Analysis of the content of phenolic compounds in *in vitro* culture of some edible mushrooms (*Basidiomycota*)

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Abstract.
Phenolic compounds, both derivatives of benzoic and cinnammic acid, possess biologically valuable properties: anti-inflammatory, antioxidant, anticarcinogenic and others. Studies of the accumulation of these compounds focused mostly on plant material. Rich sources of these compounds are representatives of Basidiomycota taxon. The aim of the study was qualitative and quantitative HPLC analysis of phenolic acids in biomass from in vitro culture of selected edible mushroom species belonging to the phylum Basidiomycota: Agaricus bisporus, Boletus badius, Cantharellus cibarius. The investigations revealed the presence of the following acids: p-hydroxybenzoic, syringic and galic acid. Both the composition and the amount of phenolic acids in biomass of A. bisporus and Boletus badius were diverse. The total amount ranged from 6.07 mg·100 g^{-1} DW in A. bisporus to 14.78 mg·100 g^{-1} DW in Boletus badius. Syryngic acid amounts fluctuated in the range of 1.75–9.66 mg·100 g^{-1} DW, with its maximum in Boletus badius. Gallic acid dominated in the biomass of the same species (5.12 mg·100 g^{-1} DW). p-Hydroxybenzoic acid was found in biomass from in vitro culture of A. bisporus at levels 0.70 mg/100 g DW. In biomass of in vitro culture of Cantharellus cibarius no phenolic compounds were found before and after hydrolysis. The results of HPLC analyses show that in vitro culture of B. badius and A. bisporus are a good dietary source of phenolic compounds with antioxidant activity.

Key words: ***Agaricus bisporus, Boletus badius, Cantharellus cibarius*, medicinal mushroom, phenolic acids**

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Introduction

Oxydo-reduction processes play an important role in the biochemistry of many diseases and in cell aging. Free radicals and reactive oxygen species influence proteins and cell membranes, they damage natural barriers and defense mechanisms and also influence gene structures allowing the groth of changed cells and the replication of wrong metabolic pathways. Therefore the search for antioxidative substances which eliminate free radicals from the organism continues. An important aspect of this research is the condition that antioxidative substances must be easily administered in the least invasive form [1-3].

It was acknowledged that mushroom fruiting bodies contain many chemically varied substances with antioxidant properties. During the research upon the quantity of these compounds in some mushroom species apart from the quantity also antioxidative potential was researched as well as the correlation between the mushroom extract composition and antioxidative properties. In the research with an extract of *Boletus edulis* fruiting bodies, it was proven that it has an ability of sweeping free radicals, inhibition of hydroxyl radicals and nitrogen oxide (NO) formation, it was shown that it can chelate metals and slow the lipid peroxidation process. Reductive potential was also established and connected with high concentration of phenol compounds and tocopherols in extracts [4–8].

Phenolic acids constitute the major percentage of phenolic compounds present in mushrooms [9–11]. They exhibit a wide spectrum of biological activities which have been attributed to their strong antioxidant power and ability to protect important cellular structures, like cell

membranes, structural proteins, enzymes, membrane lipids or nucleic acids, against oxidative damage [9–14].

Phenolic compounds are wide spread antioxidants in plants. Mushrooms contain also a wide variety of phenols, specially abundant in phenolic acids [4–14]. Up till now the acids that were determined in mushrooms can be divided in two groups: hydroxybenzoic acid derivatives (p-hydroxybenzoic, protocatechuic, vanillic, syringic and gallic acid) and cinnammic acid derivatives (p-coumaric, caffeic, ferulic, sinapic acid). Hydroxybenzoic acid derivatives occur mainly as lignan complexes, hydrolytic tannins or in connection with a sugar or organic acid. On the other hand cinnammic acid derivatives can be naturally found as esters or bound to constitutive proteins of cell walls with an ester bond [1–3]. Phenolic acids are valued antioxidants, that prevent the creation and neutralize free radicals, inhibit the peroxidases activity and inactivate metals that facilitate oxidative processes. The strongest antioxidative action is assigned to vanillic and caffeic acid [12]. In the research it was proven that reductive potential and free radicals sweeping action is bound to phenolic compounds amount in alcoholic extracts from mushrooms [7,8,13]. It was also found that the activity of iNOS and the nitrogen oxide production are impaired with consecutive increase of the concentration of phenolic acids in the extracts [11]. Due to antioxidative properties, phenolic acids may prevent degenerative disease, cardiovascular system diseases, atherosclerosis, eye diseases, inflammatory states or diseases such as rheumatoid arthritis. Furthermore phenolic acids (*p*-hydroxybenzoic, gallic and protocatechuic acid) show also antifungal, antibacterial, anti-inflammatory action as well as it increases gastric secretion. Protocatechuic acid is well known for its immunomodulatory, spasmolytic, cardioprotective and anticoagulatory action [14].

