Technology for obtaining melanin from the shells of horse chestnut (*Aesculus hippocastanum* L.), studying its composition

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Abstract. The article presents the results of a study on the development of technology for obtaining and studying the physicochemical characteristics of melanin isolated from the seed shells of horse chestnut (*Aesculus hippocastanum* L.), growing in the territory of Tashkent. A technological scheme for the production of melanin has been developed, which includes the stages of grinding the raw material (shell), the use of ultrasound in an ethyl alcohol environment, extraction, 3 times acidification with a solution of hydrochloric acid, filtration, concentration, dissolution in an alkali solution, precipitation, purification with ethyl acetate, dialysis, and melanin drying. The influence of the raw material-extractant ratio, multiplicity, and duration of extraction on the yield of melanin was studied and the optimal conditions for melanin isolation were determined. Melanin obtained from the shells of horse chestnut seeds is a brown powder, the yield was 24.5% by weight of dry raw materials. Analysis of the elemental composition showed: that the nitrogen content in the isolated melanin molecule is 0.7%, the amount of carbon is 44%, and hydrogen is 6%. IR spectral analysis of the melanin content in the shells of horse chestnut seeds showed the presence of absorption bands that correspond to melanin.

1 Introduction

Against the backdrop of the widespread use of compounds of natural origin, special attention of specialists working in the field of drug development is paid to melanins - natural cellular pigments [1,2].

Currently, the possibility of using melanin for the prevention and treatment of human diseases, as well as in the food, perfume industry, cosmetics, etc. is being actively studied. The most common sources of melanins for practical use are fungi, microorganisms, and plants. The results of studies on the search for alternative raw materials for the production of melanins that meet the requirements of availability, low cost, and rapid renewal have shown that horse chestnut is a promising source of these compounds [3-5]. However, their widespread use is hampered by poor knowledge of their structure and physicochemical properties.

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In order to determine the possibility of using melanins from horse chestnut shells on an industrial scale and highlighting the area of their use, it is necessary to carry out a complex aimed at systematizing data on the structure and physicochemical properties of these compounds.

Melanins of animal and plant origin differ in molecular composition and physicochemical properties [6-8]. Eumelanin, which contains most indole groups, absorbs strongly due to carbonyl groups in the red part of the visible region, thus giving a black color, and pheomelanin in turn contains less carbonyl groups, absorbs light differently and thus gives yellow or red color. Due to their physicochemical properties, melanins have photoprotective, antioxidant, and genoprotective activities, inhibit the formation of lipid peroxidation products, and slow down the aging process [9,10].

The purpose of this work is to develop a technology for isolating melanins from horse chestnut shells and studying their physicochemical characteristics. The information obtained will allow us to identify potential areas of application of melanins from horse chestnuts, growing in Uzbekistan.

2 Methods

Material. The object of the study is melanin isolated from the shells of horse chestnuts, growing in the territory of Tashkent.

Scheme of melanin release

Isolation of melanin from horse chestnut shells was carried out by 3 times aqueous extraction of crushed raw materials using (using ultrasound). The resulting extracts were evaporated to 1/5 of the original volume. Next, 3 times acid reprecipitation was carried out, after the separation of melanin, 2 times purification with ethyl acetate was carried out. Melanin was dialyzed and freeze-dried (PFR-1000, Biobase, China). The quantitative content of horse chestnut melanin after drying the sediment was determined gravimetrically [11].

Determination of elemental composition

Determination of the elemental composition of melanin was performed on an automatic Vario EL Cube analyzer (Netrsch EAS Hamburg, Germany) [12]. Oxygen was calculated from the difference between the ash-free anhydrous mass and the sum of carbon, hydrogen, nitrogen and sulfur atoms. Processing of the primary elemental analysis data was carried out according to the ash content and hygroscopic moisture content in the sample.

IR spectroscopy

The recording of IR spectra was carried out on an IR-Fourier spectrometer system 2000 from PerkinElmer (USA) in the frequency range 400–4000 cm^{-1} in a KBr tablet. The results were processed according to [13].

Performing qualitative reactions

Qualitative reactions to melanin were carried out according to the method [14]. Melanin solutions were added in a 1:1 (v/v) ratio: 10% H_2O_2 (solution decolorization); 0.5 M $KMnO_4$ (color change from brown to green with precipitation, discoloration of the solution); 1% FeCl3 (precipitate formation, its dissolution with additional addition of 3–5 volumes of salt solution).

Statistical data processing

Statistical processing of the results was carried out with the determination of the Student's test using the statistical program MS Excel 2010.

3 Results and discussion

Figure 1 shows a technological scheme for isolating melanin from the shells of horse chestnut seeds.

