7.1. PREFACE

Applications of any molecule are based on structural and physicochemical properties. Unique structural and physicochemical properties would result in to specific biological applications. As such the exact structural properties of melanin are not clear. However, applications of melanin in both biological and technological sectors are potential. A major application of melanin is said to be the protection of living beings against exposure to ultra violet radiations. A complex structural feature seems responsible to this important attribute which is yet to be revealed clearly. Solubility of melanin in water is utmost important to explore melanin for its various other technological and biotechnological properties. In the recent past water soluble melanin is gaining lot of importance across the world for its potential applications/functions. The basic attributes of absorption of UV radiations especially by water soluble melanin led in to several applications, such as formulation of cosmetics, paints and also glass coating agents.

The soluble melanin has an added advantage over insoluble one. A lot of literature is available regarding the fundamental applications of insoluble melanin, however very few investigations have been reported on production and applications of synthetic soluble melanin. However, the production and applications of soluble melanin from biological sources, especially microorganisms are rare.

In the present investigation, efforts were made to explore the applications of both insoluble and soluble melanin obtained from a single source, i.e., Streptomyces lusitanus DMZ3. The present chapter includes important biological applications such as antioxidant, cytotoxic, anticancer and hemolytic activities.
7.2. REVIEW OF LITERATURE

7.2.1. Catalytic properties of melanin

Melanin is brown to black colored complex natural pigment which is widely distributed throughout all living forms in nature. The presence of different types of melanin represents almost every large taxon suggesting an evolutionary importance of melanogenesis (Wood et al., 1999). Numerous literatures are available on the biology, genetics and medical applications of melanin of higher animals (Zeca et al., 2001; Halaban, 2002). Melanins are phenolic polymers and they are classified in to two main types and one least mentioned type. In a total three types of melanin can be noticed which were differentiated on the pigmentation, pathway of synthesis and components. Eumelanin is the first type and found most abundant in nature. It is a black brown colored pigment produced on oxidation of tyrosine to o-dihydroxy phenyl alanine to dopaquinone and finally to dopachrome. Eumelanin mainly made up of 5,6-dihydroxy-indole (DHI) and 5,6-dihydroxy indole-2-carboxylic acid (DHICA) as mentioned earlier in chapter III (Del marmol and Beermann, 1996; Langfelder et al., 2003). Pheomelanin is the second most abounded type of melanin found which were initially synthesized just like eumelanin, but Dopa undergoes Cysteinylation, directly or by the mediation of glutathione. In the end cysteinyln Dopa was formed which further polymerizes in to various derivatives of benzothiazines (Kobayashi et al., 1995; Nappi and Ottaviani, 2000). Allomelanin the least studied type of melanin and made up of most heterogeneous group of polymer, which emerge through oxidation/polymerization of fi-(DHN) or tetrahydroxy naphthalene via., pentaketide pathway leading through flavoline to various colored polymer of DHN-melanis, homogenetic acid (polymelanins) γ-glutaminyl-4-hydroxy benzene and catechols (Gibello et al., 1995; Kotob et al., 1995; Espin et al., 1999). Although eumelanin and pheomelanin are present in various degrees in human skin (Sarna and Plonka, 2005).
It is clear that, the function of melanins is defined by their physical and chemical properties. The properties of melanin which mainly includes feature such as antioxidant, free radical scavenging activity, broad band UV and high energy visible absorption, etc., which are defined by the molecular and supra molecular aggregate level structures. This was established the relationship between link structure and properties which is a common goal in many branches of materials physical and chemistry. The challenges faced by molecular biophysics and molecular biology are understanding how these structure-property relationships define biological function of melanin (Paul and Tadeusz, 2006). Ultimate goal on melanin research is to link all these facts together to gain a full mapping of how molecular scale structure relate to properties and functions. This will allow us to understand the clear picture of melanin related disease states on which it is possible to create meaningful medical interventions. Significant progress on the structural and property studies of melanin was observed since last decades. At this stage it is important to remember the pioneering structural work on melanin made by groups such as those of Nicolaus, Prota and Ito (to name but few) has been founded in the principles of organic and bio-organic chemistry. The major groups from photo biophysicists, photo biologists and photo chemists have played major roles in establishing and understanding key melanin photo-properties. Even though after a few very notable exceptions, the materials, structural and chemical, physics community has been largely absent from the melanin debate (Paul and Tadeusz, 2006).

