Antioxidant And Antibacterial Evaluation Of Bacterial Melanin And Study On Its Role In Photo Protection

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Abstract: Several chemical products are used for photo protection in the market due to increased beauty consciousness among people. The groundwork focuses on the synthesis of melanin which has a photo protective role on its own. The pigment extracted was analyzed for various activities. Antimicrobial activity was evaluated by well diffusion technique and inhibitory action was observed against gram-negative and gram-positive organisms. The study concomitantly intended to ascertain antioxidant activity by DPPH assay, UV Visible absorbance and sun protection factor using Mansur mathematical equation. The photo protective role of the pigment is interpreted.

Index Terms : Natural therapeutics, Melanin, Antioxidant DPPH activity, Pigment production, FTIR Analysis, SPF analysis, UV Visible absorbance

1 INTRODUCTION

Melanins are black pigments that are produced by humans, animals, and lower organisms. they are derived by a process called melanogenesis through different pathways in various organisms(1). This pigment is beneficial to humans and helps in protecting internal tissues from ultraviolet radiation. Commonly melanin is found in several areas of the human body such as skin, hair, pupils or irises of eyes, stria vascularis of the inner ear, areas of the brain, medulla and zona reticularis of the adrenal gland. In humans when this process is disturbed it leads to several pigmentation defects such as albinism, vitiligo, melasma, etc., Different types of melanin was observed such as Eumelanin (brown or black), Pheomelanin (Yellow to red), and Allomelanin. Apart from this neuromelanin is present in different areas of the brain with a neuroprotective role(2). Another is ocular melanin that protects the eyes from UV damage. The pigment has several functions such as antioxidant, antitumor, antiviral, anti-inflammatory, photoprotection, antivenin activity, and liver-protective activity, etc. melanin which also acts an immune-stimulating agent. Allomelanin produced by microbes has several distinct properties. Few producers of melanin are E.coli, K.pneumoniae, Pseudomonas sp, Streptomyces Sp, H.werneckii, etc. which provides protection against ultraviolet radiation, microbial lysis, Reactive oxygen species, extreme temperature, chemical stress, and biochemical thread. They also contribute to their virulence or enhance their virulence nature by mechanisms. A wound healing activity is also observed. Decubitus ulcers are being treated with melanin.

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bacterial pigment and its characterization by FTIR Spectroscopy. It was also evaluated for its antioxidant and antibacterial activity. The SPF was also determined by the in-vitro method.

2 MATERIALS AND METHOD

2.1 Collection of sample

The soil sample was collected in localized areas of Coimbatore district.

2.2 PROCESSING OF SAMPLE

2.2.1 Enumeration of microorganisms from soil

The soil contains different kinds of microorganisms. The microflora depends on the climatic conditions and environmental factors.

2.2.2 METHODOLOGY

Serial dilution

The nutrient agar medium was prepared and sterilized. The soil was serially diluted in sterile distilled water and plated on sterile nutrient agar using the spread plate method. The plates were incubated at 37°C for 24 hours and the colonies were observed.

2.3 SCREENING OF MELANOGENIC BACTERIA

The organism isolated from soil was inoculated onto the nutrient media supplemented with tyrosine. Tyrosine can be utilized by the organism that has the ability to produce tyrosinase. Tyrosinase is a key enzyme involved in melanin synthesis. The organisms which are able to produce melanin can utilize tyrosine. Tyrosine media was prepared and single line inoculation of the organism was made and the plates were incubated at 37°C for 2-3 days. The presence of a clear zone indicates the utilization of tyrosine and melanin production. The absence of zone indicates its inability to produce melanin.

2.4 CONFIRMATION OF MELANIN (1)

The isolates were inoculated in a 0.1% tyrosine solution with a few drops of chloroform and incubated at 37°C for 48 hours. The red colour formation indicates the presence of melanin.

2.4.1 Identification
The identification of the isolates was performed according to their morphological and cultural characteristics by following Bergey's Manual of Systemic Bacteriology. The bacterial isolate was subjected to gram staining and specific biochemical tests were performed and confirmed through MALDI-TOF.

