Chapter IV
IV. ANALYSIS OF ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF MELANIN PIGMENT FROM SELECTED STRAIN

4.1 INTRODUCTION

Natural products have been regarded as important sources that could produce potential chemotherapeutic agents. Research into pharmacological properties of marine natural products has led to the discovery of many compounds considered worthy of clinical applications. Bioprospecting from sea and marine biota has just started to bloom. Today, marine sources have the highest probability of yielding natural products with unprecedented carbon skeletons and interesting biological activities. There are several organisms that can produce pigments, one of the important classes of secondary metabolites, which are often referred as biopigments. These biopigments can be obtained from two major sources, plants (Mizukami et al., 1978; Papageorgiou et al., 1979) and microorganisms (Cross and Edinberry, 1972; Ryu et al., 1989; Parisot et al., 1990; Yongsmith et al., 1994; Kim et al., 1998; Cho et al., 2002)

The accessible authorized biopigments from plants have numerous drawbacks such as instability against light, heat or adverse pH, low water solubility and often non-availability throughout the year. The latter are of great interest owing to the stability of the pigments produced (Raisainen et al., 2002) and the availability of cultivation technology (Kim et al., 1999; Parekh et al., 2000). Biopigments, not only play an important role in bacterial life, but also possess diverse biological properties such as antibiotic, antioxidant, and anticancer activities. The latter is of special interest due to consistent requirement for chemotherapeutic drugs with high selectivity towards malignant cells.

The present study was carried out to evaluate the antimicrobial efficacy and antioxidant activity of melanin of Streptomyces sp. BI 244 which helps in the development of new, novel drugs.
4.2 MATERIALS AND METHODS

4.2.1 Bioactivity of melanin from *Streptomyces* sp. BI 244

4.2.1.1 Preparation of partially purified melanin for bioactivity

The solution of partially purified melanin was prepared by dissolving 1M NaOH for antimicrobial and antioxidant activity.

4.2.1.2 Determination of antimicrobial activity

Antimicrobial activity of pigment was determined by using disc diffusion method (Bauer *et al.*, 1966). Ten different pathogens namely *Staphylococcus aureus*, *Bacillus subtilis*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Shigella* sp., *Proteus vulgaris*, *Proteus mirabilis* and *Candida albicans* were inoculated on Trypticase Soy Broth and incubated for 24h at 37°C. The counts of pathogenic cultures were adjusted to yield $10^6$-$10^8$ CFU/mL using the McFarland Standard. Then the test pathogens were inoculated with a sterile swab on the surface of prepared, sterilized Muller Hinton Agar (MHA, g/L; Beef infusion - 300.0, Acid hydrolysate of casein - 17.5, Starch- 1.5, Agar -17.0, pH - 7.3 ±0.2 at 25°C) plates separately. The plates were and left at room temperature for 3 - 5min to allow for any moisture to be absorbed before applying the extract. Ten µL (mg/mL) of extract was impregnated with filter paper disc (6mm diameter) and placed on to the surface of MHA plates. The plates were left for 15mins to allow the extract to diffuse. The plates were incubated at 37° C for 24h and zone of inhibition was measured to evaluate the antimicrobial activity of the crude pigment. All the experiments were done in triplicates and the zone of inhibition expressed as mean ± SD (Kaewseejan *et al.*, 2012).

4.2.1.3 Estimation of DPPH radical scavenging activity

Free radical scavenging effect was estimated according to the method of Blois (1958) as modified by Zhu *et al.* (2002). Briefly, 1mM solution of DPPH (1, 1, Diphenyl-2-Picryl hydrazyl) radical solution was prepared in methanol, and then 1mL of this solution was mixed with different concentrations of methanolic extract (20-100µg/ml); the mixture was then vortexed vigorously and left for 30 min at room temperature in the dark and the absorbance (OD) was measured at 517 nm with a spectrophotometer.
DPPH radical scavenging activity was calculated as DPPH Scavenging activity % = ((Control OD – sample OD)/Control OD) x 100. The control was maintained by following the same procedure with the reaction mixture containing 1.0mL of methanol and 1mL of 1mM solution of DPPH radical solution without any extract. Solution of ascorbic acid served as positive control.

4.2.1.4 Total reducing power

This was carried out as described previously by Oyaizu (1986). Different concentrations (20-100μg/ml) of pigment extracts (1 ml)was mixed with 2.5 ml phosphate buffer(0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide, then mixture was incubated at 50°C for 20 mins. The 2.5 ml of 1% trichloroaceticacid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% FeCl₃ and absorbance measured at 700nm in Spectrophotometer. Ascorbic acid was used as standard and phosphate buffer used as blank solution. Increased absorbance of the reaction mixture indicated stronger reducing power.

