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Isolation, purification, identification of melanin from grape pomace extracts, and its application areas

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ABSTRACT

In recent years, natural pigments have gained increasing interest across various industries. Melanin is a versatile pigment with many biological effects, including antioxidant, antimicrobial, antiradical, immunostimulatory, antitumor, anti-inflammatory, and bio-stimulatory effects. Melanin can absorb heavy metal ions and remain stable over a broad pH range, with temperatures up to 130°C. These properties are primarily attributed to the high concentration of unpaired electrons (10^{17}–10^{19} spin/g).

Water-soluble melanin has many applications. This compound serves as an antioxidant, an antimicrobial, an anticancer agent in medicine, a neutralizer of free radicals in tobacco production, a bio-stimulant in agriculture, a food additive, a semiconductor in solar battery production, and a radioprotector in nuclear power plants.

Objective: This study aims to develop an efficient method to isolate and purify melanin from grape skin seeds. Once collected, its physicochemical and biological properties were determined to assess the purity yield, allowing researchers to explore this product's potential applications.

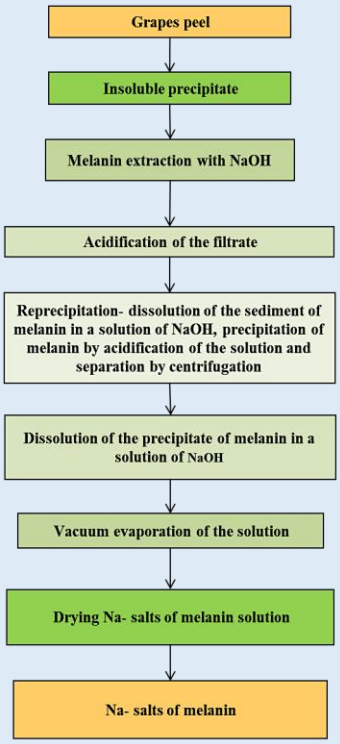
Methods: Melanin was extracted from grape pomace under static conditions (65°C, 2.5–3.0 h) using 0.7 M sodium hydroxide as an extractant. Following centrifugation, melanin was isolated by acidifying the alkaline extract with HCl to a pH of 2.0. The resulting amorphous precipitate was purified through repeated re-precipitation. To obtain water-soluble melanin, the precipitate was dissolved in NaOH, vacuum-evaporated to a viscous form, and dried at 60°C.

Results: Grape varieties analyzed (white *Itsaptuk*, red *Karmir Itsaptuk*, black *Armenia*) yielded melanin at rates of 0.114%, 0.164%, 0.148%, respectively. Infrared spectroscopy (IR) confirmed similar valency deformation vibrations across all varieties studied. HPLC analysis revealed a single symmetrical peak for each sample, indicating chromatographic purity and uniform composition. Studies concluded that melanin was effective as a food-coloring agent in beverages, baked goods, and confectionery. When applied to vegetable melon seeds, low-concentration melanin solutions exhibited high bio-stimulating activity, enhancing root development and improving crop yields.

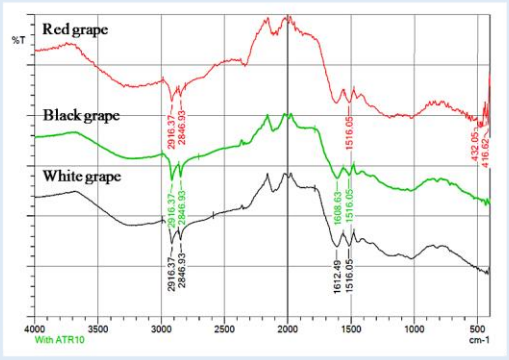
Conclusion: The extraction method within this experiment efficiently produces affordable, pure, water-soluble melanin from grape pomace. Due to its chromatographically pure composition, melanin has the potential to be used in medicine, food production, and tobacco processing. Notably, melanin can reduce the production of free radicals in tobacco smoke.

