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NATURAL DRUGS

ANALYSIS OF AQUEOUS EXTRACT OF *INONOTUS OBLIQUUS*

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Abstract: Water-soluble melanin complexes were extracted with hot water from *Inonotus obliquus* fungus. They were characterized before and after reaction with diluted hydrochloric acid. The organic components as products of degradation of melanin complexes were separated by column chromatography and analyzed by GC-MS method.

Keywords: *Inonotus obliquus*, hot water extraction, melanins, column chromatography, separation, GC-MS analysis

Inonotus obliquus fungus was used in folk medicine in East Europe and Western Siberia due to its antitumor properties (1), and some activity against cardiac, liver, and stomach diseases, as well as tuberculosis drug (2). In some cases the therapeutic efficiency of *Inonotus obliquus* has been analytically proven. Thus, the extracts from *Inonotus obliquus* were efficient in HIV-1 treatment, stomach ulcer (3) and indicated the cytostatic properties against sarcoma 180, carcinoma 755 (4), Walker 256 carcinosarcoma and MCF-7 (human mammary adenocarcinoma) growth (5,6). Medical effects were assigned to triterpenes of lanostane type, which are extractable from *Inonotus obliquus* with ethanol (6,7) or hexane (4,8,9).

The studies of water extracts of *Inonotus obliquus* were aimed at isolation and identification of melanin complexes (10-12) and other pigments (13). Due to limited solubility of these compounds in most organic solvents the major method of their characterization was the IR spectroscopy (14,15). Organic compounds, mostly phenol and carboxylate types were isolated from water or water-ethanol extracts as described in early reports (16-19). Ethanolic extract contained mainly triterpene acids, while the residue was the mixture which was separated and analyzed by column chromatography and/or fractional extraction. Using that procedure the appropriate phenols, acids, and esters were determined in particular extract fractions and the fresh fungus.

In this paper the analytical results on separation and identification of compounds in water fraction are reported.

EXPERIMENTAL

Preparation of source material

Spores of *Inonotus obliquus* (received from Dary Natury, Koryciny 71; 17-315 Grodzisk) were purchased as finely grinded pieces, which were dried at 60°C, grinded and dried again at 60°C *in vacuo* to constant mass.

Extraction

300 g of grinded spores of *Inonotus obliquus* in 2000 cm^3 of water was refluxed for 14 h. Solid residue was filtered off, while syrupy aqueous phase was concentrated on rotary evaporator to dense syrup, which was vacuum dried at 80°C to constant mass. Yield 59.9 – 61.0 g of extract **1** (19.9 – 20.3% of mass of dry fungus).

Separation procedure

10.2 g of dry extract **1** was dissolved in 100 cm^3 H_2O and 1.5 cm^3 of concentrated hydrochloric acid was added. The solution was refluxed for 5 h. The insoluble material **2** appeared (6.6 – 6.8 g), which was collected and solution was reconcentrated by vacuum distillation to give another portion of solid material **3** (3.4 – 3.6 g). The water-insoluble fraction **2** and **3** were washed with 50 cm^3 of hot ethyl acetate and 3×50 cm^3 of hot methanol and the filtrates were combined. The organic solvents were stripped out to give gum-like material **4** (2.2 g).

Column chromatography

Further separations were performed chromatographically on Kieselgel 60. The components were

eluted with: ethyl acetate, ethyl acetate – methanol (2:1, 1:1, 1:2, v/v), and finally with pure methanol. The 25 cm^3 fractions were collected and analyzed by TLC (eluent: ethyl acetate-hexane 4:1, v/v).

Methods and instruments

IR spectra (KBr pellets) were recorded on Perkin-Elmer Paragon 1000FT spectrophotometer. Atomic absorption spectra with induction generated plasma were obtained on Varian Vista – MPX instrument with horizontal CCD and simultaneously ICP--DES. The results were calculated in mg per g of dry content of extract. Thermogravimetric analysis was performed on MOM (Hungary) apparatus (registration time 100 min., temperature range 20-100°C, sensitivity of measurement DTA 1/10, DTG 1/10). Elemental analysis (C, H, N) was done on EA 1108 analyzer (Carlo Erba). GC-MS analyses were obtained with Agilent 6890N gas chromatograph (FID detector for quantitation) equipped with HP1MS column and 5973 Network mass spectrometer (carrier gas He, electron impact 70 eV). Component identification was done by comparison with spectra in the Mass Spectral Database (SDBS, Kovats and an in--house database) and in some cases direct GC-MS comparison with authentic standards.

