The impact of puffball autolysis on selected chemical and biological properties: puffball extracts as potential ingredients of skin-care products

Predrag Petrović[1,*], Katarina Ivanović[2], Aleksandra Jovanović[1], Milica Simović[3], Violeta Milutinović[4], Maja Kozarski[5], Miloš Petković[6], Anka Cvetković[7], Anita Klaus[5] and Branko Bugarski[8]

[1]*Innovation Center of the Faculty of Technology and Metallurgy, University of Belgrade*, Karnegijeva 4, 11060 Belgrade, Serbia

[2]*NIC Pharmaceutical doo*, Osme crnogorske brigade 3, 11090 Belgrade, Serbia

[3]*Department of Biochemical Engineering and Biotechnology, Faculty of Technology and Metallurgy, University of Belgrade*, Karnegijeva 4, 11060 Belgrade, Serbia

[4]*Department of Pharmacognosy, Faculty of Pharmacy, University of Belgrade*, Vojvode Stepe 450, 11221 Belgrade, Serbia

[5]*Institute for Food Technology and Biochemistry, Faculty of Agriculture, University of Belgrade*, Nemanjina 6, 11080 Belgrade, Serbia

[6]*Department of Organic Chemistry, Faculty of Pharmacy, University of Belgrade*,Vojvode Stepe 450, 11221 Belgrade, Serbia

[7]*Laboratory for Gas Chromatography, Institute for Public Health of Belgrade*, Bulevar despota Stefana 54A, 11000 Belgrade, Serbia

[8]*Department of Chemical Engineering, Faculty of Technology and Metallurgy, University of Belgrade*, Karnegijeva 4, 11060 Belgrade, Serbia

Corresponding author: bluetitwentsouth@gmail.com

Abstract: Puffballs are fungi that produce globose fruiting bodies that undergo autolysis, transforming their insides into a spore bearing, powdery mass. Mature fruiting bodies are traditionally used to treat open skin wounds. In this study, methanol extracts of two puffball species, *Handkea excipuliformis* and *Vascellum pratense*, were examined and compared in order to provide insight into the changes these mushrooms undergo during maturation, with respect to their potential use in skin care and wound treatment. Some compounds involved in skin care and regeneration were quantified, and it was found that maturation increases the concentrations of almost all of these compounds. Antioxidant activity was also more pronounced in mature fruiting body extracts, which was in correlation with the higher content of antioxidants. Tyrosinase inhibition was vastly improved with autolysis, correlating with the higher phenolic content in mature fruiting body extracts. Antimicrobial activity was negatively affected by autolysis in the case of *H. excipuliformis*, whereas autolysis had little effect on the antimicrobial activity of *V. pratense*. Autolysis generally improved the biological activity and increased the concentrations of compounds involved in skin care, which justifies the traditional use of puffballs and makes them good candidates for various potential cosmetic and medicinal skin-care products.

Keywords: puffballs; mushrooms; tyrosinase inhibitory activity; antioxidant; cosmeceutical

INTRODUCTION

Puffballs are a group of fungi which produce characteristic globose fruiting bodies [1]. They are edible when young and some species are considered a delicacy [2]. However, they are better known for their traditional uses for wound treatment, as hemostyptics and for other skin conditions. Upon maturation, the mushrooms undergo a process of autolysis, whereby their inside, the gleba, turns into a powdery, spore-bearing mass. This powder is applied directly to damaged skin [3]. There is, however, insufficient research concerning the medicinal potential of puffballs. Studies examining the chemical, nutritional and biological properties of puffballs have been limited to only a few species. The impact of autolysis on the chemical

constituents of interest is also poorly understood. In a previous studythatexamined the effect of the process of autolysis on the chemical and biological properties of mosaic puffball *Handkea utriformis*, it was shown that the maturation of the fruiting bodies improved the extraction yield of some pharmacologically active compounds, such are ergosterol (provitamin D_2), α-tocopherol (vitamin E) and *N*-acetylglucosamine; autolysis also greatly improved the antioxidant capacity of the mushroom extracts and the ability to inhibit certain enzymes involved in pathological processes[4].

In the present study, which represents a continuation of previous research, methanol extracts of immature and mature fruiting bodies of two related puffball species, *Handkea excipuliformis* (syn. *Lycoperdon excipuliforme*) and *Vascellum pratense* (syn. *Lycoperdon pratense*) were examined and compared with regard to their chemical composition (with an emphasison the compounds involved in skin care and regeneration)and selected biological activities, with the aim of providing better insight into the changes caused by autolysis and of exploring possible mechanisms that could have beneficial effects in the treatment of some skin disorders. Compounds involved in skin care and regeneration, including total phenols and various phenolic acids, ergosterol, α-tocopherol, *N*-acetylglucosamine, trehalose and free unsaturated fatty acids, were quantified. The antioxidant activities were evaluated using three *in vitro* assays and the extracts were also tested for their tyrosinase inhibitory and antimicrobial activities.