7.2.2. Applications of biologically active melanin

Apart from the discussion on the exact structural property studies, it is proved that melanin had remarkable biological and other technological properties which can be said as the applications. The biological application includes photo protection (Huang and Mau, 2006), anti-microbial (Casadevall et al., 2000), antioxidant (Goncalves et al., 2005), antitumor (El-Obeid et al., 2006), antivenin (Hung et al., 2004), anti-viral (Montefiori and Zhou, 1991), liver protecting activity (Sava et al., 2003), etc. Details on the absorption of
Ionizing radiation studies (Dadachova et al., 2007) were available with missing mechanism fundamentals. Apart from property like photo absorption, melanin is also well known for its antioxidant properties.

Numerous literatures are available reporting antioxidant activities concluded based on various tests. The main tests adopted as a part of antioxidant assay are DPPH radical scavenging activity, phosphomolybdate assay for total phenol analysis, total reducing power, $O_2$ scavenging activity, nitric oxide scavenging activity and $Fe^{2+}$ chelating activity (Manivasagan et al., 2013). Different researchers reported the different antioxidant activity assays for melanin. Melanins from different sources like fungi, Actinomycetes, bacterial and plant extracts (Casadevall et al., 2000; Manivasagan et al., 2013; Pei Hsing and Tu-fa, 2012) were proved as powerful antioxidants.

Beside their antioxidant activities of melanin, it is an extremely necessary to assess the cytotoxic activity which is a useful tool in the initial selection that can be proved as drug for anticancer. It is already proved that melanin has an excellent cytotoxic activity based on the reports available on the anticancer activity studies with regards to melanin. Hochtein and Gerald (1964) reported the cytotoxic activity of melanin and melanin precursor. Novel approach of cytotoxic studies was given by Pawelek and Murray (1986), where they observed the cytotoxicity upon increase in melanin in melanoma cell lines by phosphorylated isomers of L-Dopa. Melanin from Aspergillus fumigatus (Batol Imran et al., 2013) and synthetic melanin (Thiago et al., 2015) were shown to be one of the potent anticancer drug based on cytotoxic studies using rat embryonic fibroblast and NIH/3T3 cells respectively. Cytotoxic studies using cell lines need a sophisticated lab, well skilled handling and also deals with high cost experiments. Brine shrimps based cytotoxic studies is an alternate method of experiment which is as accurate as cell line studies and is most accepted experiments (Silva et al., 2007). But none of the reports were available on cytotoxic studies of melanin using brine shrimps.
It is evident that melanin is potential anticancer agent based on the literature available on cytotoxic studies on melanin. Monder and Waisman (1958) reported the anticancer activity of melanin obtained after non enzymatic conversion of Dopa to melanin. It is also reported that the melanin extract from *N. sativa* L. could able to inhibit the production of cancer agent accessories VEGF and produce TNF-α and IL-6 in human monocytes of PBMC and THP-1 cell lines during the transcriptional and translational levels (El-Obeid *et al.*, 2006). Since from decades a scientist named Farmer P.J. from Baylor University, Texas working on the melanoma to develop a potent anticancer melanin based drug. His group reported a melanin as a target for melanoma chemotherapy and got succeeded (Farmer *et al.*, 2003; Costa *et al.*, 2012).

In addition to above applications of melanin discussed, it is also reported for antivenin activity by Hung *et al.* (2004), potential antiviral activity in specific against HIV (Montefiori and Zhou, 1991) and liver protecting activity of melanin derived from black tea (Sava *et al.*, 2003). The biological applications discussed still are activities of melanin with irrespective of its solubility. Other than these the soluble melanin had additional biological application. Soluble melanin is used in the preparation of sunscreens to protect against harmful UV radiations, which is a major causative factor of melanoma and other cancer of the skin (Pawelek *et al.*, 1992). It is also used as a substitute for para amino benzoic acid (PABA), another component that absorb UV light because under certain conditions PABA is toxic, which may lead to adverse reactions in some individuals (Tyler A. Kokjohn and John O. Schrader, 1998).

**7.2.3. Technological applications of melanin**

It is essentially critical for melanin to be water soluble to enable their high potential applications. In general melanins are highly insoluble and require severe treatments such as boiling in strong alkali, or the use of strong oxidants such as hydrogen peroxide, which often damage the melanin. Hence, soluble melanin have not only the additional applications over
insoluble, it had a greater commercial potential over insoluble melanin (Raghukumar et al., 2009).

