III - ANTIFUNGAL ACTIVITY

3.1. INTRODUCTION

The biota of marine organisms has developed unique metabolic and physiological functions that not only ensure survival in extreme habitat but also offer an avenue for the production of novel enzymes and bioactive metabolites for potential exploitation. Various observations indicated that for every dozen organisms examined fewer than six have been shown to have chemical and biological characteristic, life saving drugs. The organisms from marine environment have been found possessing a vast array of new pharmaceutical compounds with novel activities that will provide new drugs to compact microbial pathogens currently developing resistance to conventional antibiotic therapies (Carte, 1996).

A novel antifungal peptide mytimycin, that delays the growth of *Neurospora crassa* and *Fusarium culmorum*, has been isolated and characterized in conjunctions with the defensins and mytilins from *Mytilus edulis* (Charlet et al., 1996). The works carried out on marine biota in bioactive compounds on antifungal activity of molluscs particularly gastropods revealed that only a sketchy information is available. Therefore finding out new drugs is the need of the hour and hence an attempt is made in the present investigation for in-vitro evaluations of antifungal activity on crude and column fractions obtained from the marine Prosobranchs and to identify the most potent compound. Hence the marine Prosobranchs *B.zeylanica*, *P.persica* and *C.virgineus* were screened for antifungal activity.
3.2. MATERIAL AND METHODS

In vitro antifungal activity was determined using the techniques of Kelman et al., 2001. The fungi pathogens *Aspergillus flavus, A. terreus, A. niger, A. fumigatus, Fusarium moniliforme, Trichoderma sp. Penicillium citrinum, P. oxalicum and Rhizopus sp.* were obtained from TNAU, Coimbatore. Pathogenic fungal strains were inoculated in potato dextrose agar medium and incubated at 48 hrs. In vitro antifungal activity of Prosobranchs extracts were determined against in Czapex Dox agar using inoculums of 48 hour old culture of *A. flavus, A. terreus, A. niger, A. fumigatus, Fusarium moniliforme, Trichoderma sp, P.citrinum, P.oxalicum and Rhizopus sp.* Fungal strains were gently swabbed on the surface of the sterile petridishs containing 20 ml Czapex Dox solidified nutrient agar with the help of a sterile cotton swab. Then 20ml of the crude methanol extract was pipetted out on a 6mm sterile paper disc. The solvent was allowed to evaporate and the disc was placed on the surface of the sterilized agar plate. Control disc was placed with solvents to access the effect of solvent on pathogens. Areas of inhibited fungal growth were observed after 48 hrs. Antifungal activity was measured as diameter of zone of inhibition excluding the paper disc diameter.

After initial screening, the extracts showing broad spectrum were fractionated using normal phase silica gel 160-120 mesh (Glaxo, Bombay) column chromatography with low polar to high polar solvent Hexane: Chloroform (F1), Chloroform (F2), Benzene (F3), Benzene: Methanol (F4), and Methanol (F5). The fractions thus obtained were once again evaporated, concentrated, and assayed for antifungal activity. The extracts showing broad spectrum activity was examined for MIC by testing at different concentrations viz; 1mg/ml, 10mg/ml, and 100mg/ml.
3.3. RESULTS

Antifungal activity of extracts from *B. zeylanica*, *P. persica* and *C. virgineus* are presented in Figures 25-38 and Plates 46-67. The antifungal activity varied with prosobranchs extracts and fungal species.

3.3.1. Antifungal activity of extracts from *Babylonia zeylanica*

Effects of crude extracts from *B. zeylanica* varied from 3mm (*P. oxalicum*, *A. terreus*, *Trichoderma* sp. and *Rhizopus* sp.) to 6mm (*A. fumigatus*) (Fig. 25) (Plate 46). Among the fractions, F4 and F5 showed little activity against the tested fungi. Maximum activity was noticed against *A. terreus* (4mm) and minimum in *A. flavus* and *P. oxalicum* (2mm) in F4 fraction (Fig. 26). Highest inhibition zone was developed against *F. moniliforme* (5mm) and the lowest in *Trichoderma* sp., *P. citrinum* and *Rhizopus* sp. (2mm) in F5 fraction (Fig 27). Since the activity among fractions was traceable MIC was not warranted.

