TRICHOLOMA EQUESTRE SPECIES AS A SOURCE OF INDOLE COMPOUNDS AND ZINC RELEASED INTO ARTIFICIAL DIGESTIVE JUICES

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Abstract

The research goals of presented work consisted of determination of zinc and indole compounds that are released into artificial digestive juices from Tricholoma equestre species. During the experiment freeze-dried samples of wild growing fruiting bodies and mycelia from in vitro cultures from liquid Oddoux medium were extracted to artificial digestive juices (saliva, gastric juice and intestinal juice). In the next step the determination of examined compounds was done by RP-HPLC for indole compounds and DP ASV for zinc. Furthermore it was decided to check weather this species has any beneficial properties for health.

The extraction of researched material in conditions imitating human digestive tract allows determination of true amounts of elements released to artificial digestive juices and their beneficial influence (a specially designed and constructed apparatus Gastroel-2014 was applied). The indole compound with the highest quantity was 5-hydroxy-L-tryptophan, both in fruiting bodies and in biomass from in vitro cultures of T. equestre (up to 352.47 mg/100 g d.w.). Serotonin and L-tryptophan was determined in all analysed samples, but their amounts were significantly lower than the ones found for 5-hydroxy-L-tryptophan. The amounts of zinc on the other hand showed that the biggest concentration of zinc can be obtained for fruiting bodies and biomass from in vitro cultures to both artificial saliva and gastric juice after 120 minutes of digestion (6.83 14.4 mg/100 g d.w. retrospectively) in conditions that imitate human digestive track.

Keywords: *Tricholoma equestre*, artificial digestive juices, zinc, indole compounds, *in vitro* culture, fruiting bodies

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Introduction

Recently the medicinal properties of mushrooms are more frequently researched. Consumptions of the mushrooms is increasingly popular, not only because of their taste but also because of their medicinal and nutrient properties [1,2]. Due to high protein amount and low fat mushrooms are a part of low calorie diet [1,3]. *Tricholoma equestre* (Man on Horseback) was selected for the research because it is a commonly consumed mushroom that is allowed to be sold in many countries [4,5,6,7]. T. equestre is an edible mushroom with a high amount of zinc – an element that has biological meaning and nickel [8]. This species is rich in fatty acids which are main constituent of majority of lipids, are a source of metabolic energy and enhance digestion processes. Their role in prophylaxis of hypertension and inflammatory joint diseases. *T. equestre i*s rich in oleic acid, a main constituent of olive oil and decreases total cholesterol and linoleic which is a necessary diet element and cannot be synthesized by human organism [3]. It was proven that administering this mushroom to mice increases creatinine kinase [5,9,10]. Considering that *T. equestre* is an edible mushroom, so its medicinal potential should be examined in human diet. In this research the quantity of two types of medicinal ingredients was analysed indole compounds and zinc, both of them have antioxidant properties, inhibit aging processes and positively influence mood (as they belong to neurotransmitter precursors), furthermore they have anti-inflammatory and neuroprotective action [11,12,13].

Additionally zinc has a bacteriostatic action, it stabilizes cell membranes, enhances immunological, reproductive or respiratory system action.

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Furthermore it has a beneficial influence on skin and mucous membranes, it stimulates their regeneration and wound healing [14,15,16]. Because there are researches that involve the determination of indole compounds and metals in edible mushrooms but there is no information on their release in human digestive tract this is the first analysis in which a comparative study of indole compounds and zinc released into artificial digestive fluids from *T. equestre* fruiting bodies and *in vitro* cultures was made. This experiment is innovatory because the conditions in human digestive tract (imitating peristaltic movements and the temperature) were preserved for the entire time of extractions in artificial digestive juices.

Materials and methods

Mushrooms material

In the study to presented work fruiting bodies of *Tricholoma equestre* (L.) P. Kumm. (Man on horseback) and their *in vitro* cultures were used. Fruiting bodies of this species were collected from the natural environment in mixed forests of South Poland (in the vicinity of Nowy Sącz, Alwernia and Piwniczna Zdrój) between 2012 and 2015. Taxonomic identification of the young sporocarps was made according to online keys (http;//www.mycokey.com) and to authors Knudsen and Vesterholt by prof. B. Muszyńska. Representative voucher specimens were deposited at the Department of Pharmaceutical Botany, Jagiellonian University Collegium Medicum, Kraków, Poland. Mushroom material was frozen and lyophilized (Freezone 4.5. Labconco; temperature: – 40°C) to obtain the mushroom samples for next analyses.

