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Isolation, Purification and Melanin Synthesis Pathways in *Aspergillus* Section *Nigri*

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ABSTRACT

Background and Aim: Melanin is a ubiquitous, dark, polymeric pigment found naturally in plants, animals and microbes. It has several industrial, biotechnological, and pharmaceutical applications. The study aimed to verify the presence, type, and quantity of melanin produced by eight species belonging to the *Aspergillus (A.)* section *Nigri*: *A. awamori*, *A. brasiliensis*, *A. costaricaensis*, *A. luchuensis*, *A. piperis*, *A. niger*, *A. tubingensis* and *A. welwitschiae*. We also determined the biosynthetic pathway (either 3, 4-dihydroxyphenylalanine (DOPA) or 1, 8-dihydroxynaphthalene (DHN)) responsible for melanin synthesis in these species.

Materials and Methods: Soil samples were collected from seven locations within Basrah Province. Species identification was performed through sequencing of ITS region and β-tubulin gene. The isolates were cultured on CDA and L-tyrosine PDA, and incubated for 7, 14 and 21 days for melanin extraction. Qualitative and quantitative characterizations of melanin were done using UV–Vis spectroscopy, Fourier-transform infrared (FTIR) spectroscopy, and scanning electron microscopy (SEM). The biosynthetic pathway of melanin was investigated by applying the pathway inhibitors; kojic acid and difenoconazole.

Results: The results indicated that *A. niger* produced 1.9 gr of melanin after 3 weeks on L-tyrosine PDA. DOPA pathway was identified in *A. niger* and *A. brasiliensis*, whereas the DHN pathway was active across all tested species.

Conclusion: The study revealed interspecies differences in the type and quantity of melanin produced among *A.* section *Nigri* isolates.

Keywords: *Aspergillus* Section *Nigri,* DHN-melanin, DOPA-melanin, Difenoconazole, Kojic Acid

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1. Introduction

Pigments are synthesized by majority of living organisms and contribute to diversity of colors in nature through the refraction and absorption of particular light wavelengths. There is a wide array of biological pigments including melanin **(1, 2)**. Melanin is a complex natural biopolymer produced by oxidized phenols, polymerized intermediate phenols, and the resulting quinones. Physically, melanin is amorphous and predominantly dark brown to black in color, although reddish and yellowish hues have also been reported **(3)**.

Chemically, melanin is hydrophobic polymer, negatively charged, and high molecular weight. It

exhibits remarkable stability, showing resistance to reducing agents, light, and concentrated acids, and is highly thermostable. Some forms can withstand temperatures up to 600°C without decomposition **(4)**. However, melanin is susceptible to degradation by oxidizing agents (e.g., bleaching processes) and is soluble in alkaline solutions and phenols **(5)**. It is classified into three major types according to its structural components: eumelanin, pheomelanin, and allomelanin **(6)**.

Melanin serves several critical biological functions, including photoprotection and shielding against UV radiation; it also serves as a potent free radical scavenger, exhibiting metal ion-chelating properties and contributing to thermal and drought tolerance **(6, 7)**. Additionally, melanin plays a role in maintaining cell wall rigidity and facilitates the absorption of heavy metals, enhancing an organism's resistance to environmental stressors **(8, 9)**. In the derma-cosmetic industry, melanin is widely used for its UV-protective attributes, particularly in the formulation of sunscreens and related skincare products **(10, 3)**. Moreover, melanin exhibits a range of biologically significant activities, including anti-cancer, antimicrobial, anti-inflammatory and hepatoprotective effects **(11, 12)**.

Fungi synthesize melanin through two primary biosynthetic pathways. One is a polyketide-based pathway that starts with malonyl-CoA and acetyl-CoA, leading to the formation and subsequent polymerization of 1, 8-dihydroxynaphthalene (DHN) into DHN-melanin, also referred to as allomelanin. The alternative pathway involves the metabolism of L-tyrosine and polymerization of L-3, 4-dihydroxyphenylalanine (L-DOPA) to produce L-DOPA-melanin (commonly known as eumelanin) **(13)**.

Most information related to fungal melanin is derived from studies on *Aspergillus (A.) fumigatus*, and more recent research has focused on *A. nidulans*, *A. bridgeri*, and *A. niger* **(7)**. However, information on the precise localization, quantity, type, and physicochemical characteristics of melanin across other species remains limited. The type and quantity of melanin in different species within *A.* section *Nigri* represent fundamental knowledge gaps. Addressing these gaps is essential not only for understanding the melanin biosynthesis in this group but also for aiding in species classification **(14)**. It is worth noting that this study is the first of its kind in Iraq dealing with the isolation and classification of section *Nigri* and also study the melanin biosynthesis within this section. This study was designed to verify the presence, type, and quantity of melanin formed by eight species of *A.* section *Nigri* cultured on solid media in order to assess species-specific differences in melanin production and to determine the biosynthetic pathway (either DHN or DOPA) responsible for melanin synthesis in these species.

2. Materials and Methods

2.1 Samples Collection

Soil samples were collected from seven locations within Basrah Province, Iraq namely, Al-Hartha, Shatt Al-Arab, Al-Zubair, Safwan, Al-Zubair Oil Field, Hammar Mishrif Oil Field, and Al-Faw, from October 2023 to September 2024. Approximately 250 gr of soil sample was collected from each site using a sterile spatula and placed in sterile plastic bags. The samples were then transported to the laboratory for processing **(15)**.

2.2 Isolation of *A.* Section *Nigri* Species

The dilution method described earlier by Wicklow and Wittingham **(16)** was used for the isolation of fungi from each soil sample cultured in PDA, MEA, and DRCA media.

2.3 Genomic DNA Extraction and PCR products Sequencing

Following 7 days of fungal isolates growth on PDA, genomic DNA was extracted using Presto Mini gDNA yeast kit (Geneaid, Taiwan) according to the guidelines provided by the manufacturer. The quality of the extracted DNA was assessed through gel electrophoresis, and the samples were subsequently stored at –20°C for future use. The target genes β-tubulin and internal transcribed spacer (ITS) were amplified by PCR using primers listed in Table 1. PCR amplification conditions are provided in Table 2. The amplified PCR products were sent to Macrogen Inc. (South Korea) for purification and sequencing.

