Acta Poloniae Pharmaceutica – Drug Research, Vol. 67 No. 4 pp. 397–406, 2010 ISSN 0001-6837
Polish Pharmaceutical Society

SEPARATION OF AN AQUEOUS EXTRACT *INONOTUS OBLIQUUS* (CHAGA). A NOVEL LOOK AT THE EFFICIENCY OF ITS INFLUENCE ON PROLIFERATION OF A549 HUMAN LUNG CARCINOMA CELLS

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Abstract: Aqueous extract of *Inonotus obliquus* was hydrolyzed in dilute hydrochloric acid. The products were extracted applying organic solvents, and separated chromatographically on a silica gel-packed column. Eluted fractions were analyzed by means of GC-MS. The presence of hydrocarbons, alcohols, phenols and various carbonyl compounds in analyzed fractions has been detected and quantified. Preliminarily experiments on the influence of certain separated samples on the proliferation of A549 human lung carcinoma cells were performed. Therefore, we hypothesize that the major antiproliferative effects are related to the presence of benzaldehyde, which is a benzyl alcohol metabolite formed *in situ* in the cells culture with the yield moderated by the presence of trace amounts of "high molecular mass compounds".

Keywords: *Inonotus obliquus,* extraction, hydrolysis, column chromatography, GC-MS analysis, human lung carcinoma (A549)

Therapeutic properties of *Inonotus obliquus* (chaga) are known and utilized for a long time in traditional folk medicine of Eastern Europe and Western Siberia. Traditionally, the powdered fungus fruiting bodies, or decoction made of it (called chaga tea), are used for stomach diseases or as a pain reliever. The extracts of the fungus, produced on the commercial level, are claimed to be advantageous for cure of digestive system, liver and skin diseases, tuberculosis, and for treatment of intestinal worms as well as different kinds of tumor (1, 2).

The scientific interest of the fungus dates back to the middle of the last century thanks to research of scientists representing mainly Eastern European and Northern Asian countries. In the course of the extensive research on aqueous and organic extracts from chaga a plethora of compounds have been isolated, which presence in chaga can justify its broad therapeutic properties (2, 3).

An extract from chaga sporocarp shows immunomodulatory, anti-oxidant, anti-inflammatory, anti-nociceptive, cytotoxic and anticancer properties (2, 3). Results of recent study demonstrate that chaga extracts alleviates many of the symptoms of diabetes in genetically obese mice and may offer a possibility as a therapeutic supplement for the normalization of blood glucose levels in human with hyperglycemia, and have beneficial effects in patients with non-insulin-dependent diabetes mellitus (4).

Just recently the group of Raekil Park (5, 6) have performed very extensive studies that provided

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many mechanistic details concerning chaga anticancer activity. They have shown that the water chaga extract of undefined composition exhibited anticancer activity against human hepatoma HepG2 cells (*in vitro*) and murine B16-F10 melanoma cells (*in vitro* and *in vivo*) through the inhibition of proliferation and induction of differentiation and apoptosis of cancer cells.

In this work we made an another attempt for detailed identification and quantification of the content of organic compounds present in the aqueous extracts from the sporocarp of *Inonotus obliquus*, which seem to be the most common therapeutic form of the fungus[1]. Furthermore, the influence of isolated fractions on proliferation of human lung carcinoma cells (A549) was tested. The attempt to correlate of particular chemical composition of the fractions with their anticancer activity seems important since composition can vary with a batch of the natural product, which may depend on various endogenous and exogenous factors.

EXPERIMENTAL

Plant material

Fine grinded fruiting bodies of *Inonotus obliquus* were obtained from herbal wholesaler Dary Natury (Grodzisk, Poland). Before extraction, a crude material was dried under vacuum at temperature of 60°C, till constant mass was obtained.