MATERIALS AND METHODS

Materials

Phenol, gallic acid, β-carotene, α-tocopherol, ergosterol, L-DOPA, neocuproine, the Folin-Ciocalteu reagent, linoleic acid, copper (II) chloride ($CuCl_2$) 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), triphenyltetrazoliumchloride (TTC), *o*-coumaric, *p*-coumaric, caffeic, chlorogenic, 1,3-dicaffeoylquinic, 1,5-dicaffeoylquinic, 3,5-dicaffeoylquinic, ferulic, protocatechuic, rosmarinic, sinapic and syringic acid, Supelco® 37 Component FAME Mix, and tyrosinase from *Agaricus bisporus* were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Glucose, fructose, mannose, xylose, rhamnose, arabinose, glucuronic acid, galacturonic acid, *N*-acetylglucosamine, trehalose and*p*-hydroxybenzoic acid were obtained from Acros Organics (Geel, Belgium). Potassium hydroxide (KOH), sodium carbonate (Na_2CO_3) and potassium dihydrogen phosphate (KH_2PO_4) were obtained from Fagron B.V. (Capelle aan den IJssel, Netherlands). Malt broth, malt agar, Müller-Hinton broth and Müller-Hinton agar were purchased from Biolife (Milan, Italy). Sulfuric acid (H_2SO_4), formic acid, potassium persulfate ($K_2S_2O_8$) and ammonium acetate (CH_3COONH_4) were obtained from Centrohem (Stara Pazova, Serbia). Tween 80 was purchased from Croda International (Snaith, UK). Methanol, ethanol, ethyl acetate, acetonitrile and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Loughborough, UK), while chloroform was obtained from Carlo Erba Reagents (Barcelona, Spain).

*V. pratense*fruiting bodies were collected in Bor, Serbia, and fruiting bodies of *H. excipuliformis* were collected near Bor and Belgrade, Serbia. Material identification was done by Boris Ivančević, custodian at the Natural History Museum in Belgrade, where the reference material is kept (catalogue numbers BEO21100 and BEO21121 for *H. excipuliformis* and *V. pratense* fruiting bodies, respectively). Mycelium cultures, obtained from the reference fruiting bodies, are kept at the Faculty of Technology and Metallurgy, Belgrade, Serbia.

Extract preparation

The extracts were prepared as previously described [4]. The immature fruiting bodies of *H. excipuliformis* and *V. pratense* (HEI and VPI, respectively) were sliced and lyophilized in a Christ BETA 2-8 LD plus freeze dryer (Osterode, Germany) and then powdered; The mature fruiting body's gleba (HEM – *H. excipuliformis*; VPM – *V. pratense*) is dry and has a powdery consistence so there was no need for its pretreatment. Material was mixed with methanol (0.05 g/mL) and subjected to maceration for 72h. After centrifugation, the supernatant was collected and filtered through filter paper (Whatman® No. 5); the methanol was removed under low pressure on a rotary evaporator (Heidolph Hei-VAP Value, Germany), and the extracts were additionally dried in a vacuum desiccator at room temperature for 24h.

Phenol analysis: total phenols

Total phenols were determined using a colorimetric assay [5], adapted for 96-well microplates [4]. Methanol solutions of extracts (12.5 µL, 4 mg/mL), Folin-Ciocalteu reagent water solution (25 µL, 1:5 v/v), Na_2CO_3 water solution (75.5 µL, 20% m/V) and water (175 µL) were mixed and the absorbance of the mixture was read after 2h at 630 nm, on a BioTek ELx808 microplate reader (Winooski, VT, USA). The standard curve was calculated using gallic acid and the results were expressed as mg of gallic acid equivalents per gram of extract (mg GAE/g). The analysis was applied only to the immature fruiting bodies extracts, as the results referring to the mature fruiting bodies extracts were previously published [7].

High performance liquid chromatography (HPLC) mass spectroscopy (MS) analysis of phenolic acids

HPLC-MS analysis of phenolic acids was performed on a Agilent LC/MS system 1260/6130 (Agilent Technologies, Waldbronn, Germany), with ChemStation software Rev. B.04.03-SP1, coupled with a Zorbax SB-Aq column (150×3.0 mm; 3.5 µm particle size, Agilent Technologies), UV detector (DAD) (G4212B) and single-quadrupole API-ESImass detector (6130). Methanol solutions of the samples (50 mg/mL) were centrifuged and the supernatant was used for further analysis. The column was incubated at 25°C and separation of the compounds was achieved using the mobile phase that consisted of a 0.1% HCOOH water solution (v/v) (A) and methanol (B). A linear gradient program was used (5-95% B, 30 min), with post run (5 min) and a constant flow of 0.3 mL/min. DAD spectra were recorded on 210, 254 and 330 nm. Mass spectra were recorded in negative mode (the full-scan). *p*-hydroxy benzoic, *o*-coumaric, *p*-coumaric, caffeic, chlorogenic, 1,3-dicaffeoylquinic, 1,5-dicaffeoylquinic, 3,5-dicaffeoylquinic, ferulic, protocatechuic, rosmarinic, sinapic and syringic acid were used as standards. The compounds were identified by comparing the retention times and mass spectra with those of standards.

Sugar analysis: total sugars

Total sugars were estimated by the phenol-sulfuric acid method [6]. The extracts were dissolved in water (2 mg/mL) and 200 µL of the solution was mixed with 200 µL of water and 400 µL of phenol water solution (5% w/v). Concentrated sulfuric acid was added (2 mL) and the absorbance of the mixture was read after 40 min at 490 nm on a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). Glucose was used for standard curve calculation, and the results were expressed as sugar percentages (%). The analysis was applied only to the immature fruiting bodies extracts, as the results referring to the mature fruiting body extracts were previously published [7].

HPLC analysis of mono- and disaccharides

The analysis was performed as previously described [4]. Sugars were extracted from the methanol extracts with water (50 mg/mL), using an ultrasonic bath (10 min, 320 W, 35 kHz), obtained from Sonorex Digitec Bandelin Electronic, Germany. After centrifugation of the suspension, the supernatant was collected and used for further analysis. Separation and determination of sugars was performed on a Thermo Scientific Dionex Ultimate 3000 HPLC system (Waltham, USA), using a carbohydrate column (Hyper REZ XP Carbohydrate Ca^{2+}, 300 mm×7.7 mm, 8µm) and a RI detector (RefractoMax 520, ERC, Germany). The column was incubated at 80°C and the mobile phase was water, withan elution rate of 0.6 mL/min. The data were processed using Chromeleon Software. Sugar standards (glucose, fructose, galactose, mannose, xylose, rhamnose, fucose, arabinose, glucuronic acid, galacturonic acid, *N*-acetylglucosamine and trehalose) were used to calculate the standard curves. Sugars were identified by comparing the retention times with those of the standards.

