II - ANTIBACTERIAL ACTIVITY

2.1. INTRODUCTION

Most of the pathogens are increasingly resistant to the major classes of the routinely used antibiotic. Many diseases were initially controlled exclusively by the use of antimicrobial drugs. The massive use of antimicrobial for diseases control and growth promotion in animals increases the selective pressure exerted on the natural emergence of bacterial resistance (Riguera, 1997). So there is an urgent need for the discovery of the new and novel antimicrobial drugs to effectively combat not only the drugs resistance but also the new disease producers, hence the search for active drugs from alternative sources including marine environment, obviously becomes imperative. The rich diversity of marine organism assumes a great diversity of the discovery of new bioactive substances. The ocean remains as an untapped source for many drugs and contemporary experimental studies which indicate that, pharmacologically active substances could be isolated from marine organism (Baslow, 1969).

Natural products isolated from marine organisms have increased rapidly and hundreds of new compounds being discovered every year (Faulkner, 2002; Proksch and Muller, 2006). Marine invertebrates offer good source of potential antimicrobial drugs (Bansemir et al., 2006; Mayer et al., 2007; Jayaraj et al., 2008). Studies on antimicrobial mechanisms and compounds of marine invertebrates may provide valuable information for new antibiotic discoveries and give new insights into bioactive compounds in molluscs.
Among the invertebrates, the molluscs are very good source for biomedical important products (Shenoy, 1998). Many classes of molluscs with bioactive compounds like antitumour, antileukemic, antibacterial, cytotoxic, anti inflammatory and antiviral properties have been reported (Kamiya et al., 1989; Pettit et al., 1987; Anand et al., 1997; Rajaganapathy et al., 2000; Anand and Edward, 2001). Bioactive compounds in molluscs were identified and represented specific types of activities (Balcazar et al., 2006; Blunt et al., 2006; Maktoob and Ronald, 1997). The presence of antimicrobial activity in molluscs has been reported from the mucus of the giant snail Achatina fulica (Kubota et al., 1985; Iguchi, 1982) and from the egg mass and purple fluid of the seahare Dolabella auricularia (Iijima et al., 2003). Proteins and glycoproteins with antibacterial activity have been demonstrated in the digestive organs of various molluscs (Igughi et al., 1982; Kamiya et al., 1986; Pakrashi, 2001).

A variety of antimicrobial factors, including chlorinated acetylenes, (Walker and Faulkner, 1981), terpenes (Ireland and Faulkner, 1978), indole derivates (Benkendorff et al., 2001a), glycerol derivates (Gustafson and Andersen, 1985), macrolides (Malsunaga et al., 1986), pigments (Sanduja et al., 1985), lysozymes (Nilsen et al., 1999), glycoproteins (Yamazaki, 1993), proteins (Kamiya et al., 1986; Iijima et al., 2003; Mitta et al., 1999) have been isolated from molluscs. These reports suggest that molluscs are the rich source for discovering novel lead compounds for the possible development of new types of antibiotics for pharmaceutical use. Keeping the importance of gastropods in terms of bioactive compounds with antibacterial properties, the present study has been undertaken to ascertain the antibacterial activity of extracts from B.zeylanica, C.virgineus and P.persica against various pathogenic bacteria.
2.2. MATERIAL AND METHODS

2.2.1. Collection and Preparation of Samples

The mollusc *B. zeylanica* was collected from muddy bottom of deep waters, *C. virgineus* from muddy sand bottom and *P. persica* from intertidal rocky shore of harbour area of Gulf of Mannar, near by Theraspuram Tuticorin, situated in the south east coast of India, between April 2010 and December 2010. The collected samples were rinsed with sterile sea water to remove the associated debris and salt. Test animals were first carefully removed from their shells. The flesh was cut into small pieces and air-dried. The air-dried flesh was immersed in 100% A.R. Grade methanol for 10 days at room temperature. The extract from the solvents was filtered by using Whatman no.1 filter paper and evaporated to dryness in rotary evaporator and the dried extract was stored at 0°C for further use.

2.2.2. Microbial strains used

Antimicrobial activity of tissue extracts were determined against 10 different bacterial pathogens, viz., *Aeromonas hydrophila*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella flexneri*, *Vibrio cholerae* O139, *Vibrio cholerae* Classical, *Vibrio cholerae* O1 and *Vibrio cholerae* El-Tor. These clinical strains were obtained from the Department of Basic Biomedical sciences, Bharathidasan University, Trichy.