Extraction and hydrolysis

The aqueous extract was obtained by heating of 300 g of the fungus fruit bodies suspended in 2000 mL of boiling water for 14 hours, following the procedure presented by us in (7). After separation of solid residue, aqueous solution was concentrated by evaporation of water and vacuum drying to constant mass at 80°C, obtaining ca. 20% of dry extract as black glassy precipitate **IO1** (**IO** stays for ***I**nonotus **o**bliquus)*. Thirty three grams of the precipitate **IO1** was dissolved in 300 mL of hot water then 15 mL of concentrated hydrochloric acid was added. The whole sample was heated for 7 h under reflux. After that, black solution containing visible fine suspension was stored overnight in order to collect precipitate **IO6** (19.1 g). From remaining filtrate, after evaporation of water, 10.5 g of solid material **1** was obtained. The sediments **IO6** and **1** were leached using 100 mL of ethyl acetate and 3 × 50 mL of methanol. Organic extracts were combined then, after removing of organic solvents using a rotary evaporator, the black gunk-type substance **IO7** (7.8 g) was obtained. (Scheme 1 shows the outline of the separation procedure.)

Column chromatography

The material **IO7** was subjected to the chromatography on silica gel-packed column (silica gel 60, Merck, column 3.5 × 50 cm) with eluents used in turn: hexane, toluene, ethyl acetate and methanol. The progress of the column chromatography was monitored by TLC chromatography on silica gel plates (Fluka), using the mixture of n-hexane with ethyl acetate (4 : 1, v/v) as the eluent.

GC-MS analysis

The GC-MS measurements were performed using Agilent 6890N gas chromatograph with HP1HS capillary column, FID detector and He as a carrier gas, coupled to 5978 Network mass spectrometer of electron impact 70 eV.

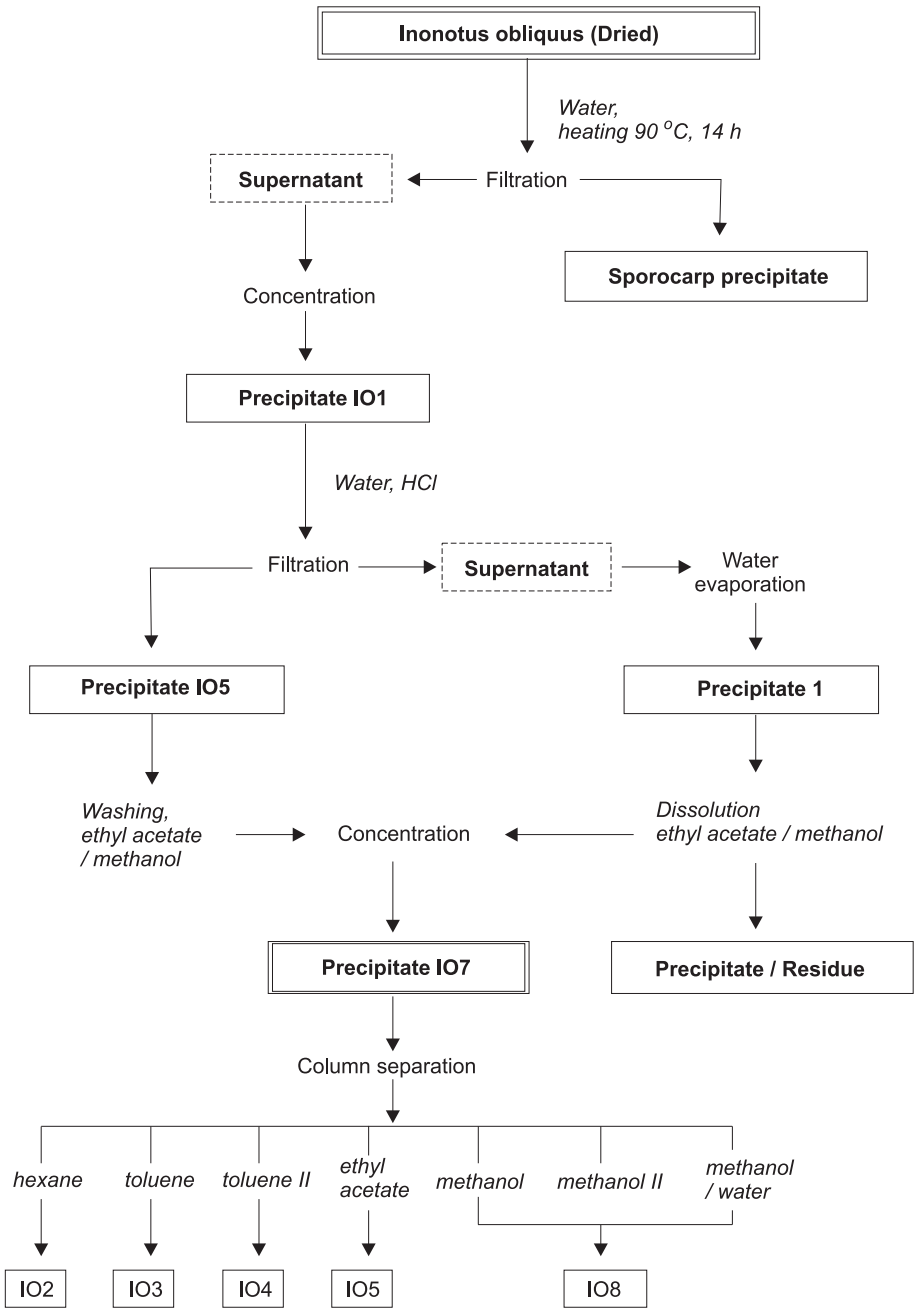
Extract for cell proliferation screening

