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Antioxidative, Antibacterial and Antibiofilm Activity of Melanin nanoparticles (MN)

Tiwari Amit and Pradhan Swapnil*

Department of Microbiology, Ramnarain Ruia Autonomous College, University of Mumbai, Mumbai, India
*Corresponding author Email : tiwari.amit8894@gmail.com

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ABSTRACT

Melanin is a complex, diverse, negatively charged, hydrophobic, amorphous and high molecular weight hetero-polymeric dark brown photosynthetic pigment synthesized by living organisms including animals, humans, and microorganisms in the course of oxidative polymerization of phenolic compounds. In this study, melanin production was carried out by Pseudomonas sp. which was isolated from soil. After production, Melanin was extracted by acid hydrolysis and characterized based on its solubility in different solvents where it was found to be water-soluble (a unique characteristic of melanin); by TLC and by NMR. After the characterization of melanin, melanin nanoparticles were formed by sonication and then characterized and evaluated for antimicrobial and antioxidative properties. The nanoparticle was found to be of discrete sizes. Melanin nanoparticles showed strong antimicrobial activity against Gram-positive organisms and also higher antioxidative properties than melanin. Melanin Nanoparticles were found to disrupt formed biofilm of *Staphylococcusaureus* by 67.39%. Thus, Melanin nanoparticles may find their utility as a coating material in medical implants and as packaging material in food products against oxidation and bacterial contamination.

Keywords: *Pseudomonas balearica* DSM 6083(T), Melanin Nanoparticles, $KMnO_4$, Titanium, Biofilm.

INTRODUCTION

Melanin is complex, diverse, negatively charged, hydrophobic, amorphous and high molecular weight hetero-polymeric dark black or brown pigments, a ubiquitous pigment produced in melanosomes, are found in bacteria (such as *Brevundimonas* sp. SGJ, *Shewanella colwelliana* D, *Aeromonas media, Pseudomonasputida* F6 , *Klebsiella* sp.GSK, *Pseudomonas stutzeri* , recombinant *Escherichia coli* W3110, etc), fungi (such as *Aspergillus fumigates*, *Aspergillus Bridgeri*, *Pneumocystis carinii*, *Cryptococcus neoformans*, *Pleurotus cystidiosus*, etc.), plants and in many human organs and tissues, including retina, mucous membrane, ovary, cerebral pia mater and skin (Liu *et al*., 2018; Pal *et al*., 2015; Shripad *et al*., 2013; Coon *et al*., 1994; Wan *et al*., 2007;

Nikodinovic-Runic *et al*., 2009; Sajjan., 2013; Kumar *et al*., 2013; Lagunas-Muñoz *et al*., 2013; Kumar *et al*., 2011; Youngchim *et al*., 2004; Plonka *et al*., 2006; Frases *et al*., 2006; Selvakumar *et al*., 2008). Melanin pigment is produced by the oxidation of tyrosine by tyrosinase in a multistep synthesis via 5,6-dihydroxyindole-2-carboxylic acid and 5,6-dihydroxyindole (Pezzella *et al*., 2013). This oxidative polymerization of these indoles gives rise to the melanin biopolymers: black-brown eumelanin and yellow/reddish pheomelanin (Xiao *et al*., 2015). Melanin is one of the most stable, insoluble, and resistant pigment (Francisco Solano, 2017). The polymer melanin is known to absorb UV radiation by redox reactions and electron transfer processes. In addition to photoreactive nature, melanin can also absorb metal ions, free radicals, etc. thereby act as a cytoprotective agent. They are an antioxidant, antitumor, and anti-inflammatory agent, as well as an immune-stimulating agent. They also find application in semiconductor and bio-electronics as well as in polarized sunglass lenses, paints, and varnishes (Francisco Solano, 2017).

