Chemistry of Natural Compounds, Vol. 48, No. 3, July, 2012 [Russian original No. 3, May-June, 2012]

PHYSICOCHEMICAL PROPERTIES AND ANTIOXIDANT ACTIVITY OF MELANIN FRACTIONS FROM *Inonotus obliquus* SCLEROTIA

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UDC 582.284:547.99

Six pigment fractions (total yield 52.13% of raw material mass) were isolated by fractionation of polymeric components of melanin from Inonotus obliquus *(Pers.) Pil. sclerotia. The isolated fractions differed in degree of aromaticity, molecular-weight distribution, and content of functional groups according to elemental analysis; UV, IR, and ^{13}C NMR spectroscopy; and gel chromatography. The dominant components were highly aromatic polymers of molecular weight 2–20 kDa with a high content of carboxylic and phenolic hydroxyls. It was found that fractions with a high degree of aromaticity and content of pyrocatechol groups exhibited pronounced antioxidant activity.*

Keywords: *Inonotus obliquus*, Hymenochaetaceae, melanin, ^{13}C NMR, antioxidant activity.

Inonotus obliquus (Pers.) Pil. is a basidial species of the family Hymenochaetaceae that is widely distributed in deciduous forests of Russia. The sterile form (sclerotia) of this species [*I. obliquus* (Pers.) Pil. f. sterilis (Vanin) Nicol], which is called Chaga, is medicinal raw material and is used in medical practice to treat various diseases. Chemical studies showed the presence in *I. obliquus* of triterpenes, phenolic compounds, polysaccharides, and essential oil [1]. A feature of the sterile form of this species is the ability to concentrate oligomeric (phelligridins, inoscavins, inonoblins, interfungins) [1] and polymeric pigments (melanin) [2–4]. Various extractants (H_2O, base solutions, EtOH) are used to isolate *I. obliquus* melanin. This enables preparations with excellent physicochemical properties (elemental composition, functional groups, molecular weight, etc.) to be prepared [2–6] and provides evidence that the polymeric part of the *I. obliquus* pigment complex is heterogeneous. The goal of the present work was to fractionate melanin from *I. obliquus* sclerotia and to compare the physicochemical properties of the resulting fractions.

Previously defatted raw material was extracted with 95% and 50% EtOH, H_2O, and KOH (5, 10, 15%) with subsequent acidic precipitation and purification of the resulting fractions in order to isolate polymeric pigment fractions of *I. obliquus* melanin. This produced six fractions, alcohol-soluble IOM-1 (95% EtOH) and IOM-2 (50% EtOH), water-soluble IOM-3, and base-soluble IOM-4 (5% KOH), IOM-5 (10% KOH), and IOM-6 (15% KOH). The yields were greatest for IOM-4 (16.86%) and IOM-6 (13.50%). The total content of the pigment fractions in *I. obliquus* was 52.13% (Table 1). Chemical analysis showed that the fractions did not contain carbohydrate and protein components. The ash index was less than 0.1%. The *I. obliquus* tissue remaining after the extraction was pigmented. However, colored components could not be removed by treatment with more concentrated base solutions, acids, and DMSO. A similar phenomenon was observed earlier for *Azotobacter chroococcum* [7] and *Ustilago maydis* [8] and indicated that part of the dark *I. obliquus* pigment was highly condensed with the structure of humic coal.

All isolated fractions gave qualitative reactions characteristic of melanin-type fungal pigments (precipitation by acids, $FeCl_3$, $AgNO_3$, bleaching with H_2O_2 and $KMnO_4$, dithionite–ferricyanide test) [9]. According to elemental analysis, the C content in *I. obliquus* melanin fractions was 48.63–60.17%; H, 4.48–5.92; O, 33.89–45.01; N, 0.19–0.49. Sulfur was not detected (Table 1). The H/C atom ratio varied from 0.96 (IOM-2) to 1.47 (IOM-5); O/C, from 0.42 (IOM-2) to 0.69 (IOM-5). An elevated H/C ratio is known to be indicative of higher aliphatic content; reduced, aromatic and condensed compounds.