2.4 Extraction and Purification of Melanin From Solid Media

Melanin extraction from solid media was performed using the methods described earlier by Rajagopal et al **(17)** and Pandey et al **(7)** with minor modifications. Three mycelial plugs (1 cm in diameter) were excised from the margins of 7, 14 and 21days old colonies grown on CDA and PDA enriched (1.5%) L-tyrosine. The plugs were boiled in 5 mL of distilled water for 5 min and subsequently centrifuged at 5000 *g* for 5 min. The resulting pellet was washed, and melanin extraction was carried out by autoclaving the pellet in 3 mL of 1 M KOH at 120°C for 20 min. The crude extract was washed three times with distilled water and dried overnight in a dehumidified atmosphere. Further purification was achieved by acid hydrolysis using 5 mL of 7 M HCl in a sealed glass vial, incubated at 100°C for 2 hr. After cooling, melanin was recovered

through centrifugation at 8000*g* for 15 min and washed with distilled water three times. Between washing steps, the tubes containing melanin were left for approximately 30 min to 1 hr to ensure that all melanin granules settle to the bottom of the tubes without any loss and dried, then extracted with 50 mL ethanol, chloroform and ethyl acetate at the ratio of 2:3:2. The resultant pellet was completely dried in a hot-air oven at 60°C. The resulting melanin was collected in powdered form. The melanin powder of each sample was analyzed using SEM, FTIR, and UV-Vis analysis.

2.5 Characterization of Fungal Melanin

2.5.1 Physiochemical Characterization

The characterization of extracted fungal melanin was conducted following the procedure described by Suwannarach et al **(18)**, in comparison with synthetic melanin (BTS, UK). The physicochemical properties of the fungal melanin, including solubility in distilled water, common organic solvents (acetone, chloroform, dimethyl sulfoxide (DMSO), ethanol, methanol and ethyl acetate) and an inorganic solvent (1 M KOH), were assessed. Briefly, 0.5 mg/mL of each melanin sample was prepared in each solvent and vortexed for 10 sec. The solubility was evaluated visually. Additionally, bleaching and precipitation tests were conducted using H_2O_2 and $FeCl_3$, respectively **(7)**.

2.5.2 UV-Vis Analysis

Synthetic and fungal melanin samples were dissolved in 1 M KOH, and four different concentrations of Synthetic melanin were prepared (10, 50, 100 and 150 ppm). Their absorption spectra were recorded by Rajagopal et al **(17)**. The UV-Vis absorption spectrum of samples was scanned at a wavelength range of 200–700 nm and strong absorption spectrum was recorded at a wavelength of 217 nm using a UV visible spectrophotometer (Shimadzu, Japan), the maximum absorption value was recorded.

2.5.3 Fourier-Transform Infrared (FTIR) Analysis

The FTIR spectroscopy was performed on synthetic and fungal melanin samples incubated on CDA and L-tyrosine PDA for 1, 2, and 3 weeks at the College of Science, University of Shiraz, Iran. The melanin samples were homogenized with analytical-grade KBr and prepared as pellets for analysis. The FTIR spectra were recorded using a Bruker FTIR spectrometer (USA) at a resolution of 4 cm^{-1} over the wavenumber range of 400–4000 cm^{-1}.

2.5.4 Scanning Electron Microscopy (SEM)

Dried melanin powder was used for surface imaging through SEM. The samples were first lyophilized and then coated with a thin layer of gold to enhance conductivity. Surface topography analysis was performed using an analytical field emission scanning electron microscope (TESCAN, Czech public) at the Taban Laboratory, Tehran, Iran.

2.6 Melanin Synthesis Pathway

To distinguish between melanin biosynthesis pathways utilized by the studied fungal species, the effects of pathway-specific inhibitors were assessed, following the method described by Pascoe and Maillard **(19)**. Difenoconazole (Sigma-Aldrich, USA) as an inhibitor of the DHN–melanin pathway and kojic acid (Sigma-Aldrich, USA) as an inhibitor of the DOPA-melanin pathway , were dissolved in ethanol and added separately to autoclaved and cooled PDA to achieve final concentrations of 60 and 120 ppm. The media were then poured in 90 mm petri dishes. Control plates containing PDA without inhibitors were prepared in parallel. All treatments were conducted in triplicate. The PDA plates were inoculated with a 0.5 cm diameter mycelial plug taken from the margin of an actively growing colony and incubated for 10 days at 25°C in dark.

2.7 Statistical Analysis

Minitab Version 16.1 was used for statistical analysis. The data were analyzed using a three-way ANOVA to assess the effects of fungal species, growth medium, and incubation time, including all possible interactions. Statistical significance was accepted at $P<0.05$. Tukey's *Post-hoc* test was performed for comparisons among fungal species.

3. Results

3.1 *Aspergillus* Section *Nigri* Identification

Eight fungal species belonging to *A.* section *Nigri* were isolated and identified using PCR molecular method. They are listed in Table 3. All identified species were registered in the DNA Data Bank of Japan (DDBJ) and assigned accession numbers.

3.2 Melanin Production in *A.* Section *Nigri* Species Showed Variation

The findings revealed variations in melanin production among different species of *A.* section *Nigri* (Table 4).

According to our data, *A. niger* and *A. tubingensis* showed the highest concentrations of melanin on CDA medium in 1 week, with values of 128.03 ppm for both. In contrast, *A. piperis* showed the lowest concentration at 34.28 ppm. On L-tyrosine PDA, *A. brasiliensis* demonstrated the highest melanin concentration at 103.03 ppm, whereas *A. piperis* showed again the lowest value at 28.03 ppm (Figure 1).

At 2 weeks of incubation, the highest melanin concentrations were recorded in *A. niger* and *A. brasiliensis*, measuring 131.15 ppm on CDA and L-tyrosine PDA, respectively. The lowest concentration on CDA was observed in *A. welwitschiae* at 40.53 ppm, and *A. awamori* exhibited the lowest concentration in L-tyrosine PDA, also at 40.53 ppm (Figure 2).

After 3 weeks of incubation, *A. niger* exhibited the highest concentrations of extracted melanin on CDA and L-tyrosine PDA, with values of 137.4 and 140.21 ppm, respectively (Figure 3).

The obtained concentrations were statistically analyzed using three-way ANOVA to evaluate the effect of fungal species, growth medium, and incubation time on melanin concentration. The analysis showed statistically significant effect of *A.* section *Nigri* species on melanin concentration ($P<0.001$, $F = 2.6$). In general, CDA showed relatively minor differences in melanin level production among the tested species.

The analysis indicated that both type of fungus and growth medium significantly influenced melanin levels, while the interaction between factors also contributed to variations. The results suggest that certain fungal species produce higher levels of melanin under specific media and time conditions. Detailed comparisons between fungal species showed that *A. niger* and *A. brasiliensis* had the highest melanin accumulation, whereas *A. welwitschiae* showed the lowest level.