2.2.3. Antibacterial susceptibility assay

In vitro antibacterial activity was assayed by the disc diffusion method of Bauer *et al.*, (1996). A known amount of crude whole body gastropod extract was dissolved in 0.6ml of solvent (methanol) and applied to 6mm sterile disc. In the same way for control 0.6 ml of methanol was soaked in sterile disc. Both the discs were
allowed to dry at room temperature. Pathogenic bacterial strains were inoculated in sterile broth and incubated at 37°C for 24 hrs. Pathogens were swabbed on the surface of sterile petri dishes in 20ml of solidified nutrient agar. The control and the experimental discs were placed in the sterile solidified nutrient agar petri plates to assess the effect of solvent and extracts on pathogens. These agar plates were incubated at 37°C for 24 hrs and the antibacterial activity was measured accordingly based on the inhibition zone around the disc impregnated with gastropod extract. Antibacterial activity was expressed in diameter zone of inhibition which was measured with the outer side of the disc to inner side of the inhibition zone. Each active extract was tested thrice for confirmation of activity.

All the three gastropod crude extracts were fractionated and elusions were made with Hexane: Chloroform (F1), Chloroform (F2), Benzene (F3), Methanol: Benzene (F4) and Methanol (F5). Eluted fractions were assayed for antibacterial activity following the above mentioned disc diffusion method. To estimate the minimum inhibitory concentration of extract three different concentrations such as 1, 10 and 100 mg/ml were prepared for all fifteen fractions and they were tested against the pathogens. After 24 hrs of inhibition, the plates were removed and observations were made for inhibition zone against the pathogens.

2.3. **RESULTS**

Antibacterial activities of extracts from *B.zeylanica, C.virgineus and P.persica* were presented in Figures 8 - 24, and Plates 4 - 45.
2.3.1. Antibacterial activity of extracts from \textit{B.zeylanica}:

The antibacterial activity of crude methanol extract was given in Fig.8 and Plates 4-7. The crude methanol extract of \textit{B.zeylanica} with a range of activity varied from 4mm (\textit{P. aerugenosa} and \textit{V. cholerae} Classical) to 8mm (\textit{A. hydrophila}) (Plate 4) (Fig.8). Of the five columns chromatographic fractions, maximum numbers of pathogens were inhibited by F4 and F5 fractions (Fig. 9&10 and Plates 8-10). The highest activity of F4 fraction was exhibited against \textit{A. hydrophila} (9mm) (Plate 8) and the least against \textit{V.cholerae} O139 (3mm) (Fig.9). F5 fraction showed maximum activity against \textit{E.coli} and \textit{S.typhi} (9mm) (Plate 9&10) and the lowest against \textit{V.cholerae} Classical (4mm) (Fig.10). MIC of various fractions revealed that \textit{A.hydrophila} (Plate 11) showed maximum inhibitory zone of 8mm at 100mg (Fig.11c) concentration when treated with F4 (Plate 11). This fraction also exhibited activity against \textit{P. aerugenosa} (Plate 12) and \textit{S. typhi} (Plate 13). In F5 fraction \textit{S. typhi} and \textit{E.coli} growths were arrested with the formation of 8mm at 100mg concentration (Fig.12c and Plates 14&15). It also has shown inhibitory activity against \textit{P. aerugenosa} (Plate 16) and \textit{A. hydrophila} (Plate 17).

Of the five fractions, the numbers of fractions active were F4 and F5 in the column as well as in MIC respectively. \textit{A. hydrophila}, \textit{E.coli} and \textit{S. typhi} were the most susceptible pathogens in concern with the \textit{Babylonia} extract.

2.3.2. Antibacterial activity of extracts from \textit{Purpura persica}

The crude extract inhibitory zone of \textit{P.persica} range varied from 2mm (\textit{P.aerugenosa}) to 7mm (\textit{S. typhi}) (Fig.13 and Plates 18-20). The crude extract was partially purified through silica gel column chromatography. Of the five fractions, F2 and F3 of \textit{P.persica} exhibited wide spectral antibacterial activity in most of the
bacterial strains tested (Fig. 14& 15). F2 fraction was highly active against *S. typhi* (19mm) (Plate 21) and it also showed inhibitory activity against *P. aerugenosa* (13mm) (Plate 22) and *S. flexneri* (12mm) (Plate 23) (Fig.14). In F3 fraction the maximum inhibitory zone was obtained in *B. cereus* (Plate 24) and *V. cholerae* Classical (11mm) (Plate 25) and minimum in *V. cholerae* O139 and *A. hydrophila* (2mm) (Fig. 15). *S. typhi* was inhibited even by F1 (17mm) and F5 (11mm) fractions (Fig.16&17). Results of the MIC were shown in Fig.18a-19c & Plates 26-32. Maximum spectrum antibacterial activity was attained at 100mg concentration against *S. typhi* (13mm) (Plate 26) and minimum against *V. cholerae* O1 (3mm) in F2 fraction (Fig.18c). This fraction also exhibited inhibitory activity against *S. flexneri* (Plate 27). Maximum inhibitory zone was exhibited against *B. cereus* (9mm) (Plate 28) and minimum against *E. coli* (4mm) by F3 fraction (Fig19c). It also showed activity against *S. typhi*, *P. aerugenosa*, *V. cholerae* Classical, and *S. flexneri* (Plates 29-32).