Experimental cultures

Young fruiting bodies of *T. equestre* were used to obtain *in vitro* culture on solid Oddoux medium. Cultures from solid medium were used to develop experimental cultures, which were run on modified liquid medium according to Oddoux. Initial *inoculum* from solid medium amounted to 0.1 g. Obtained biomass of cultures on solid medium were transferred to Erlenmeyer flasks (500 mL) containing liquid medium (250 mL). The aim of running cultures on the liquid medium was to obtain the greatest growth rates from biomass that can be used in further analyses. Biomass was obtained from cultures run on Oddoux medium. After four weeks of growth the biomass of *in vitro* cultures was separated from the liquid medium using Büchner funnel, rinsing with four-times distilled water. Obtained biomass was immediately freeze-dried using lyophilisation technique (Lyophilizer Freezone 4.5, Labconco; −40°C).

Reagents

Citric acid, $KHCO_3$, K_2HPO_4, Na_2HPO_4 ethanol were from were from Polish Company of Chemistry (Gliwice, Poland); $NaHCO_3$, NaCl were from PPH Golpharm (Kraków, Poland); $MgCl_2$ was purchased from Chempur (Kraków, Poland); $CaCl_2$ was acquired from Pharma Zentrale GmbH (Germany); bile salts, pepsin were from BTL (Łódź, Poland); pancreatic extract, HCl, HNO_3 concentrated Suprapur®, KNO_3 Suprapur® were purchased from Merck (Darmstad, Germany); Zn(II) standard was purchased from OUM-7 Łódź, Poland. Standards of indole compounds, i.e. l-tryptophan, 5-OH-l-tryptophan, serotonin, tryptamine, 5-CH_3-tryptamine, 6-CH_3-d,l-tryptophan were from Sigma-Aldrich (St Louis, Mo, USA); all these were of HPLC grade. Methanol, acetic acid, petroleum ether and dichloromethane were from Merck (Darmstad, Germany). Quadruple-distilled water with a conductivity of less than 1μS cm^{-1} was obtained using an S2-97A2 distillation apparatus (Chemland, Stargard Szczecin, Poland).

Sample preparation for zinc and indole compounds analysis

Freeze-dried mushrooms were ground in a porcelain mortar, followed by the preparation of weighed portions of approximately 500 mg. They were placed in flasks containing 10 mL of artificial saliva solution according to Arvidson's recipe, shaken for 1 minute in Gastroel-2014 apparatus at normal human body temperature 37°C (shaking time in artificial saliva similarly as in the remaining artificial digestive juices: gastric and intestinal, results from the assumed average storage time of food in the oral cavity). The suspension was then decanted, and the residue – mushrooms fruiting bodies and biomass of *in vitro* cultures after digestion in artificial saliva solution were placed in 10 mL of gastric juice. Samples were shaken in 10 mL of artificial gastric juice for: 15, 60 and 120 minutes, respectively.

The solution was again decanted, and for the recovered fruiting bodies and *in vitro* cultures of investigated species 10 mL of artificial intestinal juice solution were added and shaken for 150 minutes also in Gastroel-2014 apparatus. Solutions prepared according to such method

were filtered through membrane filters (Millex, Millipore Corporation, USA). The half of obtained extracts was designated for zinc analysis; the other half was taken for indole compound assay. The solutions for zinc determination were mineralized by an addition of nitric acid Suprapur® (1 ml : 2 ml ratio). The mineralization was done in a Magnum II microwave unit (Ertec, Poland). Each sample was mineralized with the same conditions of temperature and pressure. The mineralized solutions were transferred quantitatively to quartz evaporation vessels and evaporated to almost dry on a hot plate. The residue was diluted to 5 ml in a volumetric flask with water. The solutions were examined for zinc amount by anodic stripping voltammetry. The solutions for indole determination were evaporated. The residue was dissolved in 1.5 mL of methanol. Methanol solution was filtered with membrane filters (Millex, Millipore Corporation, USA) and transferred to Eppendorf test tubes. The samples were determined by HPLC.

Second half of filtrates was evaporated to dry. The residue was dissolved in 1.5 mL of methanol and filtrated with membrane filters (Millex, Millipore Corporation, USA) and transferred to Eppendorf test tubes. Such samples were determined with HPLC.

Preparation of artificial digestive juice solutions

The artificial digestive juices: saliva, gastric juice and intestinal juice were prepared by dissolving ingredients in quadruple distilled water and adjusting the pH according to the recipes described in FP. X, Arvidson & Johasson (1985) and Neumann et al. (2006) [17,18,19]. The procedures were described precisely in a previous work by Muszyńska et al. (2016a) [20].