Dry extracts **IO1**-**IO8** were dissolved in DMSO yielding stock solutions of concentrations 1, 10 and 100 mg/mL, respectively. The stock solutions were stored at 4°C. Prior to usage, the stock solutions were diluted in the fresh culture media supplemented with FBS (see below). The final DMSO concentrations in the culture media were non-toxic. Their cytotoxicity was examined on the cell line (A549) applying MTT assay.

Cell lines and cultures

Human lung carcinoma (A549) cells were obtained from the Institute of Immunology and Experimental Therapy (Polish Academy of Sciences, Wrocław, Poland). Cells were grown in 3:1 mixture of DMEM (Sigma, St Louis, MO, USA) and DMEM F-12 HAM (3:1) (Sigma). Culture medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma), penicillin (100 U/mL) (Sigma) and streptomycin (100 µg/mL) (Sigma). Cultures were kept at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

[1] In many countries ground sporocarps of *Inonotus obliquus* together with *Piptoporus betulinus* are distributed by herbal wholesalers as parapharmaceuticals or dietary supplements intended for preparation of aqueous and alcohol extracts strengthening the organism after anticancer chemotherapy.



Scheme 1. Preparation of *Inonotus obliquus* extracts

Cell proliferation and viability assessment

Yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) was metabolized by viable, metabolically active cells to blue formazan crystals. SDS buffer pH 7.4 was added to dissolve the insoluble blue formazan crystals into a colored solution. The absorbance of this colored solution was quantified at 570 nm wavelength using a spectrophotometer E-max Microplate Reader, as described above. The optical density was directly proportional to the amount of live or proliferating cells.

A549 tumor cells were plated on 96-well microplates at a density of 1×10^4 cells/ mL. On the

following day, the culture medium was removed and cells were exposed to serial dilutions of tested extracts in the fresh medium supplemented with 10% FBS. Cell proliferation was assessed after 96 h by means of MTT assay. Cells were incubated for 3 h with MTT (Sigma) solution (5 mg/mL). Formazan crystals were solubilized overnight in SDS buffer pH 7.4 (10% SDS (Sigma) in 0.01 M HCl and the product quantified spectrophotometrically by measuring the absorbance at 570 nm wavelength using E-max Microplate Reader (Molecular Devices Corporation, Menlo Park, CA, USA).

Statistics

Data were presented as the mean value and standard error of the mean (SEM). Statistical analysis was performed using the one way-ANOVA with Tukey *post-hoc* test, significance was accepted at $p < 0.05$.

RESULTS AND DISCUSSION

Water-soluble extract **IO1** amounts to ca. 20% of the dry content of *Inonotus obliquus* fruiting body. The extract contains melanine pigments, salts of metal ions and low-weight compounds combined together in chromogenic complexes. It is almost solely water-soluble and hardly soluble in common organic solvents. The aqueous solubility falls down after careful drying or after long-term storage under atmospheric air. For the complete dissolution of **IO1** a heating of the aqueous solution is required.

