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SHORT COMMUNICATION

Analysis of indole compounds in methanolic extracts from the fruiting bodies of *Cantharellus cibarius* (the Chanterelle) and from the mycelium of this species cultured in vitro

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Abstract Methanolic extracts obtained from the fruiting bodies of *Cantharellus cibarius* (the Chanterelle) and from the mycelium of this species cultured in vitro were analyzed for the qualitative and quantitative composition of non-hallucinogenic indole compounds. The extracts were found to contain eight indole compounds: L-tryptophan, 5-hydroxytryptophan, serotonin, melatonin, indole, kynurenine sulfate, 5-methyltryptophan, and indoleacetonitrile. The extract from the fruiting bodies also contained tryptamine. The amounts of individual compounds varied widely, ranging from 0.01 to 17.61 mg/100 g DW in the fruiting bodies, and from 0.01 to 35.34 mg/100 g DW in the biomass from in vitro cultures. The quantitatively dominating compounds included: serotonin (17.61 and 20.49 mg/100 g DW, respectively) and kynurenine sulfate (3.62 and 35.34 mg/100 g DW). In addition, the material from in vitro cultures contained a considerable amount of 5-hydroxytryptophan (12.52 mg/100 g DW). The levels of the remaining indole compounds under analysis: L-tryptophan, melatonin, indole, 5-methyltryptophan, and indoleacetonitrile in the material under study were low, below 1 mg/100 g DW.

Keywords Cantharellus cibarius · Chanterelle · Indole compounds · In vitro culture · Serotonin

Introduction

Cantharellus cibarius Fr. (Basidiomycota)—commonly known as the Chanterelle, is one of the most highly valued and most commonly harvested edible mushrooms in Europe. It is also a popular edible mushroom in Asia, Africa and in the northern part of the USA. Unfortunately, due to the fact that it is easily recognizable and harvested *en masse* from natural sites, it belongs to endangered species. Since the demand for this species rises, because of its culinary value, there are attempts to develop its culture under controllable conditions for commercial use. However, these attempts have not yet been successful. In natural habitats, *C. cibarius* occurs in deciduous and coniferous forests living in a mycorrhizal association with the pine and fir, and also with the oak, beech and hornbeam. Nevertheless, it is not a typical mycorrhizal species, since it becomes a saprophyte under certain conditions (Rangel-Castro et al. 2002). This species is rich in numerous groups of metabolites which are decisive for its dietetic and therapeutic values. They include nitrogen compounds, like assimilable proteins containing all proteinogenic amino acids (3–9 % dry weight), many free exo- and endogenous amino acids, and biogenic amines (Svrcek and Vancura 1987). The fruiting bodies of this species contain an important enzyme, homodimeric lactase, possessing lignolytic properties used in biotechnology (Ng and Wang 2004; Nakamura and Go 2005). Polysaccharides, belonging to the most important components, comprise: chitin, chitosans, and, most of all, β-glucan possessing immunostimulating properties (Kalaĉ 2009). Lipids comprise saturated and mono- and polyunsaturated fatty acids (Barros et al. 2008). Oxidation of linoleic acid in *C. cibarius* fruiting bodies yields 1-octen-3-ol responsible for the characteristic flavour of this mushroom (de Pinho et al. 2008). This reaction is particularly intensified during the process of drying the mushrooms (Kalaĉ 2009). Mention has also been made of the presence of six phenolic compounds: 3-, 4-, and 5-*O*-caffeoylquinicacid, caffeic acid,

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p-coumaric acid, and rutin, and five organic acids: citric, ascorbic, malic, shikimic, and fumaric (Valentao et al. 2005). A non-phenolic compound: cinnamic acid, a precursor of numerous phenolic acids and alkaloids, was found in *C. cibarius* (14.97 mg/kg dry weight) (Barros et al. 2009). Many of these compounds are decisive for the antioxidant properties, so they play a protective role against civilization diseases (da Silva and Jorge 2012). *C. cibarius* is a species distinguished from other mushroom species by the highest, comparable with those of baking yeasts, levels of vitamins of the B complex: B_1, B_2, B_6, H; and vitamins A, D_2, E, C. The presence of α, β, γ-tocopherol and carotenoids has also been investigated in this species (Barros et al. 2008). Due to a high level of carotenoids, this species is used as a natural source of those pigments for the food industry. *C. cibarius* is also a good source of trace elements, especially selenium, enhancing the antioxidant activity of this species (Widzicka et al. 2008). In continuation of the studies on non-hallucinogenic indole compounds in *Basidiomycota* species that had been carried out in our laboratory for several years, we also examined several typical edible species for the levels of compounds belonging to this group, which play the role of neurotransmitters or their precursors, exhibit antioxidant, anticancer, anti-inflammatory, analgesic and anti-ageing actions (Isbister et al. 2004).