Determination of ergosterol and free fatty acids

Ergosterol and free fatty acids were determined using a gas chromatography-mass spectrometry (GC-MS) method [4,7]. For ergosterol determination, the extracts were dissolved in methanol (10 mg/mL). Free fatty acids were methylated prior to analysis. The extracts (50 mg) were dissolved in 10% H_2SO_4 methanol solu-

tion (10 mL) and stirred for 3h at 40ºC. The acid was neutralized with an equimolar amount of H_2CO_3 and methanol was evaporated under low pressure using a Heidolph Hei-VAP Value Rotary Evaporator (Schwabach, Germany). Esterified fatty acids were extracted with n-hexane (2 mL).Determination of both ergosterol and free fatty acids was achieved using the same program [7]. Analysis was performed using an Agilent Technologies 6890N GC System coupled with an Agilent 5973 Network Mass Selective Detector and MSD ChemStation Software G 1701BA. An HP-5 MS column with the following dimensions: 30mx0.25mm x 0.25μm, was used. Injection was performed in pulsed splitless mode, with a splitless time of 1 min and with an injection volume of the samples of 1 μL (Agilent 7683 Injector Series autosampler). The temperature of the injector was 260ºC; helium (5.0, Messer, Bad Soden, Germany) was used as a carrier gas, with a constant flow rate of 1mL/min. The initial oven temperature was 50ºC and it was held for 2 min; the temperature was then increased to 150ºC (25ºC/min), held for 4 min, then increased further to 250ºC (4ºC/min) and finally to 300ºC (10ºC/min),which was maintained for 5 min. Mass spectra were acquired in electron impact mode. A MS quad detector was used; transfer line temperature was set at 280ºC, MS quad at 150ºC and MS source at 230ºC. The analysis was performed in full scan mode and the scan rate was 6 scan/s; the range of the scanned mass was from 50 to 600 m/z. The NIST05 MS Library Database was used to confirm the identity of ergosterol and esterified free fatty acids. A methanol solution of a standard mixture of a total of 37 fatty acid methyl esters (Supelco F.A.M.E. Mix C4-C24) was used to identify and quantify the fatty acids in the extracts, while an ethyl acetate solution of ergosterol was used for ergosterol determination and calculation of the standard curve; standards were analyzed under the same conditions as samples. The results were expressed as mg/g of extract. The free fatty acids analysis was applied only to immature fruiting body extracts, as the results referring to mature fruiting body extracts were previously published [7].

α-tocopherol determination

The analysis was performed as previously described [4]. α-tocopherol was determined by the HPLC method onan Agilent HPLC System 1260 (Agilent Technologies, Waldbronn, Germany), coupled with Zorbax SB-C18 column (250 × 4.6 mm; 5 μm particle size; Agilent Technologies) and a diode-array detector (DAD) (G4212B), with ChemStation software Rev. B.04.03-SP1. The extracts (25 mg/mL) were dissolved in a methanol:n-hexane mixture (9:1) and filtered through a membrane filter (Whatman®, 0.2 μm). The mobile phase consisted of 0.1% (v/v) formic acid in water (mobile phase A) and methanol (mobile phase B). The injection volume was 5 μL and the analysis was performed at 25ºC. 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 a flow rate of 0.8 mL/min. α-tocopherol was used for standard curve construction. The results were expressed as mg/g of extract.

In vitro antioxidant activity assessment

ABTS radical scavenging assay

The ability of the extract to neutralize free radicals was investigated by the ABTS assay [8], adapted for 96-well microplates [4]. ABTS radicals were generated by adding $K_2S_2O_8$ water solution (3.8%, 88 μL) to an ABTS water solution (0.38%, 5 mL) and keeping the reaction mixture for 16 h in a refrigerator. Methanol solutions of the samples (20 μL, 0.0625-4 mg/mL) were mixed with a diluted ABTS radical solution (200 μL, 0.0037%) and the absorbance (A_s) was read after 20 min at 630 nm on a BioTek ELx808 microplate reader (Winooski, VT, USA).A mixture of the ABTS radical solution and pure methanol was used as the control (A_c). The percentage of radical scavenging activity was calculated using the following formula: $(A_c-A_s)/A_c x100$. The concentrations that neutralized 50% of radicals (EC_{50}, mg/mL) were calculated from the standard curves created for each sample. Ascorbic acid was used as a standard. The results were expressed as the concentration of the extracts that neutralized 50% of radicals (EC_{50}).

CUPRAC reduction power assay

Cupric ion reduction ability was tested using the CURPAC assay [9]. Serial sample methanol dilutions (40μL, 0.156-10 mg/mL) were mixed with $CuCl_2$ water solution (50 μL, 10 mM), neocuproine ethanol solu-

tion (50 µL, 7.5 mM) and CH_3COONH_4 buffer (60 µL, 1 M, pH 7.0) and the mixtures incubated for 1h at 30°C. The absorbance was read at 450 nm, on a BioTek ELx808 microplate reader (Winooski, VT, USA). Trolox was used for standard curve calculation and the results were expressed as mmol/L equivalents of Trolox (Trol. mM eq.).