Biofilm can be defined as communities of microorganisms irreversibly attached to a surface, encased within a polysaccharide-rich extracellular matrix, and exhibiting an altered phenotype which has enhanced resistance to antimicrobial drugs (Naik *et al*., 2015; Mohammadi *et al*., 2013). The high density of extracellular polysaccharide-rich matrix will not only slow the diffusion of antibiotics but also hinders access of immune system defenses such as antibodies and macrophages (Naik *et al*., 2015). These biofilms are responsible for 60% of Healthcare associated infections (HAI) by corroding medical implants. Various approches have been used to disrupt or inhibit biofilm such as use of anti-virulence compounds, use of mucolytic substances, use of chelating agents, use of matrix targeting enzymes, use of phages, and recently use of nanoparticles (Percival *et al*., 2015b; Starkey *et al*., 2014; Pérez-Giraldo *et al*., 1997; Abraham *et al*., 2012; Chen *et al*., 2013; Fu at al., 2010; Algburi *et al*., 2017).

Nanomaterials have appeared as one of the most promising strategy for controlling or treating pathogenic biofilms on indwelling medical devices and implants due to their large surface to volume ratio and unique physico-chemical properties (Naik *et al*., 2015)(Ramasamy & Lee, 2016). As melanin is known to have antimicrobial and antioxidant activity, in this study melanin nanoparticles have been used for antibiofilm activity. Melanin nanoparticles can be synthesized either by physical and chemical method. Chemical synthesis of melanin nanoparticles involves reaction between either $KMnO_4$ or NaOH with melanin under specific set of conditions (Rageh & El-Gebaly, 2018; Sáez & Mason, 2009).The physical method of melanin nanoparticles involves usage of ultrasonication resulting in breaking of melanin. Biofilm formation takes place by quorum sensing signals and it has been known that melanin can inhibit these signals. Melanin inhibit acyl homoserine lactone (AHL)-regulated behaviors by binding competitively to the AHL receptor protein, thus, inhibit or disrupt biofilm formation (Bin *et al.*, 2012).

In this study, *Pseudomonas balearica* DSM 6083 isolated from soil have used for synthesis of Melanin. Synthesized Melanin was extracted by refluxing in strong acid and characterized using its biochemical properties. Melanin nanoparticles have been synthesized using ultrasonication method, a non-chemical procedure. The anti-oxidant activity of melanin and melanin nanoparticles have been performed using potassium permangnate. Well diffusion assay was performed for determining antibacterial activity of both melanin and melanin nanoparticles. Biofilm formation was allowed on titanium pieces and later it was treated with melanin and melanin nanoparticles to disrupt formed biofilm.

MATERIALS AND METHODS:

1. Sampling Site and Sample Collection

10 g of soil sample was collected from $19^{O}01'21"$ N $72^{O}50'55"$ E in a sterile glass bottle. The sample was kept in a petri dish at Room Temperature to remove moisture content i.e. clumping of soil..

2. Enrichment of the culture

1 g of the processed soil sample was inoculated in 20 ml of St. Nutrient Broth containing 0.5% Tyrosine and incubated at Room Temperature for 7 days on a shaker (adjusted to 150 rpm). The growth of the organism was indicated by turbidity in the medium followed by the brown coloration of the media.

3. Isolation and Culturing of the melanin producing organism

After the development of brown coloration of the enrichment media, a loopful of growth obtained was

streaked on St. Nutrient Agar Plates containing 0.5% Tyrosine (pH 10) and incubated at 37°C for 3 days. Colony characteristics of colonies producing brown to black pigment were selected and sub-cultured on St. Nutrient Agar Slants containing 0.5% Tyrosine (pH 10) (Pathan & Pethe, 2016).

4. Identification and Characterisation of the melanin producing organism

Isolates obtained were identified based on growth obtained on the St. Nutrient Agar Plate containing 0.5% Tyrosine (pH 10) by the production of brown to black pigment. Complete characterization of the organism was done based on cultural & morphological characteristics, and by 16 S rRNA sequencing.

- Cultural characterization was done based on colony characteristics of the isolate obtained on the St. Nutrient Agar Plate containing 0.5% Tyrosine (pH 10).
- The morphological arrangement, Gram nature, and structure of the cells were identified by performing Gram staining. A smear of culture was taken in a clean glass slide and heated gently over a flame. The smear was covered with a thin film of crystal violet for 1 min and washed gently in slow running tap water. Gram's iodine solution was flooded over the smear for 1 min and washed with tap water. Alcohol was used to decolorize the smear until the violet colour ceased to flow away. The slide was washed with water and counterstain safranin was flooded over the smear for 2 min, then the slide was washed, drained, air dried, and viewed under a microscope.
- The genetic makeup of the isolate was determined by performing 16S rRNA sequencing.
  16S rRNA gene sequence analysis has been widely used for the identification of the species. The sequence of 16S rRNA provides a measure of genetic similarity above the level of species allowing comparisons of relatedness across the genus, family, etc. In the present study, 700 bp of entire 16S rRNA from the selected isolate was sequenced.