3.3 Physiochemical Characterization Results

The physicochemical characteristics of melanin extracted from *A.* section *Nigri* species were consistent with those of the synthetic standard melanin. The results revealed that they were insoluble in distilled water, methanol, ethanol, ethyl acetate, chloroform, and acetone. However, they exhibited partial solubility in DMSO and complete solubility in 1 M KOH. In addition, both samples precipitated in 1% $FeCl_3$ solution and showed positive decolorization when treated with 30% H_2O_2.

3.4 Spectrophotometric Characterization Results

The fungal melanin extracts were analyzed spectrophotometrically compared with the synthetic standard melanin at a wavelength of 217 nm. The fungal extracts and synthetic standard exhibited similar UV–Vis absorption spectra, characterized by a strong absorption peak in the UV region and a gradual decline in absorbance toward the visible range. The maximum absorption for both samples was recorded at 217 nm, indicating comparable optical properties. The standard curve for the synthetic melanin is presented in Figure 4.

3.5 FTIR analysis Results

FTIR spectroscopy investigated the extracted fungal pigment as melanin. The FTIR spectra of the fungal melanin and synthetic standard melanin are presented in Figure 5. The spectra obtained from fungal melanin extracted after 1 week of incubation on CDA medium exhibited characteristic absorption peaks at 3732–3348 cm^{-1} (O–H stretching and aliphatic primary amines), 3268–3274 cm^{-1} (C–H stretching) and 2918–2854 cm^{-1} (N–H stretching). Additional peaks were observed at 2088–2110 cm^{-1} (C≡C stretching), 1982–1926 cm^{-1} (C=C=C stretching) and 1706–1709 cm^{-1} (unconjugated C=O adjacent to aromatic rings). Peaks at 1519–1626 cm^{-1} were attributed to C=O and C=C aromatic bonds, likely arising from conjugated quinone structures and N–O stretching vibrations. Further absorptions were detected at 1373–1377 cm^{-1} (C–OH groups from alcohols, carboxylic acids and phenols), 1026–1090 cm^{-1} (C–H stretching), 742–552 cm^{-1} (C–H out-of-plane bending in aromatic rings) and 460–452 cm^{-1} (C–I stretching). The FTIR spectrum of the synthetic standard melanin displayed similar absorption patterns, and notable differences were observed at 1255 and 755 cm^{-1} (Table 5).

After 2 and 3 weeks of incubation on CDA medium, the FTIR spectra continued to exhibit absorption peaks corresponding to the characteristic functional groups of melanin, as detailed in Table 6 and Table 7.

The FTIR analysis of melanin extracted after 1 week of incubation on L-tyrosine PDA revealed characteristic absorption peaks at the following ranges: 3644–3349 cm^{-1}, 3275–3268 cm^{-1}, 2918–2853 cm^{-1}, 2167–2108 cm^{-1}, 1987–1901 cm^{-1}, 1704–1709 cm^{-1}, 1591–1625 cm^{-1}, 1382–1200 cm^{-1}, 1028–1095 cm^{-1}, 797–575 cm^{-1} and 558–450 cm^{-1}. These peaks closely matched those of the synthetic standard melanin (Table 8). The FTIR spectra obtained after 2 and 3 weeks of incubation on the same culture medium are presented in Table 9 and Table 10, respectively.

3.6 Scanning Electron Microscopy (SEM) Results

SEM was employed in order to investigate the surface morphology of the melanin samples. The analysis revealed a granular and heterogeneous surface topology comparable to synthetic standard melanin (Figure 6). SEM images were captured at magnifications corresponding to 1 and 2 µm scales.

3.7 Melanin Synthesis Pathway

Melanin synthesis inhibition was defined as complete loss of pigmentation in fungal colonies in the presence of inhibitors. All isolates were tested with two inhibitors targeting the DOPA and DHN melanin biosynthesis pathways (Table 11). Melanin synthesis in *A. niger* and *A. brasiliensis* was inhibited by kojic acid and difenoconazole, indicating the involvement of both DOPA and DHN melanin biosynthetic pathways. By contrast, melanin synthesis in all other tested species was inhibited exclusively by difenoconazole, suggesting

the reliance on the DHN melanin pathway. The lowest effective concentration for kojic acid and difenoconazole was 120 ppm, at which complete depigmentation was observed in all tested isolates and toxicity was high. Two-way ANOVA analysis revealed statistically significant differences (P<0.01) in melanin pigmentation among the species using both inhibitors.

Table 1. Primers specifications.

Gene name	Primers	Primer Sequence	Length	References
ITS region	ITS1	5′-TCCGTAGGTGAACCTGCGG-3′	19 bases	Varga et al **(20)**
	ITS4	5′-TCCTCCGCTTATTGATATGC-3′	20 bases	
B-tubulin	Ben2f	5′-TCCAGACTGGTCAGTGTGTAA-3′	21 bases	Bian et al **(21)**
	Bt2b	5′-ACCCTCAGTGTAGTGACCCTTGGC-3′	24 bases	

Table 2. PCR cycling program.

Steps	Temperature °C		Time		Cycles	Reference
	ITS	B-tubulin	ITS	B-tubulin	ITS & B-tubulin	
Initial denaturation	95	95	1 min	5 min	1	Bian et al **(21)**
Denaturation	95	95	1 min	1 min	35	
Annealing	56	55	45 s	1 min		
Extension	72	72	1 min	1 min		
Final extension	72	72	10 min	10 min	1	

Table 3. Isolated *A.* section *Nigri* species during the study.

A. section *Nigri* Species	GenBank/DDBJ Accession Nos.	
	ITS	B-Tubulin
A. awamori	LC884984	LC884800
A. brasiliensis	LC884986	-
A. costaricaensis	LC884987	LC884804
A. luchuensis	LC884988	LC884805
A. niger	LC884985	LC884801
A. piperis	-	LC884802
A. tubingensis	LC884982	LC884798
A. welwitschiae	LC884983	LC884799

Table 4. Melanin powder level (gr) extracted from *A.* section *Nigri* species in solid media after 1, 2 and 3 weeks

Species name	After 1 weeks		After 2 weeks		After 3 week	
	CDA	L-Tyrosine PDA	CDA	L-Tyrosine PDA	CDA	L-Tyrosine PDA
A.awamori	1.1	1.1	1.2	1.2	1.3	1.2
A.brasiliensis	1.2	1.3	1.3	1.4	1.3	1.5
A.costaricaensis	1.2	1.2	1.3	1.3	1.3	1.3
A.luchuensis	1.2	1.2	1.3	1.3	1.3	1.3
A.niger	1.3	1.3	1.4	1.3	1.4	1.9
A.piperis	1.2	1.2	1.2	1.2	1.3	1.3
A.tubingensis	1.3	1.1	1.3	1.3	1.4	1.4
A.welwitschiae	1.1	1.1	1.2	1.2	1.2	1.2

Table 5. IR signals of melanin from *A.* section *Nigri* species after 1 week of incubation on CDA.