Keywords: melanin, grapes, production, identification, application

Technological scheme of melanin extraction



FTIR spectra of melanins



Antimicrobal activity of melanins

Scavenging of the DPPH radical with melanin preparations

Melanin preparations	Antioxidant activity* (IC_{50}, mg L^{-1})
Vitamin C	4.7 ± 0.29
White grape	11.8 ± 0.62
Red grape	9.4 ± 0.57
Black grape	7.2 ± 0.47

Graphical abstract: Isolation, purification, and identification of melanin from grape pomace extracts

INTRODUCTION

The demand for compounds capable of scavenging free radicals has grown significantly due to their diverse biomedical applications. Among these, melanin - a high-molecular-weight biopolymer synthesized through the enzymatic oxidation of nitrogen-containing nitrogen-free diphenols, has emerged as a promising candidate among antioxidant anti-inflammatory compounds [1–4]. Its unique unpaired electron structure contributes to properties such as electrical conductivity, solubility, and notable biological activity [5–7]. These features enable melanin to be involved in redox processes, which makes it possible to absorb electrons and neutralize reactive radicals through reversible quinone-hydroquinone oxidation-reduction transitions [8]. Additionally, melanin contains functional groups capable of forming stable complexes with metal ions, further broadening its applications [9].

Alkaline solutions are commonly recognized as effective solvents for melanin, since they promote the dissociation of ionogenic groups. This leads hydrophilic groups to orient outwards and hydrophobic groups to orient inwards, improving melanin's solubility and stability in alkaline environments [10]. Melanin has been successfully extracted from various sources, including animals, plants, microbes, and chemical synthesis [8, 10, 11]. However, isolating melanin from animal sources often results in low yields due to the complexity of raw materials, while microbial melanin extraction is energy-intensive and costly. In contrast, plant-derived melanin offers a cost-effective alternative, since plant waste typically contains fewer impurities and requires less intensive processing [12-13].

Bioactive compounds, derived from nutritive or non-nutritive natural sources, act as essential, secondary, and tertiary metabolites. They play a vital role in the management of chronic diseases. Within functional foods, bioactive compounds, such as melanin, exhibit crucial disease prevention, management, and treatment mechanisms. Their diverse functionalities compatibility with biological systems underscores their importance in promoting health overall well-being [14-18] .

There are many extraction methods to obtain melanin from plants. For example, soluble melanin has been isolated from chestnut shells, yielding three fractions with similar chemical and biological properties [19]. Melanin extracted from grape skins resulted in four fraction complexes with a total recovery of 17.3% [20, 21]. In addition, melanin has been purified from the fruit pulp of *Vitex mollis and Randia echinocarpa* using alcohol-based sonication followed by ammonium hydroxide extraction [22]. Date palm fruits (*Phoenix dactylifera* L.) also contain high levels of melanin [23]. Melanin derived from tea leaves, chestnut shells, grape, and sunflower oil demonstrates notable cosmetics, pharmaceuticals, and solar energy conversion systems [24].

Given the increasing interest in melanin’s biotechnological applications, efficient methods for its purification are necessary. This study aims to refine the extraction process for melanin from grape skin seeds, evaluating its purity, yield, and physicochemical properties. The research also explores plant-derived melanin's economic feasibility and potential biomedical applications, focusing on its role as a free radical and adaptability across various industries.

MATERIALS AND METHODS

Extraction of Melanin from Different Grape Varieties: Several mechanisms of melanin biosynthesis in plants have been proposed by the previous research [25-26]. An experiment was conducted using 1500 g of grape bunches from each variety studied to understand the temperature-dependent melanin synthesis in different

grape varieties. The bunches were autoclaved at 120 °C for 40 min. Following heat treatment, the skin seeds were separated from the pulp and thoroughly washed with water. Melanin was extracted from the skin seeds using the specified procedure when dry.

Grapes of white, red, and black varieties were selected for melanin extraction. The skin's seeds were separated through compression and washed thoroughly with water. These components were vacuumed and dried at 55–60°C. The grape skin stone samples were dried through hot air blowing at 60 °C for 4 to 6 h in an electric dryer, HS-62A (Germany). The residual moisture in dried melanin samples was 1-1.5%, with yields of seeds, skins, and melanin varying by grape variety (see Table 1).

To extract melanin, dried skin seeds were treated with 0.7 M NaOH (solid-to-liquid ratio: 1:13), heated to 65°C with stirring for 2.5 h. The liquid extract was collected after centrifugation (5000g, 20 min). Melanin was precipitated by adjusting the pH of the extract to 2.0 using HCl. The resulting precipitate was centrifuged, washed, and purified by repeated dissolution in NaOH followed by reprecipitation under the same conditions. This process yielded water-soluble melanin. The solution was concentrated by vacuum under the previously described conditions, which afforded dark brown, water-soluble melanin with a metallic sheen.