4.2.1.5 Scavenging of Hydrogen peroxide

Ability of the pigment to scavenge hydrogen peroxide was determined according to the method of Ruch et al. (1986) with the slight modification of Green et al. (1982). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Different concentrations (20- 100μg/ml) of pigment extracts were added to a hydrogen peroxide solution (0.6 ml, 40 mM). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min. against a blank solution containing phosphate buffer without hydrogen peroxide (Ilhami et al., 2005). The percentage of scavenging of hydrogen peroxide by melanin and ascorbic acid was calculated using the following formula:

\[
\% \text{ Scavenged } \text{H}_2\text{O}_2 = ((A0-A1)/A0) \times 100
\]

Where A0 was the absorbance of the control, and A1 was the absorbance in the presence of the sample and standards.
4.2.1.6 Scavenging of Nitric oxide

Nitric oxide scavenging activity was measured spectrophotometrically (Govindarajan et al., 2003). Sodium nitroprusside (5 mM) in phosphate buffered saline (pH 7.2) was mixed with different concentrations of the extract (20-100 μg/ml) prepared in methanol and incubated at 25°C for 30 min. A control without the test compound but with an equivalent amount of methanol was used. After 30 min, 1.5 ml of the incubated solution was removed and diluted with 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% N-1-naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and subsequent coupling with N-1-naphthylethylene diaminedihydrochloride was measured at 546 nm. The percentage scavenging activity was measured with ascorbic acid as reference to standard.

4.2.1.7 Scavenging of Superoxide radical

Ability of the pigment to scavenge hydrogen peroxide was determined according to the method of Misra and Fridovich (1972). To the control tube 300 μl each of potassium phosphate buffer (50 mM; pH 7.4), methionine (45 mM), riboflavin (5.3 mM), nitrobluetetrazolium (84 mM) and potassium cyanide (20 mM) were added. From that, 1.5 ml was taken and made up to 3 ml with distilled water. To the test tube 300 μl each of potassium phosphate buffer, methionine, riboflavin, nitrobluetetrazolium, potassium cyanide and pigment (20 - 100 μg/ml) were added. From that, 1.5 ml was taken and made up to 3 ml with distilled water. The tubes were then placed in aluminium foil lined box and maintained at 25°C. The tubes were exposed to light for 10 mins. and the reduced nitrobluetetrazolium was measured spectrophotometrically at 600 nm. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. Ascorbic acid was used as standard. The percentage inhibition was calculated from the formula,

\[
\text{Inhibition (\%)} = \left(\frac{A_0 - A_e}{A_0}\right) \times 100
\]

where \(A_0\) is the absorbance of the control and \(A_e\) is the absorbance of the extract/standard.
4.2.2 Statistical analysis

Experimental results were mean±SD of three parallel measurements using SPSS -15 versions. Formula \( \text{IC}_{50} = \frac{(50\%-\text{Low}_{\text{Inh}}\%)}{(\text{High}_{\text{Inh}}\%-\text{Low}_{\text{Inh}}\%)x(\text{High}_{\text{Conc}}\text{-Low}_{\text{Conc}}) + \text{Low}_{\text{Conc}}} \) used to calculate the IC50 value. Microsoft Excel was used for construction of graph with error bar.

4.3 Results and Discussion

4.3.1 Determination of antimicrobial activity

The antimicrobial activity of the pigment of marine *Streptomyces* sp. against test pathogens was determined using disc diffusion method. This study indicated that the pigment extract have shown good antimicrobial activity against *Staphylococcus aureus* (17±0.3mm; Fig. - 4.1), *Enterococcus faecalis* (16±0.041; Fig. - 4.2) and *Bacillus subtilis* (13±0.2 Fig. - 4.3) and moderate activity towards *Escherichia coli* (10±0.3; Fig. - 4.4), *Klebsiella pneumoniae* (7±0.1), *Salmonella typhi* (10±0.1), *Shigella* sp. (9±0.4) and *Candida albicans* (10±0.2). The pigment showed no zone of inhibition against *Proteus vulgaris* and *Proteus mirabilis*. The pigment showed more inhibitory activity against Gram-positive organisms than Gram-negative organisms. This is in accordance with the report of Kokare *et al.* (2004) who stated that during the screening for novel secondary metabolites, actinomycetes showed the more active antimicrobial activity against Gram-positive bacteria than Gram-negative bacteria. *Streptomyces* species showed significant antibacterial activity against *S. aureus* and *Pseudomonas aeruginosa* (Devi *et al.*, 2006).

In another report melanin extract was found to show strong antibacterial activity against both *E. coli* and *P. vulgaris* at a concentration of 1μL pigment extract with zone of inhibition ranging between 17 to 20 mm (Vasanthabharathi *et al.*, 2011) and also showed strong activity against *S. aureus, P. mirabilis, V. cholerae, S. typhi, S. paratyphi* and *K. oxytoca*. This is in accordance with our finding but the extract did not show any activity against *Proteus* sp. When about 95 microorganisms mainly *Streptomyces* were examined for pigments with antibacterial activity, *Streptoverticillium* sp. 26-1 furnished good yields of butylcycloheptlyprodiginine (I) with antimicrobial activity. Pigmented antibiotic, found to be a novel peptide antibiotic was extracted from soil *Streptomyces* showing wide antibacterial activity (Roy *et al.*, 1976). Compared to other actinomycetes,
*Streptomyces* species showed efficient antagonistic activity (Okazaki *et al*., 1972). Eighty three percent of actinomycetes isolated from Sagamy Bay were found to possess antifungal activity. Many marine microorganisms showed antifungal activity against *Aspergillus niger* but not against *C. albicans*. Nevertheless, our pigment showed moderate antagonistic activity against *C. albicans*. Actinomycetes isolated from mangrove sediments of Pichavaram Southeast Coast of India exhibiting prominent antibiotic activity against *C. albicans* (Peela *et al*., 2005).