The aim of the present studies was qualitative and quantitative HPLC analysis of phenolic compounds: caffeic acid, chlorogenic acid, ferulic acid, p-coumaric acid, p-hydroxybenzoic acid, protocatechuic acid, sinapic acid, syringic acid, vanillic acid, cinnamic acid. Materials for the study were mycelium from in vitro culture of edible mushrooms (Basidiomycota): *Boletus badius*, *Cantharellus cibarius*, and *Agaricus bisporus*. These species were chosen because they are widespread in Europe and Asia and popular among consumers. In addition, all these species contain numerous primary and secondary metabolites exhibiting significant biological activity (for example: polysaccharides, unsaturated fatty acids, water — and fat-soluble vitamins, wide spectrum of proteins, flavonoids, terpenoids, sterols, carotenoids, indole compounds and elements, e.g. selenium) [12]. As shown by Ey [15], this species contained the highest content of the latter compound in comparison with other food products. According to Turkish studies, *B. badius* was evidenced to possess excellent antioxidant properties. The percentage inhibition methanolic extracts of dried *B. badius* at 100 $\mu g \cdot ml^{-1}$ concentration on peroxidation in linoleic acid system was 99.2% [12]. *C. cibarius* is an example of a "champion" among Basidiomycota in the contents of vitamin B (comparable with its content in *Saccharomyces cerevisiae*) and vitamin C (7.2 mg·-100 g^{-1} DW, which constitutes almost one-tenth of daily requirement), These vitamins are accompanied by flavonoids (0.67 $mg \cdot g^{-1}$ DW), which increase the antioxidant power, ergocalciferol (vitamin D_2 the mean content of which approximates 1.43 $\mu g \cdot g^{-1}$), β-carotene (13.56 $\mu g \cdot g^{-1}$ DW) and tocopherols [12].

Materials and methods

Reagents and standard

Methanol and acetic acid both of HPLC-grade were from Merck (Darmstadt, Germany), chloroform and methanol both of analytical grade were from POCh (Gliwice, Poland). Phenolic acid standards of p-coumaric, ferulic, p-hydroxybenzoic, and vanillic acids were from Fluka (Chemie AG), and those of galic, caffeic, chlorogenic, cinnamic, o-coumaric, protocatechuic, sinapic, and syringic acids were from Sigma (St. Louis, USA). All standards were of HPLC-grade. Standard solutions were prepared in methanol. Water was purified by redistillation and filtered through Milipore Millex — GP, 0.22 μm.

Materials

The studies were conducted on young fruiting bodies of *Agaricus bisporus (J.E. Lange) Imbach* (White button mushroom) of commercial origin (supermarket in Poland) and *Boletus badius Pers.* (Bay bolete), *Cantharellus cibarius* Fr. – Chantarelle harvested from natural state in mixed forests of southern Poland (near Kraków 2011-2013). After taxonomic identification according to Knudsen and Vesterholt [16] (representative samples of

mushrooms were deposited in the Department of Pharmaceutical Botany, Jagiellonian University Collegium Medicum, Kraków, Poland), some of young sporocarps were used to derive culture in vitro from which were obtained mycelium formed material for further analysis.

In vitro culture

The pieces of fruiting bodies were defatted with 70 % ethyl alcohol for 15 s then sterilized in 15 % hypochlorite solution for 5 min (manufactured by Unilever, Hungry). After being rinsed several times with sterile redistilled water, mycelium fragments were transferred to Petri dishes containing agar-solidified medium with composition according to Oddoux [17].

Exerimental *in vitro* culture

After growing on solid medium, the pieces of mycelium were placed in an Erlenmeyer flask (500 mL) containing 250 mL of liquid medium with modified Oddoux medium, and the initial biomass amounted to 0.1 g. The cultures were shaken at a rate of 140 rpm (shaker ALTEL. Łódź). Cultures were incubated at the temperature 25 ±2° C under 16-h light (900 lx/8 dark). The agitated liquid cultures of *A. bisporus* were maintained for two weeks and after this time subcultured.