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 0009-3130/12/4803-0396

TABLE 1. Yield and Elemental Compositions of *I. obliquus* Pigment Fractions

Fraction	Yield, %[a]	C, %	H, %	O, %	N, %	H/C	O/C	η[b]
IOM-1	7.74	60.17	5.75	33.89	0.19	1.15	0.42	2.57
IOM-2	5.30	57.46	4.61	37.55	0.38	0.96	0.49	2.46
IOM-3	5.07	54.59	4.48	40.53	0.40	0.98	0.56	2.55
IOM-4	16.86	55.08	4.81	39.71	0.40	1.05	0.54	2.59
IOM-5	3.66	48.63	5.92	45.01	0.44	1.47	0.69	3.16
IOM-6	13.50	55.31	4.71	39.49	0.49	1.02	0.54	2.57

[a]Of raw material mass; [b]structural parameter.

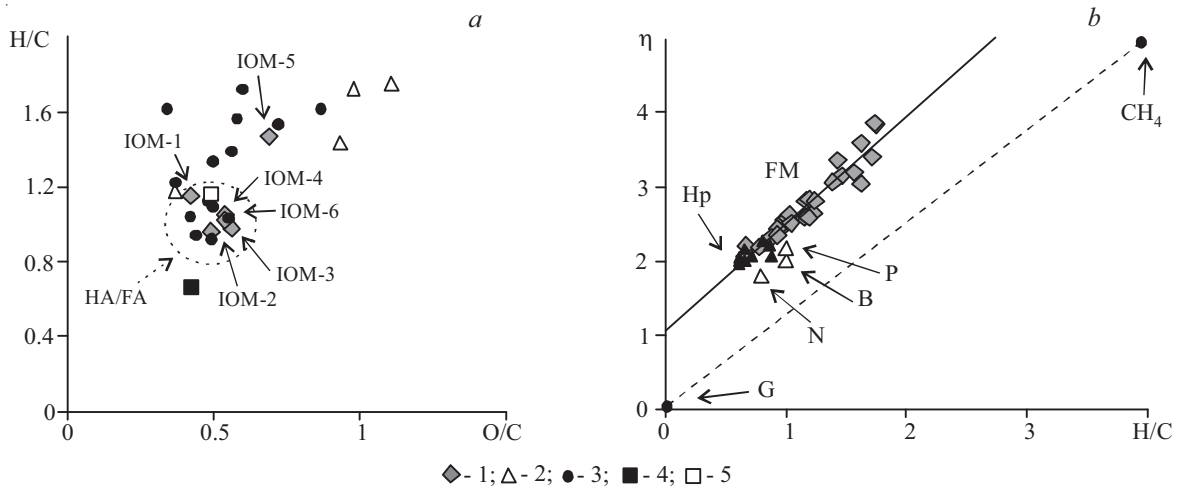

Fig. 1. Van Krevelen diagrams for fungal melanins and pure compounds (*a*) and C/H as a function of ·η (*b*): *I. obliquus* pigment fractions (1), *I. obliquus* melanin [4, 5] (2), fungal melanins [10, 11] (3), DOPA-melanin (4), pheomelanin [12] (5); HA/FA, zone of humic and fulvic acids of soils and peat; FM, fungal melanins [4, 5, 10–12]; Hp, hispidin derivatives [1]; B, benzene; G, graphite; N, naphthalene; P, phenol.

An analysis of the results and literature data for other samples of fungal melanins using Van Krevelen diagrams showed that the isolated fractions could be arbitrarily divided into groups of greater (IOM-1–IOM-4, IOM-6) and lesser degree of aromaticity (IOM-5) (Fig. 1*a*). The elemental composition of the first group was close to that of melanins from *Cerrena maxima* [10], *Aspergillus glaucus*, *Eurotium echinulatum*, and *Oidiodendron tenuissimum* [11] and synthetic pheomelanin [12]. The data for IOM-5 were similar to those for *Hendersonula toruloidea* and *Ulocladium atrum* [11] and early information on *I. obliquus* melanins [4]. The location of IOM-1–IOM-4 and IOM-6 in the zone of humic and fulvic acids of soils and peat may indicate a similarity to humic-like substances.

In addition, we used structural parameter η, which is independent of the molecular weight and was not used previously to analyze fungal pigments [13]. The range of η values for this group of compounds was 2.06–3.85; for *I. obliquus*, 2.46–3.16. A correlation analysis found that the H/C ratio was directly and linearly proportional to η. The regression equation was $\eta = 1.453 \cdot H/C + 1.057$ ($r^2 = 0.9078$), i.e., an increase of η was indicative of greater aliphatic content; a decrease, characteristic of aromatic and condensed components (Fig. 1*b*). The quantities H/C and η for oligomeric hispidin derivatives fell in the ranges 0.60–0.90 and 1.9–2.1, respectively. This was close to the lower boundary for fungal melanins and indicated that they had comparatively high aromaticity.