RESULTS AND DISCUSSION

The average 20% of solid residue has been extracted from dry fungus with hot water by 14 h. Prolongation of extraction time did not improve the yield. When the solvent was evaporated, the residue was glassy black solid of ether smell, soluble in water, and insoluble in organic solvents, even at elevated temperatures. The solid did not melt below 400°C, indicating only the release of water. Therefore, the water could not be removed completely upon heating under reduced pressure for long time. This property was noticed before (14) as well as remarkable hygroscopic properties of melanin-type products. The degradation of the material starts at 100°C and the 35% mass loss takes place within 100-400°C and later the maximum endothermic peak at 740°C is observed (Figure 1). The 60% mass loss occurs up to 1000°C, the rest is an inorganic residue.

Elemental analysis of water extract gave the following results: C 46.77-49.13, H 3.73-3.81, N 0-0.06. The IR spectrum showed intense and broad band centered at 3387 cm^{-1} corresponding to stretching vibrations of OH and NH groups and the band at 1591 cm^{-1} of CO valence band and aromatic ring. Also the band centered at 1400 cm^{-1} from deprotona-

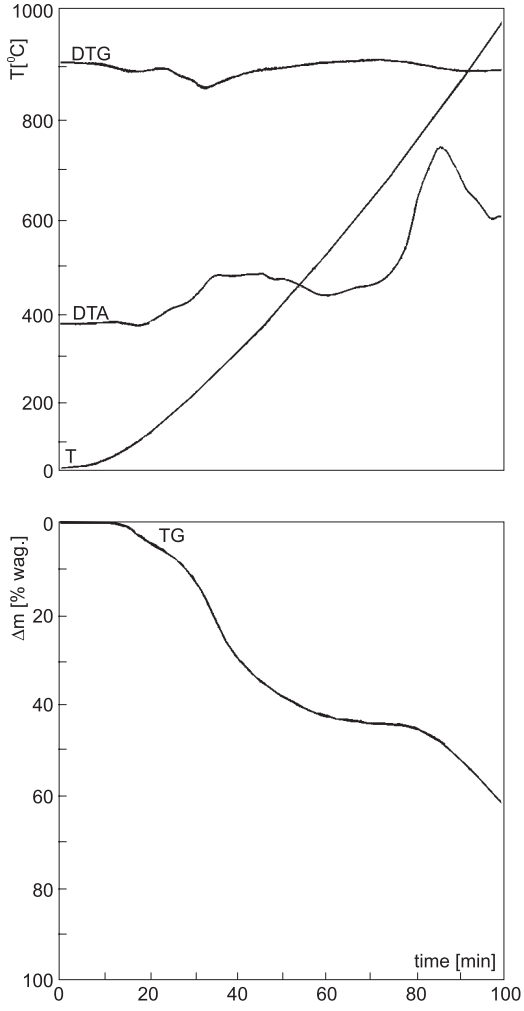

Figure 1. Thermal analysis of water extract **1**.

Table 1. AS analysis of water extract of *Inonotus obliquus*.

No.	Element	mg/g
1.	Aluminium	0.052
2.	Boron	0.033
3.	Barium	0.0108
4.	Cadmium	0.00036
5.	Chromium	< 0.00025
6.	Copper	0.0079
7.	Iron	0.035
8.	Manganese	0.15
9.	Nickel	< 0.00075
10.	Lead	< 0.00125
11.	Zinc	0.10
12.	Calcium	0.39
13.	Magnesium	2.1
14.	Sodium	0.18
15.	Potassium	55.0
16.	Organic carbon	450.0

