

From mycelium to spores: A whole circle of biological potency of mosaic Puffball

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Abstract

Methanol extracts of mosaic puffball (*Handkea utriformis*, *Bovistella utriformis*, *Lycoperdon utriforme*, *Calvatia utriformis* – current name is a subject of debate), from three different stages – mycelium (HUMIC), immature (HUI) and mature fruiting bodies (HUM) were characterized and tested for antioxidant, antimicrobial and inhibitory activity on tyrosinase, acetylcholinesterase (AChE), butyrylcholinesterase (BuChE) and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA-R). Immature, edible, fruiting bodies were shown to be a good source of antioxidants (11.5 mg/g of extract) and cholesterol-lowering agent, lovastatin (234 µg/g of extract), and exhibited significant antimicrobial activity. In addition, HUI showed good and selective AChE (4.48 mg/mL) and nonlovastatin related HMG-CoA-R inhibition (1.16 mg/mL), which all together suggests that regular consumption of it may have health benefits. Mature fruiting bodies, inedible due to powdery consistence, have been used in traditional medicine for wound treatment; their extract was relatively rich in free ergosterol (31.65 mg/g of extract), N-acetylglucosamine (24 mg/g of extract), α -tocopherol (4 mg/g of extract) and had best overall antioxidant activity, which was in correlation with its highest phenolic content (19.4 mg GAE/mL). It also exhibited significant tyrosinase inhibitory activity (0.22 mg/mL) and thus could be used in medicinal and cosmetic products for wound healing and bleaching. Mycelium, which can be easily grown in laboratory conditions did not have the same properties as, neither immature or mature fruiting bodies, although it showed prominent antimicrobial activity, notably against *Pseudomonas aeruginosa* (MIC = 0.0625 mg/mL) and could be a source of antimicrobial compounds.

Introduction

Mushrooms are source of various biologically active compounds and numerous studies found that they may exhibit beneficial effects on human health (Lindequist et al., 2005). They act as functional food – having low energy value and being rich in fibers and minerals, as well as high-quality proteins and health promoting constituents such are vitamins and β -glucans (Kalač, 2009). They may possess antiinflammatory, immunomodulating, antioxidant, antimicrobial, antiviral, antihypertensive, antihyperglycemic and other medicinal properties (Lindequist et al., 2005). Many species have been used in traditional medicine, the fact which has sparked recent mass screening of their potential use in modern medicine.

Mosaic puffball, known scientifically as *Handkea utriformis* (Bull.) Kreisel, *Lycoperdon utriforme* (Bull.), *Bovistella utriformis* (Bull.) Demoulin & Rebriv or *Calvatia utriformis* (Bull.) Jaap. (current scientific name is a subject of debate) is a mushroom species that belongs to puffballs – a group of fungi that produce enclosed, globose fruiting bodies (Larsson and Jeppson, 2008). Firm and white when young, *H. utriformis* fruiting bodies go through a process of autolysis upon maturation, which turns their inside – gleba – into a dark powdery spore-bearing mass (Læssøe and Spooner, 1994). They are edible when young and are highly rated regarding their culinary value (Coetzee and van Wyk, 2009). Puffballs are, however, better known for their use in traditional medicine, for wound dressing and treatment of various skin conditions, as mature gleba is an effective styptic. They have also been used to treat coughing, stomach ache and fever. The use of puffballs in veterinary practice was also common in some areas. Even though their use is well documented in folk medicine of many cultures (Læssøe and Spooner, 1994), they have not been a subject of extensive studies as some other mushroom species.

As for *H. utriformis*, little is known about its general chemical composition. It was reported that lipid content in (fresh) mosaic puffballs is 1.8%, and that fatty acid composition is similar to that of other mushrooms, with linoleic, oleic and palmitic acids being dominant. Detailed free fatty acid analysis of mature mosaic puffballs was given by Petrović et al. (2016). Bauer Petrovska (2001) reported protein content in *H. utriformis* (as *Calvatia caelata*) as 52% expressed in terms of dry mass, but methodology

of that study was criticized by Coetzee and van Wyk (2009). Relatively high content of copper and zinc was found in fruiting bodies of this species (Alonso et al., 2003).

Even though *H. utriformis* is widespread, and has almost cosmopolitan distribution (Kreisel and Moreno, 1996), a little is published about its pharmacological potential. *Handkea utriformis* mycelium was reported to produce calvatic acid, a potent antibiotic, and a protein named calcaelin and a ubiquitin like peptide were isolated from its fresh fruiting bodies, both of which exhibited high in vitro antiproliferative activity towards breast cancer cells (Coetzee and van Wyk, 2009). Mature fruiting bodies extracts have also been reported to exhibit antioxidant, antimicrobial, antitumor, acetylcholinesterase and angiotensin converting enzyme inhibitory activity (Petrović et al., 2016; Dulger, 2005; Akata et al., 2018).

Given the fact that *H. utriformis*, as all gasteromycetes, goes through a unique life-cycle, the goal of this study was to evaluate and compare chemical composition, emphasizing nutraceuticals, and various biological activities of methanol extracts of its mycelium (HUMIC), immature (HUI) and mature fruiting bodies (HUM). Total phenols and tocopherol (vitamin E), ergosterol (provitamin D2), proteins and sugars (including N-acetylglucosamin), were quantified in the extracts, as known physiologically active chemical species found in mushrooms. The extracts were also screened for antioxidant and antimicrobial activity, as well as inhibitory activity on several important enzymes involved in various health conditions; 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA-R) – a key enzyme in cholesterol synthesis pathway, which inhibition may cause antihypercholesterolemic effect (GilRamírez et al., 2013). The inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) is associated with relief of symptoms in Alzheimer's disease (Liston et al., 2004). Tyrosinase is involved in production of melanin, and its inhibitors are used in cosmetic products to treat hyperpigmentation (Taofiq et al., 2016). The enzyme inhibitory analysis was accompanied by quantification of known fungi-derived inhibitors, lovastatin, a well-known HMG-CoA-R inhibitor (Du Souich et al., 2017), and kojic acid, a tyrosinase inhibitor in current use in cosmetology (Ariff et al., 1996).

There are no studies about the changes that this species or other puffball species go through in their life-cycle, regarding their potential benefit for humans, as source of physiologically active products. Also, this is the first study to report activity of *H.*

utriformis extracts on HMG-CoA-R, BuChE and tyrosinase. Several pharmacologically active compounds were quantified in *H. utriformis* for the first time as well, including N-acetylglucosamin, lovastatin, α -tocopherol and ergosterol. To the best of our knowledge, this is the first report of quantification of free N-acetylglucosamin in any mushroom species.