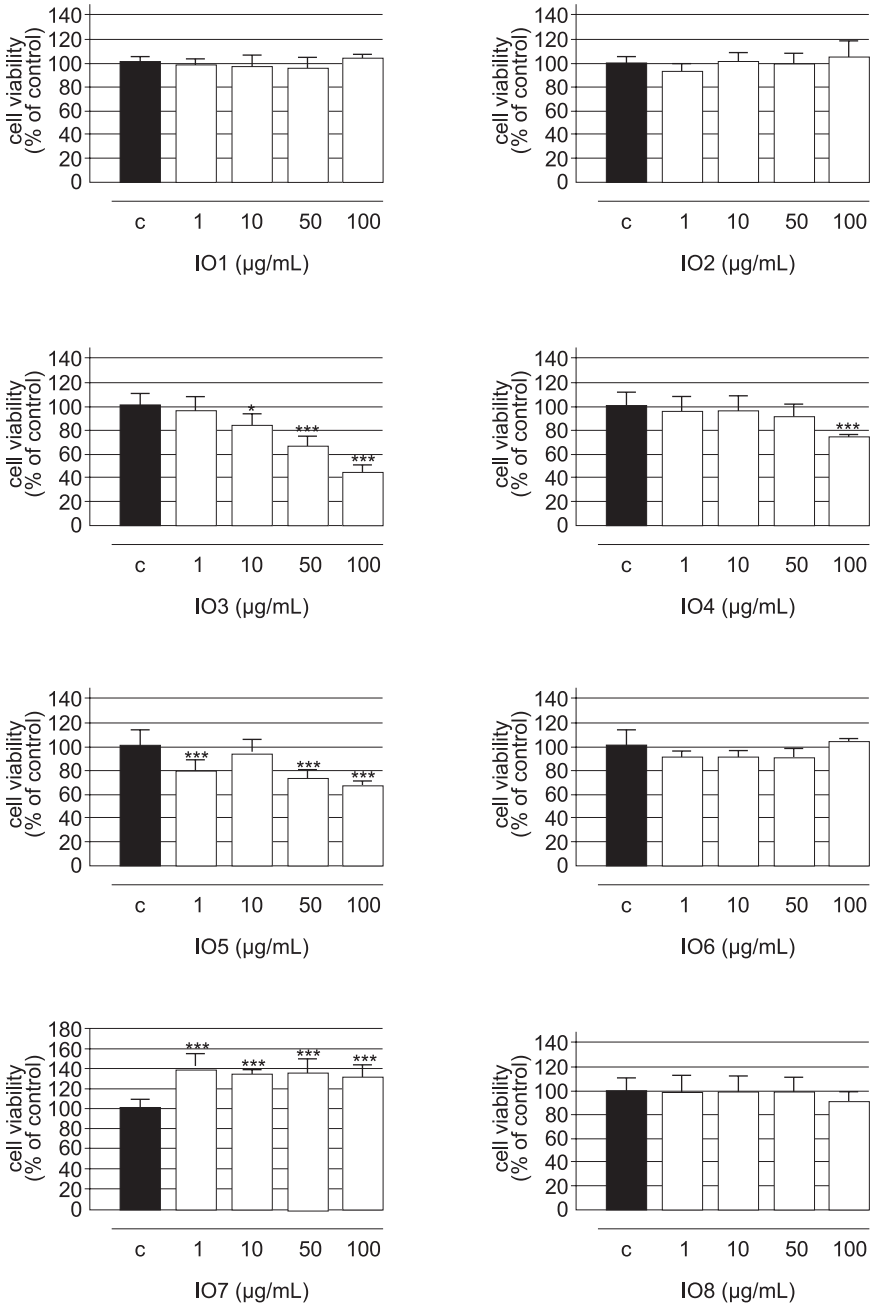
Hydrolysis of the extract in dilute hydrochloric acid results in precipitation of **IO6**, which contains mainly melanine complexes in amount of ca. 60% of dried product **IO1**. The physical and chemical properties of melanine complexes, isolated as a black powder, are similar to that already characterized in the literature, for which the elimination of *malignant melanoma* was observed (2, 8–10).