Synthetic Melanin Frequency (cm^{-1})[a]	*A. section Nigri Species* Frequency(cm^{-1}) [b]								Assignment
	A. awamori	*A. brasiliensis*	*A. costaricaensis*	*A. luchuensis*	*A. niger*	*A. piperis*	*A. tubingensis*	*A. welwitschiae*	
3574	3728 ---	3709 ---	--- ---	3732 ---	--- ---	--- ---	--- ---	3729 3598	O-H stretch
3237	3271 2922 2854	3268 2920 2851	3272 2920 2851	3274 2922 2852	3271 2919 2850	3272 2920 2851	3272 2921 2852	3348 2918 2850	O-H stretch and primary amines NH, C-H stretch
2167	2167 ---	2167 2195	2188 ---	2165 ---	2168 ---	2166 ---	2166 ---	2167 ---	C≡C stretch
2112	2109	2109	2088	2080	2110	2109	2109	2110	C≡C stretch
1991	1985	1986	1982	1987	1987	1985	1984	1984	C=C=C stretch
1898	1914	1913	1926	1916	1914	1914	1916	1920	C=C=C stretch
1713	---	---	1709	1706	1707	---	1707	1706	C=O unconjugated with benzene ring
1605	1626 ---	1619 1532	1599 ---	1622 ---	1591 ---	1622 ---	1625 1519	1595 ---	C=O; C=C aromatic (from conjugated quinone structures), N-O stretch
1255	1373 1207	1373 1299	1375 1257	1374 1259	1377 1198	1373 1205	1374 1256	1376 1202	C-OH (alcoholic, carboxylic, phenolic groups); Alcoholic C-O; C-H in-plane of aliphatic structure
---	1028	1026	1024	1026	1090	1029	1029	1090	C-H stretch
755	697	742	720	740	721	698	697	740	C-H out-of-plane in aromatic structure
640	697	697	699	667	690	552	---	695	C-H stretch
430	459	462	454	435	452	459	450	452	C-I stretch

a, b: Spectra signals of synthetic(a) and extracted(b) melanin after 1 week of incubation on CDA. Identification based on Pretsch et al **(22)**.

Table 6. IR signals of melanin from *A.* section *Nigri* species after 2 weeks of incubation on CDA.

Synthetic Melanin Frequency (cm^{-1})[a]	*A. section Nigri Species* Frequency(cm^{-1}) [b]								Assignment
	A. awamori	*A. brasiliensis*	*A. costaricaensis*	*A. luchuensis*	*A. niger*	*A. piperis*	*A. tubingensis*	*A. welwitschiae*	
3574	3643	---	---	---	3605	3641	---	---	O-H stretch
3237	3331 2919 2850	3269 2921 2853	3270 2920 2852	3272 2923 2853	3271 2920 2850	3369 2919 2850	3268 2922 2853	3296 2920 2852	O-H stretch and primary amines NH, C-H stretch
2167	2167 2190	2166 2188	2168 ---	2167 ---	2167 ---	2167 ---	2166 ---	2167 ---	C≡C stretch
2112	2103	2079	2116	2107	2112	2115	2112	2112	C≡C stretch
1991	1985	1985	---	1987	1986	1987	1986	1990	C=C=C stretch
1898	1911	1932	1932	1912	1911	1935	1924	1918	C=C=C stretch
1713	1706	---	1708	---	1709	1709	1708	---	C=O unconjugated with benzene ring
1605	1594	1621	1620	1621	1591	1591	1624	1623	C=O; C=C aromatic (from conjugated quinone structures), N-O stretch
1255	1381 1199	1373 1204	1370 1258	1375 1224	1199 ---	1200 ---	1374 1257	1373 1205	C-OH (alcoholic, carboxylic, phenolic groups); Alcoholic C-O; C-H in-plane of aliphatic structure
---	---	1025	1020	1033	---	1029	1024	1025	C-H stretch
755	722	744	744	790	735	723	742	742	C-H out-of-plane in aromatic structure
640	576	697	---	698	575	576	697	697	C-H stretch
430	459	455	454	453	452	455	453	458	C-I stretch

[a, b]: Spectra signals of synthetic[(a)] and extracted[(b)] melanin after 2 weeks of incubation on CDA. Identification based on Pretsch et al **(22)**.

Table 7. IR signals of melanin of *A.* section *Nigri* species after 3 weeks of incubation on CDA.

Synthetic Melanin Frequency (cm^{-1})[a]	*A. section Nigri Species* Frequency(cm^{-1}) [b]								Assignment
	A. awamori	*A. brasiliensis*	*A. costaricaensis*	*A. luchuensis*	*A. niger*	*A. piperis*	*A. tubingensis*	*A. welwitschiae*	
3574	--- ---	3690 ---	3732 ---	--- ---	--- ---	3609 ---	3671 3601	3621 ---	O-H stretch
3237	3270 2918 2850	3268 2921 ---	3272 2920 ---	3274 2922 2852	3344 2920 2851	3368 2917 2849	3269 2956 2852	3368 2917 2849	O-H stretch and primary amines NH, C-H stretch
2167	2167	2168	---	2167	2168	2167	2167	2167	C≡C stretch
2112	2110	2110	2116	2100	2107	2112	2106	2111	C≡C stretch
1991	1985	1984	1989	1987	1985	1986	1984	1984	C=C=C stretch
1898	1922	1932	1905	1920	1917	1932	1932	1938	C=C=C stretch
1713	1712	---	---	1707	1709	1706	1707	1703	C=O unconjugated with benzene ring
1605	1624	1621	1605	1620	1591	1592	1622	1595	C=O; C=C aromatic (from conjugated quinone structures), N-O stretch
1255	1219 ---	1374 1226	1372 1206	1373 1258	1382 1199	1381 1199	1257 1226	1204 ---	C-OH (alcoholic, carboxylic, phenolic groups); Alcoholic C-O; C-H in-plane of aliphatic structure
---	1030	1028	1035	1027	---	---	1071	1100	C-H stretch
755	776	744	755	760	736	685	695	722	C-H out-of-plane in aromatic structure
640	574	696	694	699	575	577	577	576	C-H stretch
430	452	463	460	556	437	457	459	449	C-I stretch

[a, b]: Spectra signals of synthetic[(a)] and extracted[(b)] melanin after 3 weeks of incubation on CDA. Identification based on Pretsch et al **(22)**.

