



Biologically active compounds from selected aphyllophorales mycelial cultures

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ABSTRACT

For a long time fungi belonging to *Basidiomycota* phylum have been in the center of attention because of the presence in their fruiting bodies of compounds with known therapeutic activity. Mycelial cultures of two aphyllophorales species occurring in Poland, *Hydnum repandum* L., and *Sparassis crispa* (Wulf.) Fr., were analyzed in our study. The main aim of the study was qualitative and quantitative analysis of extracts obtained from the mycelial cultures for the presence of known biologically active compounds, including phenolic acids, non-hallucinogenic indole compounds and sterols.

For analyses a reversed-phase chromatography (RP-HPLC) method was used. The presence of eight phenolic acids including gallic, gentisic, *p*-hydroxybenzoic, caffeic, *p*-coumaric protocatechuic, syringic, vanillic and cinnamic acids was confirmed in the extracts obtained from the biomass. The quantitatively predominant metabolites in biomass from *in vitro* cultures of *H. repandum* and *S. crispa* were protocatechuic acid (6.23 µg/g DW) and *p*-hydroxybenzoic acid (4.52 µg/g DW). Derivatives of indole such as indole, serotonin, tryptamine and tryptophan were measured quantitatively. Their total content was estimated as 1.28 µg/g DW and 3.07 µg/g DW in *H. repandum* and *S. crispa* extracts, respectively. The major metabolite found was tryptophan. In addition, ergosterol, one of the sterols present in the biomass of *in vitro* cultures of *S. crispa* was analyzed (700.87 µg/g DW).

The obtained results confirm the hypothesis that mycelial cultures of domestic species of aphyllophorales are able to accumulate biologically active metabolites.

KEY WORDS: Basidiomycota, indole compounds, phenolic acids, sterols

Introduction

Representatives of *Basidiomycota* are of the multidirectional therapeutic activity; inter alia, antiviral, bacterio- and an important source of active compounds and

fungistatic, antiparasitic, as well as antitumor, anti-inflammatory, vessel protective or hypoglycemic (Zjawiony 2004, Sulowska-Ziaja *et al.* 2005). More and more research has been undertaken to identify the chemical composition of not only fruiting bodies, but also the mycelium extracted from *in vitro* cultures. Studies in this area have demonstrated the existence of qualitative and quantitative differences in the production of certain groups of chemical compounds between fruiting bodies and mycelium from *in vitro* cultures. This is important for both cognitive and application reasons.

The present study involved two wild edible mushrooms commonly growing in Polish forests: *Hydnum repandum* L.

Material and methods

Fungal material

Samples of mushrooms were collected in 2012 in mixed forests of northern Poland. Taxonomic identification was conducted according to Gumińska and Wojewoda (1988). Representative voucher samples were deposited in the Department of Pharmaceutical Botany UJCM, Kraków, Poland. Mycelial cultures were derived from explants originating from the hymenial part of fruiting bodies. Fruiting

(Hydnaceae) and *Sparassis crispa* (Wulf.) Fr. (Sparassidaceae). These species belong to an artificial systematic group known as *Aphyllophorales*.

The aim of the study was to determine the levels of biologically-active compounds in extracts from the biomass of *in vitro* cultures. It is a continuation of an extensive analysis of the chemical composition of biomass from mycelial cultures to confirm their usefulness as potential sources of compounds with biological properties. As far as we know, our study is the first one where the above-mentioned groups of compounds in the biomass from *in vitro* cultures of the examined fungi species have been described.

body fragments were sterilized, placed on Petri dishes with modified Oddoux medium (Oddoux 1957), incubated at a temperature of $\pm 22^{\circ}\text{C}$ and subcultured every three weeks. Experimental *in vitro* cultures were maintained in Erlenmeyer flasks, containing 250 mL of medium and shaken at a rate of 140 rpm. The cultures were maintained for three weeks. Then, the biomass was separated from the medium, frozen and lyophilized.