Determination of Protein Constituents: The hydrochloric acid hydrolysis of melanin was carried out by contacting 1 gram of the twice re-precipitated amorphous melanin with 10 mL of a 6 M HCl solution at 120°C for 30 min. Next, the pH of the hydrolysate was adjusted to 5 with NaOH, allowing the neutralized solution to be subjected to amino acid analysis. The amino acid analysis of melanin was done using a "Shimadzu Nexera X2" amino acid analyzer (Japan), equipped with a fluorescence detector RF-20A "Shimadzu".

Spectral Analysis: UV-Vis absorption spectra of melanin were obtained using a Thermo Scientific Genesys 50 UV-Vis spectrometer. IR spectra were recorded with a SHIMADZU IRTracer-100, employing a KBr prism (range: 7800–350 cm^{-1}, resolution: 4 cm^{-1}).

HPLC Analysis:: Chromatographic analysis was performed using a Nova-Pak C18 column (3.9 mm × 150 mm, 4 µm particle size) on a Waters Alliance e2695 HPLC system. The mobile phase comprised 80% water containing 0.1% ammonium (pH 8.0, 20% acetonitrile. At a flow rate of 0.5 mL/min, 10 µL samples were injected, with detection at 220 nm using a PDA detector.

Antioxidant Activity: Antioxidant activity was evaluated using a DPPH radical scavenging assay [21]. Melanin solutions (2 mL) of varying concentrations were mixed with 2 mL of 0.2 mM DPPH solution (prepared in ethanol), homogenized, and then incubated in the dark for 30 min. Absorbance at 517 nm was measured for the reaction (A_1), control (A_2), and reduction (A_o). The scavenging activity was calculated using:

$$Scavenging\ activity(\%) = \left[1 - \frac{(A1-A2)}{A0}\right] * 100 \quad (1)$$

The antioxidant activity of melanin was compared with that of ascorbic acid (vitamin C) as an antioxidant reference. An important indicator in determining antioxidant activity by the DPPH method is the antioxidant's scavenging of 50% of the DPPH reagent (IC50). This was calculated based on the data obtained.

Statistical Analysis: Results are presented as means of three independent experiments. Statistical significance was determined using Microsoft Excel, with *p*-values <0.05 considered significant.

Test Microorganisms:: Melanin extracted from black, white, and red grapes was tested for antimicrobial activity against Escherichia coli K12 and *Bacillus subtilis* G17-89, sourced from the Microbial Depository Center (MDC), SPC "Armbiotechnology," NAS RA. Bacteria were cultured on a Nutrient Agar and an Endo Agar at pH 7.2, incubated for 16 h at 37°C, then suspended in a Nutrient Broth to a concentration of ~2.2×10^6 CFU/mL.

Determination of Antimicrobial Activity: Antimicrobial Activity was assessed using tube serial dilution [27-28] spot: The antimicrobial activity was evaluated using tube serial dilution [27-28] spot-on-lawn methods. Growth inhibition zones (Ø, mm) were measured after 24-h incubation at 30°C. Results were expressed as CFU/g or CFU/mL of the tested product. The survival rate of bacterial cells was determined by serial dilution [27]. Samples (0.1 mL) were suspended in 0.85% NaCl for 10 min., serially diluted, plated on agar, and incubated at 30°C or 37°C for 2–3 days. Colony counts were used to evaluate bacterial viability.

RESULTS AND DISCUSSION

Our study confirmed that grape pomace, a byproduct of wine production, is an excellent source of plant-derived melanin [21]. Three distinct types of grapes were selected to evaluate the melanin yield across grape varieties: white grapes (Itzapuk variety), red grapes (Karmir Itzapuk variety), and black grapes (*Armenian* variety).

Tables 1 and 2 present the results of melanin extraction without autoclave treatment. These findings provide valuable insights into how grape variety heat treatment influences melanin yield. The data reveal variations in melanin production, emphasizing the importance of optimizing extraction conditions based on the specific grape variety and pre-treatment method.