2.4.2 Gram staining method
The gram staining (1884) is a method of staining used to distinguish and classify the bacterial species into two large groups (gram-positive and gram-negative). The gram-positive bacteria retain the crystal violet dye, and thus are stained violet, while the gram-negative bacteria do not; after washing, a counterstain (commonly safranin) that will stain gram-negative bacteria a pink colour. Both gram-positive bacteria and gram-negative bacteria pick up the counterstain. The counterstain, however, is unseen on gram-positive bacteria because of the darker crystal violet stain. Using a sterile loop bacterial smear was made on a grease-free slide. The smear was air-dried and heat-fixed. Then it was subjected to the staining reagents. The slide was flooded with crystal violet stain for 1 minute and washed with running tap water. Again, the slide flooded with gram's iodine for 1 minute and washed with running distilled water. Then the slide was flooded with 95% ethanol for 30 seconds. After that, the slide was counterstained with safranin for 30 seconds and washed with running tap water. The slide was air-dried and cell morphology was checked under the microscope in oil immersion objective (100X).

2.4.3 Biochemical tests
The biochemical tests were performed according to the Cappuccino Laboratory Manual.

2.5 PIGMENT PRODUCTION
The organism producing a clear zone around the line of inoculation was inoculated into tyrosine broth and incubated in a shaker at 30°C at 200rpm for 6 to 8 days until the media become dark or opaque.

2.6 EXTRACTION OF MELANIN (3)
The broth is centrifuged at 3000rpm for 30 minutes and the supernatant was discarded. The pH was adjusted to 2 with 1M hydrochloric acid to precipitate melanin. It is again centrifuged at 3000rpm for 30 minutes and the pellet was collected. It is then hydrolyzed in 6M hydrochloric acid and centrifuged at 3000rpm for 30 minutes. The pellet was collected and washed 5 times with distilled water to remove the acid. Pellet was again washed with chloroform, ethanol, and ethyl acetate for about 3 times to wash away the lipids and other residues. The pellet is powdered and stored at -20°C.

2.7 FOURIER TRANSFORM INFRARED ANALYSIS(4)
This purified melanin is a black powder, hygroscopic that have been kept refrigerated at - 20° C to avoid any photo-chemical of photo-physical alterations. The Fourier Transform Infrared (FTIR) analysis was done on the pellet sample made from Potassium Bromide (99.99%) was mixed with melanin. In preparing samples for FTIR investigation; the amount of melanin was about 4 mg. This amount was mixed with about 1400 mg of Potassium Bromide. In order to ensure that the produced pellets enable accurate spectra, the mixture was blended using a mortar and pestle. The obtained powder was put in macro-micro Potassium Bromide pellet die and compressed into pellets by using a hydraulic press. This pellet was ready for FTIR analysis. The Mid-infrared light range is used to measure the pellet (4000 - 200 cm-1).

2.8 SOLUBILITY(1)
The solubility nature of the pigment was analyzed with different solvents such as acetic acid, ethanol, acetone, chloroform, ethyl acetate, DMSO, sodium hydroxide.

2.9 SUN PROTECTION FACTOR ANALYSIS(5)
As per the adopted method, spectra of melanin samples were collected over the spectral range 400–280nm with 1nm data point resolution on a UV-visible UV-3200 double beam spectrophotometer. The SPF values of melanin from the microbial isolate were determined using Mansur mathematical equation.

$$SPF = CF \times \rangle\ EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

2.9.1 ULTRA VIOLET-VISIBLE ABSORBANCE(6)
The UV-Visible spectrum of the purified melanin and commercially available sunscreen lotion (SunShade SPF-30) at the wavelength ranging from 200nm to 900nm was generated using UV- Visible spectrophotometer for the photoprotective role.

2.10 DIPHENYL PICRYLHYDRAZYL ASSAY(5)
The radical scavenging activity by melanin pigment was investigated by the modified method of Ju et al., (Ju et al., 2011). The absorbance of DPPH as control was measured at 516nm. Higher radical scavenging activity was evident with lower absorbance. The scavenging effect was measured using DPPH inhibition (%) = {(control absorbance – test absorbance)}$\times\times$ 100

2.11 ANTIBIOTIC SUSCEPTIBILITY TEST(7)
Antibacterial activity was tested by the well diffusion method. Pathogens like Bacillus sp., Pseudomonas sp., E.coli, Klebsiella sp. and Staphylococcus sp. were swabbed on Muller-Hinton agar and 10µl, 20µl, 30µl, 40µl of pigment extract was placed in the well and incubated at 37˚c for 24 hrs.