C. cibarius had already been included in the analysis of edible mushrooms (Muszyńska et al. 2011). The aim of the present study was to initiate in vitro cultures of *C. cibarius* to determine optimal conditions for mycelial growth and analyze the indole compounds in the cultured biomass. The fruiting bodies from which the cultures were derived were analyzed for comparison. This article is the first description of an attempt to culture *C. cibarius* in vitro and is the first report on the presence of indole compounds in this material.

Materials and methods

Materials for analysis The studies were conducted on young fruiting bodies of *Cantharellus cibarius* Fr. harvested from natural sites in mixed and coniferous forests in southern Poland (Brodła near Kraków) in August 2010 (deposited in the Department of Pharmaceutical Botany, Jagiellonian University, Collegium Medicum, Kraków, Poland). After taxonomic identification according to Knudsen (Knudsen and Vesterholt 2008), the sporocarps were used to set up in vitro cultures from which the obtained mycelium was also material for analysis.

In vitro culture Mycelial cultures were derived from explants originating from the parts of young fruiting bodies of *Cantharellus cibarius*. These pieces of fruiting bodies were sterilized with 70 % ethyl alcohol for 15 s and then in a 15 % Domestos solution for 5 min. (hypochlorite content below 15 %, manufactured by Unilever, Hungry). After being rinsed several times with sterile redistilled water, the mycelium fragments were transferred to Petri dishes containing an agar-solidified medium with a composition according to Oddoux (1957). After growing on the solid medium, the pieces of mycelium were placed in an Erlenmeyer flask (500 mL) containing 250 mL of a liquid medium with modified Oddoux medium, and the initial biomass amounted to 0.1 g. The culture was shaken at a rate of 140 rpm (shaker ALTEL, Łódź). Cultures were incubated at a temperature of 25±2 °C under 16-h light (900 lx/8 dark). The agitated liquid cultures of *C. cibarius* were maintained for 2 weeks and after that time subcultured.

After 2 weeks, the biomass was separated from the liquid medium using a filter paper on a Büchner funnel, rinsed with redistilled water. The obtained fresh biomass and fruiting bodies of *C. cibarius* were frozen and immediately dried by lyophilization (lyophilizer Freezone 4.5, Labconco; temperature: −40 °C).

Chemicals Standards of the indole compounds: L-tryptophan, 5-hydroxytryptophan, 5-metyltryptophan, serotonin, melatonin, tryptamine, kynurenic acid, kynurenine sulfate, indoleacetic acid, indoleacetonitrile and indole were from Sigma-Aldrich (St Louis, Mo, USA). Standard solutions were prepared in methanol. Methanol, ammonium acetate, both of HPLC- grade, were from Merck (Darmstad, Germany), n-propanol, ethyl acetate, petroleum ether, all of analytical grade purity, were from Polish Chemicals Company (Gliwice, Poland). Water was purified by redistillation and filtered through a Millipore filter (Millex, Millipore Corporation, USA) under reduced pressure.

Sample preparation Lyophilized fruiting bodies of *C. cibarius* and mycelium from the in vitro culture were powdered and then extracted in a percolator with petroleum ether in order to remove the lipid fraction according to the procedure developed in our laboratory (Muszyńska et al. 2007). After the extraction with ether, the material was dried and extracted with methanol in a percolator for 24 h. The extracts were combined and evaporated to dryness. To remove the remaining lipids, the concentrated extracts were frozen.