Absorption spectra of neutral solutions of all pigment fractions showed one distinct band in the range 220–230 nm (primary B-band). Weak bands in the range 390–410 nm indicative of the existence of conjugated carbonyls were seen for IOM-1 and IOM-2 [14]. The lack of bands in the long-wavelength region in absorption spectra of IOM-3–IOM-6 was consistent with a very low content of this type of chromophore. These bands were not observed in spectra of basic solutions.

Differential spectrophotometry of a version proposed earlier for studying lignin preparations was examined for possible application to spectral analysis of the *I. obliquus* pigment fractions [15]. Differential spectra of the studied melanins contained several maxima and inflections (shoulders) in the wavelength ranges 250 ± 5, 315 ± 6, 350 ± 10, and 510 ± 5 nm.

TABLE 2. Chromatic Coefficients (E_{465}/E_{665}), *a* Values in Linear Regression Equations, Specific Extinction Coefficient ($E^{1\%}_{1cm}$), and Content of Functional Groups in *I. obliquus* Pigment Fractions

Fraction	E_{465}/E_{665}	$a(r^2)$[a]	$E^{1\%}_{1\ cm}$	COOH, %	OH^{Ph}, %	OH^{PC}, %
IOM-1	7.80	–0.0038 (0.9969)	44.02	2.97	2.24	2.15
IOM-2	7.44	–0.0037 (0.9893)	63.03	5.20	4.49	1.97
IOM-3	6.26	–0.0038 (0.9968)	61.04	10.40	5.61	1.27
IOM-4	4.90	–0.0036 (0.9981)	47.04	4.46	4.21	2.08
IOM-5	6.50	–0.0039 (0.9902)	28.00	2.23	1.96	1.06
IOM-6	6.51	–0.0040 (0.9971)	48.02	5.94	5.05	1.39

[a]Determination coefficient values are given in parentheses.

TABLE 3. Molecular Weight, Relative Content, and Chromatic Coefficients (E_{465}/E_{665}) of *I. obliquus* Pigment Fractions

Fraction	Component	M_n, kDa (M_w/M_n)[a]	Content, %		E_{465}/E_{665}[b]
			in pigment fraction	in *I. obliquus*	
IOM-1	IOM-1-1	~ 90.0	2.6	0.20	7.14
	IOM-1-2	8.7 (4.02)	40.0	3.10	5.82
	IOM-1-3	2.8 (1.11)	54.2	4.20	6.22
	IOM-1-4	< 1.0	3.2	0.25	6.97
IOM-2	IOM-2-1	> 100	5.6	0.30	8.06
	IOM-2-2	6.3 (5.08)	41.7	2.21	6.33
	IOM-2-3	2.1 (1.43)	39.8	2.11	6.92
	IOM-2-4	< 1.0	12.9	0.68	7.14
IOM-3	IOM-3-1	> 100	21.6	1.10	5.81
	IOM-3-2	14.2 (1.90)	78.4	3.98	7.64
IOM-4	IOM-4-1	> 100	19.3	3.25	6.54
	IOM-4-2	7.8 (5.13)	80.7	13.61	4.95
IOM-5	IOM-5-1	> 100	22.7	0.83	11.43
	IOM-5-2	11.2 (4.29)	77.3	2.83	17.25
IOM-6	IOM-6-1	~ 90.0	29.3	3.96	7.17
	IOM-6-2	17.3 (1.56)	70.7	9.54	5.85

[a]Polydispersion values for dominant fractions are given in parentheses; [b]E_{465}/E_{665} values at elution maximum.

The presence of the first three maxima agreed well with experimental data for the effects of certain types of hydroxyls on the formation of the differential spectrum. They were due to a bathochromic shift of B-band absorption (250 ± 5 and 315 ± 6) and a shift of the K-band of phenolic structural units containing an α-carbonyl (350 ± 10). The appearance of maxima in the range 510 ± 5 for IOM-1–IOM-4 that were missing for IOM-5 and IOM-6 was the result of a bathochromic shift of long-wavelength absorption bands in the direct spectrum under the influence of base.