Determination of phenolic acids

The amount of 5 g of powdered material was hydrolysed with 2 M hydrochloric acid for 2 h at 100°C . Hydrolysates were extracted with 50 mL of ethyl acetate and concentrated to dryness. HPLC analyses were conducted using an HPLC VWR Hitachi-Merck apparatus: L-2200 autosampler, L-2130 pump, LiChrospher RP-18e column (250mm \times 4mm, 5 μm) at 25°C , L-2350 column oven, and L-2455 diode array

detector at UV range 200-400nm. 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. (Ellnain-Wojtaszek & Zgórka 1999). Phenolic acid standards were purchased from Fluka (Chemie AG) and Sigma (St. Louis, USA).

Determination of indole derivatives

Another 5 g of powdered material was extracted with 100 mL of methanol for 2 hand mixed extracts, which were then concentrated to dryness. The HPLC study was performed according to the procedure described by Muszyńska (Muszyńska *et al.* 2009). Briefly, the conditions were as follows: Hitachi

HPLC apparatus; L-7100 pump; Purospher RP-18 column (250 mm × 4 mm, 5 µm); solvent system – methanol : water : ammonium acetate 15:14:1 (v/v/v); flow rate - 1ml/min; and UV detector ($\lambda = 280\text{nm}$). Indole standards were purchased from Sigma (St. Louis, USA).

Determination of sterols

The third 5 g portion of powdered material was mixed with 100 mL of a 75:25 (v/v) mixture of methanol and dichloromethane, followed by sonification for 10 min and centrifugation at 10 000 rpm for 5 min. Combined extracts were concentrated to dryness. The HPLC method was performed according to the procedure developed by Yuan (Yuan *et al.* 2008). The mobile phase consisted of solvent A:

methanol : water 20:80 (v/v), and solvent B: methanol : dichloromethane 75:25 (v/v). A gradient procedure was used as follows: starting at sample injection, 60% of B for 5 min; a linear gradient from 60 to 100% of B for 10 min; and 100% of B for 10 min. The flow rate was 1.0 mL/min. Sterol standards, ergosterol, and ergocalciferol were purchased from Fluka (Chemie AG).

Results

The results of the analyses are shown in Table 1. Amongst the fifteen studied phenolic acids, five were detected in the extracts from the biomass from an *in vitro* culture of *H. repandum*; additionally, cinnamic acid was identified. In turn, seven phenolic acids were found in similar extracts of *S. crispa*. The total amounts of phenolic acids and cinnamic acid were 14.44 µg/g and 12.65 µg/g DW in biomass from *H. repandum* and *S. crispa*, respectively (Tab. 1). A sample chromatogram shows the separation of phenolic acids in the extract from of *H. repandum* biomass (Fig. 1).

Among the 12 indole compounds analyzed, three were detected in the extracts from *H. repandum* as well as *S. crispa*; i.e. indole, tryptamine and L-tryptophan. In addition, trace amounts of serotonin were detected in both extracts. The total amounts of indole compounds were similar (0.98 µg/g and 1.19 µg/g DW in *H. repandum* and *S. crispa* extracts, respectively) (Tab. 1). Ergosterol was the only representative of the analysed compounds with a sterol structure detected at level 700.87 µg/g DW, in *S. crispa* extracts.

Discussion

Earlier studies of the chemical composition of the fruiting bodies of the investigated species showed very similar

qualitative compositions for all investigated metabolites; however, in the biomass of the *in vitro* cultures, the level

of determined compounds was slightly lower (Sulowska-Ziaja *et al.* 2014). Phenolic acids determined in the current studies are characterized by a wide spectrum of biological activity. The strongest antioxidant activity is exhibited by vanillic acid and in a cinnamic acid derivative – caffeic acid. Gallic, *p*-hydroxybenzoic and protocatechuic acids detected in *H. repandum* and also *S. crispa* biomass are characterized by documented, multidirectional activity:

antioxidant, antibacterial, antiviral, anti-inflammatory or antifungal. Furthermore, the protocatechuic acid found in the largest amounts in the conducted studies is characterized by immunomodulatory, spasmolytic, cardioprotective and antithrombotic activity (Ferreira *et al.* 2009). A previously conducted chemical analysis of fruiting bodies also showed the quantitative predominance of this metabolite.