Indole compounds were isolated by using preparative TLC on aluminium-backed silica gel 60 (Merck, Art. 1.055540001) plates, onto which the methanol extracts were loaded. Chromatograms were developed in a mobile phase: n-propanol/ethyl acetate/water (7:1:2 v/v/v) and identification was performed at 280 nm. The obtained fractions were extracted with methanol, then filtered through a syringe-driven filter unit (Millex, Millipore Corporation, USA) and concentrated by distillation in a vacuum evaporator under reduced pressure at 40 °C. Dry

extracts quantitatively dissolved in 1.5 mL of methanol were subjected to HPLC analysis.

HPLC analysis The amounts of indole compounds: L-tryptophan, 5-hydroxytryptophan, 5-metyltryptophan, serotonin, melatonin, tryptamine, kynurenic acid, kynurenine sulfate, indoleacetic acid, indoleacetonitrile, indole and indoleacetamide were determined according to the procedure developed by Kysilka (Kysilka et al. 1985) with our modifications (Muszyńska et al. 2009, 2012). Briefly, the analytical conditions were as follows: HPLC apparatus: Hitachi; pump: L-7100; column: Purospher® RP-18 (4×200 mm, 5 μm); solvent isocratic system: methanol/water/ammonium acetate (15:14:1 v/v/v); flow rate : 1 ml/min; detector UV: λ=280 nm. The identification of the indole compounds was made by comparing the retention times of sample peaks with those of the standards. In order to confirm the presence of indole compounds in the tested extracts, the analysis was performed with standard solutions of indole compounds as internal standards. The presence of the tested metabolites in the sample showed up as an increase in peak height for the appropriate retention time. The quantitative analysis was carried out using the calibration curve method. The results are expressed in mg/100 g of dry weight, calculated by internal normalization of the chromatographic peak area (an example chromatogram of the extract from the mycelium of *C. cibarius* is presented in Fig. 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 mean values with standard deviation (SD). Statistical significance was defined at $p \leq 0.05$.

Results and discussion

After several attempts to establish an optimal sterilization method, we were successful in the initiation of *C. cibarius* mycelia in vitro culture from the hymenial part of fresh young fruiting bodies. The best biomass growth was obtained during 2-week growth cycles in shaking liquid cultures on a modified Oddoux medium. The biomass growth in the initiated cultures averaged 7.2 g DW/L of the medium. The maximum growth of *C. cibarius* mycelium biomass was observed at the initial medium pH value of 6.0 and at a temperature of 25 °C. In vitro cultures maintained under laboratory conditions optimized for maximum growth can provide a uniform mycelium which may be a repeatable and efficient source of metabolites. The obtained increases in biomass and the dynamics of mycelium growth did not differ from the results that we had obtained for *Xerocomus badius* (Fr.) Kuhn. ex Gilb—Bay Bolete and *Tricholoma equestre* (L.: Fr.) Kumm.—Man on Horseback, and for *Calocera viscosa* (Pers.: Fr.) Fr.—Yellow false coral cultures studied earlier (Muszyńska et al. 2009, 2012).

The HPLC procedure applied in the determination of the qualitative and quantitative composition of non-hallucinogenic indole compounds offered optimum conditions for the most effective separation of the metabolites analysed. The results of the analyses of the methanolic extracts of *Cantharellus cibarius* fruiting bodies and mycelia cultured in vitro revealed slight differences in the qualitative composition of the indole compounds under study and significant quantitative differences. Both extracts were found to contain eight different indole compounds: L-tryptophan, 5-hydroxytryptophan, serotonin, melatonin, indole, kynurenine sulfate, 5-methyltryptophan, and indoleacetonitrile. In addition, the fruiting body extracts