The logarithm of optical density was characteristically linearly dependent on wavelength for absorption spectra of all pigment fractions in basic solution (Table 2). Regression analysis showed that the determination coefficients (r^2) in the equations of this dependence ($\log D = a \cdot \lambda + b$) were 0.9893–0.9981 with the *a* values from –0.0039 to –0.0040. This agreed with earlier reported data for fungal melanins [16].

The chromatic coefficients (E_{465}/E_{665}) of the pigment fractions were 4.90–7.80 (Table 2). High values of E_{465}/E_{665} for IOM-1 (7.80) and IOM-2 (7.44) indicated that aliphatic and *O*-containing functional groups were present. The specific extinction coefficient ($E^{1\%}_{1cm}$) at 465 nm, which characterized the relative color grade of the solutions, was 28.00–63.03 (Table 2).

IR spectra of *I. obliquus* pigment fractions contained bands characteristic of melanin spectra at 3390–3430 cm^{-1} (OH), 2930-2970 (aliphatic C=H–, CH_2–, CH_3), 1700–1710 (carboxylic C=O), 1590–1600 (aromatic C=C– and/or C=O), 1500–1510 (aromatic ring C–C), 1450-1460 (aliphatic C–H, phenolic OH, COO^-), 1210–1230 (phenol C–H and C–O), 1125–1127 (ring vibrations and C–O), 1040–1050 (aromatic ethers), 770–890 (vibrations of H atoms of aromatic rings) [17]. IR spectra of the studied fractions were typical and differed in the relative intensity of several bands. Absorption in the range 2930–2970 cm^{-1} was greatest for IOM-1 and IOM-2. This indicated that they contained a significant amount of aliphatic groups in their structures. A weak band for a carboxylic carbonyl (1659 cm^{-1}) was characteristic of the IR spectrum of IOM-5.

TABLE 4. Relative Intensity of ^{13}C NMR Spectra Portions and Degree of Aromaticity (f_a) of *I. obliquus* Pigment Fractions, % of Total Intensity

Fraction	Range, ppm								f_a
	220–180	180–160	160–140	140–105	105–90	90–60	60–45	45–0	
IOM-1	17.7	3.4	11.9	46.6	8.5	0.5	0.2	11.2	0.59
IOM-2	21.0	5.6	8.6	40.5	4.1	1.5	3.9	14.8	0.49
IOM-3	14.1	12.7	14.4	43.3	3.1	0.8	0.6	11.0	0.58
IOM-4	7.5	6.7	18.0	34.1	3.9	1.7	3.2	24.6	0.52
IOM-5	21.6	2.6	6.3	33.9	8.7	7.7	5.4	13.7	0.40
IOM-6	11.5	3.6	12.6	43.2	3.0	1.0	0.8	24.3	0.56

Functional groups in *I. obliquus* pigment fractions were analyzed quantitatively using potentiometric and spectrophotometric methods (Table 2). It was found that the content of carboxylic groups varied from 2.23 (IOM-5) to 10.40% (IOM-3); of phenol hydroxyls, from 1.96 (IOM-5) to 5.61% (IOM-3); pyrocatechol hydroxyls, from 1.06 (IOM-5) to 2.15% (IOM-1).

The molecular-weight distribution showed that all melanin fractions were heterogeneous. The elution profiles had broad peaks. This indicated that the eluted components were polydisperse (Table 3). Alcohol-soluble fractions IOM-1 and IOM-2 were characterized by a low content of components with MW >90 kDa (2.6% for IOM-1; 5.6, IOM-2). Fractions corresponding to compounds with MW 2-9 kDa were dominant although a lower component dominated in IOM-1 (IOM-1-3, 2.8 kDa); a higher component, in IOM-2 (IOM-2-2, 6.3 kDa). Two elution portions for compounds with MW >90 kDa (19.3–21.6%) and 7-20 kDa (70.7-80.7%) were observed in water-soluble fraction IOM-3 and base-soluble fractions IOM-4–IOM-6.

Chromatic coefficients (E_{465}/E_{665}) that were determined at the elution maxima indicated that the dominant compounds in IOM-1, IOM-2, IOM-4, and IOM-6 were highly aromatic whereas the dominant compounds in fractions IOM-3 and IOM-5 had more aliphatic character (Table 3). The E_{465}/E_{665} values were in the range from 4.95 (IOM-4-2) to 17.25 (IOM-5-2).