2. Material and methods

2.1. Materials

Potassium dihydrogenphosphate (KH_2PO_4), magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), sodium dihydrogenphosphate (NaH_2PO_4), sodium hydrogen phosphate (Na_2HPO_4), sodium carbonate (Na_2CO_3), copper (II) chloride (CuCl_2), ammonium acetate ($\text{CH}_3\text{COONH}_4$), potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$), orthophosphoric acid (H_3PO_4), sulfuric acid (H_2SO_4), formic acid, glucose, fructose, mannose, xylose, rhamnose, arabinose, glucuronic acid, galacturonic acid, n-acetylglucosamine, trehalose, phenol, gallic acid, β -carotene, α -tocopherol, ergosterol, kojic acid, lovastatin, L-DOPA, neocuproine, Brilliant Blue G, Folin–Ciocalteu reagent, albumin, linoleic acid, Tween 80, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), acetylcholine iodide (AChI), butyrylcholine iodide (BuChI), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), triphenyltetrazoliumchloride (TTC), tyrosinase from *Agaricus bisporus*, acetylcholinesterase from *Electrophorus electricus* (AChE), butyrylcholinesterase from equine serum (BuChE) and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA-R) assay kit were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Merck Co. (Darmstadt, Germany). Peptone, yeast extract, malt broth, malt agar, Müller–Hinton broth and Müller–Hinton agar were purchased from Biolife (Milan, Italy). Methanol, ethanol, ethylacetate, acetonitrile, chloroform, dimethylsulfoxide k (DMSO) were obtained from LGC Promochem, Germany. Deionized water was used in all experiments unless stated otherwise. Fruiting bodies of *H. utriformis*, at different stages of development, were collected near the city of Bor, Eastern Serbia, in 2015. Identification of the specimens was done by dr Boris Ivančević (Department of Mycology and Lichenology, Natural History Museum, Belgrade). Referent specimens are held in the national fungarium, Natural History Museum in Belgrade and referent

isolated mycelium culture is kept at the Faculty of Technology and Metallurgy, Belgrade, Serbia.

2.2. Mycelium cultivation and extract preparation

Mycelium culture was isolated from an immature fruiting body of *H. utriformis*, belonging to the same collection used in the study. Mycelium was produced according to Umezawa et al. (1975). Briefly, it was cultivated in a medium consisting of glucose (2%), peptone (0.5%), yeast extract (0.3%), KH_2PO_4 (0.3%) and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.3%) for 3 weeks at 28 °C. Both mycelium and immature fruiting bodies were lyophilized in a Christ BETA 2–8 LD plus freeze dryer (Osterode, Germany) and powdered; there was no need for pretreatment of mature fruiting body's gleba as it is completely dry and powdery. Material was mixed with methanol (0.05 g/mL) and undergone to maceration for 72 h. After centrifugation of the mixture, the supernatant was collected and filtered through a filter paper (Whatman® No. 5); methanol was completely removed under low pressure (Heidolph Hei-VAP Value rotary evaporator, Germany), and the extracts were additionally dried in a vacuum desiccator at room temperature for 24 h. Dry extracts were kept in the refrigerator, protected from light and moisture. Drug to extract ratio (DER) for HUMIC, HUI and HUM was 4.3:1, 4.7:1 and 6.2:1, respectively.

2.3. Total phenols

Total phenols were estimated according to Skotti et al. (2014), adapted for 96-well microplates. Sample methanol solutions (12.5 µL, 4 mg/mL) were mixed with water (175 µL), Folin–Ciocalteu reagent water solution (25 µL, 1:5 v/v) and Na_2CO_3 water solution (20% m/V, 75.5 µL). The absorbance was read after 2 h at 630 nm on a BioTek ELx808 microplate reader (Winooski, VT, USA). Gallic acid was used for the standard curve calculation and the results were expressed as mg of gallic acid equivalents per gram of extract (mg GAE/g).

2.4. Total proteins

Total proteins were determined using Bradford protein assay (Bradford, 1976), adapted for 96-well microplates. Sample methanol solutions (25 µL, 10 mg/mL) were mixed with Bradford reagent (230 µL) and the absorbance was read after 10 min at 630

nm on a microplate reader. Albumin was used to calculate the standard curve and the results were expressed as protein percentage (%).

2.5. Sugar analysis

2.5.1. Total sugars

Total sugars were determined according to DuBois et al. (1956). Sample water solutions (200 μ L, 2 mg/mL) were mixed with water (200 μ L), phenol water solution (400 μ L, 5% w/v) and sulfuric acid (2 mL, conc.). The absorbance was read after 40 min at 490 nm on a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). D-(+)-glucose was used to calculate the standard curve and results were expressed as sugar percentage (%).

2.5.2. High performance liquid chromatography (HPLC) analysis of monoand disaccharides

Water sample solutions (50 mg/mL) were centrifuged and the supernatant collected. The analysis was performed using Dionex Ultimate 3000, Thermo Scientific (Waltham, USA) HPLC system, on a carbohydrate column (Hyper REZ XP Carbohydrate Ca²⁺, 300 mm \times 7.7 mm, 8 μ m) incubated at 80 °C. Water was used as mobile phase with elution rate of 0.6 mL/min. Detection of sugars was achieved using an RI detector (RefractoMax 520, ERC, Germany), and all data acquisition and processing was done in Chromeleon Software. In terms of constructing the calibration curves for quantification, glucose, fructose, mannose, xylose, rhamnose, arabinose, glucuronic acid, galacturonic acid, Nacetylglucosamine and trehalose were used as standards.

2.5.3. NMR analysis of mono- and disaccharides

Sugars were extracted with water (50 mg/mL), samples centrifuged and supernatant collected and lyophilized. NMR spectra were performed at 301 K on a Bruker Ascend 400 spectrometer at 100.6 MHz for ¹³C in D₂O as a solvent. The ¹³C NMR spectra were acquired with spectral width = 24.04 kHz and 16,000 scans. NMR data were processed using TopSpin version 3.2 and MestReNova version 6.0.2.

2.6. Ergosterol determination

To determine ergosterol, the extracts were dissolved in methanol (10 mg/mL). GC–MS analysis was performed using Agilent Technologies 6890N–GC System coupled with Agilent 5973 Network Mass Selective Detector and MSD ChemStation Software G 1701BA. A HP-5 MS column with dimensions of 30 m \times 0.25 mm \times 0.25 μ m was used. The conditions used were as described by Petrović et al. (2016). Injection was performed in pulsed splitless mode with splitless time of 1 min. Injection volume of the samples was 1 μ L; auto sampler used was Agilent 7683 Injector Series. The temperature of the injector was 260 °C; Helium (5.0, Messer, Bad Soden, Germany) was used as a carrier gas, with a constant flow of 1 ml/min. The initial oven temperature was 50 °C and was held for 2 min; the temperature was increased to 150 °C (at a rate of 25 °C/min), held for 4 min, then to 250 °C (4 °C/min) and finally to 300 °C (10 °C/min) which was held for 5 min. Mass spectra were acquired in electron impact mode. MS quad detector was used; transfer line temperature was set at 280 °C, MS quad at 150 °C and MS source at 230 °C. The analysis was performed in Full Scan mode and the scan rate was 6 scan/s; range of scanned mass was 50–600 m/z. NIST05 MS Library Database was used to confirm the identity of ergosterol. Series of ergosterol ethyl-acetate solutions were used to calculate the standard curve.