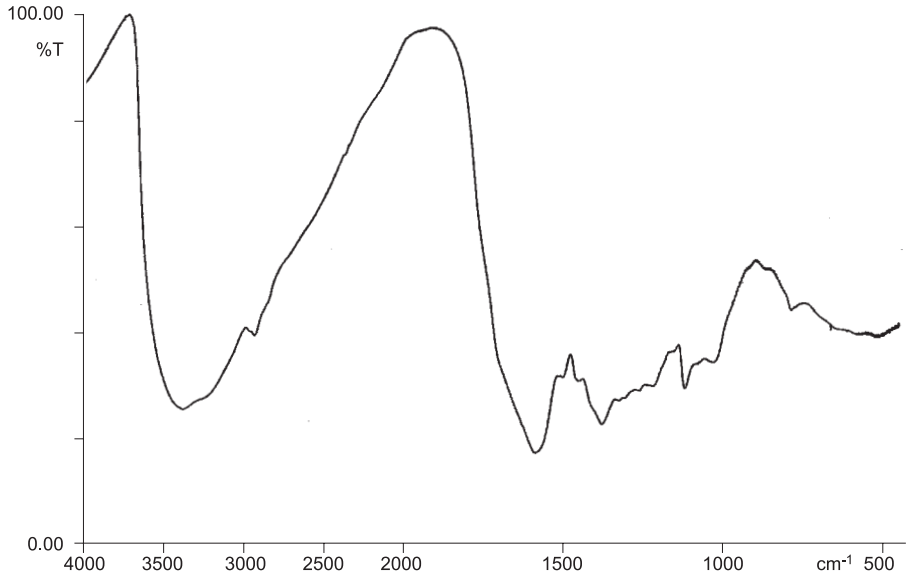

Figure 2. IR spectrum of water extract **1**.

Table 2. Some compounds extracted from *Inonotus obliquus*.

No.	Compound	Percentage content in eluate	Content [mg/g of dried fungus]
1.	Benzaldehyde	20.26	4.58
2.	Benzyl alcohol	10.87	2.46
3.	Syringol (2,6-dimethoxyphenol)	5.27	1.19
4.	Dibutyl phthalate	5.10	1.15
5.	2-(1,4,4-trimethylcyclohex-2-en-1-yl)ethyl acetate	2.59	0.59
6.	4-Oxopentanoic acid	1.15	0.26
7.	Vanillic acid	0.94	0.21
8.	Docosane	0.89	0.20
9.	Hexatriacontane	0.83	0.19
10.	Retinol (O-acetyl-all-*trans*-)	0.67	0.13
11.	Resorcinol	0.62	0.14
12.	Hexadecanoic acid	0.54	0.12
13.	Heneicosane	0.50	0.11
14.	3-hydroxy-4,5-dimethoxybenzoic acid	0.44	0.10

ted COO^- groups was visible. Although the spectrum originate from a mixture of compounds, some characteristic absorptions within the region 1263 – 1032 cm^{-1} attributed to C–O and C–O–C bonds in alcohols, ethers, and phenols can be seen (Figure 2).

Due to good solubility of extracts in water the atomic spectrometric studies of the samples were available. The results of the determination are collected in Table 1. The striking result is high content of potassium and magnesium in the extract if compared with low content of sodium and calcium. Other elements are present in trace amounts. The results confirm clearly the high affinity of melanins to metal ions. The counteranions for metal ions are carboxylic, hydroxyl and amine groups, which are able to construct biosorption centers for non-transition metal ions. Melanins are also responsible of transition metals uptake by fungi (20), in a similar way like humic acids.

Some components of aqueous extract **1** can be transformed into solids **2** and **3**, when heated with diluted hydrochloric acid. These solids are partially

soluble in ethyl acetate and methanol and can be recovered from filtrates as black solid **4**. The latter is heat-resistant black material, insoluble either in water or in organic solvents. It can be easily dissolved in =20% NaOH (aq.), however, it is further converted under these conditions accompanied by darkening and precipitation especially upon heating.

Elemental analysis for **2** gave the following percentages of common organic elements: C 57.88 –59.00, H 4.22 – 4.50, N 0.32 – 0.40, S 0.00 – 003. The solid decomposes uniformly upon heating unlike **1**. Endothermic peaks are observed within 300 – 400°C region accompanied by water release and melanin decomposition as observed morphologically under microscope. Slight shoulder on TG curve observed at *ca*. 100°C results from the release of melanine-absorbed water (Figure 3).

IR spectrum of **2** shows broad absorption centered at 3376 cm^{-1} from OH and NH. Two bands at 1707 and 1597 cm^{-1} correspond to CO and C_{ar}. The absorption within the region of 1029–1210 cm^{-1} is attributed to C–OH i C–O–C bonds (Figure 4a). The IR spectrum of **3** resembles that of **2** (Figure 4b). TLC analysis of **3** shows multicomponent mixture of overlapping species with various R_f. As can be observed, the diluted hydrochloric acid decomposes melanin complexes into primary structures with concomitant formation of low-molecular weight compounds. The aldehyde groups are present in this mixture, which are able to reduce Tollens reagent. The latter were already found (18). A straightforward reaction of **3** with 2,4-dinitrophenylhydrazine resulted in formation of the precipitate of broad melting temperature range (156-190°C).