The precipitate **1,** produced after evaporation of water from the filtrate obtained by removing residue **IO6**, is a physical mixture of inorganic salts and organic compounds. The low molecular organic components were extracted from precipitates **IO6** and **1** using ethyl acetate and methanol. Total content of organic extract **IO7** obtained from **IO6** and **1** makes up ca. 20% of water-soluble product **IO1**. The extract **IO7** is a black or dark-brown, half-dry substance soluble to various extend in organic solvents, but marginally soluble in water. The residuals obtained after organic solvent extractions are mainly inorganic salts and tough soluble organic compounds of unidentified structures, which seem to be derivatives of polysaccharides. The type and the content of cations after extraction (related to the mass of dried fungus) have been characterized by us in previously published work (7). By applying qualitative analysis**,** the presence of phenols, carboxylic acids and carbonyl compounds (aldehydes and ketones) in **IO7** was discovered. The GC-MS analysis shows the presence of more than 150 compounds, from which, in preliminary investigations only, those of high concentration such as benzyl alcohol and benzaldehyde were identified. The majority of compounds was not satisfactory separated by GC and consisted of a mixture of very complex composition, which we were unable to identify using mass spectrometry. Just after preliminary separation on the silica gel-packed column, six distinct fractions were obtained: hexane **IO2** (7.27%), toluene I **IO3** (8.59%), toluene II **IO4** (17.16%), acetate **IO5** (35.25%), methanol I (0.92%) as well as methanol II and methanol-water **IO8** (30.81%), which were in turn analyzed using GC-MS. In a course of compounds identification the Mass Spectral Databases (SDBS, Kovats, NIST and In-house database) were used. In several cases the experimental GC-MS spectra were compared to those of known standard samples.

The hexane fraction **IO2** contains mainly aliphatic hydrocarbons (both saturated and unsaturated), aromatic hydrocarbons, alcohols of long aliphatic chains, betulin and retinol acetate. The presence of antitumor betulin (11–14), as unique triterpene compound identified in aqueous extract, seems unexpected, since no other compound of that kind was detected here.

The toluene fractions I **IO3** and II **IO4** contain aromatic hydrocarbons, alcohols, phenols, aliphatic and aromatic aldehydes, ketones, and esters. Some compounds, such as benzaldehyde and benzyl alcohol, are split between two or more fractions. The largest acetate fraction **IO5** contains mainly carboxylic acids and certain aromatic esters.

The methanol elution **IO8** proceeds in a specific manner. Small part of the fraction (mainly phenols, ca. 1%), separates very quickly. The other part exceptionally slowly migrated through the column shaping of compact, black ring. We were able to increase the rate of its migration through the column, without noticeable influence on the course and quality of separation, by an addition of 5 to 10% of water to the eluent. The GC-MS chromatogram of this fraction is a series of unseparated peaks of $m/e > 250$, mainly of low intensity that indicate a presence of compounds possessing complex structure. Characteristic black color of the fraction supports



extract concentration	cell viability (% of control)							
	IO1	IO2	IO3	IO4	IO5	IO6	IO7	IO8
1 µg/mL	98.25 ± 1.899	93.05 ± 2.146	96.59 ± 4.357	96.52 ± 4.605	79.75 ± 3.162	91.25 ± 1.754	139.6 ± 5.700	99.81 ± 4.142
10 µg/mL	98.04 ± 3.432	100.8 ± 2.752	84.66 ± 3.278	96.66 ± 4.616	94.41 ± 3.836	91.25 ± 1.829	134.2 ± 1.841	98.36 ± 4.832
50 µg/mL	96.08 ± 3.080	99.10 ± 3.058	67.19 ± 2.973	92.71 ± 3.285	74.18 ± 2.361	91.61 ± 2.381	136.6 ± 4.652	99.56 ± 4.470
100 µg/mL	103.9 ± 1.276	104.5 ± 4.866	45.50 ± 2.158	74.78 ± 0.4658	67.30 ± 1.473	103.5 ± 0.6288	132.2 ± 3.900	91.84 ± 2.829