Table 8. IR signals of melanin from *A.* section *Nigri* species after 1 week of incubation on L-tyrosine PDA.

Synthetic Melanin Frequency (cm^{-1})[a]	*A. section Nigri Species* Frequency(cm^{-1}) [b]								Assignment
	A. awamori	*A. brasiliensis*	*A. costaricaensis*	*A. luchuensis*	*A. niger*	*A. piperis*	*A. tubingensis*	*A. welwitschiae*	
3574	3644	---	---	---	---	---	---	---	O-H stretch
3237	3361 2918 2849	3270 2921 2853	3274 2922 2853	3275 2922 2853	3271 2922 2852	3268 2919 2850	3271 2918 2849	3349 2918 2850	O-H stretch and primary amines NH, C-H stretch
2167	2169	2167	2167	2190	2169	2167	2167	2167	C≡C stretch
2112	2114	2108	2109	2110	2116	2110	2111	2110	C≡C stretch
1991	1986	1987	1987	1986	1987	1987	1985	1985	C=C=C stretch
1898	1938	1906	1901	1909	---	1919	1913	1909	C=C=C stretch
1713	1708	---	---	---	1708	1709	1704	1705	C=O unconjugated with benzene ring
1605	1591	1625	1604	1620	1624	1597	1597	1596	C=O; C=C aromatic (from conjugated quinone structures), N-O stretch
1255	1380 1200	1373 1208	1373 1225	1373 1258	1375 1203	1371 1245	1374 1203	1382 1203	C-OH (alcoholic, carboxylic, phenolic groups); Alcoholic C-O; C-H in-plane of aliphatic structure
---	1090	1028	1029	1029	1074	1028	---	1095	C-H stretch
755	694	744	754	797	719	720	721	742	C-H out-of-plane in aromatic structure
640	---	697	698	---	699	575	696	667	C-H stretch
430	450	435	453	554	462	457	458	456	C-I stretch

[a, b]: Spectra signals of synthetic[(a)] and extracted[(b)] melanin after 1 week of incubation in L-tyrosine PDA. Identification based on Pretsch et al **(22)**.

Table 9. IR signals of melanin of *A.* section *Nigri* species after 2 weeks of incubation on L-tyrosine PDA.

Synthetic Melanin Frequency (cm^{-1})[a]	*A. section Nigri Species* Frequency(cm^{-1}) [b]								Assignment
	A. awamori	*A. brasiliensis*	*A. costaricaensis*	*A. luchuensis*	*A. niger*	*A. piperis*	*A. tubingensis*	*A. welwitschiae*	
3574	3756	---	3734	3730	---	3658	3660	3400	O-H stretch
3237	3270 2919 2850	3273 2917 2849	3275 2917 2849	3272 2922 2853	3270 2920 2851	3272 2918 2849	3272 2920 2851	3300 2917 2849	O-H stretch and primary amines NH, C-H stretch
2167	2166	2168	2167	---	2167	2166	2167	2165	C≡C stretch
2112	2103	2110	2104	2088	2111	2111	2113	2110	C≡C stretch
1991	1986	1986	1988	1988	1989	1992	1985	1990	C=C=C stretch
1898	1942	1930	1916	1902	1934	1931	1950	1928	C=C=C stretch
1713	1716	1705	1702	---	1707	1707	---	1703	C=O unconjugated with benzene ring
1605	1602	1600	1596	1604	1615	1591	1628	1602	C=O; C=C aromatic (from conjugated quinone structures), N-O stretch
1255	1372 1226	1377 1207	1373 1201	1375 1258	1373 1221	1382 1200	1375 1258	1375 1203	C-OH (alcoholic, carboxylic, phenolic groups); Alcoholic C-O; C-H in-plane of aliphatic structure
---	1017	1075	1031	1031	1033	1088	1025	1021	C-H stretch
755	763 ---	745 ---	740 ---	750 ---	697 ---	690 ---	744 ---	790 722	C-H out-of-plane in aromatic structure
640	655	667	692	698	577	576	662	576	C-H stretch
430	460	453	440	452	---	432	467	438	C-I stretch

[a, b]: Spectra signals of synthetic[(a)] and extracted[(b)] melanin after 2 weeks of incubation on L-tyrosine PDA. Identification based on Pretsch et al **(22)**.

Table 10. IR signals of melanin from *A.* section *Nigri* species after 3 weeks of incubation on L-tyrosine PDA.

Synthetic Melanin Frequency (cm^{-1})[a]	*A. section Nigri Species* Frequency(cm^{-1}) [b]								Assignment
	A. awamori	*A. brasiliensis*	*A. costaricaensis*	*A. luchuensis*	*A. niger*	*A. piperis*	*A. tubingensis*	*A. welwitschiae*	
3574	3732	---	---	---	3607	---	3602	3623	O-H stretch
3237	3271 2918 2850	3272 2922 2852	3273 2921 2852	3273 2922 2852	3272 2919 2850	3341 2918 2849	3271 2917 2849	3269 2917 2850	O-H stretch and primary amines NH, C-H stretch
2167	2167	2167	2170	2165	2168	2169	2166	2167	C≡C stretch
2112	2107	2114	2110	2099	2112	2112	2112	2106	C≡C stretch
1991	1988	1986	1988	1986	1986	1985	1985	1986	C=C=C stretch
1898	1924	1936	1936	1925	1922	1929	1930	1950	C=C=C stretch
1713	1703	1710	1709	---	1707	1707	1703	---	C=O unconjugated with benzene ring
1605	1615	1625	1621	1625	1592	1596	1596	1604	C=O; C=C aromatic (from conjugated quinone structures), N-O stretch
1255	1374 1203	1372 1258	1370 1258	1373 1258	1381 1200	1379 1202	1385 1206	1375 1224	C-OH (alcoholic, carboxylic, phenolic groups); Alcoholic C-O; C-H in-plane of aliphatic structure
---	1029	1030	1021	1029	1069	1036	1081	1030	C-H stretch
755	743	---	755	742	723	723	722	745	C-H out-of-plane in aromatic structure
640	696	698	590	698	696	576	672	654	C-H stretch
430	461	436	454	455	449	454	455	573	C-I stretch

[a, b]: Spectra signals of synthetic[(a)] and extracted[(b)] melanin after 3 weeks of incubation on L-tyrosine PDA. Identification based on Pretsch et al **(22)**.

Table 11. Effect of melanin inhibitors (120 ppm) on pigmentation of *A.* section *Nigri* species grown on PDA medium (*P*<0.01).