Table 1. Amount of Isolated Skin, Seeds, and Melanin from Different Colored Grapes (Initial Grape Weight: 1000 g)

Grape color	Weight of the obtained skin, g	Weight of the obtained seed, g	Weight of the obtained melanin from skin, g	Weight of the obtained melanin from seeds, g	Melanin yield from grapes, %	Weight of obtaining melanin, g
Black	9.54±0.72	2.37±0.15	1.42± 0.104	0.064±0.0045	0.098±0.0052	1.484±0.11
Red	13.4±0.89	8.2±0.61	1.53± 0.091	0.104±0.0088	0.109±0.0071	1.634±0.14
White	5.8±0.41	13.8±1.03	0.86± 0.062	0.28±0.021	0.076±0.0053	1.14±0.078

Table 2: Amount of Isolated Skin, Seeds, and Melanin from Different Colored Grapes after Autoclaving (Initial Grape Weight: 1000 g)

Grape color autolysis mode	Weight of skin, g	Weight of seed, g	Weight of melanin from skin, g	Weight of melanin from seed, g	Melanin yield from grapes, %	Weight of obtaining melanin, g
Red, 120 °C, 40 min	13.86±0.84	8.33±0.63	1.34±0.084	0.124±0.01	0.097±0.007	1.464±0.089
Black, 120°C, 20 min	10.4±0.82	2.2±0.15	1.28±0.086	0.086±0.003	0.061±0.007	1.366±0.071
White, 120 °C, 40 min	6.0±0.42	11.0±0.77	0.19±0.014	0.092±0.006	0.02±0.0012	0.282±0.023

As shown in Tables 1 and 2, autoclaving resulted in an 11–12% reduction in the weight of melanin extracted from grapes compared to non-autoclaved samples. In contrast, the melanin yield from white grapes decreased drastically, by a factor of four, following the autoclaving process.

Previous studies suggest [25-26] that the reduction in white grapes is likely due to the inactivation of polyphenol oxidase, an enzyme critical for oxidizing phenols into quinones. These quinones subsequently undergo spontaneous polymerization to form melanin.

Melanin synthesis predominantly occurs intracellularly in red and black grapes. Melanin is already present in the cells before autoclaving. Notably, the pigmentation in red grapes is influenced by other natural pigments, such as polyanthocyanins, resveratrol, and flavonoids. During the reprecipitation process, melanin is efficiently separated from these pigments, which exhibit limited solubility in alkaline solutions, thereby minimizing their interference in melanin extraction.

As highlighted in Tables 1 and 2, 75–97% of the melanin extracted from grapes originated from the skins, depending on the array.

IR Spectra of Melanin: The infrared (IR) absorption spectrum is an essential characteristic of melanin. Figure 1 depicts the IR spectra of the melanin preparations derived from various grape varieties.