The technological applications of soluble melanin includes, it is used in paints, varnishes and other surface protection formulations to provide greater UV protection to the surfaces (Gallas, 1991). Gallas and Eisner (2006) conjugates the solid plastic films of polyvinyl alcohol containing soluble melanin with other plastics to make laminated sheets and lens, making them UV protective and against photochemical damages from electromagnetic radiations. Last but not the least, soluble melanin had great affinity towards metals, chemicals and drugs, hence it is used in the detection of low levels of compounds and metabolites and in elimination of these toxic targets (Tolleson, 2005). It is unfortunate state of affairs that we do not have a clear understanding of how UV and visible radiation interacts with the macromolecule to generate properties such as radiation absorbance. To explain the exact mechanism of these most fundamental of issues requires a detailed and correct structural model at the molecular and aggregate level which is lacking (Paul and Tadeusz, 2006).

**General applications of melanins**

- **Soluble melanin**
  - Photo absorber (UV and Visible)
  - Antioxidant
  - Cytotoxicity
  - Anti-cancer
  - Antivenin
  - Antiviral

- **Insoluble melanin**
  - Sunscreens
  - Skin lotions
  - Lens coating
  - Glass coatings
  - Paints/Varnishes
  - Detoxifier
7.3. EXPERIMENTAL METHODS

7.3.1. Antioxidant activity of melanins

Antioxidant activity of melanins, as biomedical attributes were determined as per standard methods. Antioxidant activity was carried out by following 2,2’-diphenyl-1-picrylhydrazyl-DPPH (Bersuder et al., 1998), Phosphomolybdate (Umamaheswari and Chatterjee, 2008) and reducing power (Oyaizu, 1986) methods.

**DPPH radical scavenging activity:** Various concentrations (25, 50, 100, 200, 400 and 800 µg/mL) of 500 µl insoluble and soluble melanin separately, in duplicate was mixed with 500 µl of 99.5% ethanol and 125 µl of 0.02% DPPH in 99.5% ethanol. The mixture was incubated at 30 ºC in dark for 30 minutes. The reduction of DPPH was measured at 517 nm using UV-vis spectrophotometer and the percentage inhibition of the DPPH radical was calculated. The IC$_{50}$ value, based on concentration at which 50% inhibition occurred was also calculated. A lower absorbance of the reaction mixture indicates a higher DPPH radical scavenging activity. Ascorbic acid was used as a standard.

**Phosphomolybdate assay (total antioxidant capacity):** The total antioxidant capacity of the insoluble and soluble melanin was determined. The sample of 0.1 mL at various concentrations (25, 50, 100, 200, 400 and 800 µg/mL) was mixed with 1 mL reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and incubated in a water bath at 95 ºC for 90 minutes. The UV-vis absorbance of the mixture at 30 ºC was recorded at 765 nm against a blank. A typical blank contained 1 mL reagent solution and appropriate volume of the solvent. The antioxidant capacity was estimated using ascorbic acid as standard.

**Reducing power:** The reducing powers of insoluble and soluble melanin were assessed. Insoluble and soluble melanin at different concentrations (25, 50, 100, 200, 400 and 800 µg/mL) were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 7.6) and potassium ferricyanide (2.5 mL,
1% separately. The mixture was incubated at 50 ºC for 20 minutes. Trichloroacetic acid (10%) of 2.5 mL was added to the mixture and centrifuged for 10 minutes. The upper layer of the solution (2.5 mL) was separated and mixed with distilled water (2.5 mL) and FeCl₃ (0.5 mL, 0.1%). The UV-vis absorbance of the mixture was recorded at 700 nm. The increase in the absorbance of the reaction mixture indicates increased reducing power.