5. Pigment Production, Extraction, and Purification of melanin pigment

Isolate was inoculated in 10 ml of St. Nutrient Agar Broth containing 0.5% Tyrosine (pH 10) and incubated for 24 h at 37°C for activation. 10 ml of activated culture was inoculated in 200 ml of St. Nutrient Agar Broth containing 0.5% Tyrosine (pH 10) and incubated at 37°C for 3 weeks to produce melanin pigment (V, V., *et al* ., 2011).

After 3 weeks of incubation, chloroform was added into samples at 10 % concentration and kept for 2 days to kill viable cells. Then samples were centrifuged at 12000 rpm for 15 min. The cell pellet was discarded and melanin-containing supernatant was then acidified to pH 3 with 5 N HCl to ensure precipitation of the melanin. The precipitated melanin was centrifuged at 12000 rpm for 15 min, washed three times with deionized water, and allowed it to dry at Room Temperature and then weight was determined (Pal *et al*., 2015).

Melanin produced was purified using the dialysis method. In the dialysis method, melanin powder was kept in the dialysis tube which itself was kept in 2000 ml distilled water which was changed every 4 hours for 2 days.

6. Characterization of melanin pigment

6. A. Solubility Test

Purified melanin was identified based on its solubility in different solvents. Purified melanin was dissolved in 3 ml of various organic and inorganic solvents (Hou *et al*., 2019).

Organic solvents: Ethanol, Diethyl ether, Acetone (warm and cold), Chloroform (warm and cold), Benzene, Dimethyl sulfoxide, Hexane, n-butanol, and Phenol.

Inorganic solvents: Distilled water, 0.1 N NaOH solution (pH 9) and Conc. HCl.

6. B. Thin Layer Chromatography

10 mg of Standard melanin (Purchased from Sigma Aldrich) and purified melanin was dissolved in Phosphate buffer (pH 8) and applied to silica gel TLC (Thin layer chromatography) plates and a chromatogram was developed using the solvent system of n- butanol: acetic acid: water (70:20:10). Plates were then sprayed with the Iodine solution and kept for drying in a hot air oven. Spots were obtained and the retention factor was calculated (Prajapati *et al*., 2017).

6. C. $_1$H NMR

NMR is used in research for determining the content and purity of a sample as well as its molecular structure. The 1H NMR spectrum of melanin using DMSO as solvent was obtained by Nuclear Magnetic Resonance spectrometer in 5-mm NMR tubes at 25°C.

7. Synthesis of Melanin Nanoparticles

The purified melanin was dissolved phosphate buffer (pH 8) to attain a concentration of 300 µg/ml. 1% Trisodium citrate was added in melanin solution in a ratio of 20:1. Trisodium citrate acts as a capping agent and prevents the aggregation of Melanin Nanoparticles. The mixture of trisodium citrate and melanin was sonicated, with the help of probe sonicator, pulsing for 10 seconds with a gap of 15 seconds between each pulse for a total of 60 min. Formed melanin nanoparticles were stirred vigorously on a magnetic stirrer for 40 minutes at 300 rpm. The stirring helps in the dispersion of nanoparticles in solution (Kiran *et al*.,2017). (Note: Synthesis of nanoparticles should be carried out in 16°C)

8. Characterization of Melanin Nanoparticles

The size of the melanin nanoparticles (300 µg/ml) formed by sonication was determined by the particle size analysis. The particle size distribution (volume percent) was produced by the computer-controlled instrument according to the principle of light scattering.

9. Antibacterial Activity of Melanin and Melanin Nanoparticles

Each of Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacterial culture were inoculated in 10 ml of St. Nutrient broth and incubated at 37°C for 18 hours. The antibacterial activity of melanin, as well as melanin nanoparticles, was determined by using a well diffusion assay. 0.1 ml of each 18 h old enriched cultures were spread on St. Nutrient Agar Plate. Wells were made with the help of sterile steel cork-borer and 100 µl of both melanin (300 µg/ml) and melanin nanoparticles (300 µg/ml) suspension was added to the wells. The plates were then incubated at 37°C for 24 h, and zone of inhibition was monitored (Zerrad *et al*., 2014).