Results and discussion

Mushrooms ale well-known for their ability for accumulation of metals i.a. zinc. Due to that property the amount of zinc released to artificial digestive juices was determined in an apparatus that imitated the human digestive tract. The amount of zinc was determined by DP ASV a method that allows quick determination with acceptable accuracy and precision. In this experiments the samples were mineralized in a microwave digestion unit what increased the effectivity of mineralization and determinations. The results were recalculated to mg/100 g of dry weight. In this work the quantity of zinc released to artificial saliva and gastric juice was summed because in natural conditions zinc is not absorbed in oral cavity and the food is transferred to further parts of digestive tract. DP ASV was previously used in zinc determination in mushroom material what allowed precise preparation of analysis conditions what positively influenced the results precision. For the research both fruiting bodies and in vitro cultures of the same species were used to show zinc accumulation ability and the release of this element. The accumulation ability is species specific ability, and depends on the fruiting bodies structure as well as on the zinc amount in the mushroom environment. This research may confirm the ability of zinc accumulation from the environment because *in vitro* cultures made on Oddoux liquid medium (with an addition of 1.5mL of 0.2% zinc sulphate for 1L of medium) released more zinc than fruiting bodies from natural environment. In *T. equestre* fruiting bodies zinc amounts varied between from 1.11 to 6.83 mg/100 g d.w., and in biomass from *in vitro* cultures the quantities were higher and ranged from 1.52 to 14.4 mg/100 g d.w. (Table 1). Both for fruiting bodies and *in vitro* cultures the summed amounts of zinc from arterial saliva and gastric juice were higher from these in intestinal fluid.

Tab. 1. The content of zinc in lyophilized biomass of *T. equestre* extracted into artificial digestive juices in different time periods.

Extraction time *T. equestre* species	Mean content of zinc [mg/100 g d.w.] ± SD
in vitro cultures	
saliva	
+	9,88±0,24
gastric juice	7,25±0,26
15 min	14,4±0,62
60 min	2,65±0,15
120 min	1,74±0,08
intestinal juice	1,52±0,10
150 min	
fruiting bodies	
saliva	
+	3,22±0,09
gastric juice	4,95±0,21
15 min	6,83±0,25
60 min	1,11±0,03
120 min	1,71±0,13
intestinal juice	1,27±0,06
150 min	

n=3 repetitions; $p \leq 0.05$; by Statistica 10 (StatSoft, Poland)

The highest amounts of zinc were released from mushrooms incubated in artificial gastric juice for 120 min. The biggest amount released from *in vitro* cultures was 15.92 mg/100 g d.w. (daily zinc requirement of human organism), and from fruiting bodies 8.1 mg/100 g d.w. what is shown in Fig. 1. The worst variant of incubation was 15 minutes incubation in gastric juice for fruiting bodies (4.33 mg/100 g d.w.), and 60 minutes incubation for *in vitro* cultures (8.99 mg/100 g d.w.). In previous experiment by Yamaç et al. (2007) the zinc amount was higher and was 17.38 mg/100 g d.w. but represented the amount in fruiting bodies not the one that is released [8].

In the analysis of indole compounds by RP-HPLC five indoles were determined serotonin, 5-hydroxy-L-tryptophan, L-tryptophan, 5-methyltryptamine and 6-methyl-D,L-tryptophan (Table 2.). In this experiment also, the amounts of indoles released to artificial saliva and gastric juice were summed alike to zinc. Tryptamine was not determined in any of the extracts. 5-Hydroxy-L-tryptophan, serotonin and L-tryptophan were determined in all samples. L-tryptophan amounts were the lowest (each time lowered than 0.001 mg/100 g d.w.). 5-Hydroksy-L-tryptophan on the other hand had the highest amounts (up to 352.47 mg/100 g d.w. when summed from saliva and gastric juice during 120 minutes extraction in gastric juice from *in vitro* cultures of *T. equestre*). 5-Methyltryptamine and 6-methyl-D,L-tryptophan had the highest amounts released in artificial saliva and gastric juice. In extracts from fruiting bodies no 6-methyl-D,L-tryptophan was present. The highest summed amounts of indole compounds was found for 15 minutes extracts in artificial gastric juice (537.15 mg/100 g d.w for fruiting bodies and 492.62 mg/100 g d.w. from *in vitro* cultures) what can be observed in Fig. 2. The lowest amounts were shown for 120 minutes extraction in gastric juice from fruiting bodies (469.15 mg/100 g d.w.) and for *in vitro* cultures in 60 minutes extracts (464.71 mg/100 g d.w.), after 120 minutes the amounts were slightly higher (466.60 mg/100 g d.w.). *T. equestre* proved to be a species in which by every time the released amounts of indole compounds were higher for natural fruiting bodies than for *in vitro* cultures. The quantities do not vary significantly from these previously observed an determined for *Agaricus bisporus* and *Boletus badius* species [21,22].