The agitated liquid cultures of *A. bisporus, Boletus badius* and *Cantharellus cibarius* on Oddoux medium with addition of L-tryptophan (0.5 g/L) were maintained for two weeks. After two weeks the biomass was separated from the liquid medium using a filter paper on Büchner funnel, rinsed with redistilled water. The obtained fresh biomass: mycelium from in vitro cultures of *A. bisporus, B. badius, C. cibarius* (50 g of each species) and mycelium of the same species from in vitro cultures with addition tryptophan to medium (50 g) were frozen and immediately dried by lyophilization (lyophilizer Freezone 4.5. Labconco; temperature: −40°C) for quantitative analyses.

Sample preparation

The lyophilized materials were weighed (5 g of each species) and ground in a mortar, and then they were subjected to acid hydrolysis (2 mol·dm^{-3} HCl) at 100°C for 4 h. The obtained hydrolysates were filtered through paper filters (Munktell) and were shaken in a percolator with a 5-fold excess of ethyl acetate. The organic fractions obtained after shaking were combined. The extracts were concentrated by distillation in a vacuum evaporator (Rotavapor R-114, Büchi) under reduced pressure at 40°C. The residues were quantitatively dissolved in methanol (1.5 ml), filtered through Milipore Millex — GP, 0.22 μm and were subject to HPLC analysis.

HPLC analyses

The obtained extracts were analyzed for contents of phenolic acid by HPLC method. These analyses were carried our according to the procedure developed by Ellnain-Wojtaszek and Zagórka [18] with some modifications. HPLC analyses were conducted using an HPLC VWR Hitachi-Merck apparatus: autosampler L-2200, pump L-2130, LiChrospher RP-18e column (250mm·4mm, 5μm) thermostated at 25°C, column oven L-2350, diode array detector L-2455 at UV range 200–400 nm. The mobile phase consisted of solvent A: methanol/0.5% acetic acid 1:4 (v/v), and solvent B: methanol. The gradient was as follows: 100:0 for 0–25 min; 70:30 for 35 min; 50:50 for 45 min; 0:100 for 50–55 min; 100:0 for 57-67 min. The comparison of UV spectra and retention times with standard compounds enabled the identification of phenolic acids presented in analysis samples. The quantitative analysis of phenolic acids was performed with the use of a calibration curve with the assumption of the linear size of the area under the peak and the concentration of the reference standard. The results were expressed in mg·100 g^{-1} dry weight (DW). A chromatogram of phenolic acid standard is presented in Figure 1.

Statistical analysis

For each mushroom three samples were used for the determination of every quality attribute and all the analyses were carried out in triplicate. The results were expressed as the mean values and relative standard deviation (RSD). The experimental data were analyzed for completely random design to determine the least significant difference at the level 0.05.

Results and discussion

It was established that mycelial mass growth of *A. bisporus, B. badius And C. cibarius* could be obtained in agitating liquid cultures on modified Oddoux [17] medium at 25 ±2°C under a 16 h photoperiod (900 lx/8 h dark). A 25 fold growth in liquid cultures was obtained within a typical 21-day growth cycle (the preparatory phase, the logarithmic growth phase, the stationary phase). The

Tab. 1. Comparison of phenolic acids amounts in extracts from fruiting bodies and mycelia from *in vitro* cultures of examined mushroom species

Species	Determined phenolic acids [mg·100 g^{-1} DW]								
	Gallic acid	Syringic acid	*p*-hydroxybenzoic acid	Protocatechuic acidy	Vanillic acid	*p*-coumaric acid	Sinapic acid	Cinnammic acid	Ferulic acid
Agaricus bisporus Fruiting bodies	1.11	—	—	—	—	—	—	—	—
Agaricus bisporus Mycelia from *in vitro* cultures	3.62	1.75	0.70	—	—	—	—	—	—
Cantharellus cibarius Fruiting bodies	—	—	0.23	0.15	0.33	—	0.30	0.13	—
Cantharellus cibarius Mycelia from *in vitro* cultures	0.62	—	—	—	—	—	—	—	—
Boletus badius Fruiting bodies	—	—	0.13	2.14	—	1.39	0.15	0.87	0.15
Boletus badius Mycelia from *in vitro* cultures	5.12	9.66	—	—	—	—	—	—	—