10. Antioxidant Activity of Melanin and Melanin Nanoparticles

The percentage of antioxidant activity (AA %) of melanin and melanin nanoparticles was assessed by using KMnO4 described by Amponsah *et al*. (2016) with modifications. 0.1 ml each of melanin (300 µg/ml) and melanin nanoparticles (300 µg/ml) was placed in a test tube with 3.9 ml of 0.25 mg/ml KMnO4 solution. The resulting system was incubated at 25°C for 30 min and the absorbance of the residual $KMnO_4$ was determined at 530 nm using a colorimeter. 0.1 ml of Phosphate buffer solution added in 3.9 ml of KMnO4 and incubated at 25°C for 30 min was used as a control. Vitamin C (300 µg/ml) was used as a positive control. The Antioxidant activity percentage (AA %) was determined according to Priya *et al*.:

$$\text{Antioxidant Activity \%} = Ac - At/Ac * 100$$

11. Antibiofilm Activity of Melanin Nanoparticles

After cleaning Ti6Al4V pieces with ethanol, they were transferred in 30 ml of St. Tryptone soy broth containing 1% glucose. 3 ml of 18 h old *Staphylococcus aureus* culture was inoculated in broth bottles and incubated at 37°C for 5 days to allow the formation of biofilm. After incubation, established biofilm on Ti6Al4V was treated with Melanin and Melanin nanoparticle at 37ºC for 1 h. After treatment, each piece was washed thrice with St. D/W and then incubated with 5 mL of crystal violet (O.D. adjusted to 1.0) for 30 min at 37ºC. After incubation, Crystal violet was removed and the pieces of the Ti6Al4V were washed thrice by St. D/W to remove unbound cells. After washing, 5mL of ethanol was added and kept at room temperature for 15 mins, the reaction mixture was then read colorimetrically at 590nm and the percentage of biofilm disruption was determined using (Namasivayam *et al*., 2013).

$$\text{\% of disruption} = \frac{\text{OD in control - OD in treatment}}{\text{OD in control}}$$

RESULTS AND DISCUSSION

1. **Isolation and Characterization of Melanin Producing Organism**

A total of 3 distinct bacteria were isolated from the soil on Nutrient agar containing 0.5% tyrosine (pH 10) plates. All the isolates were screened for melanin production, with isolate PP1 identified as the best melanin producer. The isolate PP1 was identified as a rod-shaped Gram-negative bacterium. The 16 S rRNA sequence of the isolate showed the highest similarity of 99.76% with *Pseudomonas balearica* DSM 6083(T).

2. Production and Characterization of Melanin

After a week of incubation, it was found that *Pseudomonas balearica* DSM 6083(T) was able to produce 0.95 mg of Melanin/ml of production media. Melanin was characterized based on its chemical properties. The pigment was found to be insoluble in

ethanol, acetone, chloroform, benzene, hexane, butanol and phenol. The pigment was only soluble in alkaline solvents like sodium hydroxide, Dimethyl sulfoxide (DMSO), pyridine and water. This insoluble nature of melanin is because of its aromatic structure. Melanin produced from *Pseudomonas balearica* DSM 6083(T) was found to be water-soluble which is a unique characteristic of melanin. Water insoluble melanin requires treatment with strong alkali or strong oxidants which often damages the structure of melanin, thus, interferring with antimicrobial and antioxidant activity of melanin (Aghajanyan *et al*., 2011).

Melanin was characterized by Thin Layer Chromatography using the solvent system of n-butanol: acetic acid: water (70:20:10). The Rf value of Extracted Melanin was found to be 0.4 ± 0.062 and that of Standard Melanin (purchased from Sigma) was found to be 0.38 ± 0.064.