7.3.2. Cytotoxic activity of melanins

The Cytotoxic activities of insoluble and soluble melanins were determined by following the standard protocol of Meyer et al. (1982) using brine shrimps (Artemia Salina). Artificial sea/saline water was prepared, dissolving 20 g of NaCl per liter and pH was adjusted to 8.5 with 0.1 M Na₂CO₃. 1 g eggs of brine shrimp was added to the 1 L seawater and incubated at 28 ºC for 48 h with constant air supply and light. The hatched brine shrimps were collected and rinsed in fresh seawater. The insoluble and soluble melanin concentrations (0, 1, 2, 4, 8, 16, 32 and 64 µg/mL) were diluted in 5 mL seawater in separate tubes and incubated at 28 ºC. Sample with zero concentration of melanins was considered as control. The mortality number of brine shrimps for every 6 h up to 24 h was recorded. The percentage of mortality and lethal concentration value (LC₅₀ - µg/mL) of melanins were calculated. The mortality end point of the bioassay was referred as the absence of controlled forward motion during 30 seconds of observation and the concentration that killed 50% of brine shrimps as LC₅₀. Criterion of toxicity for fractions was categorized as nontoxic (LC₅₀ values >1000 µg/mL), poor toxic (500-1000 µg/mL) and toxic (<500 µg/mL) according to Deciga-Campos et al. (2007).

7.3.3. Anticancer attribute of soluble melanin

The soluble melanin was exploited for its anticancer attribute as per standard methods, namely clonogenic survival assay (Hamburger and Salmon, 1977), DNA ladder assay (Gavrieli et al., 1992) and analysis of apoptosis (Nicoletti et al., 1991).
Clonogenic survival assay: A375 cell lines (Appendix 2) were plated (2x10^3 cells/dish - p35 mm) containing Dulbecco’s Modified Eagle Medium – DMEM (Appendix 1.2) and incubated at 37 °C with 5% CO_2 for 24 h. After 24 h, cells were treated with various concentrations of soluble melanin from 0 to 1200 µM and allowed to grow for fourteen days. Fresh DMEM medium was again added on the seventh day of growth. On the fourteenth day, after attaining colonies of >50 cells/colony, medium was removed from the dishes and washed once with ice-cold PBS (Appendix 3.2). The colonies were stained with 1 mL of 1% crystal violet in 80% methanol for 30 minutes on a rocking platform. The dishes were rinsed three times with PBS and air-dried, and the colonies were counted under 4x by using inverted microscope.

DNA Ladder Assay: After the microscopic counting as mentioned earlier, adherent and detached cells were harvested and washed with phosphate buffered saline (1X PBS). Approximately 2x10^5 cells were lysed with a lysis buffer containing 50 mM Tris-HCl, 10 mM ethylene diamine tetra acetic acid (EDTA)-4Na and 0.5% sodium-N-lauroylsarcosinate (pH 7.8). The lysates were incubated in the lysis buffer containing 0.33 mg/mL RNase A at 50 °C for 30 minutes and then further incubated in the lysis buffer containing 0.33 mg/mL proteinase K at 50 °C for 30 minutes. 5 µL of lysate was electrophoresed on 2.0% agarose gel. Gels were stained with 0.5 mg/mL ethidium bromide for 15 minutes and visualized under UV light.

Analysis of apoptosis: The Acridine orange (AO)/Ethidium bromide (EB) staining as fluorescent probes method was used to observe the apoptotic cell death morphologic changes. A375 cells were suspended at a final concentration of 1×10^5 cells/well and cultured in a 6-well flat-bottomed plate and allowed to adhere to the bottom of the wells for 24 h before the treatment with soluble melanin. Cells were exposed to 400 and 800 µM concentrations of soluble melanin for 24 h. Plain media containing 1% DMSO were used as a control. After designed time, AO/EB mixture [25 µL, containing 100 µg/mL AO and 100 µg/mL EB in PBS (pH 7.4)] was added to cells treated with
extract/compounds and allowed to stain for 5–10 minutes at room temperature. Thereafter, 2–3 drops of the stained cells were placed on each slide with a coverslip. Photographs were captured at random locations under a Nikon 80i fluorescence microscope equipped with Nikon BR software. Untreated cells were used as the negative control. These experiments were facilitated by M/S Skanda Life Sciences, Bangalore.

7.3.4. Hemolytic activity of soluble melanin

The hemolytic activity of melanin was assessed for its usage in medicine as per standard method (Dillingham et al., 1983).

**Isolation of erythrocytes:** Five mL of human blood was obtained from the blood bank in the tubes along with anticoagulants and centrifuged at 1000 rpm for 10 minutes at 4 ºC. Plasma was removed carefully and the white buffy layer was completely removed by aspiration with a pipette with utmost care. The erythrocytes were then washed for additional three times with 1X PBS, pH 7.4. Washed erythrocytes were stored at 4 ºC and used within 6 h for the haemolysis assay.