β-carotene bleaching assay

The ability of the extracts to prevent oxidation of linoleic acid was investigated using a β-carotene-linoleic acid model system [10], adapted for 96-well microplates [4]. β-carotene was dissolved in chloroform (0.2 mg/mL) and 2 mL of the solution were subjected to evaporation under low pressure until the chloroform was completely removed. The working emulsion was prepared by adding linoleic acid (40 mg), Tween 80 (400 mg) and water (100 mL) in small portions, with constant mixing. Sample methanol solutions (20 µL, 0.156-10 mg/mL) were mixed with the emulsion (250 µL) and incubated for 1h at 50°C, in a water bath. A mixture of methanol (20 µL) and the emulsion (250 µL) was used as the control. The absorbance was read at 470 nm, on a BioTek ELx808 microplate reader (Winooski, VT, USA), immediately after mixing and incubation. The following formula was used to calculate the percentage of β-carotene bleaching inhibition: $[(A_{c0}-A_{c1})-(A_{s1}-A_{s0})]/(A_{c0}-A_{c1})$, where the letters "c" and "s" in the subscript indicate the absorbance of control and samples, respectively, while numbers "0" and "1" indicate the absorbance before and after incubation, respectively. Ascorbic acid was used as a standard. The results were expressed as the concentration of the samples that prevented 50% of β-carotene bleaching.

Tyrosinase inhibitory assay

The ability of the extracts to inhibit tyrosinase activity was examined using an *in vitro* assay [11], with slight modifications [4]. Tyrosinase (46 U/mL) and L-DOPA (2.5 mM) solutions were prepared in a phosphate buffer (0.067M, pH 6.8). The buffer was prepared by dissolving KH_2PO_4 in water and adjusting pH with a KOH aqueous solution (1M). Samples (0.156-5 mg/mL) were dissolved in a DMSO/phosphate buffer solution (5% v/v).The samples and reagents were mixed as follows: A: phosphate buffer (120 µL), enzyme (40 µL); B: phosphate buffer (160 µL); C: phosphate buffer (80 µL), sample (40 µL), tyrosinase (40 µL); D: phosphate buffer (120 µL), sample (40 µL). After 10 min, 40 µL of L-DOPA solution was added and the microplates were incubated for 20 min at 25°C. The absorbance was read on a BioTek ELx808 microplate reader (Winooski, VT, USA) at 490 nm. Enzyme inhibition (%) was calculated according to the formula: $[(A-B)-(C-D)]/(A-B)\times100$; results were expressed as the sample concentration that inhibited 50% of the enzyme reaction (IC_{50}, mg/mL), with kojic acid used as the control (1 mg/mL).

Broth microdilution antibacterial assay

Minimum inhibitory (MIC) and bactericidal/fungicidal concentrations (MBC/MFC) of the extracts were determined by the broth microdilution method [12,13]. Antimicrobial activity was tested against five 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 one clinical strain of methicillin-resistant *Staphylococcus aureus* (MRSA), obtained from the City Institute of Public Health (Belgrade, Serbia). The extracts were prepared in a 5% (v/v) DMSO water solution. Microbial suspension density was set at 10^5 colony forming units, using the Müller Hinton broth for bacterial strains and malt broth for *C. albicans*. 2,3,5-triphenyltetrazolium chloride (TTC) was added as the growth indicator, at a concentration of 0.0075%. The extract dilutions (50 µL, 0.01-20 mg/mL) and microbial suspensions (50 µL) were mixed in 96-well microplates and incubated for 24h at 37°C (bacterial strains) or for 48h at 30°C (*C. albicans*). A positive control containing only the microbial suspensions and DMSO water solution (5%) was included. 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 the MIC value. MBC/MFC was determined by serial subcultivation 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 the MBC/MFC. Amoxicillin was

used as a standard for bacterial strains (0.05-50 μg/mL) and fluconazole for *C. albicans* (0.1-100 μg/mL). The analysis was applied only to the immature fruiting bodies extracts, except in the case of MRSA, as the results referring to the mature fruiting body extracts were previously published [7].

Statistical analysis

All measurements were performed in triplicate, and the data are expressed as the mean±standard deviation, if not stated otherwise. The experimental data were subjected to one-way analysis of variance (ANOVA), and Tuckey's HSD test was used to detect significant differences (α=0.05). MS Excel (Microsoft Office 2010 Professional) was used to calculate EC_{50} and IC_{50} values, and OriginPro 8 was used for the statistical analyses.

RESULTS

Extraction yields and chemical analysis

The yields of the extractions were very similar for both species, with the drug:extract ratio (DER) being 4.6 and 5.1 for HEI and VPI, respectively. Of all of the tested compounds, sugars made up the largest part of the extracts (Table 1), reaching values between 25 and 30%; the total sugar content of HEM and VPM was previously published [7]. Trehalose was the dominant sugar in all four extracts, making up about 90% of the sugar content of HEI and the entire sugar fraction of HEM. The sugar fraction of *V. pratense* extracts contained less trehalose, about 60% in VPI and 50% in VPM. The rest of the *V. pratense* sugar fraction remains unknown; oligosaccharides could also be present in the extracts. The extracts of the mature fruiting bodies contained another sugar of interest, *N*-acetylglucosamine, although in relatively low amounts (5.34 and 10.24 mg/g for HEM and VPM, respectively).