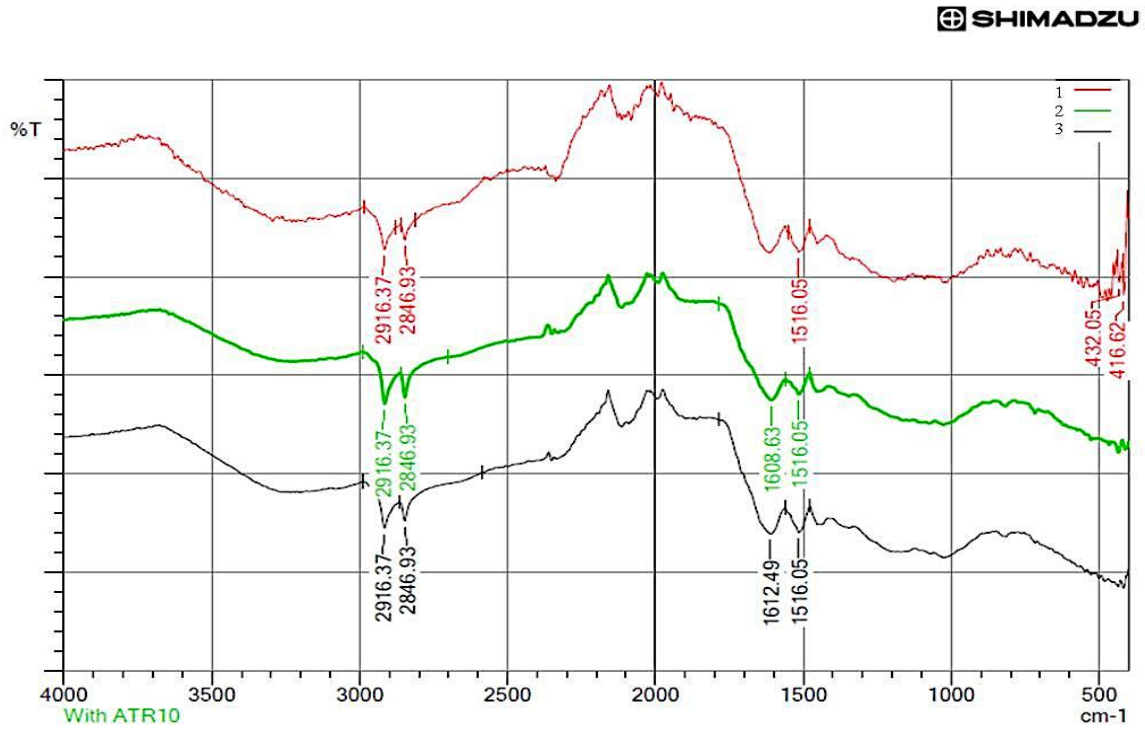


Figure 1. IR spectrum of melanin isolated from 1- red grape, 2 - black grape, 3 - white grape

The IR spectra of purified plant melanin exhibit characteristic absorption bands similar to observations made in other studied melanin compounds [5, 8, 21]. The broad band at 3330 cm-1 corresponds to the stretching vibrations of -OH -NH groups, of hydroxyl amino functionalities. Bs observed at 2916 and 2846 cm^{1} are attributed to the stretching vibrations of aliphatic – CH_2-CH_3 and 2846 cm^{1} are attributed to the stretching

vibrations of aliphatic–CH_3 groups, respectively. The absorption band at 1610 cm^{-1} is associated with the stretching vibrations of conjugated double bonds (C=C C=O), characteristic of secondary amides. The region spanning 1520–1450 cm^{-1} corresponds to phenolic compounds' C–O bonds. Furthermore, bs in the 1200–1030 cm^{-1} range are attributed to stretching vibrations of ether (C–O–C) hydroxyl (C–O) groups. However, no bands are visible in that range. Increased magnification may distinguish, although visibility is unlikely.

Significant similarities in the leading absorption bands are observed when comparing the IR spectra of melanin pigments derived from various plant sources to those of synthetic microbial melanin compounds. This underscores their shared chemical features and functional groups [5].

Due to the amorphous nature of melanin polymers and the spontaneous polymerization of their monomers, there is currently no precise understanding of melanin's structure and chemical formulas.

UV-Vis Spectra of Melanin. Natural pigments' absorption of light is a fundamental property that defines their function. UV-Vis spectrophotometry was employed to analyze the pigment in aqueous solutions.

The absorption spectra of melanin have the form of flat curves with a gradual decrease in optical density as the wavelength increases from 200 to 600 nm, which is typical for all melanin regardless of their origin. At the same time, the absorption contours of individual melanin had noticeable inflections. The literature describes examples of inflections at 220-240, 260-270, 310, 325 nm in the melanin spectra, which are considered a specific characteristic or indicate the presence of impurities in the preparation [1, 13, 21]. Maximum absorption is observed at a wavelength of 200 nm. A slight inflection is observed in the spectrum at 260-280 nm due to protein pigment in the polymer.