3 RESULT
The samples were subjected to macroscopic observation, screening for tyrosine utilization, confirmation of melanin production, microscopic observation, production and extraction of pigment, FTIR analysis, solubility tests, SPF, UV visible Spectroscopy, antioxidant and antibacterial assay. The pigment coated onto cotton gauze.

3.1 MACROSCOPIC OBSERVATION(1)

3.1.1 COLONY MORPHOLOGY
Irregular, mucoid, creamy colonies were observed on nutrient agar (figure 1).

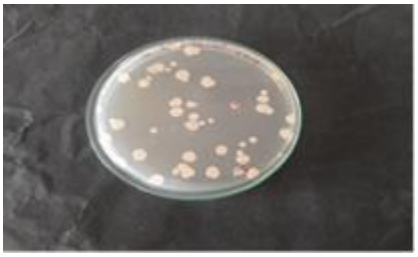

Fig. 1*. The figure showing colonies isolated by serial dilution of a soil sample.*

3.2 SCREENING OF TYROSINE UTILISING MICROORGANISM(6)

The bacteria K.pneumoniae was isolated from soil and was indicative of tyrosine utilization.

3.2.1 ZONE CLEARANCE METHOD

A clear zone was observed around the line of inoculation on tyrosine media when incubated at 37°c for 2-3 days (figure 2). Primary screening involved spot inoculating on tyrosine basal agar plates with 2g L-tyrosine added as the sole source of carbon and nitrogen. The selection was based on clear zone formation around the colonies.

Fig. 2*. The figure showing the production of melanin by utilization of tyrosine in media.*

3.2.2 CONFIRMATION OF MELANIN PIGMENT(1)

The red colour was formed which confirms the presence of melanin pigment (figure 3). The isolates were inoculated into 50 mL of 0.1% tyrosine substrate solution with a few drops of Chloroform and incubated at 37℃ for 48hrs. The deep red colour shows a positive result

Fig. 3*. The figure showing the confirmation of melanin produced by the bacterial isolate..*

3.3 MICROSCOPIC OBSERVATION

3.3.1 Gram staining

The pink rod-shaped bacteria was observed which indicates the presence of gram-negative organism.

3.3.2 Biochemical test

The biochemical results were observed and tabulated.

TABLE 1
RESULTS FOR BIOCHEMICAL TEST

BIOCHEMICAL TEST	*RESULT*
Motility	*Negative*
Hydrogen sulfide production	*Negative*
Capsule	*Positive*
Indole	*Negative*
Methyl red	*Negative*
Voges–Proskauer	*Positive*
Citrate	*Positive*
Urease	*Positive*
Starch	*Negative*
Catalase	*Positive*
Oxidase	*Negative*
Triple sugar iron	*A/A*
Nitrate reduction	*Positive*

TABLE 2
CARBOHYDRATE FERMENTATION

SUGARS	*RESULT*
Sucrose	*Positive*
Mannitol	*Positive*
Lactose	*Positive*
Glucose	*Positive*

The bacterium was identified by Gram staining method and biochemical characteristics. Biochemical tests included: Sugar fermentation, IMViC test, TSI, Nitrate reduction, Catalase & Oxidase test

3.4 PRODUCTION OF MELANIN PIGMENT(8)

The dark color was observed which indicated the pigment production (figure 4). Autoclaved 500ml nutrient broth media enriched with L-tyrosine inoculated the isolate into the media and incubated at 37°C until the media became opaque due to the pigment production.

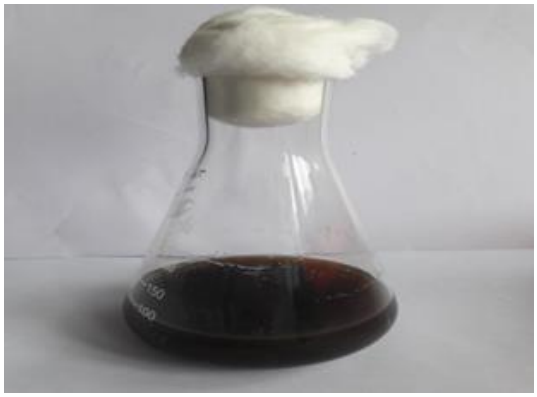

Fig. 4*. The figure showing the production of pigment by the bacterial isolate in tyrosine media (dark colour indicates the melanin pigment production).*