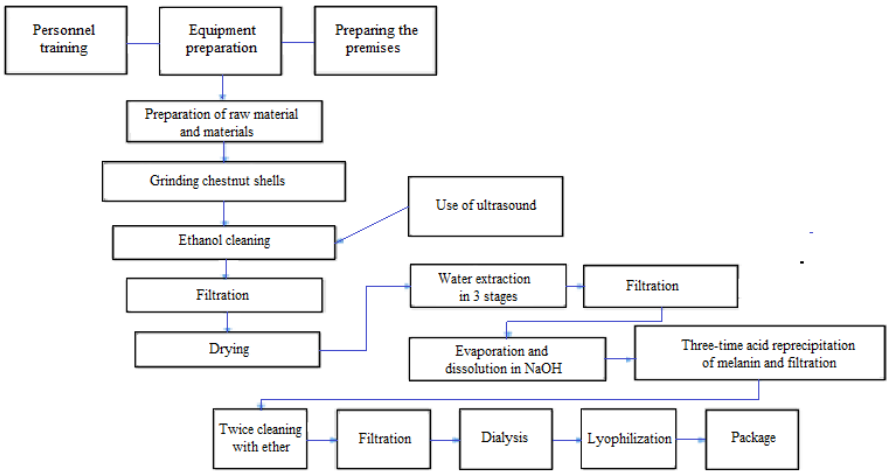

Fig.1. Technological scheme for obtaining melanin from horse chestnut shells (*Aesculus hippocastanum* L.)

To obtain melanin, horse chestnut seeds were collected during the period 2020-2021, growing on the territory of the Tashkent botanical garden. The shells are cleared of fruits and crushed to a particle size of no more than 2 mm.

To extract melanin as completely as possible, as well as to remove low-molecular impurities and lipids from the feedstock, the extraction process was carried out using ultrasonication for 10 minutes twice in an environment of 96% ethyl alcohol, which improves hydrodynamic conditions in large pores of the raw material and reduces extraction time, as well as increase product yield.

To determine optimal extraction conditions, we studied the dependence of melanin yield on the raw material-extractant ratio, duration, and frequency of extraction. It has been established that three-fold extraction of raw materials at a ratio of 1:20, 1:15, and 1:10 with distilled water as an extractant at 100 °C for 2 hours for each extraction stage is optimal. When the lower limit of the raw material-extractant ratio (1:10) is reduced, there is no increase in yield, and exceeding the upper limit of the ratio (1:20) leads to a decrease in product yield. Reducing the duration of extraction to less than 2 hours leads to a decrease in the yield of melanin; increasing the duration to more than 2 hours does not lead to an increase in the yield. The extraction frequency also affects the yield of melanin. The resulting aqueous extracts were combined and filtered, evaporated to 1/5 of the original volume. Next, the evaporated extract was acidified with 1N HCl to pH 1.5, and the precipitated melanin was centrifuged. Then melanin was dissolved in 0.1-0.5N NaOH solution. The use of sodium hydroxide solution increases the solubility of melanin, which improves its quality. To purify melanin from accompanying impurities, acid reprecipitation was carried out three times. A decrease in acid reprecipitation by less than two times leads to an increase in impurities; an increase by more than three times is irrational since it does not lead to a higher degree of purification. Next, melanin was subjected to double liquid-phase treatment of the aqueous residue with ethyl acetate in a volume ratio of aqueous and organic phases of 1:2. The ratio of melanin: ethyl acetate 1:2 is optimal for obtaining a homogeneous mixture that is stable under normal conditions. After mixing and settling, the mixture is divided into two layers: organic and aqueous. The top layer of the organic extract (which contains hydrophobic (lipid) components of melanin) is separated, and part of the ash substances passes into the dispersion

medium of the aqueous layer, due to which the ash content of melanin is reduced. After purification, the melanin was dialyzed and freeze-dried.

As a result of extraction, melanin was obtained - a dark brown powder. To determine whether the obtained samples belonged to the melanin category, qualitative reactions were carried out. It was found that melanin isolated from horse chestnut shells becomes discolored in the presence of a 10% solution of H_2O_2, $KMnO_4$, and, under the influence of $FeCI_3$, precipitates, which dissolves when $FeCI_3$ is added in excess. This behavior of the pigment under study is characteristic of melanins and indicates the presence of quinoid and phenolic components in the structure [15]. Determination of yield, ash content, and solubility for all obtained melanins was carried out according to standard methods given in the State Pharmacopoeia of the USSR.

Three-stage extraction leads to an increase in the yield of melanin due to a more complete extraction of melanin from the raw material. The analysis was performed in five replicates. The results are presented in Table. 1.

Table 1. Yield and ash content of melanin from chestnut shells before and after treatment with ether

Raw material	Yield, %	Humidity, %	Ash content, g/100 g		Melanin solubility in phosphate buffer, %	
			Before treatment with ether	After treatment with ether	pH 6,8	pH 7,2
1	25,1	10,5±0,1	3,59±0,1	1,2±0,1	99,1±0,1	93,1±0,1
2	24,5	11,4±0,1	3,29±0,1	1,16±0,1	98,6±0,1	90,3±0,3
3	24,6	10,2±0,1	3,18±0,1	1,14±0,1	97,1±0,1	91,3±0,1
4	23,3	11,8±0,1	2,98±0,1	0,9±0,1	98,9±0,1	94,2±0,1
5	24,6	11,3±0,1	3,44±0,1	1,09±0,1	96,8±0,1	92,1±0,1

As can be seen from Table 1, the ash content of melanin decreases after treatment with ether.