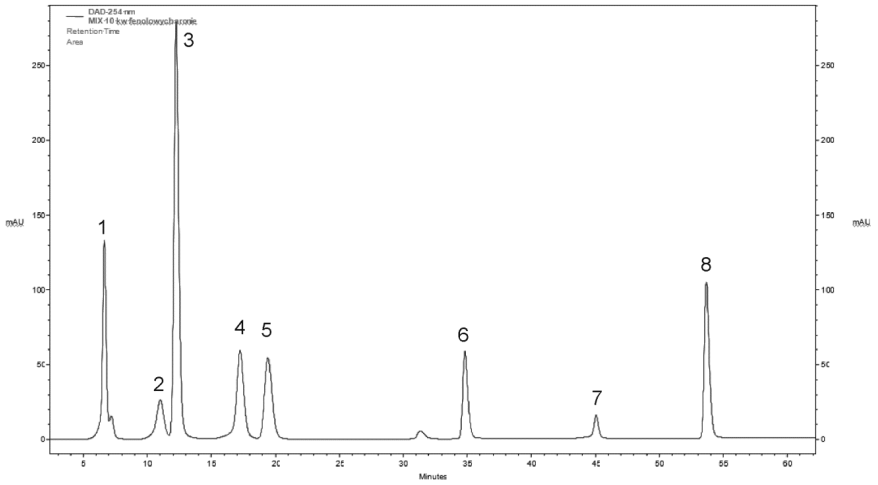

Fig. 1. Chromatogram of standards used for analysis of phenolic acids in examined mushroom species; 1 – protocatechuic acid, 2 – chlorogenic acid, 3 – p-hydroxybenzoic acid, 4 – caffeic acid, 5 – syringic acid, 6 – ferulic acid, 7 – rosmarinic acid, 8 – cinnammic acid

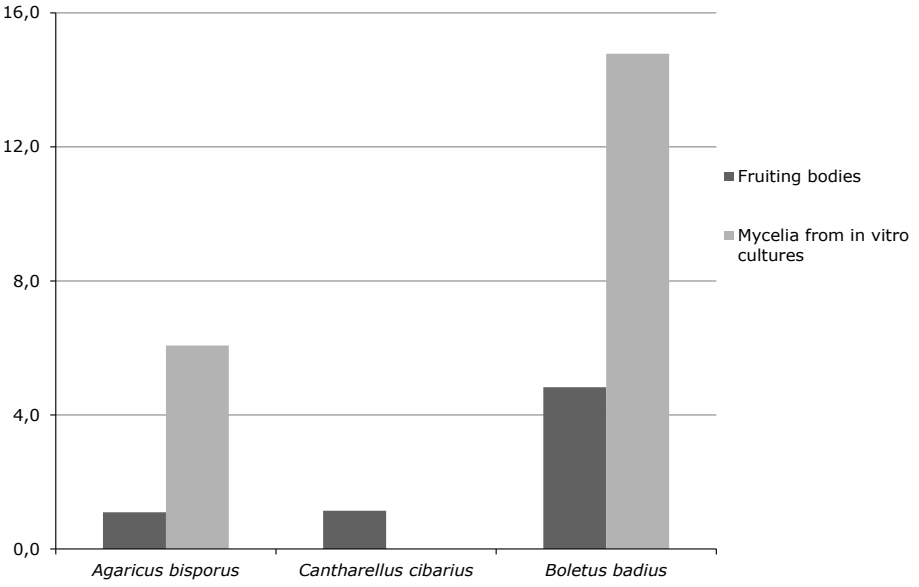

Fig. 2. Comparison of total phenolic acid amount [mg·100 g-1 DW] in fruiting bodies and mycelia