3.3.2. Antifungal activity of extracts from *Purpura persica*

Antifungal activity of *P. persica* was presented in Figures (28- 33) and Plates (47-61). Crude methanol extract of *P. persica*’s inhibitory range varied in between 2mm (*A. flavus* and *Rhizopus* sp.) and (*A. fumigatus*) 6mm (Fig. 28) (Plate 47). Among the silica gel fractions, maximum activity was observed in fraction F2. Maximum inhibition zone was obtained against *A. fumigatus* (24mm) (Plate 48) and minimum against *A. niger* (5mm) in fraction F2 (Fig 29). It also showed higher degree of inhibition zone against *A. terreus* (23mm) (Plate 49), *F. moniliforme* (22mm) (Plate 50), *Trichoderma* sp. (20mm) (Plate 51), *P. citrinum* (19mm) (Plate 52), *A. flavus* (18mm) (Plate 53) and *P. oxalicum* (17mm) (Plate 54). Fractions F1 (Fig. 30)
and F5 (Fig. 31) also shown inhibition zone against *A.terreus* (21mm) and *Trichoderma* sp. (16 mm) respectively. In MIC maximum antifungal activity was exhibited against *A.fumigatus* (13mm) (Plates 55), *F. moniliforme* (12 mm) (Plate 56), *A.terreus* (12 mm) (Plates 57), and minimum against *Rhizopus* sp. (2 mm) at 1 mg level (Fig 32a) and maximum against *A.fumigatus* (19mm) (Plate 55) and minimum against *A.niger* (1mm) at 100mg (Fig. 32c) level in F2 fraction. It also has shown activities against *P. oxalicum* (Plate 58), *P. citrinum* (Plate 59) and *S.flexneri* (Plate 60). F5 fraction also showed maximum activity against *Trichoderma* sp., (10mm), at 100 mg level (Fig.33c) (Plate 61).

As a result, potential antifungal activity was found in *P.persica’s extract* against *A.fumigatus, A.terreus, Trichoderma sp.* and *F.moniliforme.*

### 3.3.3. Antifungal Activity of extracts from Chicoreus virgineus

The obtained results of antifungal activity of *C.virgineus* extracts are presented in Fig. (34-38) and Plates (62-67). Crude extracts of *C.virgineus* inhibitory range varied from 1 (*A.niger*) to 7mm (*Trichoderma* sp) (Fig. 36) (Plate 62). Among the silica gel fractions F1 and F2 were more significant. Maximum inhibition zone was expressed against *Trichoderma* sp. (Plate 63) and *F.moniliforme* (19 mm) (Plate 64) and minimum against *A.fumigatus* (3mm) in F1 fraction (Fig. 35). The highest inhibition zone was exhibited against *P.citrinum* (18mm) (Plate 65) and the lowest against *Rhizopus* sp. (4mm) when treated with F2 fraction (Fig. 36). It also showed activity against *F. moniliforme* and *A. fumigatus* (Fig. 36). *F.moniliforme* (Plate 66) and *Trichoderma* sp. growth were inhibited by 10 mm even at 1 mg level by F1 fraction and minimum against *Rhizopus* (1 mm) of the same concentration (Fig. 37a). Maximum inhibition zone was exhibited against *P.citrinum* (11 mm)
(Plate67) and minimum against *P. oxalicum* (3 mm) at 100mg level by F2 fraction (Fig. 38c).

The crude and partially purified extract of *C. virgineus* showed significant activity against *P. citrinum*, *Trichodema* sp. and *F. moniliforme*. In conclusion among the tested prosobranch molluscs *P. persica* showed significant antifungal activity against most of the tested fungal pathogens.