Considering the information on the content of pigment fractions in *I. obliquus* raw material, it could be concluded that base-soluble components IOM-4-2 (13.61%) and IOM-6-2 (9.54%) were dominant. The dominant alcohol- and water-soluble compounds were IOM-1-3 (4.20%), IOM-3-2 (3.98), and IOM-1-2 (3.10). The total content of components with MW >90 kDa in *I. obliquus* was 9.64%; with MW 2–20 kDa, 41.58%.

Next *I. obliquus* pigment fractions were studied using ^{13}C NMR spectroscopy. Spectra of all studied preparations were similar and contained three groups of resonances in regions corresponding to absorption of aliphatic fragments (0–90 ppm), aromatic components (90–160 ppm), and carbonyls (160–220 ppm). Spectral portions in the range 105–160 ppm were the strongest. This indicated that the pigments were aromatic in nature. Use of a quantitative version of ^{13}C NMR found that the intensity of *O*-containing aromatic fragments (160–140 ppm) varied from 8.6 (IOM-2) to 14.4% (IOM-3) (Table 4). The intensity was much higher for aryl fragments without *O*-functional groups (105–140 ppm), from 33.9 (IOM-5) to 46.6% (IOM-1). The intensity of this group of resonances was greatest for all pigment preparations. It should be noted that distinct resonances for carbonyls of quinoids (180–190 ppm) and ketones (190–220 ppm) were present. The total content of this type of resonances was from 7.5 (IOM-4) to 21.6% (IOM-5). A resonance at 170 ppm that belonged to a carboxylic carbonyl (2.6–12.7% intensity) was present in all spectra. Portions of spectra corresponding to oxidized alkyl functional groups (105–60 ppm) and methoxyls were weak. The content of alkyl fragments (0–45 ppm) in the pigment fractions was from 11.24 (IOM-1) to 24.6% (IOM-4). The degree of aromaticity (f_a) fell in the range 0.40 (IOM-4) to 0.59% (IOM-1). Information from ^{13}C NMR spectroscopy confirmed data from elemental analysis, absorption spectra, IR spectroscopy, and functional group analysis.

The total antioxidant capacity (TAC) of the *I. obliquus* pigment fractions was from 150.51 (IOM-5) to 569.40 mg/g (IOM-1) (Table 5). The studied samples had the most pronounced activity for free radicals ($DPPH^{\bullet}$, $ABTS^{\bullet+}$) and certain active oxygen species ($O_2^{\bullet-}$, $OH^{\bullet}$, H_2O_2). The $IC_{50}^{DPPH^{\bullet}}$ values were 6.85–54.17 μg/g; $IC_{50}^{ABTS^{\bullet+}}$, 6.69–28.07 μg/g; $IC_{50}^{O_2^{\bullet-}}$, 6.11–67.24 μg/g; $IC_{50}^{OH^{\bullet}}$, 0.98–7.37 μg/g; and $IC_{50}^{H_2O_2}$, 5.21–11.92 μg/g. Significant amounts of chelating Fe^{2+} (Fe-CA) and Fe^{3+}-reducing/antioxidant power (FRAP) were found, 2.07–33.91 Fe^{2+}/mg and 0.96–3.73 mM Fe^{2+}/mg, respectively. High activities of IOM-1 (IC_{50} 14.62 μg/mL) and IOM-3 (IC_{50} 18.61 μg/mL) that were comparable with the effectiveness of gallic acid (IC_{50} 12.30 μg/mL) were found for a β-carotene oxidative degradation model. The studied fractions had a less noticeable influence on NO binding, from 335.24 (IOM-2) to >1000 μg/mL (IOM-5). Regression analysis established that fractions with a high content of pyrocatechols (OH^{PC}) and *O*-containing functional groups (O/C ratio) and degree of aromaticity (H/C ratio) showed high antioxidant activity (Table 6).

