mg/ml
1	*E.Coli*	6	10	7	8
2	*S. aureus*	12	16	10	14
3	*P. aeruginosa*	8	10	8	11
4	*V. parahaemolyticus*	7	11	8	10

PLATE-1

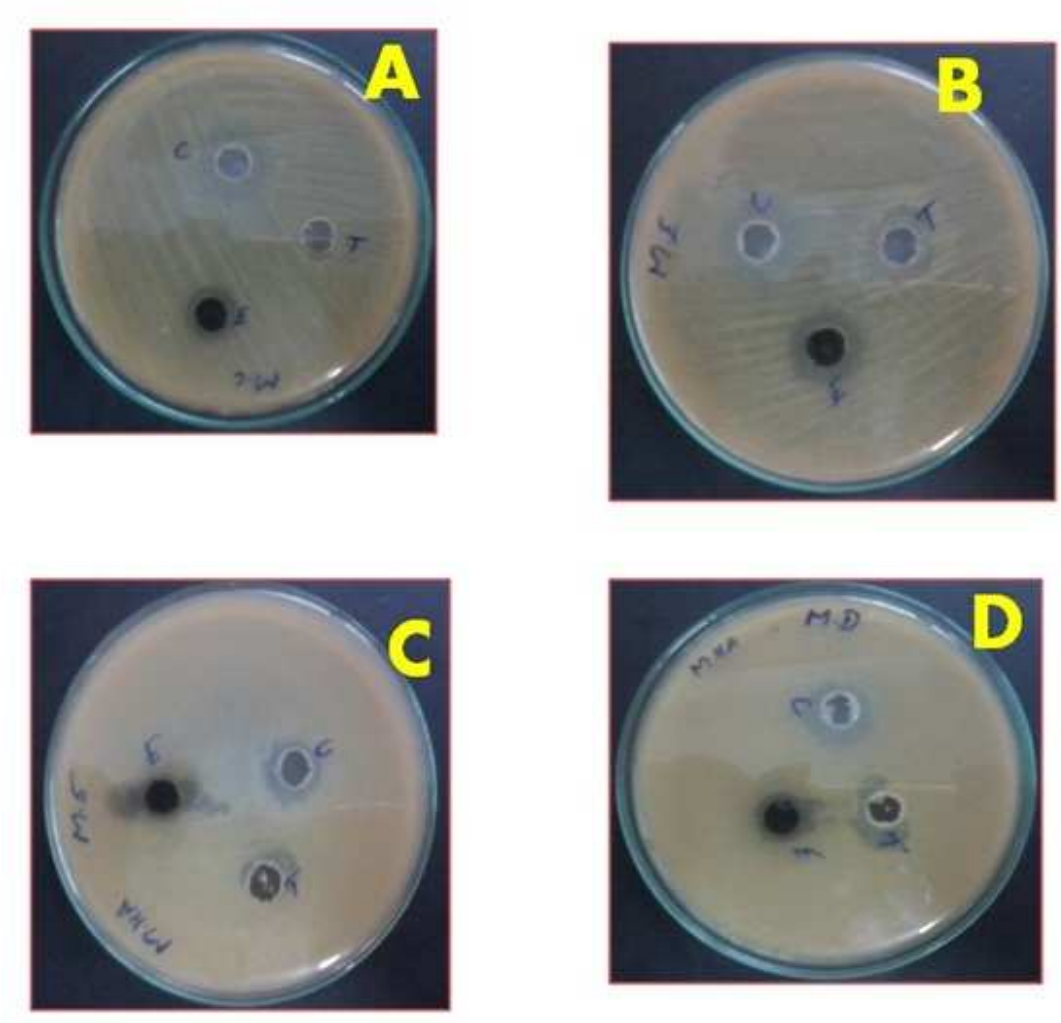

Plate- 1; Zone formation of biofilm forming bacteria
A) *E. coli*, B) *S. aureus*, C) *P. aeruginosa*,
D) *V. parahaemolyticus*

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