Table 1. Contents of biologically active compounds in mycelial cultures (µg/g DW).

Metabolites	<i>Hydnum repandum</i>	<i>Sparassis crispa</i>
Indole compounds		
Indole	0.018±0.05	0.07±0.02
Serotonin	*	*
Tryptamine	0.02±1.05	0.05±0.11
L-Tryptophan	0.94±0.09	1.07±0.17
Phenolic acids		
Gallic acid	4.16±0.12	2.27±0.34
Gentisic acid	2.03±0.11	0.10±0.01
<i>p</i> -Hydroxybenzoic acid	nd	4.52±0.4
Caffeic acid	nd	0.10±0.16
<i>o</i> -Coumaric acid	nd	1.45±0.21
Protocatechuic acid	6.23±0.02	3.92±0.3
Syringic acid	0.41±0.21	0.29±0.02
Vanillic acid	0.60±0.56	nd
Cinnamic acid	1.01±0.01	nd
Sterols		
Ergosterol	nd	700.87±0.3
Ergocalciferol	nd	nd

Values are means of three experiments ± SD, nd - not detected, *- traces.

Indole-derived non-hallucinogenic compounds are also an interesting group of secondary metabolites found in fungi. Estimating the content of this group of metabolites in the investigated species seems to be important due to their biological activity as well as from a toxicological viewpoint (Muszyńska *et*

al. 2011a, b). Within the scope of the study, the determined compounds have important biological functions as natural neurotransmitters and neuromodulators (serotonin, tryptamine), and are involved in the regulation of circadian rhythms, body temperature, mood, blood pressure, and appetite (5-hydroxy-tryptophan,

serotonin), as well as blood clotting (serotonin, melatonin). L-tryptophan and 5-hydroxytryptophan exhibit a hypnotic, antidepressant activity. In turn, melatonin and hydroxyindole derivatives have antioxidant potential.

The data concerning the appearance of non-hallucinogenic indole compounds in the representatives of Basidiomycota mainly refer to tryptophan, a biogenetic progenitor of all other indole compounds. Tryptophan is one of the diet supplements used as antidepressants. It can cross the brain-blood barrier and in the central nervous system can be converted to serotonin and melatonin, important neurotransmitters and neuromodulators controlling the circadian rhythm. A rich source of these

metabolites are edible mushrooms e.g. *Suillus bovinus* where tryptophan content is 25.9 mg/100g d.w.

The last study demonstrates the occurrence of a biogenic amine, tryptamine in many basidiomycetes. Tryptamine, synthesized from tryptophan is a direct precursor of different active compounds, including serotonin. Considerable quantities of tryptamine were detected in *Suillus luteus* (34/100d d.w.) and *Leccinum rufum* (31.71 mg/100g d.w.) (Muszyńska *et al.* 2011a, b). It has multi directional pharmacological activity i.a. it takes part in body temperature regulation, moodiness, organism maturation but also tissue regeneration and cell senescence.