The contents of phenolic compounds, estimated using the Folin-Ciocalteu reagent, were very similar for both species (HEI=11.22 mg/g; VPI=13.67 mg/g); the phenolic content in mature fruiting body extracts was previously published [7].α-tocopherol (vitamin E) was detected in all of the extracts, except for HEI; however, it was present in the mature gleba of *H. excipuliformis* (HEM=0.49 mg/g),and in both the immature and mature fruiting body extracts of *V. pratense* (VPI=0.29 mg/g, VPM=0.34 mg/g, respectively).Another fungal

Table 1. Content of selected molecular fractions/compounds of the methanol extracts of the immature and mature fruiting bodies of *Handkea excipuliformis* (HEI and HEM, respectively) and *Vascellum pratense* (VPI and VPM, respectively), expressed as mean±SD

	HEI	HEM	VPI	VPM
Total carbohydrates (%)	26.83±1.01[a1]	[9.46±0.74[b2]]	28.64±1.19[a]	[9.01±0.87[b2]]
Trehalose (mg/g)	238.00±10.79[a]	105.20±6.07[c]	160.80±6.92[b]	47.20±2.31[d]
N-acetylglucosamine (mg/g)	nd[3]	5.34±0.09[b]	nd	10.24±0.23[a]
Total phenols (mgGAEs/g)[4]	11.22±0.43[b]	[14.93±0.81[b2]]	13.67±0.58[b]	[20.25±0.92[a2]]
α-tocopherol (mg/g)	nd	0.49±0.07[a]	0.29±0.02[b]	0.34±0.08[ab]
Ergosterol (mg/g)	0.36±0.04[c]	0.47±0.12[c]	1.11±0.08[b]	2.85±0.07[a]

[1]Different letters in the rows stand for statistically different values (p=0.05, ANOVA, Tukey's HSD test);
[2]Previously published data [7].
[3]Not detected/found in traces.
[4]mg of gallic acid equivalents per g of extract

Table 2. Free fatty acid composition of the methanol extracts of immature and mature fruiting bodies of *Handkea excipuliformis* (HEI and HEM, respectively) and *Vascellum pratense* (VPI and VPM, respectively), (mg/g); C14 – myristic acid; C15 – pentadecylic acid; C16:1 – palmitoleic acid; C16 – palmitic acid; C17 – margaric acid; C18:2 – linoleic acid; C18:1 – oleic acid; C18 – stearic acid; C22 – behenic acid; Tri – tricosanoic acid; C24 – lignoceric acid.

	HEI	VPI
C14	0.04±0.00[b1]	0.06±0.02[a]
C15	0.24±0.04[b]	0.44±0.09[a]
C16:1	0.24±0.07[b]	0.98±0.06[a]
C16	0.97±0.08[b]	1.43±0.20[a]
C17	0.10±0.01[b]	0.17±0.02[a]
C18:2	4.60±0.21[a]	4.90±0.36[a]
C18:1	0.15±0.03[b]	0.23±0.02[a]
C18:0	0.67±0.08[b]	0.88±0.04[a]
C22	nd[3]	nd
C24	0.04±0.01[a]	0.04±0.00[a]
Total	7.05±0.46[b]	9.13±0.81[a]

[1]Different letters in different rows stand for statistically different values (p=0.05, ANOVA, Tukey's HSD test).
[2]Not detected

Table 3. The results of antioxidant and tyrosinase inhibitory activities (mean±SD) of the methanol extracts of the immature and mature fruiting bodies of *Handkea excipuliformis* (HEI and HEM, respectively) and *Vascellum pratense* (VPI and VPM, respectively), expressed as EC_{50}/IC_{50} (concentration that 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)	Tyrosinase inhibition (IC_{50}, mg/mL)
HEI	1.93±0.13[a1]	8.41±0.22[a]	5.11±0.2[a]	>5
HEM	1.13±0.04[b]	8.74±0.34[a]	<0.156	<0.156
VPI	2.08±0.19[a]	6.19±0.14[b]	1.26±0.17[b]	>5
VPM	0.55±0.00[c]	8.81±0.30[a]	<0.156	0.28±0.02

[1]In the same column, means followed by different letters are significantly different (p = 0.05, ANOVA, Tukey's HSD test).

metabolite with (pro)vitamin activity, ergosterol (pro-vitamin D_2), was also quantified in the extracts; once more, higher concentrations were found in the mature gleba extracts of both species. The amount of extracted ergosterol was significantly higher in the case of *V. pratense* (HEI=0.36 mg/g, HEM=0.47 mg/g, VPI=1.11 mg/g and VPM=2.85 mg/g).The total free fatty acid contents (Table 2) in the immature fruiting body extracts were similar, although slightly higher in the case of *V. pratense* (HEI=7.05 mg/g; VPI=9.13 mg/g). Linoleic acid was the most abundant component of the free fatty acid fraction; palmitic and stearic acid were also found in relatively high concentrations. Palmitoleic acid was found in an unusually high amount in VPI (>10% of the free fatty acid fraction); free fatty acid analysis of mature fruiting body extracts was previously published [7].