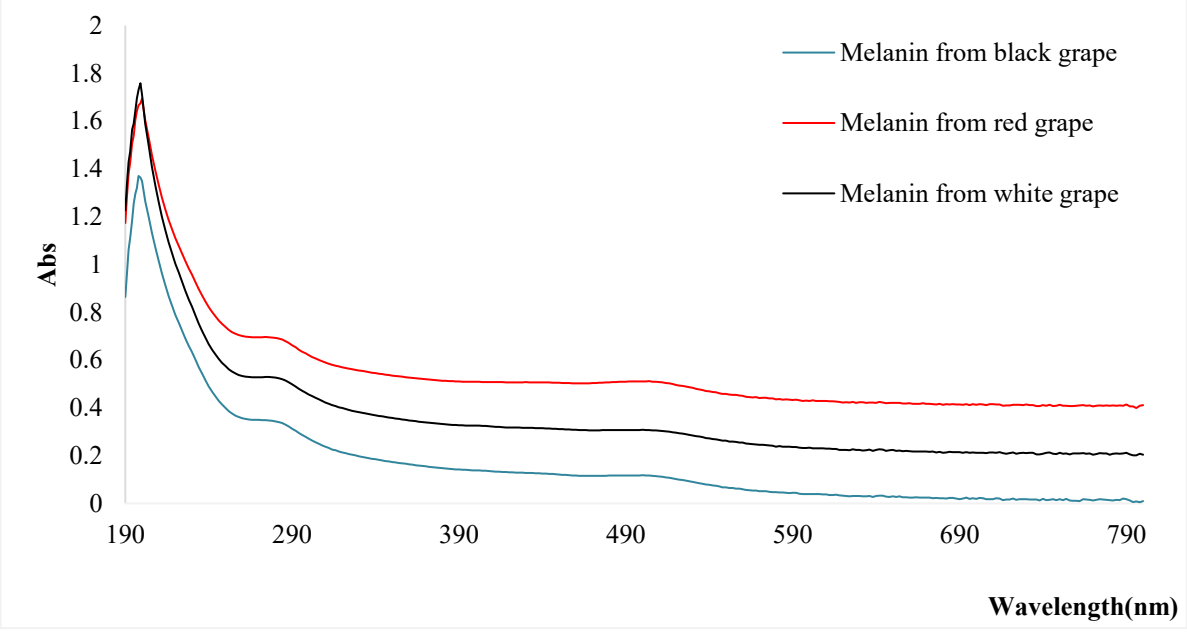


Figure 2. Absorption spectra of melanins from black grape, red grape, and white grape

HPLC analysis: Melanin compounds obtained from different colored grapes are chromatographically pure (Fig.3) and have the same valency deformation vibrations at various wavelengths (Fig. 1).

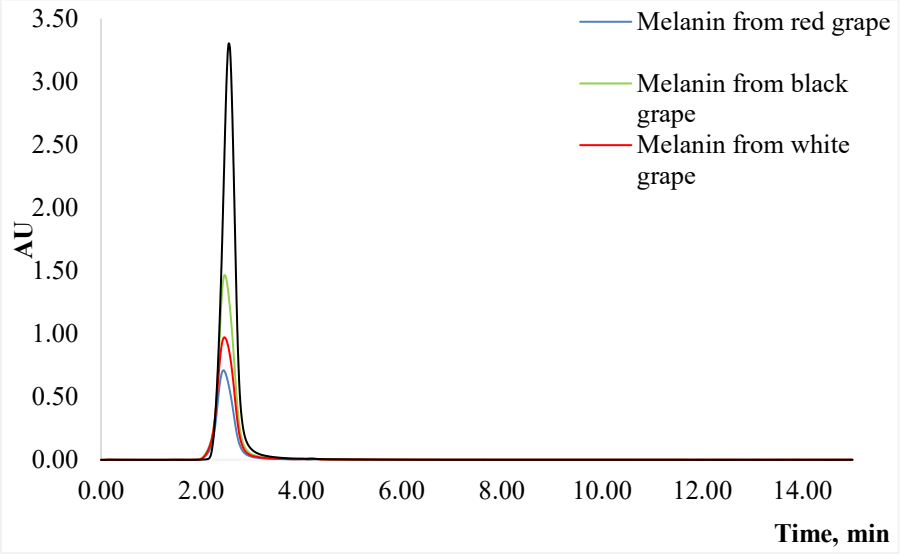


Figure 3. HPLC chromatogram of melanin from Red, Black, and White grape

HPLC analysis revealed that melanin extracted from red, white, and black grapes exhibited consistent chromatographic profiles, each presenting a single, well-defined peak. These findings indicate a high degree of similarity in melanin composition across the three grape varieties. The absence of additional peaks in the chromatographic analysis confirms that the The melanin obtained is chromatographically pure.