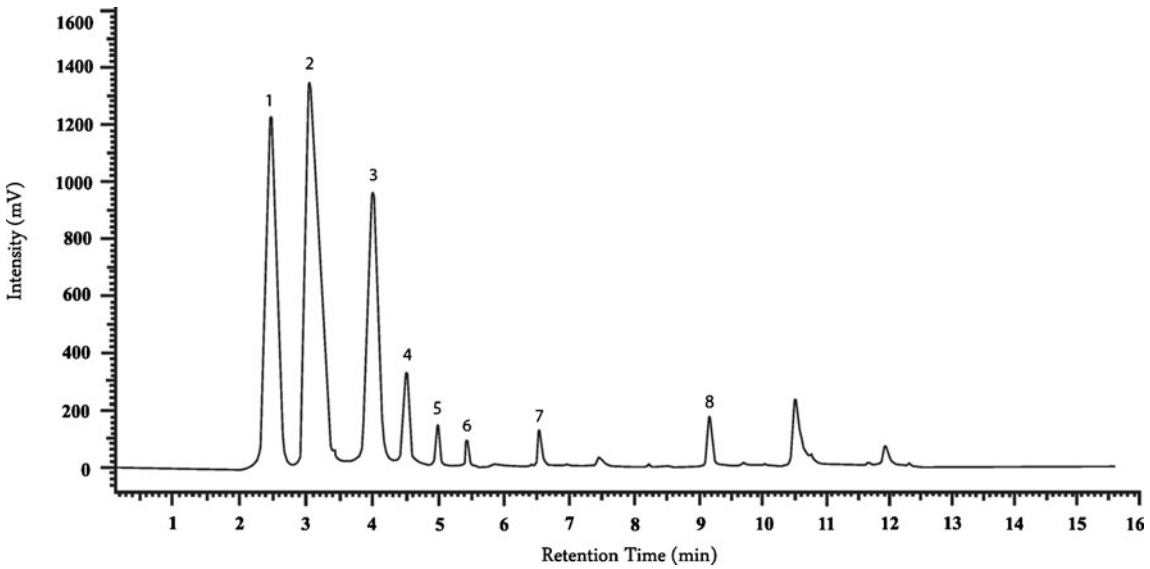

Fig. 1 HPLC chromatogram of the extract from the mycelium of *Cantharellus cibarius*: (1) - serotonin, (2) - kynurenine sulfate, (3) - 5-hydroxytryptophan, (4) - L-tryptophan, (5) - 5-metyltryptophan, (6) - melatonin, (7) - indoleacetonitrile, (8) - indole

contained tryptamine. The amounts of individual compounds varied widely, ranging from 0.01 to 17.61 mg/100 g DW for the fruiting bodies and from 0.01 to 35.34 mg/100 g DW for the mycelium cultured in vitro. The amounts of indole compounds in the methanolic extracts of the fruiting bodies and mycelium of *C. cibarius* are presented in Table 1. Serotonin and kynurenine sulfate (the last product of serotonin metabolism) were the quantitatively dominant compounds in both extracts. However, the mycelium from in vitro cultures contained greater amounts of these compounds. The levels of serotonin were of the same order of magnitude, but were slightly higher in the mycelium extracts (17.61 and 20.49 mg/100 g DW, respectively). On the other hand, kynurenine sulfate levels were almost 10 times higher in the material from in vitro cultures compared with the fruiting bodies (3.62 and 35.34 mg/100 g DW, respectively). The extracts from in vitro cultures were characterized by a much higher amount of 5-hydroxytryptophan (12.52 mg/100 g DW). This was also the highest level of 5-hydroxytryptophan obtained from all the species of *Basidiomycota* investigated in our laboratory so far. The amounts of the remaining indole compounds under analysis in the fruiting bodies and mycelium from in vitro cultures: L-tryptophan, melatonin, indole, 5-methyltryptophan, indoleacetonitrile were low, below 1 mg/100 g DW. The levels of indole compounds in the extracts of *C. cibarius* fruiting bodies presently under study do not diverge from the results obtained earlier for the fruiting bodies harvested from a natural site in Poland in 2007 (Muszyńska et al. 2011). The extracts studied earlier also contained a higher amount of serotonin (29.61 mg/100 g DW) and kynurenine sulfate (4.81 mg/100 g DW), and low (below 1 mg/100 g DW) amounts of other indole compounds: L-tryptophan, 5-hydroxytryptophan, melatonin, tryptamine and indoleacetonitrile. A comparison of the present data with the results obtained with methanolic extracts of other

Table 1 Amounts of indole compounds under study (mg/100 g d. w.) in extracts from the fruiting bodies and mycelium of *Cantharellus cibarius*