2.7. α -tocopherol determination

Quantification of α -tocopherol was performed on an Agilent HPLC System 1260 (Agilent technologies, Waldbronn, Germany) equipped with ChemStation software Rev. B.04.03-SP1, quaternary pump (G1311B/1260), autosampler (G1329B) and diode-array detector (DAD) (G4212B), using Zorbax SB-C18 column (250 \times 4.6 mm; 5 μ m particle size, Agilent technologies). The extracts (25 mg/mL) were dissolved in a methanol:n-hexane mixture (9:1) and filtered through a membrane filter (Whatman®, 0.2 μ m). Elution was achieved with 0.1% (v/v) formic acid in water (mobile phase A) and methanol (mobile phase B). The column was operating at 25 °C and the gradient program was as follows: 10–90% B (15 min), 90–100% B (5 min), isocratic elution with 100% B (5 min), 100–10% B (5 min), with the total run time of 32 min, flow rate of 0.8 mL/min, and the injection volume of 5 μ L. DAD was operating at 280 nm. α -tocopherol was used to construct the standard curve.

2.8. Lovastatin determination

Samples were dissolved in DMSO (50 mg/mL) and filtered through a membrane filter (Whatman®, 0.2 µm). Sample analysis was done by a Dionex Ultimate 3000 Thermo Scientific (Waltham, USA) HPLC system and a reverse phase column (Hypersil GOLD C18, 150 mm × 4.6 mm, 5 µm). Elution system was water and 0.1% (v/v) formic acid (mobile phase A) and acetonitrile and 0.1% (v/v) formic acid (mobile phase B). It was conducted as follows: 0–10 min isocratic step of 75% B, then a gradient step from 75 to 100% B, then 15–20 min 100% B, 20–20.1 min gradient from 100 to 75% B, and 20.1–25 min isocratic step of 75% B. The elution flow rate was 1 mL/min and the column was thermostated at 30 °C. Detection was achieved by a UV detector at 238 nm. Lovastatin was used to calculate the standard curve.

2.9. Kojic acid determination

Kojic acid was determined by HPLC method described by Ariff et al. (1996). Samples (50 mg/mL) were dissolved in DMSO and filtered through a membrane filter (Whatman®, 0.2 µm). The same HPLC system was used as for lovastatin determination, with the UV detector set at 265 nm. Mobile phase was mixture of phosphate buffer (50 mM, pH 3) and methanol (95:5), with a flow rate of 1 mL/min. Kojic acid was used to calculate the standard curve.

2.10. In vitro antioxidant activity

2.10.1. ABTS radical scavenging assay

Radical scavenging ability of the extracts was tested by ABTS assay (Re et al., 1999), adapted for 96-well microplates. K₂S₂O₈ water solution (3.8%, 88 µL) was added to ABTS water solution (0.38%, 5 mL) and left for 16 h to produce ABTS radicals; the working solution was prepared by diluting the stock solution 100x with methanol (A_{734nm} = 0.700). Sample methanol serial dilutions (20 µL, 0.0625–4 mg/mL) were mixed with ABTS radical solution (200 µL) and the absorbance (A_s) was read after 20 min on a microplate reader at 630 nm. Mixture of methanol (20 µL) and ABTS solution (200 µL) was used as a control (A_c). For each sample concentration, percentage of ABTS radical neutralization was calculated according to the formula: (A_c – A_s)/A_c × 100. The results were expressed as a sample concentration which neutralizes 50% of ABTS radicals (EC₅₀, mg/mL). Ascorbic acid was used as standard.

2.10.2. CUPRAC reduction power assay

The ability of extracts to reduce cupric ions was determined according to the method described by Özyürek et al. (2011). Solutions of Cu²⁺ (50 µL, 10 mM), neocuproine (50 µL, 7.5 mM), CH₃COONH₄ buffer (60 µL, 1 M, pH 7.0) and serial dilutions of samples prepared in methanol (40 µL, 0.156–10 mg/mL) were added to a 96-well microplate and incubated for 1 h, at 30 °C. The absorbance was measured on a microplate reader at 450 nm. The results were expressed as mmol equivalents of Trolox (Trol. mM eq.).

2.10.3. β -carotene bleaching assay

The ability of extracts to prevent (per)oxidation of linoleic acid was determined by the β -carotene -linoleate model system using the procedure given by Barros et al. (2007a), adapted for 96-well microplates. β carotene chloroform solution (2 mL, 0.2 mg/mL) was evaporated under low pressure, until chloroform was completely removed. Then, linoleic acid (40 mg), TWEEN 80 (400 mg) and water (100 mL) were added to make the emulsion. Emulsion (250 µL) was mixed with serial methanol dilutions of samples (20 µL, 0.156–10 mg/mL). The mixture of methanol (20 µL) and emulsion (250 µL) was used as control. The absorbance of the control and sample/emulsion mixtures was read immediately (Ac0 and As0, respectively) and after 1 h (Ac1, As1), i.e. after control was completely bleached, on a microplate reader, at 470 nm. The percentage of β -carotene bleaching inhibition was calculated according to the following formula: $[(Ac0 - Ac1) - (As1 - As0)] / (Ac0 - Ac1) \times 100$, and the results were expressed as sample concentration which prevents 50% of β -carotene bleaching (EC₅₀, mg/mL). Ascorbic acid was used as standard.

2.11. Tyrosinase inhibitory assay

The assay was performed according to the procedure given by Alam et al. (2012), with some modifications. Tyrosinase (46 U/mL) and LDOPA (2.5 mM) were dissolved in phosphate buffer (0.067 M, pH 6.8). Sample dilutions (40 µL, 0.156–5 mg/mL) were prepared in DMSO/ phosphate buffer (5% v/v). The reagents and samples were mixed in 96-well microplates as following: A: phosphate buffer (120 µL), enzyme (40 µL), L-DOPA (40 µL); B: phosphate buffer (160 µL), L-DOPA (40 µL); C: phosphate buffer (80 µL), sample (40 µL), tyrosinase (40 µL), LDOPA (40 µL). D:

phosphate buffer (120 μ L), sample (40 μ L), L-DOPA (40 μ L). The microplates were incubated for 10 min before adding L-DOPA and for another 20 min after the addition of L-DOPA, at 25 °C. The absorbance was read at 490 nm. The inhibition (%) was calculated using following formula: $[(A - B) - (C - D)] / (A - B) \times 100$; the results were expressed as a sample concentration which inhibits 50% of enzyme reaction (IC₅₀, mg/mL) and kojic acid was used as a positive control.