Among the tested pathogens *S. typhi*, *P. aerugenosa*, *S. flexneri* and *B. cereus* showed inhibition in growth by crude, F2 and F3 fractions of *P. persica*.

2.3.3. Antibacterial activity of extracts from *C. virgineus*

The result of antimicrobial activity of crude methanol extracts of *C. virgineus* was given in Figure 20 and Plates 33 & 34. The level of crude extract activity, which was measured by inhibition zone, showed variation in between 2mm (*P. aerugenosa*, *B. cereus*, *V. cholerae* Classical and *V. cholerae* O1 and 7mm (*S. typhi*) (Fig.20). The crude extract obtained with methanol was fractionated using silica gel column chromatography. In the present investigation, among the five fractions F1 and F2 fractions exhibited maximum activity. *S. typhi* was the most sensitive pathogen (20mm) (Plate 35) and the least was *E. coli* (2mm) when concern with F1 fraction.
(Fig. 21). It also showed inhibitory activity against *P. aeruginosa* (11mm) (Plate 36) *B. cereus* (13mm) (Plate 37) and *S. flexneri* (12mm) (Plate 38) (Fig. 21). In F2 fraction profound zone of inhibition was exhibited against *S. flexneri* (22mm) (Plate 39) and minimum against *E. coli* (3mm) (Fig. 22). This fraction also showed inhibitory activity against *P. aeruginosa* (20mm), *S. typhi* (15mm), *B. cereus* (14mm) and *V. cholerae* El-Tor (11mm) (Fig. 22). *S. typhi* exhibited 10mm inhibition zone even at 1mg (Fig. 23a) (Plate 40) concentration of F1 fraction while *B. cereus* and *V. cholerae* Classical developed 10mm zone at 10mg (Fig. 23b) (Plate 41 & 42) concentration. Maximum inhibition zone was obtained in F2 fraction against *S. flexneri* (16mm) at 100mg level (Fig. 24c) (Plate 43). This fraction also exhibited activity against *P. aeruginosa* (Plate 44) and *B. cereus* (Plate 45).

*C. virgineus* exhibited significant effect against *S. flexneri*, *S. typhi* and *V. cholerae* Classical in crude, F1, F2 and MIC.

### 2.4. DISCUSSION


Anand *et al.*, (1997) studied the antibacterial activity of common marine molluscs from Parangipettai coast and reported that the methanol extract of molluscs exhibited significant activity against *Escherichia coli*. This finding corroborates the results of the present study since methanol extract of *B. zeylanica* showed pronounced
activity against *E. coli*. Antibacterial activity of opercular extracts of *Chicoreus ramosus* and *Pleuroloca trapezium* against six bacterial pathogens was reported by Murugan and Ayyakkannu (1997). Thilaga (2005) screened the antibacterial activity of a marine mollusc *Babylonia spirata* against bacterial pathogens. The inhibitory action of the methanol fractions of *Perna viridis* was reported by Chandran et al., (2009) against bacterial and fungal strains. Similar result was also reported in four bivalves against few pathogens and found that methanol extracts showed significant activity against *Bacillus subtilis* (Jayaseeli et al., 2001). Anand and Edward (2002) noted that the crude methanol extracts of *Cypraea errones* exhibited promising antibacterial and antifungal activity against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Aspergillus niger* and *Candida albicans* and very good activity against *Shigella flexneri* in heptane fraction (Prem Anand and Patterson Edward, 2002). Santhana Ramasamy and Murugan (2003) have reported that the crude methanol extract of *Didemnum psammathodes* inhibited the growth of bacteria. Mohammed Hussain and Anandhan (2009) reported that the methanol extract of *Didemnum candidum* showed maximum antibacterial activity against *Salmonella typhi*, *Pseudomonas aeruginosa* and *Vibrio cholerae*. In the present study in *B. zeylanica*, the crude methanol extract was well within the range of activity reported earlier.