Figure 1. The influence of *Inonotus obliquus* extracts on human lung carcinoma (A549) cells proliferation. Cells were exposed to culture medium alone (control) or tested extracts (1–100 µg/mL) for 96 h; viability was measured photometrically by means of the MTT assay. Data are expressed as a percentage ± SEM of 8 trials, *** at least $p < 0{,}001$ *vs.* control, one-way ANOVA, Tukey *post-hoc* test, c = control

Table 1. Chemical compounds from organic extract **IO7**

Compound	**Content in extract (%) (fraction presence)**
Hydrocarbons – *saturated*	
Docosane	0.14 (**IO2, IO3**)
Hexatriacontane	0.12 (**IO2, IO3**)
Pentadecane	0.08 (**IO2**)
Heneicosane	0.07 (**IO2**)
2,6,10-Trimethyltetradecane	0.05 (**IO2**)
Dotriacontane	0.04 (**IO2**)
Hydrocarbons – *unsaturated*	
Tetradecene	1.25 (**IO2, IO5**)
Octadecene	1.12 (**IO2, IO5**)
Eicos-1-ene	0.80 (**IO2**)
Hexadecene	0.76 (**IO2**)
Docos-1-ene	0.23 (**IO2**)
Nonadec-1-ene	0.02 (**IO2**)
Hexacos-1-ene	< 0.01 (**IO2**)
Hydrocarbons – *aromatic*	
Mesitylene	1.74 (**IO5**)
3,4'-Dimethyl-1,1'-biphenyl	0.24 (**IO4**)
(1-Octyldecyl)benzene	0.23 (**IO2**)
2-Methyldiphenylmethane	0.22 (**IO3**)
3-Methyldiphenylmethane	0.20 (**IO3**)
3-Methyl-1,1'-biphenyl	0.19 (**IO3**)
(1-Methylundecyl)benzene	0.18 (**IO2**)
(1,1,4,6,6-Pentamethylheptyl)benzene	0.12 (**IO2**)
3-Propyltoluene	0.01 (**IO2**)
o-Xylene	< 0.01 (**IO8**)
Alcohols and diols	
Benzyl alcohol	14.54 (**IO3, IO4, IO5**)
3,5-Dimethylbenzyl alcohol	0.82 (**IO3, IO4**)
Nonacosan-1-ol	0.59 (**IO2**)
Non-2-en-1-ol	0.48 (**IO3**)
α-Phenylbenzeneethanol (1,2-diphenylethanol)	0.13 (**IO3**)
2-Methyloct-2-en-4-ol	0.11 (**IO3**)
Oxiranemethanol	0.08 (**IO8**)
Cyclohexane-1,2-diol	0.06 (**IO8**)
3-Chloropropane-1,2-diol	0.01 (**IO8**)
p-Butoxybenzyl alcohol 0.01	(**IO8**)
Phenols	
2,6-Dimethoxyphenol (syringol)	2.11 (**IO4**)
2,4-Di-*tert*-butylphenol	1.70 (**IO8**)
Resorcinol	0.66 (**IO8**)
Cresol	0.37 (**IO3, IO4**)
Mequinol + guaiacol	0.02 (**IO8**)
4-(1,1,3,3-Tetramethylbutyl)phenol	0.02 (**IO8**)