2.12. Acetylcholinesterase (AChE)/butyrylcholinesterase (BuChE) inhibitory assay

The assays were performed according to Fadaeinasab et al. (2013), with a slight modification. AChE/BuChE (0.09 U/mL), AChI/BuChI (0.014 M), DTNB (0.01 M) and sample solutions (0.156–5 mg/mL) were prepared in a phosphate buffer (0.1 M, pH 8, except for DTNB for which pH was set at 7) and mixed as following:

A: phosphate buffer (160 μ L), AChE/BuChE (20 μ L);

B: phosphate buffer (180 μ L);

C: phosphate buffer (140 μ L), AChE/BuChE (20 μ L), sample (20 μ L);

D: phosphate buffer (160 μ L), sample (20 μ L).

Plates were incubated at 25 °C for 15 min, with gentle stirring and DTNB (10 μ L) and AChI/BuChI solutions (10 μ L) were added. Incubation was continued for 40 min at 25 °C and the absorbance was read at 405 nm. Percentage of enzyme reaction inhibition was calculated for each sample concentration, according to the following formula: $[(A - B) - (C - D)] / (A - B) \times 100$; the results were expressed as a sample concentration which inhibits 50% of enzyme reaction (IC₅₀, mg/mL) and galantamine was used as a positive control.

2.13. HMG-CoA reductase (HMG-CoA-R) inhibitory assay

HMG-CoA-R inhibitory assay was performed using a commercial assay kit with a slightly modified procedure. Serial dilutions of the extracts were prepared in 10% DMSO water solution (0.313–5 mg/mL), while buffer, enzyme (1.5 U/mgP), coenzyme

and substrate solutions were prepared as described in the manufacturer's procedure. The reagents and samples were mixed as following:

A: buffer (346 μ L), enzyme (2 μ L), substrate (24 μ L), coenzyme (8 μ L), 10% DMSO (20 μ L);

B: buffer (348 μ L), substrate (24 μ L), coenzyme (8 μ L), 10% DMSO (20 μ L);

C: buffer (346 μ L), enzyme (2 μ L), substrate (24 μ L), coenzyme (8 μ L), sample (20 μ L). The absorbance was read immediately and after 10 min at 340 nm, at 37 °C, on a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan) and percentage of enzyme inhibition was calculated using following formula:

$$\frac{\{(A_0-A_{10})-(B_0-B_{10})\}-[(C_0-C_{10})-(B_0-B_{10})]}{(A_0-A_{10})-(B_0-B_{10})}$$
, where numbers in subscript indicate the absorbance at the start (“0”) and 10 min (“10”) after the reaction commenced. The results were expressed as a sample concentration which inhibits 50% of enzyme reaction (IC₅₀, mg/mL) and pravastatin was used as control.

2.14. Broth microdilution antibacterial assay

Minimum inhibitory (MIC) and bactericidal concentrations (MBC) of the extracts were determined using broth microdilution method (CLSI, 2005; Klaus et al., 2015).

Antimicrobial activity was tested against 5 microbial strains of the American Type Culture Collection (ATCC): *Staphylococcus aureus* 6538, *Enterococcus faecalis* 29212, *Pseudomonas aeruginosa* 27853, *Escherichia coli* 25922, *Candida albicans* 10259 and a clinical strain of methicillin-resistant *Staphylococcus aureus* (MRSA), obtained from the City Institute of Public Health (Belgrade, Serbia). The antimicrobial assay was performed in 96-well microplates (Sarstedt, Germany). The extracts were dissolved in DMSO aqueous solution (5% v/v), covering the concentration range from 0.01 to 20 mg/mL. The density of microbial suspensions was set at 10⁵ CFU and TTC (0.0075%) was added as growth indicator. Suspensions (50 μ L) were added to each well containing previously prepared sample dilution (50 μ L). Growth control was prepared by mixing microbial suspension (50 μ L) with DMSO water solution (5% v/v, 50 μ L).

All determinations were performed in duplicates. Bacterial strains were incubated 24 h at 37 °C, while yeast strains were incubated 48 h at 30 °C. The concentration of the sample at which there was no visible microbial growth in both replications (absence of red color for bacterial strains and absence of visible colonies for *C. albicans*) was taken as a MIC value. MBC was determined by serial sub-cultivation of the samples taken from each well on a solid medium (Müller–Hinton agar for bacterial strains and malt agar for *C. albicans*); the lowest concentration of the sample without any visible growth after repeated incubation was considered as a MBC. Amoxicillin was used as a standard for bacterial strains (0.05–50 µg/mL) and fluconazole for *C. albicans* (0.1–100 µg/mL).

2.15. Statistical analysis

All measurements were done in triplicate, and data were expressed as mean \pm standard deviation, if not stated otherwise. The experimental data were subjected to one-way analysis of variance (ANOVA) and Tuckey's HSD test were calculated to detect significant difference ($\alpha = 0.05$) between the mean values. MS Excel (Microsoft Office 2010 Professional) was used to calculate EC₅₀ and IC₅₀ values and OriginPro 8 for statistical analyses.

3. Results and discussion

Protein content (**Table 1**) was relatively low in all three extracts (b1%), but carbohydrates made significant part of them, about 20% of HUM (previously published, Petrović et al., 2016), 29% of HUI and over 50% of HUMIC. Most of the analyzed monosaccharides were either not detected or present only in traces. Di-, tri- and oligosaccharides (and possibly low molecular polysaccharides) make most of the carbohydrate content in all three extracts. Glucose, however, makes about 50% of HUMIC carbohydrate content (~25% overall). Trehalose is abundant in both HUMIC and HUI, making about 50% and almost 100% of their sugar content, respectively, but only 11.8% of sugar content of HUM.

Table1

Content of selected molecular fractions/compounds of *Handkea utriformis* mycelium (HUMIC), immature (HUI) and mature fruiting bodies (HUM) methanol extracts expressed as mean \pm SD. Only sugars that were detected in at least one of the samples are given in the table

	HUMIC	HUI	HUM
Total proteins (%)	0.69 \pm 0.04 ^{b1}	0.84 \pm 0.02 ^a	0.90 \pm 0.01 ^a
Total carbohydrates (%)	53.48 \pm 2.84 ^a	29.02 \pm 0.83 ^b	19.57 \pm 0.82 ^c
Trehalose (mg/g)	256.81 \pm 5.83 ^b	287.91 \pm 2.07 ^a	23 \pm 0.4 ^c
Glucose (mg/g)	246.23 \pm 8.12	nd ²	nd
N-acetylglucosamine (mg/g)	nd	nd	26.32 \pm 0.08
Total phenols (mgGAEs/g) ³	3.21 \pm 0.21 ^{c2}	11.52 \pm 0.25 ^b	19.75 \pm 0.95 ^a
α -tocopherol (mg/g)	nd	2.29 \pm 0.08 ^b	4.00 \pm 0.36 ^a
Ergosterol (mg/g)	7.70 \pm 0.08 ^b	1.22 \pm 0.05 ^c	31.65 \pm 0.41 ^a
Lovastatin (μ g/g)	4.95 \pm 0.17 ^c	234.44 \pm 8.67 ^a	25.04 \pm 1.13 ^b
Kojic acid	nd	nd	nd

¹ Different letters by rows stand for statistically different values ($\alpha = 0.05$, ANOVA, Tukey's HSD test);

² Not detected/found in traces.