3.5 EXTRACTION OF MELANIN PIGMENT (3)

The organism showed the production of melanin and was extracted by centrifugation method and stored at -20°c (figure 5). The broth was centrifuged at 6000 rpm. The mixture was first adjusted to pH 2.0 with 1M HCl to precipitate melanin, followed by centrifugation at 6000 rpm for 10 min and the pellet was collected. The pellet was hydrolyzed in 6M HCl (90 °C, 2 h), centrifuged and washed by distilled water five times to remove the acid. The pellet was washed with chloroform, ethyl acetate, and ethanol three times to wash away lipids and other residues. Finally, the purified melanin was dried, ground to a fine powder in a mortar and stored at -20 °C.

Fig. 5*. Figure showing the powdered form of extracted melanin pigment.*

3.6 FTIR ANALYSIS(7)

The peaks of FTIR were observed (Table 3 & graph 1) and interpreted.

TABLE 3
RESULTS FOR FTIR ANALYSIS

S.NO	*WAVENUMBER (CM-¹)*	*FUNCTIONAL GROUP*
1	*3278*	*Carboxylic group(9)*
2	*2931*	*Carbyne (10)*
3	*1651*	*Amide (10)*
4	*1450*	*Aldehyde (9)*
5	*1365*	*Carboxylic (4)*
6	*1111*	*Amide (11)*
7	*1000*	*Amide (11)*
8	*779*	*Alkene CH (12)*

The IR spectrum of the bacterial melanin pigment showed a broad absorption at 3278.99 cm¹, 3209.55 cm-¹, 3178.69 cm-¹, 3070.68 cm-¹ revealed the presence of the carboxylic group. Absorption at 2931.80 cm-¹, 2376.30 cm-¹ which indicates as CH. Absorption at 1728.22 cm-¹, 1651.07 cm¹, was attributed to the amide group. These characteristics properties of the IR spectrum prove that the pigment is melanin.

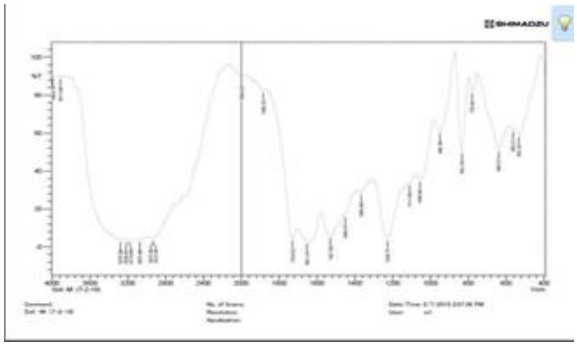

Fig. 6*. Graph showing the peak which confirms the presence of melanin in the sample.*

3.7 SOLUBILITY (1)

The extracted melanin pigment was tested for solubility in various solvents.

TABLE 4
RESULTS FOR SOLUBILITY TEST

SOLVENT	*SOLUBILITY*
Hot distilled water	*Soluble*
Distilled water	*Insoluble*
Acetic acid	*Sparingly soluble*
Ethanol	*Insoluble*
Acetone	*Sparingly soluble*
Chloroform	*Insoluble*
Ethyl acetate	*Insoluble*
DMSO	*Sparingly soluble*
Methanol	*Insoluble*
1N sodium hydroxide	*Soluble*
1M hydrochloric acid	*Insoluble*

The solubility nature of the pigment was analyzed with different solvents such as acetic acid, ethanol, acetone, chloroform, ethyl acetate, DMSO, sodium hydroxide. The solubility is maximum in hot distilled water.

3.8 SUN PROTECTION FACTOR ANALYSIS (5)

The results are tabulated.

TABLE 5
RESULTS FOR SPF

Nm	*Melanin solution*	*Lotion*
280	*0.45*	*1.052*
300	*0.378*	*1.276*
320	*0.370*	*0.813*
340	*0.483*	*1.178*
360	*0.515*	*1.371*
380	*0.524*	*1.473*
400	*0.271*	*1.282*

The SPF of the extracted melanin is found to be 6.952

4.9 UV-VISIBLE ABSORBANCE(6)

Maximum absorbance was seen in the UV range (200nm-400nm) and decreases towards the visible range (Table 6 & figure 6).