An important characteristic of melanins is their elemental composition, based on which their type and structural features can be established. The nitrogen and sulfur content of melanin allows them to be classified into eumelanins, pheomelanins, and allomelanins. Table 2 shows the data from the analysis of the elemental composition of melanin in horse chestnut shells, carried out in triplicate.

Table 2. Elemental composition of melanin from chestnut shells

Melanin	Estimated concentrations of elements, % wt.				
	C	H	N	S	O
1	44,10	6,23	0,71	0,35	48,60
2	43,81	6,34	0,69	0,38	47,80
3	44,01	6,27	0,72	0,38	47,43

Analysis of the elemental composition showed: that the nitrogen content in the isolated melanin molecule is 0.7%, which is significantly less than that of most other melanins, the amount of carbon is 44%, and hydrogen is 6%. Assessing the atomic ratios of these elements, it can be assumed that the proportion of aromatic structures in them does not exceed half the weight of the substance. Thus, based on these properties, by Britton's classification of melanin, the resulting melanin can be classified as a group of allomelanins.

The IR spectroscopy method made it possible to identify several functional groups in the obtained samples. The wide absorption band at 2926-3260 cm^{-1} refers to the stretching

vibrations of the OH groups of alcohols and phenols connected by inter- and intramolecular bonds. The structure of melanins contains phenolic fragments, which are represented on the graph by absorption bands at 1014-1097, 1141-1280, and 1320-1400 cm^{-1}, which correspond to stretching and bending vibrations of the C-O and OH groups of phenols. The presence of aromatic fragments in the structure of the studied melanins is evidenced by absorption bands of stretching vibrations of aromatic C-C bonds at wave numbers 1519-1602, and 1327-1409 cm^{-1}. Absorption bands in the range of 2850-2926 cm^{-1} confirm the presence of $-CH_2-$ fragments in the structure. In the frequency range 1519-1600 cm^{-1}, absorption bands corresponding to amino acid fragments are observed.

4 Conclusions

A technology has been developed for isolating melanins from non-traditional raw materials, horse chestnut shells. The differences in the technological scheme for isolating melanins from the standard method are the use of ultrasound in an ethyl alcohol environment to purify the raw materials and the most complete extraction of melanin, the use of 2-time purification with ethyl acetate, which leads to a decrease in the ash content of melanin. The total yield of melanin according to the developed scheme was 24.5%. The results of elemental analysis established that the isolated melanin contains 0.7% nitrogen, 44% carbon, and 6% hydrogen. The isolated melanin belongs to allomelanins. Absorption bands corresponding to melanins were observed in the IR spectra. The identified characteristics make it possible to justify the further use of the obtained melanin in pharmaceuticals for the development of highly effective dietary supplements and medications.

References

1. D.A. Bogaeva The scientific heritage, **65**, 3-4 (2021).
2. G.A. Arsakhanova Integral, **6**, 38-39 (2020).
3. M. Ekor Frontiers in Pharmacology, **4(177**) (2014).
4. T.V. Sambukova Phytopharmacology, **15(2),** 56-63 (2017).
5. V.A. Kurkin Fundamental research, **11,** 366-371 (2014).
6. E.A. Prutenskaya Biotechnology: reality and prospects: Materials of the international scientific and practical conference (Saratov), 68-70 (2014).
7. F. Solano Department of Biochemistry and Molecular Biology B & Immunology, 1-28 (2014).
8. M.A. Sysoeva Highly active antioxidants based on the lnonotus obliquus mushroom. Kazan: Publishing house: KazGTU. 2009. P. 294.
9. S.R. Pombeiro-Sponchiado, G.S. Sousa, J.C.R. Andrade, H.F. Lisboa, R.C.R. Gonçalves In book: Melanin. Ed.: M. Blumenberg. InTechOpen, 47-75 (2017), DOI: 10.5772/67375.
10. T.A. Puchkova, V.V. Trukhonovets, N.V. Ikonnikova Proceedings of the international scientific and practical conference Biotechnology of microorganisms, 27-29 (2019).
11. A.V. Filatova, D.T. Djurabaev, L.B. Azimova, A.S. Turaev Izv. Universities. Chemistry and chem. Technology, **65(7),** 88–93 (2022), DOI: 10.6060/ivkkt.20226507.6605.
12. D.S. Orlov Soil chemistry. M.: Moscow State University Publishing House, 527 (2005).

13. D.N. Olennikov Structural and functional study of biopolymers of plant and fungal origin and improvement of methods for their analysis. Ulan-Ude, 2012. P. 40.
14. A.S. Bukhalo, V.G. Babitskaya, N.A. Bis'ko, S.P. Vasser Collection of scientific papers in two volumes, **V.2,** 459 (2012).
15. T. Masuko, A. Minami, N. Iwasaki, T. Majima, S.I. Nishimura, Y.C. Lee Analytical Biochemistry, **339(1),** 69-72 (2004), DOI: 10.1016/j.ab.2004.12.001.