³ mg of gallic acid equivalents per g of extract.

NMR identification of carbohydrates were made by comparison with spectra of standards and ¹³C spectra confirmed the presence of trehalos in all three samples. This disaccharide is known as a stress protectant in fungi, which was demonstrated by its high content in *H. utriformis* mycelium and young fruiting bodies, the stages in which mushroom has to survive in the conditions of different stress factors (Jorge et al., 2006). It is probably consumed for energy during the spore production and autolysis. The physicochemical properties of trehalose make it a promising candidate for both food and cosmetic products (Dey and De, 2015).

N-acetylglucosamine (GlcNAc), an amino-sugar and a building block of the fungal polysaccharide chitin, was present only in HUM (26 mg/g of the extract) as it is a product of chitinolysis which occurs during maturation of the fruiting bodies (Lim and Choi, 2009). Its presence was specifically targeted by NMR, as it's not commonly found as monomer in mushrooms. The ¹³C spectra of HUM indicated characteristic signals for carbonyl (δ 174.54), anomeric (δ 94.99 and 90.90) and methyl carbons (δ 22.23 and 21.96) of GlcNAc. Besides its importance for fungi, it also has multiple functions in the human organism, most notably being a building block of hyaluronic acid, a glycosaminoglycan of human connective tissues. Dietetic supplements containing GlcNAc are used to treat conditions affecting connective tissues, such as arthritis.

Importance of hyaluronic acid in the skin's health and integrity prompted the potential use of GlcNAc in products for wound healing and wrinkle treatment (Pedrali et al., 2015). In addition, GlcNAc was shown to inhibit tyrosinase by inhibiting tyrosinase glycosylation, thus preventing activation of the enzyme and production of melanin (Ortonne and Bissett, 2008), so it was proposed that it could be used in the treatment of hyperpigmentation caused by aging (Pedrali et al., 2015).

If chitinolysis in mushrooms was complete, GlcNAc, would be expected in higher amounts than 0.39% of dry weight, since studies showed that chitin makes significantly greater part of mushroom tissue (Nitschke et al., 2011); this suggests that either chitinolysis was incomplete, leading to a formation of both GlcNAc monomers and oligomers or that chitin is mostly localized in the thin outer layers of the mushrooms (endo- and exoperidium). N-acetylglucosamine oligomers are found to have biological potential as well, showing immunopotentiating and antitumor activity (Harish Prashanth and Tharanathan, 2007) and further investigation would be needed to determine their presence in mature puffballs' gleba.

Ergosterol, a steroid component of fungal cell membranes with a provitamin D2 activity (Heleno et al., 2016), makes about 3% of HUM (31.65 mg/g); its content in mature fruiting bodies, expressed on dry weight (~0.5%), is similar to that found in other mushrooms (Kalač, 2009). On the other hand, ergosterol makes only 0.12% of HUI, suggesting that autolysis, which causes destruction of cell walls, increases availability of free ergosterol, making it more easily extractable; the extraction of ergosterol from mushroom tissue is otherwise much more complex (Jasinghe and Perera, 2005). Apart from its provitamin D activity, ergosterol was reported to possess antioxidative (Amirullah et al., 2018) and anti-inflammatory activity (Kuo et al., 2011). Mycelium extract contained significantly lower amount of ergosterol than HUM (7.70 mg/g of extract), but higher than HUI.

Similarly, α -tocopherol (vitamin E) was found in greater amount in HUM (4.00 mg/g of extract) than in HUI (2.29 mg/g extract); in plants, tocopherols are involved in keeping cell membrane integrity by preventing (per)oxidation of unsaturated fatty acids, thus their levels change in response to stress factors (Saini and Keum, 2016). Its higher content in mature fruiting bodies could be, again, linked to autolysis and better availability for extraction. Interestingly, α -tocopherol was not detected in mycelium

extract. β -tocopherol was reported in even higher amount than α -tocopherol from several mushroom species (Heleno et al., 2010), but, α -tocopherol is the biologically most active form, which, apart from possessing strong antioxidant activity, seems to also manifest direct stimulating effect on fibroblasts and keratinocytes and could be used in wound healing products (Hobson, 2014). The presence of considerable amount of α -tocopherol, ergosterol and GlcNAc makes mature *H. utriformis* a promising candidate for both cosmetic and wound healing products (Taofiq et al., 2016; Ding et al., 2016).

Phenolic compounds are well known for plenty of biological activities, most notably antioxidant, anti-inflammatory, anti-atherogenic, antimicrobial, cholinesterase-inhibitory and many more, therefore being important as nutraceuticals in food products (Shahidi and Ambigaipalan, 2015; Singh et al., 2017; Vuong et al., 2013; Kocak et al., 2017), but also pharmaceutical and cosmetic products (Kumar et al., 2018; Demoliner et al., 2018). Phenolic content in the extracts of the fruiting bodies (HUI and HUM) is similar to that other authors found in mushrooms (Butkhup et al., 2017); in HUMIC however, phenols are present only in traces (3.21 mg GAE/g). Mature fruiting bodies have the highest phenolic components amount (even if difference in DER is considered), about 19.75 mg GAE/g of the extract (previously published, Petrović et al., 2016) and immature fruiting bodies about 11.52 mg GAE/g, which may come to surprise as they go through a rapid autolysis process upon maturation. However, Lim and Choi (2009) found that mushroom cell wall disintegration during autolysis of an ink cap species, *Coprinellus congregates*, increases the availability of phenolic compounds which are associated with cell wall carbohydrates. Apparently that same process leads to increase of compounds with phenolic groups in puffballs as well, the same as in the case of ergosterol and α -tocopherol. These compounds seem to remain stable as autolysis in puffballs leads to very quick loss of water, making suitable conditions for preservation of various molecular groups sensitive to hydrolysis. Also, fruiting bodies are enclosed and gleba's exposure to air is drastically reduced.

Radical scavenging ability (Table 2, Fig. 1), examined by ABTS assay, appeared to be positively correlated with total phenolic content (HUM N HUI N HUMIC, $R^2=0.9976$). Other authors also reported a positive linear correlation between total phenolic compounds and ABTS radical neutralization (Islam et al., 2016).

Table2

The results of antioxidant activity (mean \pm SD) of *Handkea utriformis* mycelium (HUMIC), immature (HUI) and mature fruiting bodies (HUM) methanol extracts, expressed as EC50 (concentration of the extract which exhibited 50% of the activity) or Trolox mM eq.