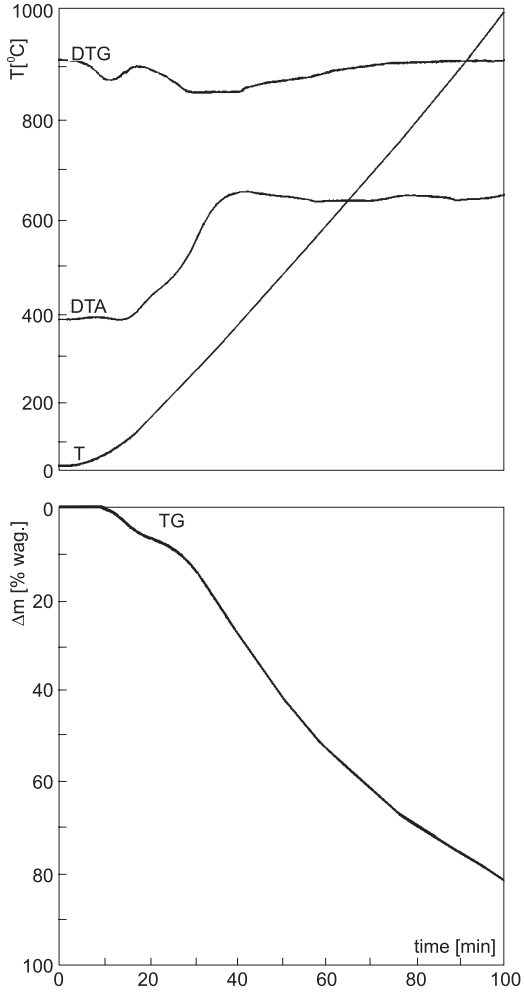

Figure 3. Thermal analysis of melanin precipitate **2**.

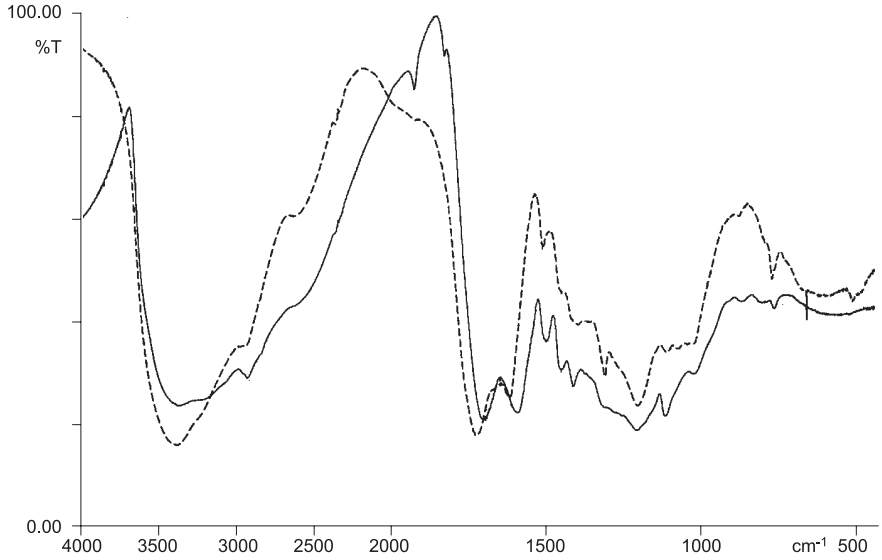

Figure 4. IR spectra of products **2**(a) and **3**(b).

Organic fraction **4** was preliminarily separated by column chromatography. Using the ethyl acetate/methanol mixtures six fractions were collected by grouping aliquots. Single components were present in one or at most two aliquots of total number of 50 aliquots. The collected fractions had different masses; the weight % of particular fractions were:
fraction I – 75.65; fraction II – 10.80; fraction III – 4.49
fraction IV – 3.43; fraction V – 4.51; fraction VI – 1.12

The best resolution of separation was further obtained for fractions I and II, eluted with pure ethyl acetate. A majority of identified compounds (phenols, free acids, and esters) were found in those fractions. Some hydrocarbons were found in fraction V.

The percentage of compounds in **4** was calculated based on integral intensity of peaks in GC. The content of particular components varied between trace to almost 20%. The identified compounds are listed in Table 2. The identity of major components was confirmed by MS analysis. Further studies on identity of some minor components are currently in progress.

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