Species name	Kojic acid	Difenoconazole
A. awamori	-[a]	+
A. brasiliensis	+[b]	+
A. costaricaensis	-	+
A. luchuensis	-	+
A. niger	+	+
A. piperis	-	+
A. tubingensis	-	+
A. welwitschiae	-	+

[a]: - = No inhibition of pigmentation, [b]: + =Inhibition of pigmentation

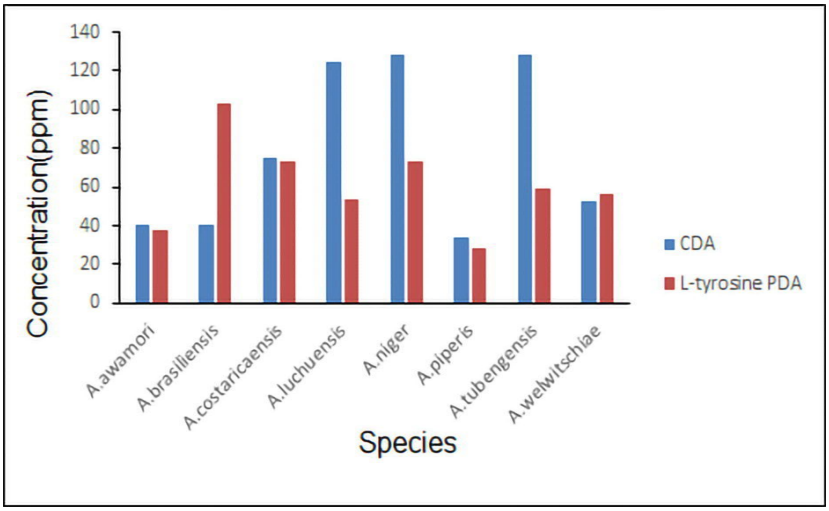


Figure 1. Melanin concentration (ppm) in *A.* section *Nigri* species after 1 week (*P*<0.001, *F* = 2.6) **(Prepared by Authors, 2025)**.

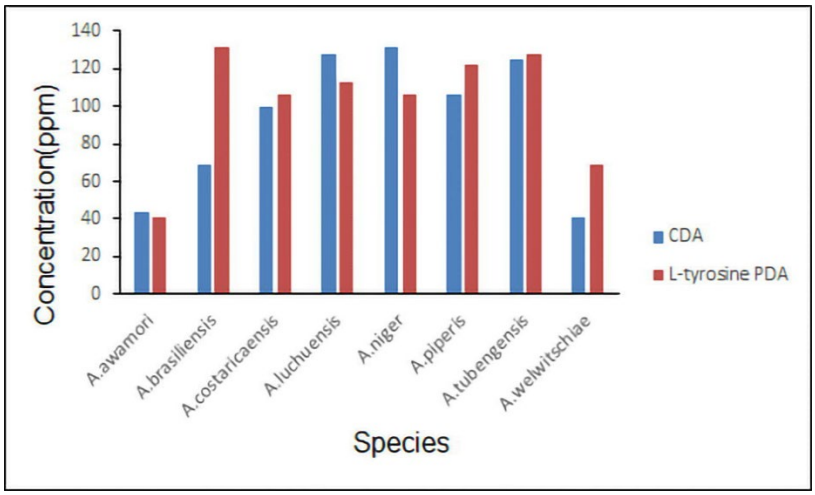


Figure 2. Melanin concentration (ppm) in *A.* section *Nigri* species after 2 weeks (*P*<0.001, *F* = 2.6) **(Prepared by Authors, 2025)**.

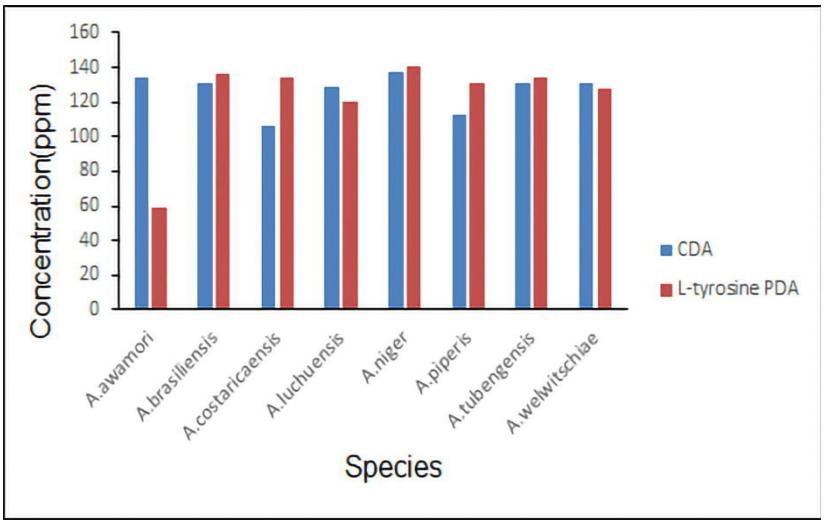


Figure 3. Melanin concentration (ppm) in *A.* section *Nigri* species after 3 weeks (*P*<0.001, *F* = 2.6) **(Prepared by Authors, 2025)**.