The process of extraction of mushroom

Tab. 2. The content of indole compounds in lyophilized biomass of T. equestre extracted into artificial digestive juices in different time periods.

Extraction time *T. equestre* species	5-hydroxy-L-tryptophan	Serotonin	L-tryptophan	5-methyl-tryptamine	6-methyl-D,L-tryptophan
	[mg/100 g d.w.] ± SD				
in vitro cultures saliva					
+	206.41±5.69	132.29±2.97	*	5.58±0.02	16.05±0.12
gastric juice	217.12±6.66	138.81±1.68	*	4.89±0.15	13.77±0.04
15 min	352.47±12.70	41.11±3.62	*	2.07±0.00	11.96±0.02
60 min	48.40±2.67	70.04±0.21	*	5.96±0.00	7.88±0.03
120 min	36.98±1.55	45.20±2.63	*	–	7.94±0.01
intestinal juice 150 min	31.33±0.08	19.86±1.26	*	–	7.79±0.01
fruiting bodies saliva					
+	281.56±4.09	62.37±2.15	*	9.27±0.02	7.77±0.01
gastric juice	189.19±4.71	195.65±5.64	*	3.04±0.02	11.41±0.01
15 min	190.73±1.69	66.53±1.77	*	12.18±0.02	–
60 min	81.99±4.93	88.13±6.95	*	6.06±0.02	–
120 min	47.74±1.28	71.98±2.10	*	–	–
intestinal juice 150 min	56.28±1.40	137.42±4.40	*	6.00±0.00	–

n=3 repetitions; *less than 0.001 mg/100 g d.w. $p \leq 0.05$; by Statistica 10 (StatSoft, Poland)

material and release is crucial for the next experiments which will allow the evaluation of bioavailability of substances from fruiting bodies. The quantity of compounds and elements alone does not determine the way in which biological material will act in human digestive tract. The key feature of this research was the description of phenomena that take place in conditions of human digestive tract and pointing the true advantages of *T. equestre* consumption. *In vitro* cultures proved to be a better source of zinc, their preparation on a well fit medium could influence the production of some beneficial compounds and both the accumulation and release of bioelements. The fruiting bodies on the other hand showed higher quantity of indole compounds and a better release of these substances. Then it was proven that *T. equestre* is not only a rich source of examined compounds but it also releases them to digestive tract where these substances can be absorbed to the human organism and express their beneficial action.

Resumo

La celo de la prezentita laboro estas determino de zinko kaj indolaj kemiaj komponaĵoj, kiuj estas liberigitaj en artefaritajn digestivajn sukojn de Tricholoma equestre specio. Dum la eksperimento frostitaj sekigitaj specimenoj de sovaĝe kreskantaj korpoj de fungoj kaj micelo de *in vitro* kulturoj kreskantaj en Oddoux likvaĵo estis uzitaj kun tri artefaritaj digestivaj sukoj (salivo, stomata suko kaj intesta suko) por ricevi ekstraktojn. En la sekva paŝo oni determinis la indolajn kemiajn komponaĵojn uzante RP-HPLC metodon kaj por determini jonojn de zinko uzante DP ASV metodon. La celo de tiu ĉi laboro estis indiko de porsanaj proprecoj de tiu ĉi fungo. La ekstraktado de esplorita materialo en kondiĉoj de homa artefarita digesta dukto ebligas pritaksi faktajn kvantojn de liberigitaj substancoj havantajn porsanajn eblecojn (oni aplikis speciale por tiu ĉi eksperimento konstruitan aparaton, t.n. Gastroel-2014). La indolaj kombinaĵoj en plej alta kvanto estis 5-hydroxy-L-triptofano, ambaŭ en fungaj korpoj kaj en biomaso de en vitro kulturoj de T. equestre (ĝis 352,47 mg/100 seka maso). Serotoninon kaj L-triptofanon oni determinis en ĉiuj esploritaj provaĵoj, sed ilia kvanto estis malpli alta kompare al determinita kvanto de 5-hydroxy-L-triptofano. La determinita kvanto de zinko montris, ke la plej granda koncentriteco de zinko povas esti ricevita entute de fungaj korpoj kaj biomaso de in vitro kulturoj post eltiro el artefaritaj salivo kaj stomaka suko post 120 minutoj de digesto (respektive 6.83 14.4 mg/100 g seka maso) en kondiĉoj, kiuj imitas homan digestan organon.\

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