TABLE 5. Antioxidant Activity of *I. obliquus* Pigment Fractions

Index	Melanin fraction, compound							
	IOM-1	IOM-2	IOM-3	IOM-4	IOM-5	IOM-6	Gallic acid[e]	Trolox[e]
TAC[a]	569.40±23.35	495.55±18.82	242.64±10.19	447.31±17.44	150.51±6.02	312.85±12.83	1000	354.92±14.51
DPPH$^{\bullet}$[b]	6.85±0.20	8.19±0.25	27.31±0.81	10.95±0.33	54.17±1.63	13.35±0.40	0.98±0.03	16.37±0.49
ABTS$^{\bullet+}$[b]	10.00±0.23	6.69±0.16	12.95±0.34	8.75±0.21	28.07±0.67	10.75±0.30	0.76±0.02	12.61±0.32
NO [b]	352.61±18.33	335.24±17.76	453.61±22.60	690.63±34.52	>1000	345.23±17.60	268.35±12.95	148.93±7.44
$O_2^{\bullet-}$ [b]	6.11±0.24	11.63±0.44	16.27±0.65	21.04±0.84	67.24±2.68	27.15±1.08	7.63±0.31	24.61±0.98
OH$^{\bullet}$[b]	1.23±0.06	2.02±0.10	2.36±0.12	0.98±0.04	7.37±0.32	5.00±0.21	0.62±0.03	2.55±0.12
H_2O_2[b]	5.21±0.22	6.11±0.26	8.14±0.35	6.91±0.30	11.92±0.51	7.05±0.30	6.27±0.27	7.11±0.30
Fe-CA[c]	33.91±1.56	25.44±1.14	2.07±0.09	20.73±1.06	1.01±0.05	15.16±0.69	<0.01	<0.01
FRAP[d]	3.73±0.17	3.14±0.11	1.70±0.06	2.18±0.08	0.96±0.03	2.13±0.07	8.42±0.29	2.18±0.07
CBA[b]	14.62±0.44	24.29±0.73	18.61±0.53	29.09±0.87	92.38±2.77	32.48±0.97	12.30±0.37	29.15±1.02

[a]mg gallic acid/g; [b]IC_{50}, μg/mL; [c]mg Fe^{2+}/g; [d]mM Fe^{2+}/g; [e]reference compound.

TABLE 6. Determination Coefficients (r^2) of Linear Structure–Activity Relationships*

Activity index	Structure index					
	COOH	OH^{Ph}	OH^{PC}	H/C	O/C	η
TAC	0.0568	0.0057	**0.9445**	0.2254	**0.8391**	0.4642
DPPH$^{\bullet}$	0.0151	0.1313	**0.6904**	**0.6248**	**0.8382**	**0.8290**
ABTS$^{\bullet+}$	0.1083	0.3047	**0.5664**	**0.8273**	**0.7240**	**0.9486**
NO	0.1830	0.2636	0.2038	**0.6685**	**0.7056**	**0.8028**
$O_2^{\bullet-}$	0.0019	0.0105	0.4819	0.1391	**0.6639**	0.3212
OH$^{\bullet}$	0.0666	0.0876	**0.7013**	0.4907	**0.6514**	**0.6553**
H_2O_2	0.0182	0.0875	**0.6908**	**0.5754**	**0.9455**	**0.8226**
Fe-CA	0.1452	0.0358	**0.8613**	0.1296	**0.7827**	0.3224
FRAP	0.0270	0.0052	**0.7328**	0.2251	**0.9239**	0.4813
CBA	0.2490	0.2936	0.4103	**0.7760**	**0.7710**	**0.9255**

*Values with $r^2 > 0.5$ are shown in bold.

A comparison of data for the antiradical activity of hispidin derivatives [18] and the results of the present work showed that low-molecular-weight metabolites were less effective for the inactivation of free radicals. This could possibly be explained by a more complicated network structure for polymeric pigments that enabled them to act as free-radical traps.

The spectral properties of *I. obliquus* pigment fractions and hispidin derivatives indicate that they may be structurally similar and suggest that the low-molecular-weight compounds are biogenetic precursors of the polymeric compounds. The biosynthesis of the hispidin derivatives presumably goes from the acetate or 3,4-dihydroxybenzaldehyde through several additions of acetyls with subsequent decarboxylation and dimerization of the resulting intermediates [19]. This process is probably not completed by the formation of oligomers and eventually leads to the appearance of the more complicated structures that form the *I. obliquus* pigment complex.

Thus, the studies showed that the method for isolating melanin from *I. obliquus* sclerotia affects the physicochemical properties of the obtained preparations and their activity. The polymeric part of the *I. obliquus* pigment complex was a heterogeneous system, the components of which differed in degree of aromaticity, molecular-weight distribution, and functional group content. The dominant components were phenol polymers of molecular weight 2-20 kDa with a high content of carboxylic and phenol hydroxyls. All pigment fractions had pronounced antioxidant activity with the alcohol- and water-soluble components showing the highest effectiveness.