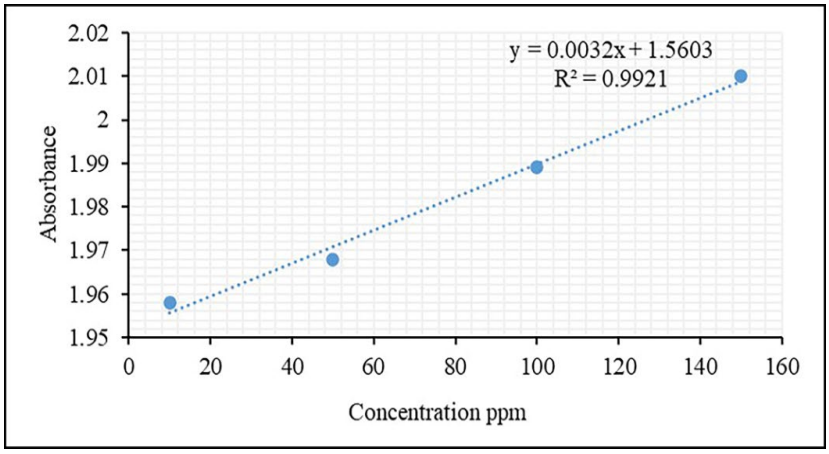
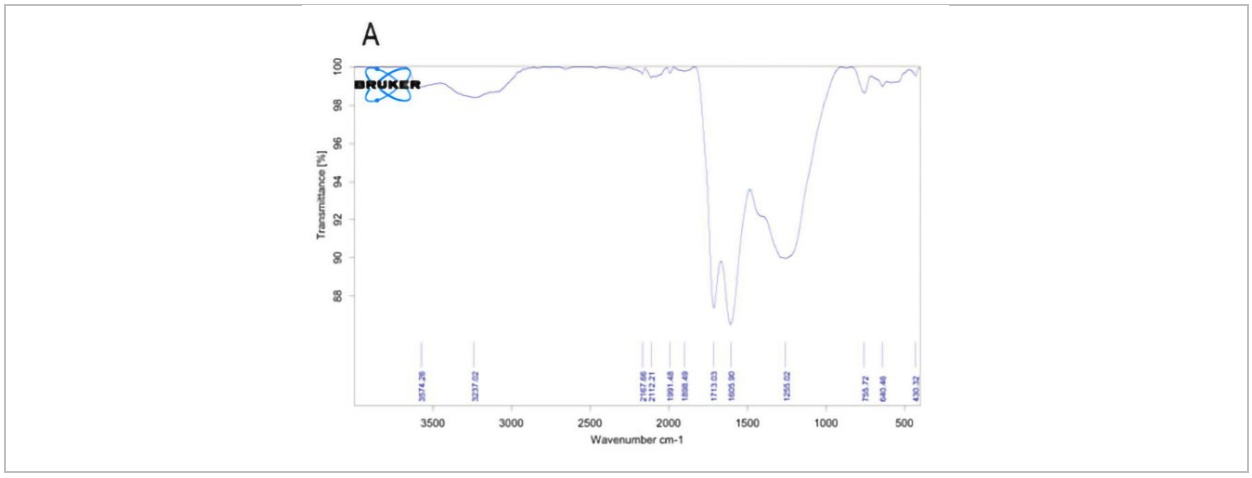


Figure 4. Synthetic melanin concentration measurement standard curve **(Prepared by Authors, 2025)**.



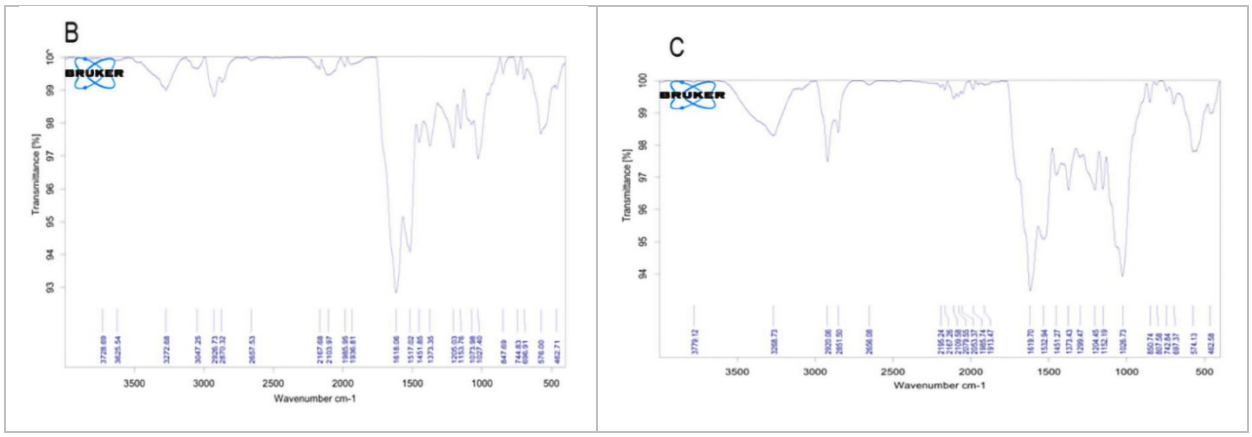


Figure 5. Graphs show various peaks of synthetic standard melanin **(A)** and extracted melanin from *A. niger* and *A. tubingensis*, respectively after 3 weeks **(B-C)** showing functional groups generated via FTIR **(Prepared by Authors, 2025)**.

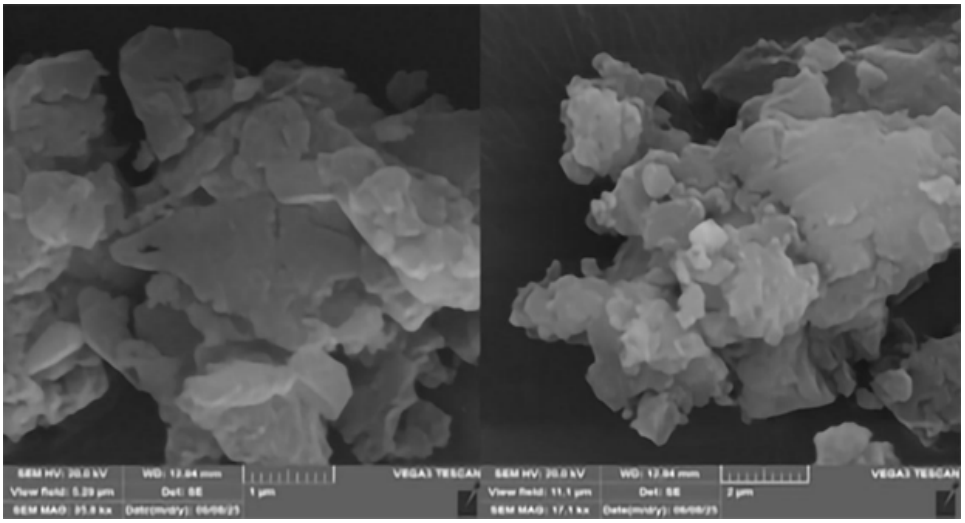


Figure 6. The images show surface topology of fungal extracted melanin (left) and synthetic standard melanin (right) analyzed by scanning electron microscopy **(Prepared by Authors, 2025)**.

4. Discussion

Researchers are actively investigating innovative strategies for the large-scale production of melanin **(3)**. Microorganisms, in particular, are considered sustainable and economically viable biological resources for melanin synthesis, with promising applications across biotechnological, environmental and pharmaceutical domains **(10, 9)**. As far as we are aware, the specific melanin biosynthetic pathways that have never been characterized for most species exist within *A.* section *Nigri*. This study represented the first comprehensive investigation in this area.