	ABTS (EC ₅₀ , mg/mL)	CUPRAC (Trolox mM eq at 10 mg/mL)	β -Carotene bleaching assay (EC ₅₀ , mg/mL)
HUMIC	5.24 \pm 0.19 ^{a1}	7.2 \pm 0.01 ^b	3.20 \pm 0.22 ^b
HUI	2.68 \pm 0.11 ^b	6.16 \pm 0.14 ^c	9.97 \pm 0.82 ^a
HUM	0.55 \pm 0.07 ^c	8.44 \pm 0.04 ^a	<0.16

¹ Within the same column, means followed by different letters are significantly different (α = 0.05, ANOVA, Tukey's HSD test).

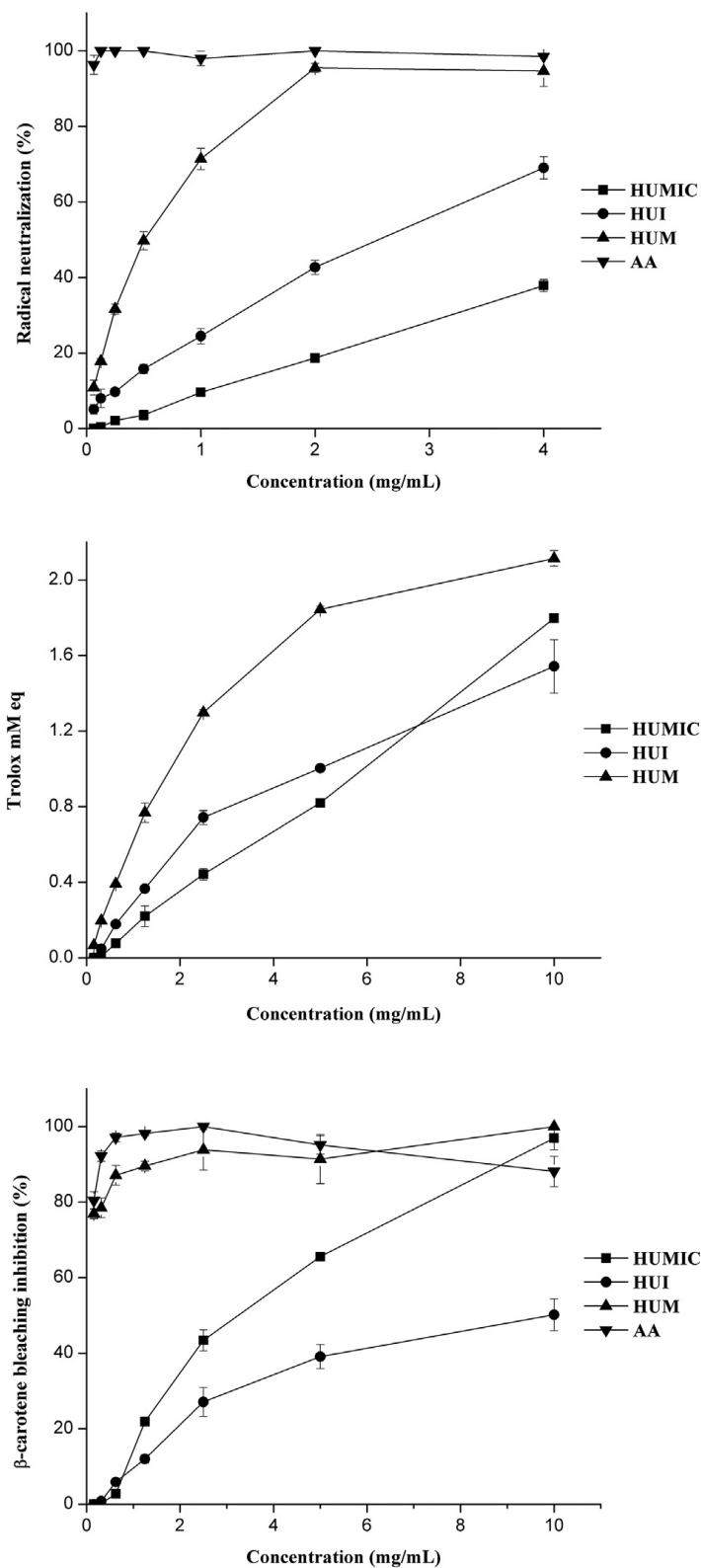


Fig. 1. In vitro antioxidant activity of *Handkea utriformis* mycelium (HUMIC), immature (HUI) and mature fruiting bodies (HUM) methanol extracts; a – ABTS radical scavenging assay (AA = ascorbic acid), b – CUPRAC reduction power assay, c – β -carotene bleaching assay (AA = ascorbic acid). Results are presented as mean \pm SD.

Phenolic content affects the ability of extracts to reduce Cu²⁺ ions as well, as it also correlates with the activity, at lower extract concentrations.

The order of activity is however different at the highest concentration used (10 mg/mL), with HUMIC being more effective than HUI. The dependence of concentration and reducing ability of HUMIC was almost linear (instead of logarithmic, seen in other two samples, Fig. 1), pointing that the examined ability of metal ion reduction was dependent on some other compound, most probably glucose (which is a reducing sugar and makes a considerable part of HUMIC, ~25%), not on the chemical fraction of biological importance. In β -carotene bleaching assay, HUM exhibited the best activity again (b0.16 mg/mL), while HUMIC expressed significantly higher activity than HUI, at all concentrations used. Several authors found correlation between total phenolics and α -tocopherol particularly and the neutralization of free linoleic radicals (Cheung et al., 2003; Barros et al., 2007b; Froufe et al., 2011), which isn't the case here; HUI has higher content of phenolics than HUMIC and α -tocopherol is present in HUM and HUI, but is absent from HUMIC. Although presence of other tocopherols in HUMIC cannot be excluded, the order of activity correlates well with ergosterol content (HUM > HUMIC > HUI), which was also reported to have antioxidant activity, as stated earlier. Other related lipophilic compounds found in mushrooms, like triterpenoids are found to act as antioxidants (Yang et al., 2007). The extracts show various inhibitory activity on tyrosinase, AChE, BuChE and HMG-CoA-R (Table 3, Fig. 2). The correlation of phenolic content and both tyrosinase- and AChE/BuChE-inhibitory activity is strong, which has also been confirmed by other authors (Taofiq et al., 2016; Zengin et al., 2015; Hasnat et al., 2013; Szwajgier and Borowiec, 2012; Paun et al., 2016; Arumugam et al., 2018). HUMIC and HUI are very weak inhibitors of tyrosinase, even at the highest concentration (5 mg/mL), but HUM seems to be a very potent inhibitor of the enzyme, with IC₅₀ value being only 0.22 mg/mL. This is in a line with findings of Park et al., 2015, who also confirmed that ethanol mycelium extracts of different mushroom species are not very effective tyrosinase inhibitors. Alam et al. (2012) used the similar assay procedure, differing only in enzyme activity (0.31 U/mL against 0.46 U/mL used in this study) to investigate tyrosinase inhibition of *Pleurotus ferulae* methanol extract. It showed 60.1% of tyrosinase inhibition at concentration of 1 mg/mL; methanol extract of mature *H. utriformis* displays much more promising results, and thus may be used as a melanin production inhibitor in cosmetics. Kojic acid, a fungal compound currently used in dermatological products (Ariff et al., 1996) was not detected in the extracts, but

our current knowledge about tyrosinase inhibitors suggests that the active compounds should be searched for in the phenolic fraction of the extract. In addition to direct inhibition of tyrosinase, HUM is also a source of free N-acetylglucosamine, as already stated, which is an indirect inhibitor of the enzyme and could act synergistically with other direct inhibitors present in the extract.