Indole compounds	*Cantharellus cibarius* fruiting bodies Mean ± SD	*Cantharellus cibarius* mycelium from cultures Mean ± SD
L-Tryptophan	0.02±0.003	0.64±0.013
5-Hydroxytryptophan	0.01±0.001	12.52±0.671
Serotonin	17.61±0.455	20.49±0.670
Melatonin	0.11±0.006	0.01±0.006
Tryptamine	0.02±0.002	_[a]
Indole	0.02±0.001	0.19±0.017
Kynurenine sulfate	3.62±0.032	35.34±1.332
5-Metyltryptophan	0.68±0.006	0.05±0.007
Indoleacetonitrile	0.02±0.003	0.02±0.002

Each observation mean=SD of three replicate experiments (n=3)

[a] Content lower than 0.001 mg/100 g d. w.

popular edible mushrooms (*Agaricus bisporus* (J.E. Lange)—White bottom mushroom, *Boletus edulis* Bull. ex Fr.—King Bolete, *Lactarius deliciosus* (L. Fr.) S.F. Gray—Saffron milk-cap, *Leccinum rufum* (Schaef.) Kreisel—Roudh-stalked boleti, *Pleurotus ostreatus* (Jacq.: Fr.) Kummer—Oyster mushroom, *Suilus luteus* L. ex Fr.—Slipery Jack, *Xerocomus badius*) and conditionally edible mushrooms (*Armillaria mellea* (Vahl.) Karst s. l.—Honey mushroom, *Lactarius deterrimus* Groger—False saffron milkcap, *T. equestre*), previously studied in our laboratory showed that the amounts of indole compounds were also very diverse and had a very wide variability range from 0.01 to 39.20 mg/100 g DW (Muszyńska et al. 2009, 2011). Only one of all the indole compounds under study, serotonin, was present in all the species investigated either previously or at present (except for *L. deterrimus*). The lowest serotonin content was determined in the fruiting bodies of *T. equestre* (0.18 mg/100 g DW), and the highest in *S. luteus* (34.11 mg/100 g DW). The amounts of serotonin in *L. rufum, L. deliciosus* and *B. edulis* were of the same order of magnitude as in *S. luteus* (31.71, 18.42, 10.14 mg/100 g DW, respectively) and these results are similar to the amount of this compound in the fruiting bodies and mycelium of *C. cibarius*. The levels of this compound in the commercial species of *A. bisporus* and *P. ostreatus* were slightly lower (6.52, 5.21 mg/100 g DW). The amounts of serotonin in the conditionally edible species were low and were estimated at a maximum of 2.20 mg/100 g DW in *A. mellea* and 0.18 mg/100 g DW in *T. equestre*. However, the amounts of tryptamine and tryptophan were much higher (from 2.01 to 4.46 mg/100 g DW) than in the above mentioned typical edible species. High levels of indole compounds degradation products, kynurenic acid and kynurenine sulfate, in the fruiting bodies of *S. luteus, L. deliciosus* and *A. bisporus*, ranging from 2.63 to 39.20 mg/100 g DW, appear to be of interest from a practical perspective. The amounts of the remaining indole compounds: melatonin, 5-hydroxytryptophan, indoleacetic acid and indoleacetonitrile in the fruiting bodies of the tested species were of the same order of magnitude as in the species analyzed earlier, i.e. below 1 mg/100 g DW.

Conclusions

The obtained results indicate that in vitro cultures of *Cantharellus cibarius* can be a good model for studies on the accumulation and metabolism of indole compounds in mushrooms. High levels of serotonin and its precursor, 5-hydroxytryptophan, in the fruiting bodies of *C. cibarius* and in its mycelium cultured in vitro also indicate a potential for the use of this material as a source of this physiologically important compound for humans. Among the indole compounds, the high level of kynurenine sulfate, which is the final product of kynurenic acid metabolism, also improves

the dietetic potential of this species. Kynurenic acid is an NMDA receptor antagonist, and is responsible for adaptive processes in the human organism. Further optimization of the conditions for in vitro cultures may allow them to become an alternative for commercial cultivation of this species. This is needed, since it may be expected that the mycelium cultured in vitro is also a source of other important metabolites, possessing both culinary and medicinal values, characteristic of the fruiting bodies.

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