biomass growth in the initiated cultures averaged 9 g DW ·L^{-1} of medium. The obtained biomass increments and dynamics of mycelium growth did not differ from the results that have previously been obtained for *Sarcodon imbricatus* L., *Xerocomus badius* (Fr.) Kühn. ex Gilb., *Tricholoma equestre* (L.: Fr.) Kumm., *Calocera viscosa* Pers., *and Cantharellus cibarius* Fr. mycelial cultures [19–21]. The methods of extraction used here proved to obtain the optimal conditions for the qualitative and quantitative determination of phenolic compounds in the test material. The method of HPLC analysis developed in the present study allows identification and simultaneous quantitation and in consequence revealed the presence of the following acids: p-hydroxybenzoic, syringic and galic acid. Both the composition and the amount of phenolic acids in biomass of *A. bisporus and Boletus badius* were diverse. The total amount ranged from 6.07 mg·100 g^{-1} DW in *A. bisporus* to 14.78 mg·100 g^{-1} DW in Boletus badius. Syringic acid amounts fluctuated in the range of 1.75–9.66 mg·100 g^{-1} DW, with its maximum in *Boletus badius*. Galic acid dominated in the biomass of the same species (5.12 mg·100 g^{-1} DW). p-Hydroxybenzoic acid was in biomass from in vitro culture of A. bisporus at levels 0.70 mg·100 g^{-1} DW. In biomass of in vitro culture of *Cantharellus cibarius* were not presented any phenolic compounds. In the opposite the results of analyses show that in vitro culture of *B. badius* and *A. bisporus* are a good dietary source of phenolic compounds with antioxidant activity. The levels of indole compounds in mycelia from in vitro cultures determined in this work are similar to the ones that were previously reported in fruiting bodies of *A. bisporus* i *B. badius* [12]. Contents of individual phenolic compounds is shown in Table 1. In fruiting bodies of *C. cibarius*, from which the cultures were developed five phenolic acids were found but in very low quantity, from 0.13 to 0.33 mg·100 g^{-1} DW. Total amount was low (1.11 mg·100 g^{-1} DW) but similar to the total amount of phenolic compounds in *A. bisporus*, (although only one phenolic compound was found in it 1.10 mg·100 g^{-1} DW) contrary to biomass from in vitro cultures where the quantity was four times bigger (6.07 mg·100 g^{-1} DW). Biomass from *in vitro* cultures of *B. badius* contains three times more of indole compounds than fruiting bodies. In biomass of *B. badius* six phenolic compounds were found but their total quantity was three times lower than in biomass from in vitro cultures. Other compounds were also determined in all examined materials. In case of *A. bisporus* fruiting bodies gallic acid was determined but in smaller amount than in extracts from biomass of *in vitro* cultures (1.11 mg·100 g^{-1} DW). The quantities of gallic acid, syringic acid and *p*-hydroxybenzoic acid were found to be 0.70–3.62 mg·100 g^{-1} DW, also in biomass from *in vitro* cultures *of B. badius* gallic and syringic acid were found to be (5.12 mg·100 g^{-1} DW and 9.66 mg·100 g^{-1} DW respectively). Comparing the total amount of phenolic compounds found in biomass from *in vitro* cultures of *A. bisporus and* fruiting bodies, five times bigger amount was discovered (6.07 mg·100 g^{-1} DW), in case of biomass from *in vitro* cultures of *B. badius* three times bigger amount was found (14.78 mg·100 g^{-1} DW). Biomass of *C. cibarius* and fruiting bodies had nearly the same amount (about 1mg·100 g^{-1} DW). Ferulic acid was found only in fruiting bodies of *B. badius* (0.15 mg·100 g^{-1} DW).

Summarizing edible mushrooms contain phenolic acids that influence their antioxidative properties. *In vitro* cultures of *A. bisporus, B. badius* synthetize and accumulate considerably bigger amounts of these compounds, this phenomenon can be used in dietary supplementation.

Resumo

Fenoldevenaj kemiaj kombinaĵoj, ambaŭ derivaĵoj de benzoesana kaj cinamona acidoj, posedas biologie valorajn ecojn: kontraŭinflamajn, antioksidantanjn, kontraŭkancerigajn kaj aliajn. Esploroj de la akumulado de tiuj enhavaĵoj plej ofte enfokusigis la plantan materialon. Riĉaj fontoj de tiuj enhavaĵoj estas la reprezentantoj de la *Basidiomycota* taksono. La celo de tiu ĉi esploro estis kvalita kaj kvanta HPLC-analizo de fenoldevenaj acidoj en la biomaso de *in vitro* kulturado de elektitaj fungospecoj, kiuj apartenas al la grupo *Basidiomycota*: *Agaricus bisporus, Boletus badius, Cantharellus cibarius*. La esploroj malkovris la ĉeeston de la jenaj acidoj: p-hidroksobenzoesa, siringa kaj gala acidoj. Kaj la komponado kaj la kvanto de fenoldevenaj acidoj en la biomaso de *A. bisporus* kaj *Boletus badius* estis diversaj. La totala kvanto variis inter 6.07 mg·100 g^{-1} Seka Maso en *A. bisporus* kaj 14.78 mg·100 g^{-1} SM en *Boletus badius*. Kvantoj de siringa acido fluktuis inter 1.75 – 9.66 mg·100 g^{-1} SM, kun maksimumo en *Boletus badius*. Gala acido superregis en la biomaso de la sama speco (5.12 mg·100 g^{-1} SM). *p*-Hidroksobenzoesa acido troviĝis en biomaso de *in vitro* kulturado de *A. bisporus* sur la nivelo de 0.70 mg·100

g^{-1} SM. En la biomaso de *in vitro* kulturado de *Cantharellus cibarius* neniuj la fenoldevenaj kemiaj kombinaĵoj troviĝis antaŭ kaj post la hidrolizo. La rezultoj de la HPLC analizoj montras, ke *in vitro* kulturado de *B. badius* kaj *A. bisporus* estas bonaj dietaj fontoj de la fenoldevenaj kemiaj kombinaĵoj havantaj antioksidantan aktivecon.

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