**Hemolytic assay:** Erythrocytes suspension of 50 µL of 10 dilution (100 µL Erythrocytes suspension: 1000 µl 1XPBS) was taken into 2 mL eppendorff tube and 100 µL of different concentration (50, 100, 200, 400 and 800 µg/mL) of melanin was added. 100 µL of 1XPBS was maintained as negative control. 100 µL of 1% Triton X-100 and 100 µL of 1% SDS were used as standard positive controls. Reaction mixture was incubated at 37 ºC in water bath for 60 minutes. The volume of reaction mixture was adjusted to 1 mL by adding 750 µl of 1XPBS and additionally 100 µl of 10 mM H₂O₂ was added to this mixture as free radical initiator. Finally centrifuged at 300 rpm for 3 minutes and the resulting supernatant was measured at 540 nm by spectrophotometer to determine the concentration of hemoglobin.

\[
\text{% haemolysis} = \left[ \frac{\text{Control} - \text{Sample}}{\text{Control}} \right] \times 100
\]
7.4. RESULTS AND DISCUSSION

7.4.1. Antioxidant property of insoluble and soluble melanins

Use of melanin for biomedical activity is fast progressing. In this study, antioxidant and cytotoxic activities of insoluble and soluble melanins were assessed. Normally antioxidant activity of a compound is assessed by DPPH method which reveals scavenging activity of free radicals (Bersuder, 1998). The DPPH radical scavenging activity of melanins at different concentrations was determined (Figure 7.1). Insoluble and soluble melanins revealed a promising radical scavenging activity with an IC\textsubscript{50} value of 137.5 and 115 µg/mL respectively. Phosphomolybdic acid assay (Umamaheswari and Chatterjee, 2008) reveals phenolic contents of the compound that would determine the total antioxidant activity. Insoluble and soluble melanins showed a good antioxidant activity based on phosphomolybdic acid assay and the total antioxidant activity was increased (Figure 7.2) upon the increases in the concentration of melanins. The half of the effective concentration (EC\textsubscript{50}) of insoluble and soluble melanins was 100 and 77.5 µg/mL. The reducing power technique (Oyaizu, 1986) exhibits the antioxidant activity of a compound based on the degree of reducing power of iron provided in the reaction. The melanins exhibited encouraging reducing properties, which have increased upon the increase in the concentration (Figure 7.3).

Figure 7.1: Antioxidant attribute of insoluble and soluble melanin by DPPH assay

[Graph depicting the antioxidant activity of melanin by DPPH assay]
A promising and relatively enhanced scavenging activity exhibited by insoluble and soluble melanins may be attributed to a greater medical value of water soluble melanin rather than insoluble. Insoluble melanin was reported (Manivasagan et al., 2013) as a reducing agent and water soluble melanin reveals the higher activity compared to insoluble melanin. Ascorbic acid which was used as a standard and control in all three methods found to be more prominent. Evaluation and understanding of antioxidant
activity of insoluble and soluble melanins employing all three methods may be a unique criterion. Numerous literatures are available reporting antioxidant activities concluded based on various tests. The main tests adopted as a part of antioxidant assay are DPPH radical scavenging activity, phosphomolybdate assay for total phenol analysis, total reducing power, O$_2$ scavenging activity, nitric oxide scavenging activity and Fe$_{2+}$ chelating activity (Manivasagan et al., 2013). Different researchers reported the different antioxidant activity test for melanin. Melanins from Fungi like Cryptococcus neoformans (Casadevall et al., 2000), Aspergillus nidulans (Goncalves et al., 2005), Lachnum singerianum (Ming Ye et al., 2011), Aspergillus bridgeri (Kumar et al., 2011) and Lachnum sp. (Gengyi Guo et al., 2012); Actinomycetes like Streptomyces sp. (Preeti and Ashok, 2013) and Actinoalloteichus sp. (Manivasagan et al., 2013) and among bacterial, Klebsiella sp. (Shrishailnath et al., 2013), plant extracts of Coccinia grandis L., extracts of black tea, black soybean and black bone silky fowl (Pei Hsing and Tu-fa, 2012) and synthetic Dopa melanin (Thiago et al., 2015) were proved to be powerful antioxidant.