The crude whole body methanol extracts of *P. persica* showed wide spectrum antibacterial activity against seven pathogens out of ten pathogens tested. Raja ganapathi, (2000) also reported that the methanol extract from the whole body of *Hemifuses pugilinus* exhibited activity against *Bacillus subtilis*, *Escherichia coli* and *Klebsiella pneumonia*. Similarly Santhana Ramasamy and Murugan (2005) experimentally analyzed the methanolic extract of *C. virgineus* and *C. ramosus* and they also observed the broad spectrum antibacterial activity of body tissue extract.
Highest activity was observed against *Klebsiella pneumoniae* and *Staphylococcus epidermis* by the extract of acetone and against *Salmonella paratyphi* by the extract of chloroform column purified whole body extract of the winged Oyster *Pteria chinensis* were reported by Chellaram *et al.*, (2004). Dyrynda *et al.*, (1995) reported antimicrobial and cytotoxic activities in the eggs of opisthobranch mollusc.

Apart from methanol extract in the present investigation hexane: chloroform and chloroform fractions of *C. virgineus* and chloroform and benzene fraction of *P.persica*, inhibited *S.flexeri, S.typhi, V.cholerae* Classical and *P.aerugenosa, S.flexneri and B.cereus* respectively. Similar result was reported by Chellaram *et al.*, (2004) in chloroform extract of *Pterai chinensis* which inhibited eight fish pathogens and the acetone extract in the same animal showed broad spectral activity against all the fish pathogens tested. Anbuselvi *et al.*, (2009) found that acetone column purified fractions of *Trochus tentorium* shown highest antibacterial activity. Abraham *et al* (2002), studied the antibacterial activity in alcoholic extracts of holothurians species inhibited *S.typhi*. The present study with test animals *C.virgineus and P.persica* also corroborates the earlier findings of suppressing the activity of *S.typhi*. Even then also difference in antibacterial activity found in the molluscan extracts may depend on the solvents used for extracts and the compounds extracted (Annamalai *et al* 2007). Higher degree of inhibition of *C.virgineus* was shown by low and medium polar compound in the present study. On contrary Anand *et al.*, (2002) reported that non polar solvent of *Cypraea erronus* extract inhibited human pathogens.

*C.virgineus* and *P.persica* column fractionated extract at 100mg/ml concentration inhibited *S.typhi, B.cereus, S.flexneri P.aerogenosa* and *V.Cholerae Classicial* with more than 10mm inhibitory zone. Gnanambal *et al.*, (2005) found that 0.07 mg of *Trochus radiatus* inhibited *Proteus mirabilis* and 0.15 mg of extract
inhibited Serratia marcescens. Some of the peptides obtained from oysters inhibited E.coli at 330 mg/ml and Vibrio alginolyticus at 162 mg/ml. (Gueguen et al., 2006; Seo et al., 2005). Antibacterial activity of various form of marine animals and the bioactive products at molecular level have been studied by various authors and the molecules responsible for antibacterial activities were identified and characterized (Pawlik, 1992; Lijima et al., 1995; Silvestri et al., 1995; San-Martin et al., 1996; Pouliquen et al., 1997; Chatterji et al., 1999; Ogawa et al., 1999; Gnanambal et al., 2005; Annamalai et al., 2007). Aplysianin-A isolated from albumin gland of Aplysia kurodai inhibited Bacillus subtilis (Kamiya et al., 1986), glycoprotein isolated from Achacina fulica inhibited P.fluobrescens, E.coli, Streptococcus faecalis, Staphylococcus epidermidis, S.aureus and Vibrio angulliarum (Ogawa et al., 1999) and Lectin isolated from Modiolus modiolus inhibited Vibrio angaillarian and Vibrio salmonicida (Tunkijianukerji and Olafsen, 1998).