Biological activity

As regards antioxidant activity (Table 3, Fig 1), there was a significant difference between the immature and mature fruiting body extracts. In the radical scavenging activity, young fruiting body extracts showed very similar activities, while there was a significant difference between HEM and VPM, with VPM being more active in free radical neutralization. The mature fruiting body extracts were more active in the metal ion reduction assay (CUPRAC) as well, although the difference was noticeable at concentrations higher than 1 mg/mL. There was no significant difference in the activities of HEM and VPM, however HEI showed significantly higher activity than VPI at concentrations ≥5 mg/mL, reaching almost the same reduction capacity of the mature gleba extracts at the highest concentration used (10 mg/mL).The superiority of the mature fruiting body extracts in terms of antioxidant capacity is best seen in the β-carotene bleaching assay, with
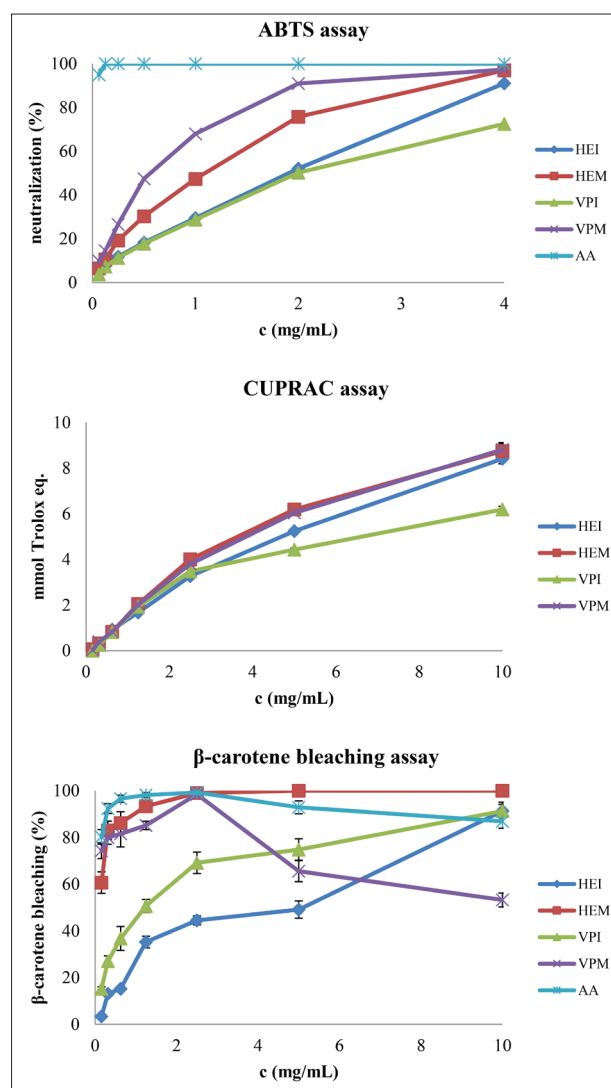
Fig. 1. *In vitro* antioxidant activity of methanol extracts of the immature and mature fruiting bodies of *Handkea excipuliformis* (HEI and HEM, respectively) and *Vascellum pratense* (VPI and VPM, respectively). Ascorbic acid (AA) was used as a standard in the ABTS and β-carotene bleaching assay. Results are presented as the mean±SD.
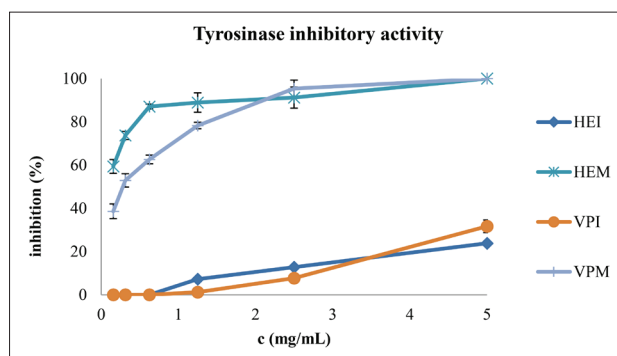
Fig. 2. *In vitro* tyrosinase inhibitory activity of the methanol extracts of the immature and mature fruiting bodies of *Handkea excipuliformis* (HEI and HEM, respectively) and *Vascellum pratense* (VPI and VPM, respectively). Results are presented as the mean±SD.

both of the extracts exceeding EC_{50} values at the lowest concentration used; at 0.156 mg/mL, HEM and VPM prevented oxidative degradation of ~60 and ~75% of β-carotene, respectively, and reached almost 100% of the activity at the concentration of 2.5 mg/mL. However, in the case of VPM, the activity decreased with further increase in concentration, and fell to ~53% at the concentration of 10 mg/mL, while HEM reached the plateau of activity and showed no change.

Autolysis had a huge impact on the ability of puff-ball extracts to inhibit tyrosinase as well (Table3, Fig 2). HEI and VPI only weakly inhibited tyrosinase, reaching 24 and 32%, respectively, of the activity at the highest concentration (5 mg/mL). On the other hand, HEM and VPM showed excellent inhibitory activity, attaining more than 90% of enzyme inhibition at the concentration of 2.5 mg/mL; in the case of HEM, the IC_{50} was below the lowest concentration used (<0.156 mg/mL), while for VPM it was higher, but still significant (0.28 mg/mL).

The extracts exhibited varying activities towards bacterial strains but no activity against *C. albicans* (Table 4). HEI had the best overall activity, displaying inhibitory activity on the growth of alltested bacterial strains, which was slightly more pronounced against Gram(-) bacteria (*E. coli*and*P. aeruginosa*, MIC=1.25 mg/mL).Significant activity was achieved against MRSA (MIC=5 mg/mL; MBC=20 mg/mL). The same MIC was obtained for the ATCC strain of *S. aureus*, and the sample was shown to be even more active against *E. faecalis* (MIC=2.5 mg/mL). On the other

Table 4. Antimicrobial activities of the methanol extracts of immature and mature fruiting bodies of *Handkea excipuliformis* (HEI and HEM, respectively) and *Vascellum pratense* (VPI and VPM, respectively), 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	
		HEI	VPI	AMX	FLU
Staphylococcus aureus 6538[1]	MIC	5	20	0.0002	
	MBC	-[2]	-	0.0014	
Staphylococcus aureus MRSA*	MIC	5	20	-	
	MBC	20	-	-	
Enterococcus faecalis 29212	MIC	2.5	-	0.0003	
	MBC	-	-	0.0027	
Pseudomonas aeruginosa 27853	MIC	1.25	20	0.0217	
	MBC	-	-	0.0434	
Escherichia coli 25922	MIC	1.25	5	0.0054	
	MBC	-	-	0.0217	
Candida albicans 10259	MIC	-	-		0.0125
	MBC	-	-		0.0500

[1]ATCC number of the microbial strain.
[2]Not achieved.
*Clinical isolate.

hand,VPI was less active against all examined strains, with best activity achieved against *E. coli* (MIC=5 mg/mL) and no activity against *E. faecalis* and *C. albicans*. The growth of other strains was inhibited only at the highest concentration used (20 mg/mL), and the sample showed no bactericidal activity. HEM and VPM were tested only against MRSA(MIC=20 mg/mL), as their antimicrobial activity against other ATCC strains was the subject of a previous study.