Previous studies [23, 30, 34] have demonstrated that melanin extracted from grape pomace was covalently bound to proteins. Meaning, the melanin from the different grape varieties underwent acid hydrolysis, and their amino acid content was analyzed using an amino acid analyzer (Table 3)

Table 3. Amino acid composition of melanin hydrolysate obtained from black, red, and white grapes

Concentration of Amino Acids in Protein in Melanin Hydrolysate (mg/mL)			
	Black grape	**Red grape**	**White grape**
L-Asp	0.95	0.95	0.94
L-Glu	1.34	1.73	1.08
L-Ser	0.54	0.26	0.35
Gly	0.84	1.15	0.77
L-Ala	0.95	0.64	0.76
L-Arg	0.03	0.27	0.48
L-Tyr	0.29	0.29	0.31
L-Phe	0.53	0.48	0.38
L-Ile	0.49	0.29	0.18
L-Leu	0.88	0.59	0.49
L-Lys	0.35	0.28	0.38
L-His	0.36	0.51	0.36
L-Val	0.83	0.13	0.23
Total Protein, %	8.38	7.63	6.71

The hydrolysis conditions: melanin 0.2 g; 2 mL 6 M HCl; 120 °C; 30 min.

The protein content in the melanin samples, calculated from these analyses, ranged from 6.7% to 8.4%.

Antioxidant Activity of Grape Melanin: The antioxidant properties of natural plant-derived compounds hold immense potential for biomedical applications [20, 35]. Among the methods used to evaluate antioxidant activity, the DPPH assay is widely recognized for its ability to measure the' free radical scavenging capacity of biological samples. This method, which employs a stable radical, is a standard technique for assessing the efficacy of antioxidants [20, 33].

This study evaluated the antioxidant activity of purified plant melanin using the DPPH assay. Melanin solutions' free radical scavenging capacity was evaluated against ascorbic acid (vitamin C), a benchmark antioxidant known for its potent activity. The results demonstrated effective free radical scavenging across all grape-derived melanin samples, highlighting their potential as natural antioxidants (Table 4).

Table 4. Scavenging of the DPPH radical with melanin preparations.

Melanin preparations	Antioxidant activity* (IC_{50}, mg L^{-1})
Vitamin C	4.7 ± 0.29
White grape	11.8 ± 0.62
Red grape	9.4 ± 0.57
Black grape	7.2 ± 0.47

* 50 % scavenging activities for 0.1 mM DPPH

Melanin extracted from black grape skin exhibited the highest antioxidant activity, with an IC50 value of 7.2 mg/L for 50% scavenging of 0.1 mM DPPH, compared to 4.7 mg/L for ascorbic acid (vitamin C). In contrast, the IC50 values for melanin from red and white grapes were higher, with levels of 9.4 mg/L, and white grapes were higher, with 9.4 mg/L and 11.8 mg/L, respectively. The free radical scavenging ability of melanin derived from grape waste presents a promising avenue for developing cost-effective antioxidants, anti-inflammatory agents, and other biomedical products.

Based on the data in Table 3, the antioxidant capacity of melanin obtained from white, red, and black grapes is 40; 50.6, and black grapes are 40; 50.6, and 65.3%, respectively, of the equivalent of vitamin C.

Antimicrobial Activity: The antimicrobial activity of melanin extracted from black, white, and red grapes was evaluated over time against foodborne pathogens, including Escherichia coli and Bacillus subtilis. The results are summarized in Table 5 and Figure 4.

Table 5. Evaluation of antimicrobial activity of melanin

Melanin source	Incubation time			
	24 h		48 h	
	***B. subtilis* G17-89**	***E. coli* K12**	***B. subtilis* G17-89**	***E. coli* K12**
	Cell count, 10Log CFU/ml			
Control	$7x10^{10}$	$1,5x10^{11}$	$2x10^{10}$	$7x10^{12}$
Black grape	$4x10^{6}$	$1x10^{8-9}$	$1.2x10^{7}$	$4.7x10^{10}$
Red grape	$1x10^{9}$	$6.6x10^{10}$	$5x10^{10}$	$7.5x10^{11}$
White grape	$1.5x10^{9}$	$9.5x10^{10}$	$6x10^{9}$	$1.4x10^{12}$

The antimicrobial effect of melanin was found to vary based on the source of isolation of the bacterial strains tested. Melanin derived from black grapes exhibited the strongest and most potent antibacterial activity against Bacillus subtilis and *Escherichia coli*. After 24 h of incubation, the cell counts of *Bacillus subtilis* decreased by four orders of magnitude. In contrast, the cell counts of *Escherichia coli* decreased by three orders of magnitude compared to the control. In comparison, melanin isolated from red and white grapes showed weaker effects.