In this study, the extracted melanin exhibited positive results across all physical and chemical tests commonly used to confirm melanin identity. Solubility assays demonstrated insolubility of pigment in organic solvents, partial solubility in DMSO and full solubility in alkaline solutions. Furthermore, it precipitated in 3N HCl, indicating its sensitivity to acidic environments. This solubility profile was consistent with known characteristics of melanin, which is generally insoluble in organic solvents but soluble under alkaline conditions. These findings are in full agreement with previous reports **(12, 18, 23)**, which described similar physicochemical properties of melanin.

The results obtained in this study are consistent with those reported in previous investigations **(12, 18, 24, 25)**, which demonstrated prominent absorption peaks around 217 nm in the UV–Vis spectrum of extracted melanin. This absorption pattern is characteristic of melanin pigments and is attributed to the presence of aromatic structures and conjugated double bonds within the melanin polymer. These findings further confirm the presence and identity of melanin in the

fungal extracts, in agreement with previous observations **(23)**.

FTIR spectroscopy is a widely utilized analytical technique for identifying functional groups within a compound by correlating characteristic absorption bands to specific molecular vibrations **(12)**. In the present study, the FTIR absorption spectra of the extracted melanin demonstrated prominent peaks corresponding to hydroxyl (–OH), amine (–NH) and aromatic groups (C=C, CH and COO). The fungal melanin spectrum exhibited broad absorption features, particularly indicative of hydrogen bonding involving hydroxyl groups and aromatic ring C=C stretching vibrations. These spectral characteristics closely matched those observed in synthetic melanin standards and are consistent with findings reported in earlier studies **(12, 18, 26, 27)**.

SEM images captured at 1 and 2 µm resolutions revealed the surface morphology of the extracted melanin. The samples exhibited a granular and heterogeneous structure, which is characteristic of melanin aggregates. These observations were consistent with previously reported findings **(19)**, further confirming the typical morphological features of fungal melanin.

The concentration of extracted melanin from all isolates exhibited clear quantitative differences in melanin production, both between the two culture media and across different incubation periods. These findings align with previous studies that identified *Aspergillus* species as prolific producers of melanin **(28, 29)**.

The present study investigated melanin content in eight species of *A.* section *Nigri* and determined whether differences in melanin content exist among the species when cultivated on two solid media at different incubation periods. Quantitative data on melanin production in species belonging to section *Nigri* were limited. It was previously reported only for *A. carbonarius* **(30)**, *A. niger* and *A. tubingensis* **(31)**. In this study, melanin was extracted from all isolates grown on CDA and L-tyrosine PDA using alkaline extraction followed by acid precipitation. This method is widely applied for melanin isolation in *Aspergillus* species **(31-34)**. PDA and CDA were selected based on prior reports indicating their capacity to enhance pigment production **(17, 23, 30)**.

The current study revealed minimal differences in melanin production among the tested species after 1 and 2 weeks of incubation on both culture media. However, after 3 weeks, distinct differences in melanin level were observed, particularly on L-tyrosine PDA across all species. In contrast, CDA showed only slight variations in melanin yield among the species. Both CDA and L-tyrosine PDA were effective in supporting fungal growth and consequently melanin production **(35)**. Furthermore, the organization of melanin granules appeared to be influenced by salt concentration in the culture medium. Metal ions played a critical role in melanin biosynthesis; specifically, the supplementation of KCl and $MgSO_4$ in culture media enhanced melanin production, likely due to their involvement in the proper functioning of melanin-synthesizing enzymes **(36)**. The melanin yields obtained in this study were higher than those reported in previous researches, which may be attributed to the improved extraction techniques with minor modifications applied, culture media composition, and optimized cultivation conditions employed. Quantitative differences in melanin production among *A.* section *Nigri* species may also reflect the kinetics of colony differentiation and species-specific variations in melanin synthesis, which can differ based on the solid media used **(30)**. Moreover, the natural variability in melanin production across species and even among different strains within the same species contributes to the observed differences **(12, 18)**.

The significant *P*-value ($P<0.05$) indicates that the observed differences in melanin production was not due to random variation. The F value (2.6) suggests a moderate effect of the factor tested. The interaction effects imply that the influence of growth medium or time on melanin production varies depending on the fungal species. This highlights that melanin biosynthesis is species-specific and it can be modulated by the environmental conditions such as nutrient composition and incubation period.

Kojic acid and difenoconazole are known inhibitors of the DOPA and DHN pathways, respectively. Our results demonstrated that the DOPA and DHN pathways are both present in *A. niger* and *A. brasiliensis*, while only the DHN pathway was identified in the other tested species. These findings were consistent with previous studies **(23, 31)**, which reported similar pathway distributions among *A.* section *Nigri* species.

5. Conclusion

The study successfully demonstrated that species within this section are emerging as significant producers of melanin. Physicochemical characterization and analytical methods confirmed its identity as melanin. The present study demonstrated variation among *A.* section *Nigri* species in their ability to produce melanin and in their response to melanin biosynthesis inhibitors. The observed differences suggest that melanin production in these fungi may involve multiple biosynthetic pathways and regulatory mechanisms. These findings provide additional insights

into the diversity of melanin production within this group of fungi. Furthermore, understanding the variability of melanin synthesis among *Aspergillus* species may contribute to a better interpretation of their ecological adaptation and may also offer useful perspectives for potential industrial, biotechnological, and pharmaceutical applications involving fungal melanin production. Further investigations are needed to further substantiate these findings through the characterization of genes encoding enzymes involved in both melanin biosynthesis pathways, use of additional inhibitors, and identification of pathway intermediates.

6. Declarations

6.1 Acknowledgment

The authors gratefully acknowledge the Department of Ecology, College of Science, University of Basrah, Iraq, for their assistance and laboratory facilities required for the successful completion of this study.

6.2 Ethical Considerations

The study was approved by the Ethical Committee of the Institutional Review Board (IRB), University of Basrah, Basrah, Iraq (Approval No.6422-54-07, dated November 30, 2023). Written informed consent was obtained from all participants prior to their inclusion in the study.

6.3 Authors' Contributions

S.S.R.: Data curation, investigation, methodology, formal analysis, writing-original draft preparation and Corresponding author. M.K.M: supervision, writing-review and editing, Statistical analysis. M.A.A.: supervision, writing-review and editing. All authors reviewed the manuscript.

6.4 Conflict of Interests

The authors declare no conflict of interest.

6.5 Financial Support and Sponsorship

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

6.6 Using Artificial Intelligence Tools (AI Tools)

All authors declare that there is no use of AI Tools in this study, including the writing of this manuscript.

6.7 Data Availability

Data generated for this study are presented in the accompanying tables and figures. The sequenced data set is publicly available at NCBI (GenBank IDs LC884982 to LC884988 and LC884798 to LC884805).

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