DISCUSSION

When compared with the extraction yields previously obtained for mature fruiting bodies (DER=6.1 and 5.9 for HEM and VPM, respectively [7]), the extraction yield for the immature fruiting bodies differed significantly. The slightly greater yield obtained for HEI and VPI was probably due to the fact that mature gleba represents a spore-bearing mass, with thick-walled spores being more difficult to extract; this could be overcome by modification of the extraction conditions.

The autolysis clearly led to a decrease in the sugar content in the extracts, as the total sugar content in the immature fruiting body extracts was between 25-30

%, and in the mature fruiting body extracts less than 10% [7]. Sugars can be used for energy during autolysis or, in the case of trehalose, they can accumulate in the spores [14], which would make the extraction difficult. Trehalose is an important stress protectant in fungi, helping them to survive periods of unsuitable conditions, such is extremely dry or cold weather [15]. It is therefore used in medicine and science as cryoprotectant for various types of cells, tissues, proteins and lipid membrane systems, as well as in cosmetic products [16].

The extracts of the mature fruiting bodies contained another physiologically active sugar, *N*-acetylglucosamine, the monomer of the polysaccharide chitin, which is one of the constituents of fungal cell wall. *N*-acetylglucosamine is also one of the building units of hyaluronic acid, the polysaccharide of human connective tissue, which is important for maintaining skin integrity and which has acquired growing usage in anti-aging skin products. It was proposed that *N*-acetylglucosamine itself could be used in wound healing and wrinkle treatment products as well [17]. This sugar is not present in young mushrooms, since it is the product of chitinolysis. In a previous study, it was speculated that the total *N*-acetylglucosamine content in the extract of the mature gleba of another puffball species, *Handkea utriformis* (26 mg/g), would have been higher [4] since chitin contributes to a considerable percentage of fungal tissue [18]. However, if hydrolysis was incomplete, there could be oligomers of *N*-acetylglucosamine present in the mature gleba [4]. *N*-acetylglucosamine oligomers were found to be biologically active as well, possessing immunomodulating properties [19].

The extracts of the mature fruiting bodies have higher contents of phenolic compounds (HEM=14.93 mg/g; VPM=20.25 mg/g), as was previously reported [7], although the difference was not as significant when considering DER differences. The phenolic content in young puffball extracts is very similar to that determined for other mushrooms [20] and for *Lycoperdon molle* and *L. perlatum* puffballs (11.48 and 10.57 mgGAEs/g of extract, respectively) [21]. None of the tested ubiquitous phenolic acids were detected in the extracts, indicating that different structures were present in these mushrooms. Phenolic acids were not detected in *L. perlatum* either, while only traces

of *p*-hydroxybenzoic and vanillic acid were found in *L. molle* (41.66 and 39.99 mg/kg dry mass, respectively) [22]. Phenolic compounds and antioxidants in general have been shown to have beneficial effects on the skin, especially in preventing premature skin aging and wrinkling [23].

The difference in the total amount of methanol-extractable α-tocopherol present in young and mature fruiting bodies of *V. pratense* practically does not exist when the difference in DER is considered. In a previous study, the higher content of α-tocopherol was found in the methanol extract of the mature fruiting bodies of *H. utriformis* [4], as in the case of *H. excipuliformis*. Since tocopherols are responsible for maintaining cell membrane integrity in plants by preventing lipid oxidation, their concentrations may vary depending on different stress factors [24], and their role in fungi could be the same. Autolysis does not affect α-tocopherol concentration negatively and may actually cause its production and/or better availability for extraction. In a previous study, several mushroom species were tested for the presence of α-, β- and γ-tocopherols, and only α-tocopherol was found in two puffball species (*Lycoperdon perlatum* and *L. molle*), while most of the other tested mushrooms contained higher levels of β-tocopherol [21]. Autolysis leads to higher availability of extractable ergosterol, which is associated with cell membranes, although much higher contents of both ergosterol and α-tocopherol were reported for the methanol extract of the mature fruiting bodies of *H. utriformis* [4]. Higher amounts of total free fatty acids were also reported earlier for the extracts of the mature fruiting bodies of *H. excipuliformis* and *V. pratense* [7], especially in the case of *V. pratense* (HEM=9.64 mg/g; VPM=20.27 mg/g). Linoleic acid, which was found to be important in wound healing and skin regeneration processes [25], was the most abundant component of the free fatty acid fraction in HEM and VPM as well, and autolysis led to its synthesis or/and higher availability. Another unsaturated fatty acid, oleic acid, which was shown to have similar properties [26], was also found in higher concentration in the extracts of mature fruiting bodies.

In general, the process of autolysis in puffballs leads to the production and/or improved availability of certain compounds that may benefit skin health (*N*-

acetylglucosamine, α-tocopherol, ergosterol, linoleic acid and phenolics), and many of them are probably involved in skin regeneration [23,26-28]. However, the extraction processes need to be optimized as these compounds have different hydrophilic/hydrophobic characteristics. Methanol extracts of *H. utriformis* were shown to follow the same pattern [4], which may be universal for all puffballs, or even for all "gasteromycetes". In the case of *H. utriformis*, all tested compounds were found in significantly higher amounts in the mature gleba extract, which may be due to interspecific differences, or it could be linked to the degree of autolysis. The mature gleba of the aforementioned species, which was the subject of a previous study [4], has a more powdery consistence, a property hard to quantify, which may be due to the higher activity of hydrolytic enzymes involved in autodigestion.