HUM exhibited the strongest inhibitory activity towards AcHE as well, while the mycelium's inhibitory action against AcHE was the least. However, the activity of the extracts doesn't differ as much as in the case of the tyrosinase-inhibitory activity, with HUM being approximately twice as effective as mycelium and about 33% more effective than HUI. The obtained results are in the range for those found from methanol extracts of *Agaricus* species (Öztürk et al., 2011). It was found that all tested samples showed selective inhibition towards AChE when compared to BuChE; this was least pronounced in the case of the most active HUM, which was shown to be approximately as twice as active against AChE at the highest concentration (near 70% compared to 32% inhibition of BuChE at 5 mg/mL). Even greater selectivity is shown in HUY and HUMIC; HUY inhibited over 50% of AChE activity at 5 mg/mL and only ~9% of BuChE activity, while HUMIC showed slightly weaker AChE inhibition (~40% at 5 mg/mL) and no BuChE inhibition whatsoever. When several wild mushroom species from Turkey were screened for anti-AChE activity, *H. utriformis* (as *Lycoperdon utriforme*) showed the highest activity (Akata et al., 2018).

Table 3

The results of the enzyme-inhibitory assays (mean±SD) of *Handkea utriformis* mycelium (HUMIC), immature (HUI) and mature fruiting bodies (HUM) methanol extracts, expressed as IC₅₀ (concentration of the extract which exhibited 50% of the activity).

	Tyrosinase inhibition (IC ₅₀ , mg/mL)	AChE inhibition (IC ₅₀ , mg/mL)	BuChE inhibition (IC ₅₀ , mg/mL)	HMG-Co-R inhibition (IC ₅₀ , mg/mL)
HUMIC	>5.00	5.98 ± 1.06 ^{a1}	>5.00	>5.00
HUI	>5.00	4.48 ± 0.10 ^b	>5.00	1.16 ± 0.11
HUM	0.22 ± 0.01	2.84 ± 0.11 ^c	>5.00	>5.00

¹ Within the same column, means followed by different letters are significantly different ($\alpha = 0.05$, ANOVA, Tukey's HSD test).

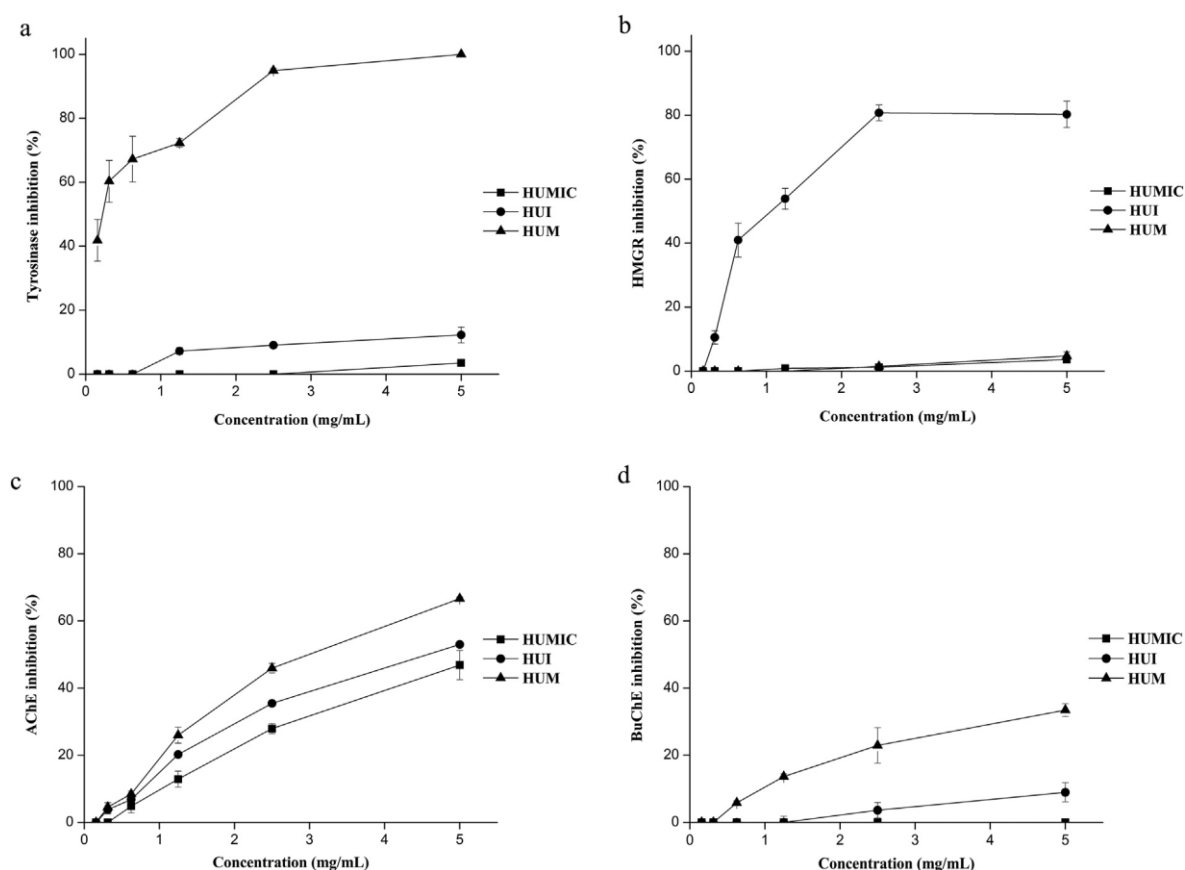


Fig. 2. In vitro inhibitory activity of *Handkea utriformis* mycelium (HUMIC), immature (HUI) and mature fruiting bodies (HUM) methanol extracts on tyrosinase (a), HMG-CoA reductase (b) acetylcholinesterase (c) and butyrylcholinesterase (d). Results are presented as mean \pm SD.