TABLE 6
RESULTS FOR UV ANALYSIS

Nm	Melanin solution	Lotion
200	1.24	1.525
300	1.15	1.473
400	1.03	1.371
500	0.85	1.331
600	0.68	1.282
700	0.37	1.276
800	0.271	1.178
900	0.239	1.052

The UV-visible spectrum of the melanin extract obtained from the bacterial isolate and commercially available sunscreen lotion (SunShade SPF-30) was analyzed from 200nm to 900 nm using UV-Visible spectrophotometer.

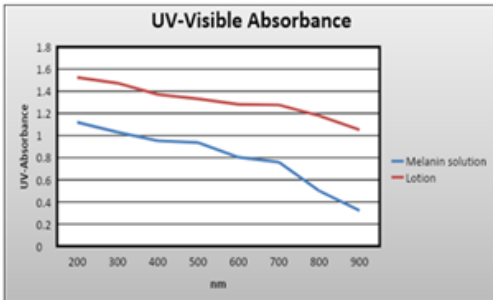

Fig. 7*. Graph showing the decrease in absorbance spectrum with an increase in wavelength interpreting its role in photoprotection in comparison with the commercially available lotion.*

3.10 DIPHENYL PICRYLHYDRAZYL ASSAY (5)
DPPH inhibition % was calculated using the formula
DPPH inhibition (%) = [control absorbance – test absorbance] x 100Before incubation: DPPH inhibition (%) = [0.85 – 0.48] x 100 = 37%

After incubation :(At 37˚C for 30 mins)
DPPH inhibition (%) = [1.02 – 0.45] x 100 = 57%
Increase in DPPH inhibition % was seen after incubation at 37˚C for 30 mins.

The presence of a reducing agent in this solution pairs the odd electrons of DPPH radical and furthers the solution losses colour and also the absorbance of the solution decreases at 516 nm.

3.11 ANTIBIOTIC SUSCEPTIBILITY TEST (7)

The antibiotic sensitivity patterns were observed.

TABLE 7
RESULTS FOR ANTIBIOTIC SUSCEPTIBILITY TEST

TEST ORGANISMS	CONCENTRATIONS			
	10µl	20µl	30µl	40µl
Bacillus sp.	-	13mm	18mm	23mm
Pseudomonas sp.	11mm	13mm	20mm	23mm
E.coli	-	-	23mm	24mm
Klebsiella sp.	11mm	14mm	22mm	24mm
Staphylococcus sp.	-	13mm	21mm	23mm

The antibacterial activity of melanin was carried out by agar well diffusion technique against 24 h old cultures of Gram-positive bacteria and Gram-negative bacteria. Zone clearance was found to be maximum for E.coli and Klebsiella sp.

4 CONCLUSION

The microorganisms from soil were isolated by serial dilution using the spread plate method. The isolated organisms were screened for the production of melanin. These are called as melanogenic bacteria, isolated by using a specialized media containing tyrosine-based on the utilization of tyrosine. This is because tyrosine is the substrate for melanin biosynthesis. The melanin producing organism was found to be K.pneumoniae. The isolate was confirmed for melanin production. The red colour formation indicated that the bacterium was capable of producing melanin. The isolate was then inoculated into a selective broth containing tyrosine and was incubated until the media become dark or opaque due to pigment production. The pigment produced was extracted and purified by centrifugation and was further powdered and stored at -20°C for future use. The pigment powder was analyzed by FTIR in order to determine the chemical groups present in it. Later the powder was dissolved in different solvents to check its solubility nature. The photoprotective role of melanin pigment was determined by using Mansur mathematical equation and UV-Visible spectroscopy. The antioxidant activity was evaluated using DPPH assay and was found to be high which showed the pigment have effective radical scavenging activity. The antibacterial activity was evaluated by a well diffusion method against a set of organisms and was found to be highly effective against Klebsiella sp. and E.coli. The pigment was also coated onto cotton gauze to find its effectiveness. It showed maximum activity against Pseudomonas sp. In conclusion, microbial strains from the collected samples were successfully isolated with the potential to produce melanin (Eumelanin). K.pneumoniae was found to produce melanin and has effective anti-microbial activity. These findings have combined application in wound healing and cosmetic products.

5 ACKNOWLEDGEMENT

The authors would really like to convey thanks to the Management, the Principal of Sri Ramakrishna College of Arts & Science for Women, Coimbatore for kind support towards the completion of this work.

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