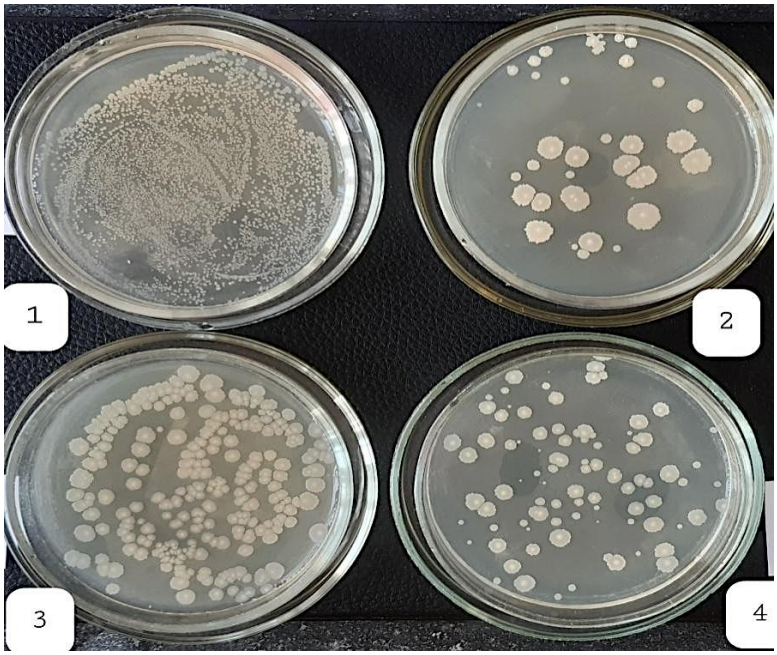


Figure 4. The antimicrobial activity of melanin against *Bacillus subtilis* G17-89
1-control, 2 - Black grape, 3 - White grape, 4 - Red grape

The antibacterial activity of melanin can be attributed to several key mechanisms. One of the primary factors is the presence of catechol groups, which facilitate the production of reactive oxygen species (ROS) through electron transfer during phenolic quinone isomerism. This ROS generation plays a crucial role in inducing bacterial cell death. Melanin's photothermal properties also enhance its antimicrobial efficacy by increasing localized heat, further contributing to bacterial inactivation. These combined mechanisms highlight melanin's potential as a powerful agent in the fight against bacterial infections [27, 33, 35].

The notably high antimicrobial properties of melanin derived from black grapes can be associated with its superior antioxidant activity (Table 3). For example, the concentration of black grape melanin solution required to neutralize 50% of DPPH free radicals is only 7.2 $mg \cdot L^{-1}$, compared to 9.4 $mg \cdot L^{-1}$ for red grape melanin and 11.8 $mg \cdot L^{-1}$ for white grape melanin. Although melanin from grapes of different colors exhibits similar spectral characteristics (Figs. 1–4), the variations in antioxidant capacities are likely due to differences in their molecular structures. Despite its amorphous nature, the specific structural features of melanin, which remain largely undefined, are thought to influence these antioxidant properties.

CONCLUSION

This research's scientific novelty lies in a standardized method for separating and purifying melanin from grape varieties. This study not only characterized the properties of the extracted melanin samples but also identified potential applications for these bioactive compounds.

The findings demonstrate that effective processing of grape pomace from various wine varieties enables the extraction of valuable bioactive compounds. For example, solvent extraction of grape pomace yields colored natural compounds, including poly anthocyanins, resveratrol, and flavonoids. The remaining insoluble residue is a source of poly anthocyanins, resveratrol, and flavonoids. It is an excellent raw material for producing water-soluble melanin with diverse biological properties.

Abbreviations: DPPH: 2,2-Diphenyl-1-picrylhydrazyl; MDC: Microbial Depository Center; IR: Infrared spectroscopy

Conflict of Interest: The authors declare no conflicts of interest.

Author Contributions: AA led the project, designed and performed experiments, analyzed results, and wrote the manuscript. EM and TS conducted experiments, analyzed results, and prepared the original draft. KK, GM, GH, and KY contributed to experimental execution, and AT supervised the project. All authors approved the final manuscript.

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