Table 1. continued

Compound	**Content in extract (%) (fraction presence)**
Carbonyl compounds – *aldehydes*	
Benzaldehyde	5.78 (**IO3, IO4, IO5**)
2-Ethylidene-6-methylhepta-3,5-dienal	0.27 (**IO4**)
3-Methoxy-4-phenylmethoxybenzaldehyde	0.05 (**IO3**)
Carbonyl compounds – *ketones*	
Benzoin	0.72 (**IO3, IO4**)
6-Methylhept-5-en-2-one	0.56 (**IO4, IO5**)
4-Methylhept-3-en-2-one	0.13 (**IO3**)
5-Hydroxy-2,3-dimethylcyclopent-2-en-1-one	0.09 (**IO3**)
5,9-Dimethyldeca-5,8-dien-2-one	0.08 (**IO3**)
3,6-Dimethyloctan-2-one	0.05 (**IO3**)
Carbonyl compounds – a*cids*	
4-Oxopentanoic acid	0.86 (**IO5**)
Trimethoxybenzoic acid	0.80 (**IO5**)
Non-2-enoic acid	0.71 (**IO5**)
Vanillic acid	0.62 (**IO5**)
Oleic acid	0.56 (**IO5**)
Hexadecanoic acid	0.37 (**IO5**)
3,4,5-Trihydroxybenzoic acid (gallic acid)	0.29 (**IO5**)
3-Hydroxy-4,5-dimethoxybenzoic acid	0.22 (**IO8**)
Dec-2-enoic acid	0.01 (**IO8**)
Carbonyl compounds – *esters*	
Benzyl benzoate	1.78 (**IO3, IO4**)
Methyl 1-benzyl-5-oxo-5-pyrrolidinecarboxylate	1.34 (**IO5**)
Methyl dodecanoate (methyl laurate)	0.92 (**IO3**)
Methyl linoleate	0.42 (**IO8**)
Methyl 14-methylpentadecanoate	0.24 (**IO4**)
2-Methoxyphenyl benzoate	0.22 (**IO3**)
Benzyl acetate	0.19 (**IO3, IO5**)
2,3-bis((trimethylsilyl)oxy)propyl octadeca-9,12,15-trienoate	0.17 (**IO2**)
Isopropyl dodecanoate (isopropyl laurate)	0.14 (**IO2**)
Methyl hexadec-1-enoate	0.14 (**IO2**)
Methyl hexanoate (methyl palmitate)	0.13 (**IO3, IO8**)
Methyl 12-methyltridecanoate	0.13 (**IO3**)
Methyl 3-methoxypropanoate	0.08 (**IO8**)
Cyclohexane-1,2-diol monoacetate	0.06 (**IO8**)
Methyl octadec-16-enoate	0.05 (**IO2**)
Methyl octadecanoate (methyl stearate)	0.01 (**IO8**)
Others	
m-Phenylethylbenzonitrile	1.34 (**IO8**)
2-Aminopurine-6,8-diol	0.64 (**IO5**)
Betulin	0.43 (**IO2**)
β-1,5-Dibenzoyl-2-deoxyribofuranose	0.22 (**IO3**)
Retinol(O-acetyl-all-trans)	0.20 (**IO2**)
3-Methoxypropanenitrile	0.04 (**IO8**)
Tetrahydropyran-4-ol (traces)	< 0.01 (**IO8**)

the presence of melanine dyes together with compounds of large molecular mass, among which triterpenes and steroids may be expected. Although derivatives of such kind are only marginally soluble in water, during the long term extraction by boiling water they migrate to the water layer in the form of water-soluble complexes.

The other compounds of similar structure are expected here in vestigial concentrations. Some of them, mainly alcohols, had been previously identified in the organic extracts of moderate polarity (15–21). In the water extract, the presence of vanillic acid and indirectly pyrogallol (through gallic acid) and syringol was confirmed. The presence of syringic and *p*-hydroxybenzoic acids that supposedly is produced during degradation of the melanine-like structures (22, 23), was not detected.

The main components of water extract are benzyl alcohol (14.54%) and benzaldehyde (5.78%), the content of remaining components (excluding syringol) does not exceeds 2% (see Table 1). (In the interpretation process, the signals from butyl and octyl phthalates were omitted since the septum melted in the chromatograph injector seems to be their most probable source.)

Antiproliferative effect of extracts isolated from *Inonotus obliquus* assessed in human lung carcinoma cells, which were exposed to tested extracts in concentrations ranging from 1 to 100 μg/mL for 96 h, was observed in the concentration-dependent fashion only for extracts **IO3**, **IO4** and **IO5,** whereas, extract **IO7** increased A549 cells proliferation. The other extracts (**IO1**, **IO2**, **IO6**, **IO8**) did not influence proliferation (see Figure 1).