7.4.2. Cytotoxic activity of insoluble and soluble melanins

The cytotoxic effect of insoluble and soluble melanin on brine shrimps were summarized in Table 7.1 and 7.2. The number of brine shrimps survived and the percentage of mortality for 6 h, 12 h and 24 h was noted. After 24 h, the total mortality was 100% in the highest concentration (64 µg/mL) of soluble melanin and 95% mortality was observed in insoluble melanin. The LC$_{50}$ value was 0.40 µg/mL for soluble melanin and 0.80 µg/mL for insoluble melanin.

Assessment of cytotoxicity of chemicals using cell lines is not an uncommon procedure and is accurately correlated (Carballo et al., 2002) with the assessment of cytotoxicity using brine shrimps. The brine shrimp assay method is considered as an excellent alternate option to assess the cytotoxic activity of the biological product (Quignard et al., 2003). From the beginning of its introduction to standardization (Meyer et al., 1982) this in vivo test had
successfully adopted for the bioassay of active cytotoxic and antitumor agents (Pisutthanan et al., 2004). Further the lethal concentration of brine shrimp can be correlated with the lethal dose in mice and was explained using medicinal plants earlier (Parra et al., 2001). Both pigments exhibit higher cytotoxic activity, as LC$_{50}$ value of both showed less than 500 µg/mL (Déciga-Campos et al., 2007). However, soluble melanin could reveal 100% mortality and greater cytotoxic activity with half of the LC$_{50}$ value, when compared to insoluble melanin.

It is already proved that, melanin has an excellent cytotoxic activity based on the reports available on the anticancer activity studies with regard to melanin. Hochstein and Gerald (1963) reported the cytotoxic activity of melanin and melanin precursor.

Table 7.1: Cytotoxic activity of the insoluble melanin

<table>
<thead>
<tr>
<th>Concentration of Melanin (µg/mL)</th>
<th>Survived brine shrimps</th>
<th>Percent mortality</th>
<th>Lc 50 (µg/mL) at 24 h</th>
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<tr>
<td>01.0</td>
<td>19 11 06</td>
<td>10 45 70</td>
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<td>16.0</td>
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<td>32.0</td>
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<td>64.0</td>
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Table 7.2: Cytotoxic activity of the soluble melanin

<table>
<thead>
<tr>
<th>Concentration of Melanin (µg/mL)</th>
<th>Survived brine shrimps</th>
<th>Percent mortality</th>
<th>Lc 50 (µg/mL) at 24 h</th>
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Novel approach of cytotoxic studies was given by Pawelek and Murray (1986), where they observed the cytotoxicity upon increase in melanin in melanoma cell lines by phosphorylated isomers of L-Dopa. Melanin from *Aspergillus fumigates* (Batol Imran *et al.*, 2013) and synthetic melanin (Thiago *et al.*, 2014) were shown to be one of the potent anticancer drug based on cytotoxic studies using rat embryonic fibroblast and NIH/3T3 cells respectively. After comparing the potentialities of insoluble and soluble melamins for different biological applications, the soluble melanin can be concluded as the most prominent. Hence, the soluble melanin was considered for anti-cancer attributes and assessed for its hemolytic activity.

### 7.4.3. Anticancer attributes of soluble melanin

The colonies of A375 melanoma cell lines treated with different concentrations (400, 800 and 1200 µM) of soluble melanin were as presented in Plate 7.1. Decrease in the colony count with increase in the concentration of soluble melanin was observed. Frequency of inhibition was observed on comparing the melanin treated cells with the control. The fluorescent micrographs of healthy and apoptotic A375 cells were as observed in Plate 7.2. The melanoma cells treated with soluble melanin of 400 and 800 µM concentrations were incubated separately and stained using acridine orange and ethidium bromide and observed under fluorescence microscope. The soluble melanin of 400 and 800 µM treated cells showed apoptosis in a dose dependent manner by cells stained with red color (due to staining with ethidium bromide) but the untreated or control cells stained with green color (due to staining with acridine orange). It was observed that, the treatment of cells with higher concentration increased the death of the cells which were red/orange and healthy/survived cells were green. Hence, the melanin exhibited more cytotoxicity/apoptosis against cancer cells at 800 µM. The results of DNA fragmentation analysis of soluble melanin on A375 cells using agarose gel electrophoresis were as shown in Plate 7.3. A375 melanoma cell lines treated with 1% DMSO (control) and different concentrations (400, 800, and 1200 mM) of soluble melanin were lysed and separated on
agarose gel electrophoresis. It was observed that, no fragmentation of DNA of cells treated with DMSO (lane 1) and the increase in the fragmentation of DNA was observed in lane 2, 3 and 4 containing the DNA of cells treated with 400, 800 and 1200 mM of soluble melanin respectively.