^{1}H Nuclear Magnetic Resonance (NMR) Spectroscopy was also used to characterize the melanin pigment. ^{1}H NMR peaks of melanin had shown similarity with the earlier reports (Arun *et al*., 2015; Guo *et al*., 2014a). The ^{1}H NMR spectrum of melanin in DMSO showed signals in both the aromatic and aliphatic regions. Peaks in the absorption region from 6.5 to 8.5 ppm corresponds to the benzene structure in the molecule. Peaks in the region between 0.9 to 1.8 ppm correspond to the protons attached to –CH3 groups. Absorption at 4.344 and 5.458 ppm is attributable to an alkene group and at 1-3 ppm range probably corresponds to protons attached to an amine group.

Figure 1: Isolation of isolate PP1 on St. Nutrient agar plate and St. Nutrient agar plate containing 0.5% Tyrosine.

Table 1: Colony Characteristics of PP1 Isolate

Characteristics	Size	Shape	Colour	Opacity	Elevation	Surface	Margin	Consistency	Gram Nature & Morphology
PP1 Isolate	2mm	Irregular	Colourless	Translucent	Flat	Smooth	Smooth	Butyrous	Gram Negative Rods

Table 2: Solubility test of Melanin.

	Solvents	Standard Melanin	Test Melanin
Organic Solvent	Ethanol	Insoluble	Insoluble
	Pyridine	Sparingly soluble	Sparingly soluble
	Acetone	Insoluble	Insoluble
	Chloroform	Insoluble	Insoluble
	Benzene	Insoluble	Insoluble
	Dimethyl sulfoxide	Sparingly soluble	Sparingly soluble
	Hexane	Insoluble	Insoluble
	n-butanol	Insoluble	Insoluble
	Phenol	Soluble	Insoluble
Inorganic Solvent	Distilled water	Insoluble	Soluble
	NaOH solution (pH 9)	Soluble	Soluble
	Conc. HCl	Soluble	Soluble

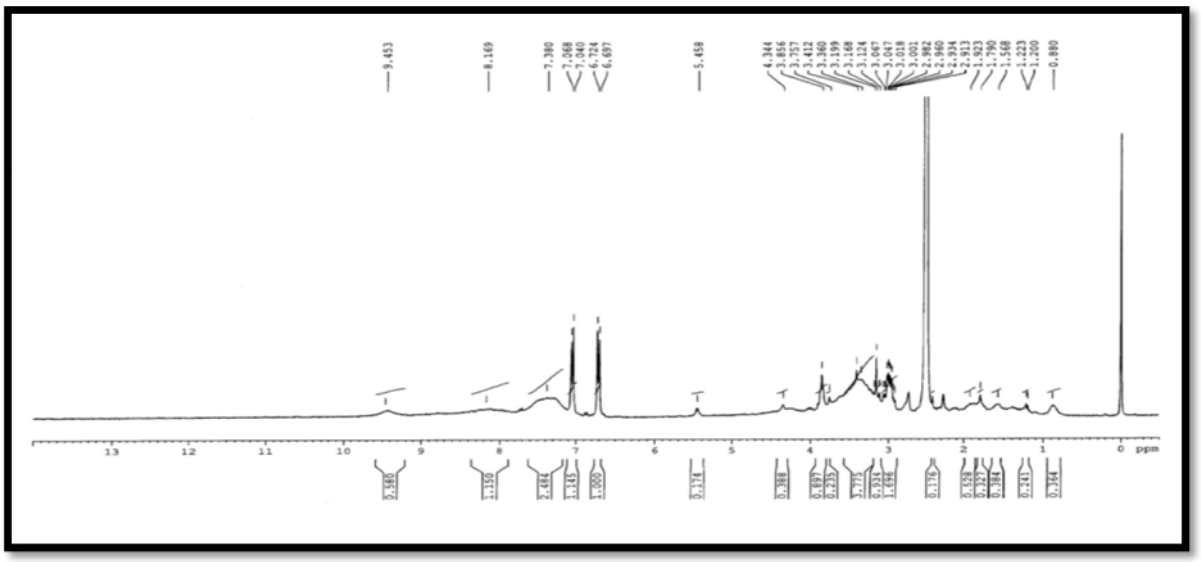

Figure 2: ^{1}H NMR spectrum of Melanin showing peaks in the absorption region similar to earlier reports.