### 3.4. DISCUSSION

In the present investigation crude and column fractionated extracts of *P. persica* and *C. virgineus* had distinct antifungal activity against most of the pathogenic fungi tested than *B. zeylanica*. Prem Anand and Patterson Edward (2002) reported moderate antifungal activity from the extract of various bivalve molluscs. Highest inhibitory activity was observed against *Aspergillus niger* with methanol extract of *Microcosmus curvus* (Karthikeyan et al., 2009). Similar result was obtained by Chandran et al., (2009) in *Perna viridis* methanol extract against *Aspergillus flavus* and *Mucor* sp. In the present study the chloroform extract of *P. persica* inhibited *A. flavus*, *F. moniliforme*, *A. terreus*, *Trichoderma* sp. *P. citrinum*, *A. fumigatus*, and *P. oxalicum* but no inhibitory activity was noticed against *A. niger*. Comparable work was that of Mohamed Hussain and Ananthan (2009) who observed that the chloroform extract of *Didemnum psammathodes* and *Didemnum candidum* inhibited *Penicillium* sp. whereas no antifungal activity was noticed against *A. flavus*, *A. fumigatus* and *A. niger*.

Similarly antifungal activities of various marine organisms and plants have been reported by many workers. Shanmugam et al., (2008) noticed in the methanol extract of *Sepia aculeata* which exhibited the maximum activity against *A. flavus*. 

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Bhosale et al., (1999) in their study reported that the methanol extract of polychaeta *Sabellaria cementifera* showed maximum activity against *Aspergillus flavus* and minimum against *Aspergillus niger* also support the present study. Elayaraja et al., (2010) reported that the methanol extract of polychaete *Perinereis cultifera* exhibited highest inhibitory zone against *Rhizopus* sp. Padmakumar and Ayyakannu (1997) screened about 80 species of marine algae of which none showed activity against *A.flavus* using any of the algal extracts but the present study showed activity against *A.flavus*.

In the present study the extract of *P.persica* inhibited *F.moniliforme*, *Trichoderma sp.*, *P.citrinum*, *A.terreus*, *A.fumigatus*, *P.oxalicum* and *M. virgineus* inhibited *F.moniliforme*, *A.terreus at 100mg level*. Several marine natural products showed significant antifungal activity; Callipeltins J and K, MIC at 1 µm (D’Auria et al., 2007); the triterpene glycoside holothurin B, MIC at 1.56µg/ml (Kumar et al., 2007); the macrolids neopeltolide MIC at 0.62 µg/ml (Kunze et al., 2008); and pseudoceratins A&B, MIC at6.5-8.0 µg/disc (Jang et al., 2007). Charlet et al., (1996) isolated Mytimyein from *Mytilus edulis* a noval antifungal Cys-rich polypeptide of 6.2 KDa which hindered the growth of fungi. Myticin B isolated from haemocytes of *Mytilus galloprovincialis* showed antifungal activity against *F.oxysporum* (Mitta et al., 1999). A polypeptide type AMP (Antimicrobial peptide) isolated from the Chilean scallop *Argopecten purpuratus*, showed antifungal activity against *F. oxysporum* and *Saprolegnia parasitica* (Arenas et al., 2009).

Okuda and Shever (1985) isolated Latrunclin-A from *Chromodoris elisbenthina* that showed inhibition activity against *Candida albicans*. Charlet et al., (1996) isolated antifungal peptide from the blood of immune challenged and untreated
*Mytilus edulis*. Dolastin 10, from *Dolabella auricularia* was shown to be highly antifungal against *Cryptococcus neoformans* (Pettit *et al*., 1998). Dolabellin B2, from the body wall of the sea hare *D. auricularia* is fungicidal against *S. cerevisiae*, while it is fungistatic against *Candida albicans* (Iijima *et al*., 2003). Ulapualide-A, a sponge derived macrolide isolated from the nudibranch *Hexabranchus sanguineus* exhibited antifungal activity (Rorsener *et al*., 1986).

In the present study the activity of the *Purpura persica* was found to be high when compared to *Chicoreus virgineus and Babylonia zeylanica*, may be due to species specific characteristics. The results showed that potent antifungal activity could be detected in two of the investigated prosobranchs. Compared to *C. virgineus*, *P. persica* from their broad spectral activity, considered to be a promising source of antifungal source, which definitely expected to be a potential producer of new antibiotics. Extraction of this biologically active compound from marine resources will certainly be helpful in protecting and treating various fungal diseases in human beings.