Some of the identified compounds, present in the aqueous extract, have already known diverse therapeutic properties. Several of them, especially phenols and carboxylic acids, are enumerated among the most known and applied. For example, the group of therapeutic phenols includes: resorcinol (in **IO8**) – applied internally for the relief of nausea, asthma, whooping cough and diarrhea; guaiacol (in **IO8**) – applied as expectorant; syringol (in **IO4**)– prohibits platelet aggregation, causes decrease of N-nitrosomorpholine (NMOR) in stomach and blood (24), and 2,4-di-*tert*-butylphenol (in **IO8**) – which shows antioxidant effects (25). Therapeutic properties of carboxylic acids such as vanillic (in **IO5**), oleic (in **IO5**), and gallic acids (in **IO5**), and some esters are well documented in the literature (26–29). Many polyphenolic compounds, including triterpenoids, steroids, and ergosterol peroxides from *Inonotus sclerotia*, have shown various biological activities, including anti-bacterial (30), hepato-protective (31), and antitumor (32–37) effects. But, we did not observe their presence in a significant amount in the extracts **IO3**-**IO5** showing antiproliferative activity. On the contrary, their small amount (peaks of m/e > 250) may be present in the fractions showing virtually a lack of antiproliferative activity (i.e., **IO8**) or even boost of A549 cells proliferation (i.e., **IO7**). On the other hand, in the samples (fractions) where is a lack of compounds of unambiguously documented antitumor properties (i.e., **IO3**-**IO5**) we observed noticeable antiproliferative activity.

Based on our results, one may hypothesize that antiproliferative effects of fractions **IO3**-**IO5** are caused by the metabolites of benzyl alcohol, the major compound present there but not in inactive fraction **IO2**. Although benzyl alcohol alone may induce some cellular and biochemical effects (38), being a cell membrane "fluidizer" that affects lipid bilayer structure (39), as it was demonstrated on membranes of erythrocytes (40, 41) and hepatocytes (42), it seems unlikely that it is the direct cause of the extracts cytotoxicity against carcinoma. On the contrary, benzaldehyde (the second major compound in the extracts) demonstrates well documented cytotoxicity (43, 44). Benzaldehyde was even preclinically evaluated for purpose of antitumor chemotherapy (45). Since benzaldehyde is a volatile substance and can be readily oxidized in the air, which presents a serious problem in administering it during clinical trials, for purpose of chemotherapy new pharmaceutical forms of it were invented and evaluated (46, 47). In our case, benzaldehyde can be formed *in situ* from benzyl alcohol in metabolic processes (48–50) or in aerobic autoxidation (51) that may be accelerated by the presence of reactive oxygen species (*ROS*) under the conditions of oxidative stress accompanying carcinogenesis (52).

Assuming the simplified metabolic pathway of benzyl alcohol: benzyl alcohol (moderately active) → benzaldehyde (active) → benzoic acid (moderately active) → hippuric acid (moderately or non-active) → *excretion*, an inhibition of the first two steps seems important for the fractions antiproliferative activity. In order to keep antitumor activity, the benzaldehyde level should remain relatively high, therefore the second metabolic step should be inhibited but the first not[2]. It seems that high molecular

[2] The base-induced disproportionation of benzaldehyde, the Cannizzaro reaction (53), to benzyl alcohol and benzoic acid require strongly basic condition, therefore, is improbable in physiological environment.

mass compounds (see above) that are present in fraction **IO8** (and extracts **IO1**, **IO6** and **IO7** as well) can effectively inhibit both benzyl alcohol and benzaldehyde oxidation, on both enzymatic and autoxidative / *ROS* pathways, making the extracts inactive. On the other hand, some aromatic, unsaturated and carbonyl compounds found in active fractions **IO3**-**IO5** may inhibit benzaldehyde oxidation. However, we have to be conscious of the limits of such interpretation, since the synergic effect of the compounds and their influence on pharmacological efficiency of the extracts can be far more complicated. Therefore, fractions isolated by us from *Inonotus obliquus* are subjected to further, more advanced and carefully crafted biochemical experiments.

Acknowledgments

The financial support and highest purity samples of betulin provided by Mr. Radosław Piela (Sylveco® Poland) are greatly appreciated.

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Received: 04. 11. 2009