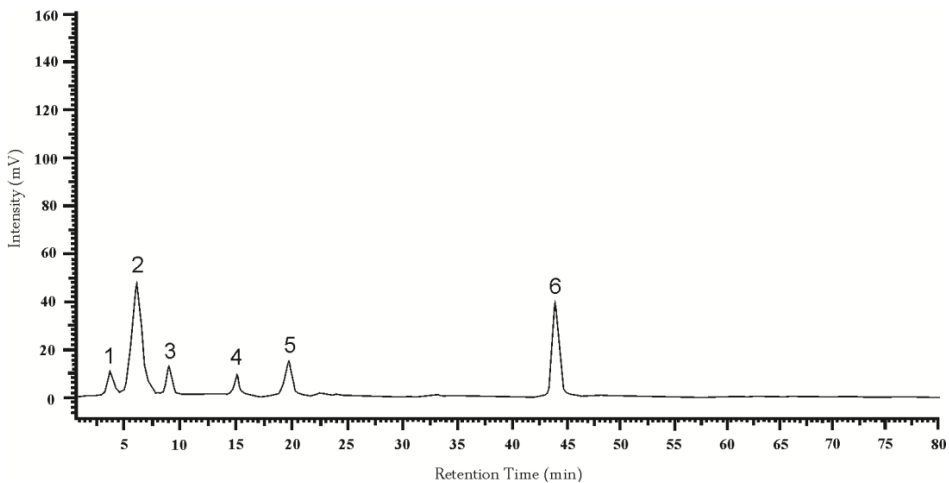


Figure 1. HPLC chromatogram of phenolic acids in biomass of mycelial culture of *Hydnum repandum* (1-gallic acid, 2-protocatechuic acid, 3-gentisic acid, 4-vanillic acid, 5-syringic acid, 6-cinnamic acid).

One of the main components of fungal cell membranes – ergosterol – is converted into vitamin D in the presence of sunlight or another ultraviolet light source (Mattila *et al.* 2002). This vitamin plays an essential role in the prevention of cancer, through increasing tumor cells

phagocytosis and facilitating other immunomodulatory functions. According to the latest studies, vitamin D also blocks the angiogenesis in tumors and impairs their growth progress (Kopczyński 2012, Mraz *et al.* 2010). The highest levels of ergosterol have

been noted in saprotrophic fungi and may constitute up to 83–89% of the entire amount of sterols. In addition, it has promising anti-allergic and immunostimulatory properties. Therefore, the search for new sources of this compound is becoming more and more popular (Yuan *et al.* 2006). Our research has shown that *S. crispa* is one

of the fungi with significant ergosterol levels in mycelial cultures. The data presented in this paper confirm the significant potential of chemical components with recognized antioxidant activity. The species can be considered an alternative source of phenolic acids and ergosterol.

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Streszczenie

Grzyby z gromady *Basidiomycota* od dawna budzą zainteresowanie ze względu na występowanie w ich owocnikach szeregu związków o uznanych właściwościach leczniczych. Obiektem przeprowadzonych badań były kultury mycelialne dwóch gatunków grzybów aphyloforoidalnych występujących na terenie Polski *Hydnum repandum* L. oraz *Sparassis crispa* (Wulf.) Fr. Celem podjętych badań była jakościowa i ilościowa analiza ekstraktów otrzymanych z kultur mycelialnych pod kątem

występowania związków o udokumentowanej aktywności biologicznej: kwasów fenolowych, niehalucynogennych związków indolowych oraz steroli.

Do oznaczeń wykorzystano wysokosprawną chromatografię cieczoową faz odwróconych (RP-HPLC). Na podstawie analizy stwierdzono w ekstraktach z otrzymanej biomasy obecność ośmiu kwasów fenolowych: galusowego, gentyzynowego, *p*-hydroksybenzoesowego, kawowego, kumarowego, protokatechowego, syringowego, wanilinowego oraz kwasu cynamonowego. Ilościowo dominującym związkiem był kwas protokatechowy w ilości 6,23 µg/g s.m. (*H. repandum*) oraz kwas hydroksybenzoesowy w ilości 4,52 µg/g s.m. (*S. crispa*). Spośród związków pochodnych indolu ilościowo oznaczono: indol, serotoninę, tryptaminę i tryptofan. Całkowita ich zawartość wynosiła 1,28 µg/g s. m. (ekstrakty z *H. repandum*) oraz 3,07 µg/g s.m. (ekstrakty z *S. crispa*). Ilościowo dominującym metabolitem był tryptofan. Spośród steroli oznaczono ergosterol w biomacie z kultur *in vitro* *S. crispa* (700,87 µg/g s.m.).

Uzyskane wyniki wskazują, że przebadane kultury mycelialne krajowych gatunków grzybów aphyloforoidalnych są zdolne do akumulacji metabolitów aktywnych biologicznie.