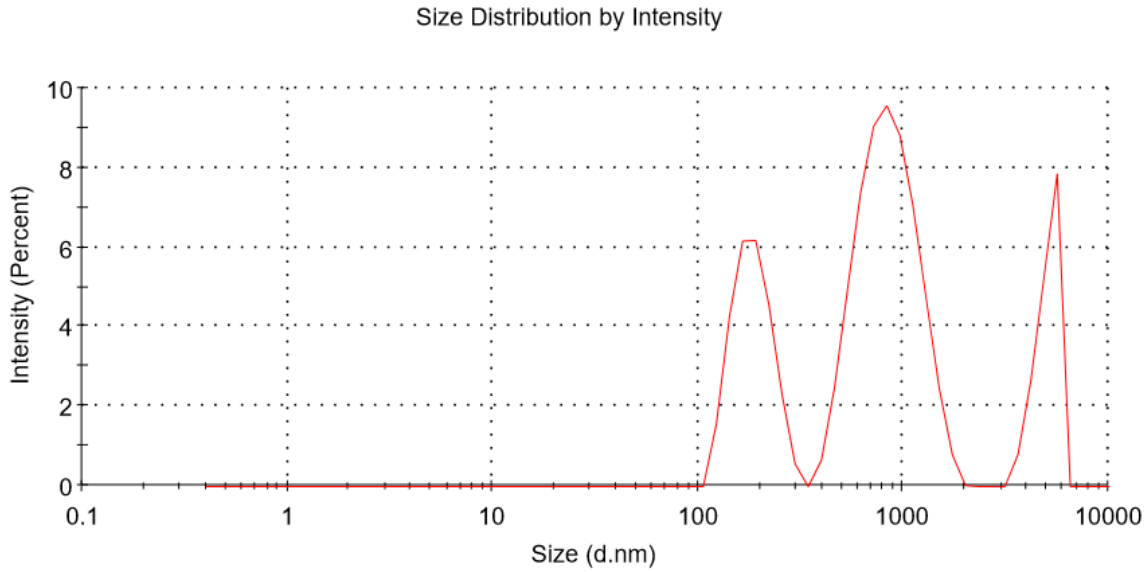

Figure 3: Melanin nanoparticle size analysis.

3. Characterization of Melanin Nanoparticles

Melanin Nanoparticles were formed by a "top-to-bottom" approach. As melanin is a highly complex polymer nanoparticles can be made by breaking it down to smaller sizes using the ultrasonication method. As nanoparticles tend to aggregate after some time, Trisodium citrate was used as a capping agent which resist aggregation. This study does not involve the use of any chemical or metal during Nanoparticle synthesis which were involved in conventional studies and responsible for toxicity in environment. The size of melanin nanoparticles produced by ultrasonication method using melanin produced by *Pseudomonas balearica* DSM 6083(T) was determined using Particle Size Analysis.The average size of melanin nanoparticles were found to 459.4 nm.

4. **Antioxidant Activity of Melanin Nanoparticles**

$KMnO_4$ is an oxidizing agent and therefore can mimic the ROS (Reactive Oxygen Species) present in the body. Melanin and melanin nanoparticles can capture electrons causing the reduction of the MnO_4^- to MnO_4^{2-} and mops up ROS (Reactive Oxygen Species) produced by organisms metabolism or due to UV radiations and thus, protects from oxidative stress (Zerrad *et al*., 2014; Bilgihan *et al*., 1995; Geng *et al*., 2008). The reduction results in a change of colour from purple to light yellow as a result of the gain of electrons with a

consequent bathochromic shift, depending on the concentration of the antioxidant used. The free radical scavenging effect of melanin and melanin nanoparticles was assessed using the $KMnO_4$ assay. The antioxidant activity of melanin nanoparticles was found to be 93.10 ± 0.00 much higher than that of melanin (11.16 ± 1.41) and Vitamin C (36.78 ± 7.18).