### 4.3.2 Determination of DPPH activity

Free radicals are chemical species containing one or more unpaired electrons that make them highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability. In recent years, much attention has been devoted to natural antioxidants and their association with health benefits (Rajan *et al*., 2012). In the present study, the DPPH free radical scavenging activity of the pigment extract was found to increase in concentration dependent manner (Fig.-4.5). The addition of an antioxidant resulted in decrease of absorbance proportional to the concentration and free radical scavenging activity of the compound and it indicated an increase of the DPPH radical scavenging activity (Williams *et al*., 1995). The IC$_{50}$ value of the pigment extract was found to be 100.70µg/mL.

### 4.3.3 Total reducing power

The reducing power was determined by the reduction of Fe$^{3+}$ to Fe$^{2+}$ in the presence of different concentrations of pigment and ascorbic acid. The absorbance of reaction mixture at 700nm increased with the increase in concentration of extract indicating reducing potential of extract (Chung *et al*., 2006). The gain in absorbance at 700nm with the increase in concentration showed the reducing capability of standard and the pigment. Ascorbic acid showed absorbance values of 0.638 and the pigment showed 0.3 absorbance units at concentrations of 100µg/ml respectively (Fig.-4.6). Nevertheless, the reducing potential of pigment was lesser than that of substance reference. Similar observations have been made in previous studies of Manasa *et al*., 2012, Rakesh *et al*., 2013, Gautham and Onkarappa (2013).
4.3.4 Scavenging of Hydrogen peroxide

Scavenging of Hydrogen peroxide by extracts may be ascribed to their phenolics, which can provide electrons to H$_2$O$_2$, thus neutralizing it to water (Nabavi et al., 2008; Ebrahimzadeh et al., 2009). Hydrogen peroxide scavenging activity of pigment at 100µg/mL was found to be 39.28± 1.0776 %. On the other hand, ascorbic acid exhibited 82.24 ± 1.7651%, hydrogen peroxide scavenging activity respectively at the same concentration (Fig.- 4.7). The IC$_{50}$ value of the extract was found to be 129.91 µg/mL.

4.3.5 Scavenging of Nitric oxide

Nitrogen oxide (NO) is a serious chemical inter mediator generated by endothelial cells, macrophages, neurons etc. It is embroiled in the regulation of varied physical processes (Halliwell, 1989). Nitric oxide and superoxide radicals are engaged in host defense, still over production of these radicals contributes to the pathogenesis of whatsoever inflammatory diseases (Gautham and Onkarappa, 2013). In the present study, Scavenging of Nitric oxide of Streptomyces sp. BI 244 and ascorbic acid were 48.27±1.8356 and 77.80± 2.2002 % at 100µg/mL concentration respectively (Fig.-4.8). The IC$_{50}$ values of the pigment extract was found to be 103.54µg/mL.

4.3.6 Scavenging of superoxide radical

The percentage of radical scavenging activity of ascorbic acid and pigment at 100µg/ml was 77.35 ± 2.1487 and 53.43 ± 1.4208% respectively (Fig.-4.9). Superoxide radicals harm biomolecules directly or indirectly by forming hydroxyl radical, the superoxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical, and various lipid peroxides. All are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes, and other small molecules, resulting in cellular damage (Yen and Duh, 1994; Ross et al., 2000; Smirnova et al., 2000). The IC$_{50}$ value of the pigment extract was found to be 91.77µg/mL.

4.4 CONCLUSION

The present study revealed the antioxidant property of melanin from Streptomyces sp. BI 244. Melanin could serve as free radical inhibitors or scavengers acting possibly as primary antioxidants, which can help in preventing, or slowing down the progress of
various oxidative stress related disorders and bacterial contaminations. The use of these antioxidants as a replacement of conventional synthetic antioxidants in food and food supplements has been employed owing to the fact that natural products are considered a promising and safe source.

Fig. 4.1 Antibacterial activity of partially purified melanin against *Staphylococcus aureus*

Fig. 4.2 Antibacterial activity of partially purified melanin against *Enterococcus faecalis*
Fig. 4.3 Antibacterial activity of partially purified melanin against *Bacillus subtilis*

Fig. 4.4 Antibacterial activity of partially purified melanin against *Escherichia coli*
Fig.-4.5 DPPH radical scavenging activity of partially purified melanin and ascorbic acid

Fig.-4.6 Total reducing power of partially purified melanin and ascorbic acid
Fig.-4.7 Scavenging activity of Hydrogen peroxide of partially purified melanin and ascorbic acid

Fig.-4.8 Scavenging activity of Nitric oxide of partially purified melanin and ascorbic acid
Fig.-4.9 Scavenging activity of superoxide radical partially purified of melanin and ascorbic acid