EXPERIMENTAL

I. obliquus sclerotia were collected in Pribaikal District, Republic of Buryatiya (Goryachinsk, 14 Aug., 2010, 52°98′84″ N, 108°28′95″ E). The collected raw material was cut into pieces, dried to constant mass at 60°C, and ground to particle size 0.5 mm. The species was determined by Cand. Pharm. Sci. G. V. Chekhirova (IGEB, SB, RAS). Specimens of *I. obliquus* are preserved in the herbarium of IGEB, SB, RAS (No. HY/fb-42/03-12/0810).

Elemental composition was determined on a 2400 Series II elemental analyzer (Perkin–Elmer). Spectrophotometric studies were carried out on an SF-2000 spectrophotometer (OKB Spektr). IR spectra were recorded from films on ZnSe windows on an FT-801 IR-Fourier spectrometer (Simeks) in the range 4000–600 cm^{-1}. Potentiometric studies were performed using a pH-410 pH-meter (Aquilon). ^{13}C NMR spectra were recorded from NaOD solutions (1%) of compounds on a VXR 500S NMR spectrometer (Varian) at operating frequency 125.7 MHz. Quantitative analysis of ^{13}C NMR spectra used the MestReC 4.9.9.6 program set (Mestrelab Research). The degree of aromaticity (f_a) was calculated as before [20].

Qualitative reactions were performed according to the literature [9]. The carbohydrate content was determined by the anthrone–H_2SO_4 method and recalculated for glucose [21]. Protein was estimated by the Bradford method [22]. Ash content was measured gravimetrically after ashing at 600°C. Carboxylic (COOH) and phenolic hydroxyls (OH^{Ph}) were found potentiometrically [10]; pyrocatechol hydroxyls (OH^{PC}), spectrophotometrically recalculated as pyrocatechol (Fluka) [23].

Isolation of Pigment Fractions. Dried and ground *I. obliquus* raw material (120 g) was extracted in a Soxhlet apparatus successively by C_6H_{14} and $CHCl_3$. The defatted raw material was dried for complete removal of the solvents and extracted by EtOH (96%) on a boiling-water bath (1:50, 7×, 120 min). The EtOH extracts were filtered, diluted with H_2O (~10:1), and concentrated in vacuo at 30°C to ~500 mL. The aqueous residue was extracted with $CHCl_3$, acidified with H_2SO_4 (10%) until the pH was 2.0, and left at room temperature for 6 h. The resulting precipitate was centrifuged (30 min, 6,000 g), suspended in HCl (20%), and left for 5 d at 5°C. The resulting mixture was centrifuged (30 min, 6,000 g). The solid was washed with H_2O, dried, and stored in a desiccator over P_2O_5 to afford fraction IOM-1 (9.288 g). Then, raw material was worked up analogously with EtOH (50%) to afford fraction IOM-2 (6.368 g).

After the alcohol extraction, raw material was worked up with H_2O on a boiling-water bath (1:20, 10×, 60 min). The aqueous extract was centrifuged (30 min, 9,000 g). The resulting supernatant was acidified with H_2SO_4 (10%) until the pH was 1.0 and left at 4°C for 12 h. The resulting precipitate was centrifuged (20 min, 6,000 g), washed with H_2SO_4 (2%) and HCOOH (5%), worked up with HCl (20%) as described above, and dried to afford fraction IOM-3 (6.080 g). The raw material remaining after alcohol and water extractions was treated successively with KOH solution (5%) containing $NaBH_4$ (1%) at 20°C (1:10, 5×, 180 min), KOH (10%) containing $NaBH_4$ (2%) at 20°C (1:10, 7×, 60 min), and KOH (15%) containing $NaBH_4$ (5%) at 90°C (1:10, 5×, 60 min). The basic extracts were acidified with H_2SO_4 (20%) until the pH was 1.0 and left at 4°C for 12 h. The resulting precipitates were centrifuged (20 min, 6,000 g), washed with H_2SO_4 (2%) and H_2O, worked up with HCl (20%), and dried to afford fractions IOM-4 (20.240 g, 5% KOH, 20°C); IOM-5 (4.384 g, 10% KOH, 20°C); and IOM-6 (16.196 g, 15% KOH, 90°C).

IOM-1. UV spectrum (λ_{max}, nm), pH 6.0: 226, 284sh, 409; pH 12.0: 224; diff. 221, 246, 330, 515. IR spectrum (ν, cm^{-1}): 3399, 2967, 2935, 1703, 1592, 1504, 1461, 1417, 1374, 1327, 1294, 1228, 1166, 1127, 1087, 1044, 878, 822, 773, 616.