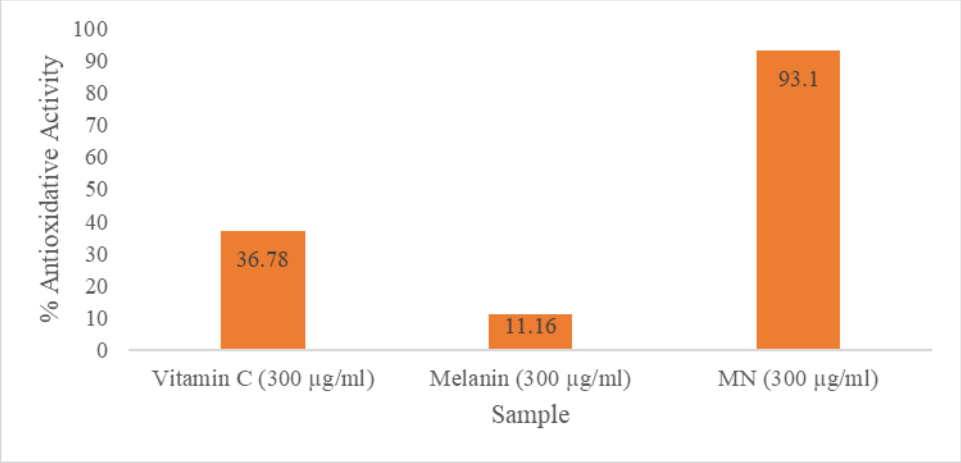

Figure 4: Antioxidant activity of melanin and melanin nanoparticles using $KMnO_4$ assay. Both melanin and melanin nanoparticles at 300 µg/ml concentration were found to scavenge ROS (Reactive Oxygen Species).

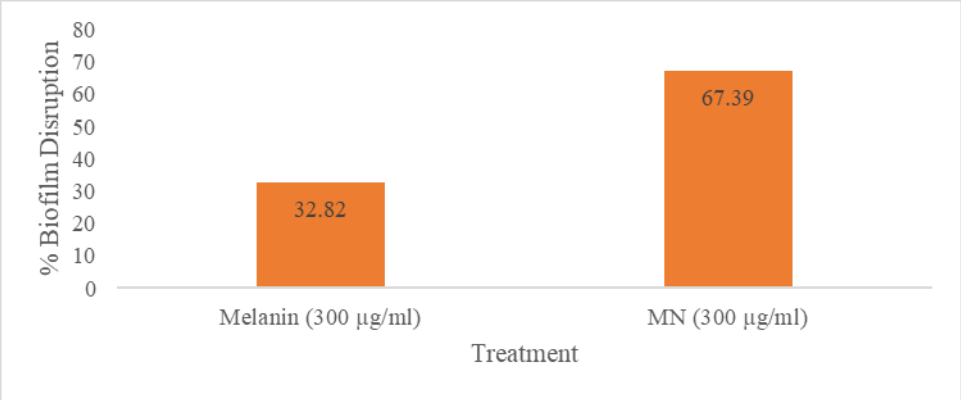

Figure 5: Antibiofilm activity of Melanin and Melanin Nanoparticles using crystal violet assay. The tested concentration of melanin and melanin nanoparticles (300 µg/ml) disrupted formed biofilm of *Staphylococcus aureus*.

5. **Antibacterial Activity of Melanin and Melanin nanoparticles**

Well diffusion assay has been used for evaluation of antibacterial activity of melanin and melanin nanoparticles against gram positive *Staphylococcus aureus* and gram negative *Escherichia coli*. It was observed that both melanin and melanin nanoparticles showed antibacterial activity against gram positive *Staphylococcus aureus* at a concentration of 300 µg/ml. Melanin showed 10.67 ± 0.5 mm of zone of inhibition whereas melanin nanoparticles showed 21 ± 0 mm of zone of inhibition.

6. Antibiofilm Activity of Melanin and Melanin nanoparticles

The crystal violet assay has been used for evaluation of disruption of preformed *Staphylococcus aureus* by Melanin and Melanin nanoparticles. The treatment of Melanin nanoparticles on the preformed biofilm showed greater reduction in the biofilm mass

compared to Melanin. The treatment of the preformed biofilm with Melanin nanoparticles resulted in a reduction in the biofilm mass of 67.39 ± 3.82 %, whereas a decrease in biofilm of 32.82 ± 5.68 % was observed when treated with Melanin.

CONCLUSION

Melanin nanoparticles exhibited activity only against Gram positive organism in planktonic form. Melanin nanoparticles were found to disrupt established biofilm of *Staphylococcus aureus* on Ti6Al4V pieces suggesting they can be used as antimicrobials. Further research and development are necessary to find its use in preventing biofilm formation and translation of this technology into therapeutic and preventive strategies.

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Conflict of Interest

The author declares that there is no conflict of interest.

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