The secretion from hypobranchial gland of muricid Rapana rapiformis was found to inhibit bacteria (Murugan et al., 1991). Similarly, the hypobranchial gland of the Chicoreus ramosus exhibited a broad spectrum activity against ten bacterial strains (Kagoo and Ayyakannu, 1992). Haemocyte of the marine bivalve mollusc Pinna nobilis exhibited antibacterial activity. Abraham et al., (2002) studied on the antibacterial activity of alcoholic extracts of holothurians species and found that among the bacteria as observed presently Salmonella typhi showed highest activity. The egg capsules of Rapana rapiformis extracted with polar solvents have been reported to show wide spectral activity (Prem Anand et al., 1997). Antibacterial activity of Opercula extracts of Chicoreus virgineus fractions gave very promising results against Bacillus subtilis and Vibrio cholerae (Anand and Edward, 2001).
The acetone extract of the winged Oyster, *Pteria chinensis* had a broad spectral activity inhibiting all the fish pathogenic strains tested and the extracts of chloroform inhibited eight pathogens and little inhibition in the fractions of methanolic phase was also noted (Chellaram *et al.*, 2004). In the present investigation also chloroform extract of *P. persica* and *C. virgineus* dissented the growth of pathogens. The acetone column purified fractions of *Trochus tentorium* was found to have highest antibacterial activity (Anbuselvi *et al.*, 2009). These results indicate that *C. virgineous* contains a variety of molecules, active against pathogenic microorganisms. As antibacterial activity was detected in most of the pathogens tested it is reasonable to assume that multiple factors are responsible for the antibacterial activity (Tor Haug *et al.*, 2002). Apart from methanolic extract chloroform, benzene extract of *P. persica* and hexane, chloroform extract of *C. virgenius* exhibited wide spectral activity against *S. typhi, P. aerugenosa, S. flexneri V.cholerae Classical* and *B. cereus*.

In the present investigation higher degree of inhibition was confined to F2 and F3 fractions of *P. persica* indicating the substances involved in producing the antibacterial effect could be a medium polar compound, *Cypraea erronus* was reported to have antibacterial activity at the non polar end of the step gradient by the column-purified fractions (Anand *et al.*, 2002). Difference in antibacterial activity found with prosobranchs extracts may depend on extracting capacity of the solvents and the compounds extracted (Annamalai *et al.*, 2007). Many antimicrobial screening studies have shown that Gram-negative bacteria are more sensitive than Gram-positive bacteria (Tadesse *et al.*, 2008). The ethyl acetate extracts of *Trochus radiatus* was found to possess MIC values of 0.07 and 0.15 mg for *Proteus mirabilis* and *Serratia marcescens, was reported by Gnanambal, et al.*, (2005). Antibacterial MICs
data obtained with peptides from oysters against *E. coli* and *V. alginolyticus* MICs of 330 µg/ml and 161-92 µg/ml respectively (Gueguen et al., 2006; Seo et al., 2005).

Antibacterial activity has been described in the haemolymph of several molluscan species including several sea hares, sea slug, oysters and mussels species (Gueguen et al., 2006; Maktoob and Ronald, 1997; Olicard et al., 2005; Roch et al., 2008). Some of the molecules responsible for these activities have been identified and characterized. Proteins and glycoprotein with antibacterial activity have been demonstrated in the digestive glands of various molluscs (Iguchi et al., 1982; Kamiya et al., 1986; Pakrashi, 2001). The European and Pacific oyster *Ostrea edulis* and *Crossostrea gigas* expressed antibacterial activity attributed to small proteins (Hubert et al., 1996). An antibacterial factor, “Aplysianin-A” from albumin gland of the sea hare *Aplysia kurodai* was found to inhibit *Bacillus subtilis* (Kamiya et al., 1986). Antibacterial glycoprotein was reported from reproductive organs of hare sea hare *Aplysia juliana* (Kamiya et al., 1989), and ‘dolebellannin- Dolebella A’ was purified from the albumen gland of the sea hare, *Dolabella auricularia* (Ryosuke Iijima et al., 2003).

Among the test animals the crude extract of *B. zeylanica* showed maximum activity but the inhibitory effect of column fractionated extract of the same animal on tested pathogens was not remarkable when compared to other two species. The antibacterial activity was greater in *M. virgineus* than *P. persica*. Lesser degree of inhibition by the column fractionated extracts in comparison to the crude in *B. zeylanica* could be concluded that the active compound may be degraded or modified during the fractionation process as reported by Cannel (1998). F4 and F5 fractions of *B. zeylanica*, F2 and F3 fractions of *P. persica* and F1 and F2 fractions of
C. virgineus extracts of prosobranchs used in the present study showed potent antibacterial activity. In the present study the activity of all the three Prosobranchs experimental animals exhibited very promising activities against pathogenic strains. This finding is very significant and may pave way for the discovery of new potent drugs against these dangerous pathogens. Among the test animals on count of their broad spectrum antibacterial activity and the previous available literature C. virgineus and P. persica were expected to be a new potential producer of new antibiotics.