IOM-2. UV spectrum (λ_{max}, nm), pH 6.0: 224, 391; pH 12.0: 223; diff. 220, 314, 510. IR spectrum (ν, cm^{-1}): 3427, 2943, 1704, 1594, 1511, 1460, 1417, 1294, 1224, 1126, 1038, 876, 817, 774, 618.

IOM-3. UV spectrum (λ_{max}, nm), pH 6.0: 226; pH 12.0: 224; diff. 219, 314, 349, 507. IR spectrum (ν, cm^{-1}): 3412, 2944, 1706, 1596, 1502, 1452, 1416, 1285, 1213, 1126, 1048, 883, 773, 611.

IOM-4. UV spectrum (λ_{max}, nm), pH 6.0: 223; pH 12.0: 224; diff. 222, 321, 353, 509. IR spectrum (ν, cm^{-1}): 3412, 2941, 1709, 1596, 1502, 1461, 1420, 1325, 1222, 1126, 1045, 871, 831, 773, 616.

IOM-5. UV spectrum (λ_{max}, nm), pH 6.0: 210; pH 12.0: 219; diff. 250, 354. IR spectrum (ν, cm^{-1}): 3219, 3085, 2929, 2855, 1659, 1590, 1501, 1454, 1421, 1330, 1226, 1125, 1047, 835, 773, 616.

IOM-6. UV spectrum (λ_{max}, nm), pH 6.0: 224; pH 12.0: 224; diff. 215, 253, 360. IR spectrum (ν, cm^{-1}): 3401, 2942, 1709, 1603, 1503, 1454, 1421, 1322, 1221, 1127, 1048, 870, 773, 614.

Spectroscopy in the UV and Visible Spectral Regions. A weighed portion (10 mg) of pigment fraction was dissolved in DMSO (1 mL), treated with EtOH (50%, 20 mL), filtered through a quartz filter (Universil) into a 25-mL volumetric flask, and adjusted to the mark with EtOH (50%) (stock solution). Working solutions were prepared by transferring stock solution (2 mL) into two 25-mL volumetric flasks and adjusting to the mark with buffer solutions at pH 6.0 (495 mL of 0.2 M KH_2PO_4

+ 113 mL of 0.1 M NaOH + 1392 mL of distilled H_2O; solution A) and pH 12.0 (400 mL of 0.1 M $Na_2B_4O_7$ + 600 mL of 0.1 M NaOH; solution B). The reference solutions for determining absorption spectra were the corresponding buffer solutions. The test solution for recording the differential spectrum was solution B; the reference solution, solution A. Calibration curves of the logarithm of optical density as a function of wavelength were constructed from the resulting absorption spectra in basic solution. Regression analysis was carried out using the Advanced Grapher 2.11 software (Alentum Software Inc.). The chromatic coefficients (E_{465}/E_{665}) and specific extinction coefficient ($E^{1\%}_{1cm}$) were determined as described earlier [24].

Gel-chromatography. The studies were carried out over a column of Sephadex G-100 (2 × 90 cm, 0.5% $NaHCO_3$ eluent, flow rate 200 μL/min; Pharmacia) connected to an SF-2000 flow detector (OKB Spektr) at 280 nm. Quantitative analysis of the gel-chromatograms was performed using the Leonardo 1.01 program (Nauka Plyus).

During the study of the antioxidant activity of *I. obliquus* melanin fractions, we determined the total antioxidant capacity (TAC) [25]; antiradical activity relative to the radical 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$, MP Biomedicals Inc.) [26] and the cation-radical 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$, Sigma) [27]; binding of nitric oxide (NO) [28]; inactivation of superoxide radicals ($O_2^{\bullet-}$) [29], hydroxyl radicals ($OH^{\bullet}$) [30], and hydrogen peroxide (H_2O_2) [31]; Fe^{2+}-chelating activity (Fe-CA) [32]; Fe^{3+}-reducing/antioxidant power (FRAP) [33]; and oxidative destruction of β-carotene (CBA) in the system β-carotene:oleic acid:DMSO:H_2O_2 [32]. Gallic acid and trolox (Fluka) were used as standards.

Regression analysis was carried out using the Advanced Grapher ver. 2.2 software (Alentum Software Inc.).

ACKNOWLEDGMENT

The work was supported financially by the Lavrent′evskii Competition of Young Scientists, SB, RAS.

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