Plate 7.1: Anticancer attribute of soluble melanin by clonogenic survival assay

Plate 7.2: Anticancer attribute of soluble melanin by visual observation of apoptotic cells under florescent microscopy
In all, regarding the attributes of anticancer, soluble melanin showed an impressive observation in which an increase in the activity was found along the increase in the concentration. In the clonogenic assay, the soluble melanin at 1200 µM concentration succeeded the maximum inhibition of A375 melanoma cell lines. Soluble melanin at 800 µM could able to kill about 90% melanoma cells, which was visualized as orange fluorescence spots (Plate 7.2). This reveals that soluble melanin is a potent anticancer agent. In specific, soluble melanin inhibit and kill the melanoma cells by fragmenting their DNA. The observation on DNA fragmentation (Plate 7.3) illustrates this phenomenon. The fragmentation of DNA was increased on increase in the concentration of melanin and the maximum fragmentation was observed (lane four) containing the DNA of melanoma cells treated with soluble melanin at 1200 mM concentration. Therefore, it is evident that melanin is a potential anticancer. Monder and Waisman (1958) reported the anticancer activity of melanin obtained after non enzymatic conversion of Dopa to melanin. It was also reported that, the melanin extract from N. sativa L. could able to inhibit the production of cancer agent accessories VEGF and produce TNF-α and IL-6 in human monocytes of PBMC and THP-1 cell lines during the transcriptional and translational levels (El-Obeid et al., 2006). No available
literature reports the anticancer activity of soluble melanin. Present investigation reveals a significant anticancer attribute of soluble melanin over insoluble melanin.

7.4.4. Hemolytic activity of soluble melanin

The hemolytic activity of soluble melanin was plotted in Figure 7.4. The activity of different concentration (50, 100, 200, 400, and 800 µM) of soluble melanin on hemoglobin was assessed keeping 1% SDS as positive control and 1XPBS as negative control. The standard positive control exhibited activity of 60.477% hemolysis, whereas none of the above concentration of soluble melanin showed the hemolytic activity.

Non hemolytic activity of soluble melanin, directs soluble melanin as a potential anticancer agent. Therefore, soluble melanin produced by *Streptomyces lusitanus* DMZ3 can be fearlessly explored as an anticancer agent.

CONCLUSION

Melanins are well known for broad range of biological and non-biological applications. However, among melanins, soluble melanin has greater credentials in applications when compared to insoluble melanin, due to its typical physicochemical properties. Melanins are proved to be more valuable in medical usage due to its higher antioxidant and cytotoxic
activities. Assessment of cytotoxicity of natural products is normally determined by employing either specific cell lines or experimental animals. In the present study, cytotoxicity of melanin was investigated using brine shrimps successfully for the first time. Both insoluble and soluble melanins have been proved to be highly toxic, but soluble melanin was more biologically active (0.40 µg/mL) as compared to insoluble melanin (0.80 µg/mL). Antioxidant assay also reveals that the soluble melanin is prominent showing more activity in DPPH (IC$_{50}$ - 115 µg/mL) and phosphomolybdate assay (EC$_{50}$ - 77.5 µg/mL), compared to insoluble melanin. Further, only soluble melanin was preferred to assess for anticancer attribute and hemolytic activity, as it had shown higher cytotoxic and antioxidant activities. Impressive anticancer activities were exhibited by soluble melanin, which was revealed by clonogenic, apoptotic and DNA fragmentation analysis of melanoma cell lines. Irrespective of methods, soluble melanin has showed a maximum activity at 800 mM and 1200 mM concentrations. The soluble melanin was also assessed for hemolytic activity to ensure its safer anticancer attribute. Naturally, soluble melanin proved to be non-hemolytic on human erythrocytes even at higher concentrations of 800 mM and above. This has revealed that, soluble melanin is unable to damage the hemoglobin of human blood indicating its non-hemolytic nature on human erythrocytes.