However, the results cannot be compared, as authors didn't provide details about the method they used to measure enzyme-inhibitory activity. AChE inhibitors represent the main group of drugs used for treatment of Alzheimer's disease and dementia, as increased concentration of neurotransmitter acetylcholine in CNS leads to improvement in cognitive function. It is however recognized that current therapy options are limited and that natural products are promising field of search for new active compounds (Kulshreshtha and Piplani, 2016). Until recently, selectivity towards AChE was considered beneficial in treatment of Alzheimer's, leading to a more favorable therapeutic index (Liston et al., 2004), but novel studies showed that expression levels of BuChE rise significantly in the late stages of Alzheimer's disease (Kumar et al., 2018); selectivity of the extracts towards one of the cholinesterase enzymes thus cannot be regarded as significant for potential medical use, at this point.

The activity towards HMG-CoA-R, the enzyme responsible for endogenous cholesterol synthesis, correlates with lovastatin content in the extracts (Tables 1 and 3), i.e. mycelium and mature fruiting bodies, which contain only traces of lovastatin, do not show significant activity towards the enzyme even at the highest concentrations used (5 mg/mL). On the other hand, immature fruiting bodies do contain lovastatin in considerable amount (given the fact that statins are potent at very low concentrations) and show good activity (IC₅₀=1.16mg/mL). However, lovastatin acts as a prodrug, which means that it needs to be metabolized by liver enzymes to its active, hydroxyl acid form (Du Souich et al., 2017). Therefore, it is not lovastatin that is responsible for the activity but other similar structures, apparently positively correlated with lovastatin content, such as fore-mentioned lovastatin acid form, which is also present in fungi (Lisec et al., 2012). The same observation came from other authors who examined *Pleurotus* spp. samples (no statins were detected but inhibition of HMG-CoA-R activity was present), so there may be other structures that contribute to the activity (Gil-Ramírez et al., 2013).

Table 4

Antimicrobial activity of *Handkea utriformis* mycelium (HUMIC), immature (HUI) and mature fruiting bodies (HUM) methanol extracts, amoxicillin (AMX) and fluconazole (FLU), expressed as minimum inhibitory (MIC, mg/mL) and minimum bactericidal concentration (MBC, mg/mL), determined by the broth microdilution method.

Microbial strain		Sample			Standard	
		HUMIC	HUI	HUM	AMX	FLU
<i>Staphylococcus aureus</i> 6538 ¹	MIC	2.5	5	20	0.0002	
	MBC	- ²	-	-	0.0014	
<i>Staphylococcus aureus</i> MRSA ³	MIC	5	2.5	20	-	
	MBC	-	-	-	-	
<i>Enterococcus faecalis</i> 29212	MIC	2.5	2.5	20	0.0003	
	MBC	-	-	-	0.0027	
<i>Pseudomonas aeruginosa</i> 27853	MIC	0.625	1.25	-	0.0217	
	MBC	-	-	-	0.0434	
<i>Escherichia coli</i> 25922	MIC	10	1.25	20	0.0054	
	MBC	-	-	-	0.0217	
<i>Candida albicans</i> 10259	MIC	20	-	-		0.0125
	MBC	-	-	-		0.0500

Mycelium and immature mushroom extracts show very similar and significant antibacterial activity (Table 4); mature mushroom extract shows much weaker activity (MIC=20mg/mL for all bacterial strains; the results were previously published, except for MRSA, Petrović et al., 2016), indicating that maturing probably leads to a loss of the active compounds. Both HUMIC and HUI show good activity towards Gram-positive strains (*E. faecalis* and *S. aureus*), which includes MRSA, a particularly dangerous *S. aureus* strain resistant to methicillin. There are some differences in the activity towards Gram-negative bacteria (*P. aeruginosa* and *E. coli*) between mycelium and immature fruiting bodies; HUMIC seems to be very active towards *P. aeruginosa* (MIC = 0.625 mg/mL), stopping the growth of this bacteria as a 0.0625% solution. The pronounced activity towards this opportunistic pathogen may be because *P. aeruginosa* is a terrestrial, therefore being a direct competition to fungi growing in the soil. Calvatic acid, a potent antibiotic, was isolated from broth cultures of several puffball species and it might be present in mycelium as well. HUI also exhibits excellent activity towards *P. aeruginosa* (MIC= 1.25 mg/mL), but also towards *E. Coli* (MIC = 1.25 mg/mL), 8 times greater than that of HUMIC (MIC = 10 mg/mL). HUMIC is the only sample that showed inhibitory activity against *C. albicans* (MIC=20 mg/mL) and none of the extracts showed bactericidal or fungicidal activity, in the range of tested concentrations.

4. Conclusion

Mosaic puffball is a great source of biologically active compounds; young, edible fruiting bodies are good source of antioxidants and lovastatin, making them a functional food which may have health benefits in people with dyslipidemia, especially hypercholesterolemia, as lovastatin inhibits synthesis of endogenous cholesterol. It also possesses in vitro, non-lovastatin mediated inhibitory activity of HMG-CoA-R, prompting conclusion that in vivo effect may be even greater than expected. Novel studies linked brain HMG-CoA-R inhibition with possible neuroprotective effects (Sparks, 2011). Both antioxidative (especially antiradical) and AChE inhibitory activity may further increase this effect. The methanol extracts also possess broad-spectrum antimicrobial activity. All these findings make mosaic puffball a functional food with potential health benefits if regularly consumed. More interesting is, however, that mature fruiting bodies of mosaic puffball, which are inedible due to powdery

consistence, have even greater overall medicinal potential. They are richer in phenolics, and show better antioxidative activity than immature fruiting bodies in all conducted assays. Also, they have greater inhibitory effect on AcHE, and are potential source of compounds that may find use in treatment of neurodegenerative diseases such as Alzheimer's. However, they seem to be more prospective for use in wound healing and cosmetic products, as they are a good source of provitamin D2, vitamin E, N-acetylglucosamin, phenolics and exhibit great antioxidative activity. Furthermore, they are natural and potent inhibitors of tyrosinase, and may have antihyperpigmentation effect. And although its antimicrobial activity wasn't as good as other extracts', it cannot be ignored. All these findings validate puffballs' traditional use but they also point out that they are more than just “effective styptics” as autolysis of the fruiting bodies leads to production and easier extraction of biologically active compounds.

Mycelium, which is easily cultivated in laboratory conditions, does not have the same properties as immature or mature fruiting bodies and thus cannot replace mushrooms collected from the wild. However, mycelium is a good source of trehalose, ergosterol, and is superior in some antioxidative tests to immature fruiting bodies, namely inhibition of linoleic acid (per)oxidation. Also, mycelium extract showed inhibitory activity on AcHE, and although HUI and HUM were more active, mycelium can be easily produced and could yield higher amount of active compounds. Mycelium's antibacterial activity was the greatest, with *P. aeruginosa* being most susceptible. This activity is even more significant, given the fact that more than 50% of mycelium extract are sugars (trehalose and glucose) with no antimicrobial activity in low concentrations. Notable growth inhibition was obtained against *S. aureus*, both ATCC strain and clinical methicillin-resistant strain (MRSA), so it could be a great source of antimicrobial compounds.

Conflict of interest

There is no conflict of interest to declare.

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