Mature fruiting body extracts possessed better antioxidant activity, in general. Radical scavenging activity correlates well with the total phenolic content of the samples (R^2=0.8), whereas the reduction ability of the extracts clearly depends on other types of molecular groups as well, since HEI showed almost the same activity as HEM and VPM at higher concentrations. The greatest difference in activity between the extracts of immature and mature fruiting bodies was detected in the β-carotene bleaching assay, with EC_{50} values of HEM and VPM being below the lowest concentration used (<0.156 mg/mL), indicating that these extracts could provide great protection of lipid membranes against damage caused by (per)oxidation. However, at higher concentrations, there was a decline in the activity of VPM. It was shown that antioxidants, such are phenolics, may also have prooxidant activity, depending on the circumstances, and VPM might contain such molecular groups [29].

Phenolic compounds, α-tocopherol and ergosterol all possess antioxidant activity [24,29-31] and are found in higher concentrations in the extracts of mature fruiting bodies; however, many other unknown metabolites are probably involved. Although phenolics may prevent lipid peroxidation [32], lipophilic compounds such are tocopherols and ergosterol may show a greater effect in the β-carotene bleaching assay.

Tyrosinase inhibitory activity correlates with the total phenol content, and since the aromatic amino acids, tyrosine and L-DOPA are tyrosinase substrates[33], it was found that other natural phenolic compounds found in plants may act as tyrosinase inhibitors as well. Phenolic acids such are ferulic, caffeic, ellagic and *p*-coumaric acid are known inhibitors of tyrosinase and are found in many extracts of different mushroom species [34-37], but none of these compounds were detected in our extracts. As the extracts of mature fruiting bodies possess much more pronounced activities than those obtained from young fruiting bodies(with HEM and VPM reaching EC_{50} at 0.156 and 0.28 mg/mL, respectively, while the extracts of the immature fruiting bodies did not reach EC_{50} even at the highest concentration used (5 mg/mL)), it is probable that specific phenolic compounds that are created during the maturation process could be responsible for the activity; however, they are yet to be isolated and characterized. These compounds may be methanol-soluble puffball pigments, as the gleba turns dark brown during autolysis of puffballs and the methanol extracts of the mature fruiting bodies are darker in color. There are only a few studies about mushroom inhibitory activity towards tyrosinase, and mature gleba extracts have been shown to be much more potent inhibitors than extracts from most mushroom species, except for petroleum and butanol extracts of the medicinal mushroom chaga (*Inonotus obliquus*) [11,34,38]. A phenoxazine compound (2-amino-3H-phenoxazin-3-one) isolated from the white button mushroom (*Agaricus bisporus*) was shown to be a potent tyrosinase inhibitor, but this compound does not act as a direct inhibitor of the enzyme, it acts as a gene expression regulator [39]. Tyrosinase inhibitors such is kojic acid isolated from *Aspergillus* spp. are used in medicinal and cosmetic products to treat hyperpigmentation [40]. Retinoids are also used as they downregulate enzyme synthesis [41] but may cause an adverse reaction, known as "retinoid dermatitis" [42]. Hyperpigmentation occurs as a consequence of several medical conditions, most important being postinflammatory hyperpigmentation, solar lentigos and melasma [41]. The mature puffball extracts are good candidates to be used in the treatment of such conditions as they might regulate pigmentation on several levels. Aside from direct inhibition of the enzyme, these extracts contain free *N*-acetylglucosamine, which downregulates tyrosinase activity by inhibiting enzyme glycosylation [41]. Also,

the extracts possess very strong radical scavenging activity and reactive oxygen species (ROS) are among the known stimulators of melanocytes [41]. The copper reduction ability may lower the available Cu^{2+} ions, which are required as tyrosinase cofactors [43].

Maturation affected the antimicrobial activity of the extracts differently. In *H. excipuliformis* it caused a decrease in antibacterial activity, which was several times lower for HEM, with MIC values against most bacterial strains being 20 mg/mL (previously published, except for MRSA [7]). Also, the slight selectivity of HEI towards two Gram(-) strains was not observed in HEM. Similar results were found in previous studies in which the antimicrobial activity of another puffball species, *H. utriformis,* was examined [4,7]. On the other hand, there wasa little difference in the activity of VPI and VPM (the resultsof antimicrobial activity of VPM were previously published [7], except for MRSA), which were not active against all tested strains, and the obtained MIC values were relatively high (mostly 20 mg/mL for both samples). There was, however, a trend of decrease in the activity against Gram(-) strains upon maturation, the same as in the case of *H. excipuliformis*.While VPI was active against *P. aeruginosa* (MIC=20 mg/mL), VPM did not show any activity. Also, VPI was most active against *E. coli* (MIC=5 mg/mL) and maturation of the fruiting bodies caused some decrease in the activity (MIC=20 mg/mL for VPM). On the other hand, VPM was most active against*E. faecalis* (MIC=5 mg/mL), the strain against which VPI showed no activity at all. Calvatic acid is an antibacterial agent isolated from several species of puffballs [44,45], although it could not be detected or quantified in the extracts. The gleba of mature fruiting bodies, despite the fact that it has been used in traditional medicine to treat open wounds, doesnot seem to possess significant antimicrobial activity.

All samples were active against MRSA, which is a multiresistant strain that causes complications in hospitalized patients (mostly skin infections), in particularHEI, which exhibited bactericidal activity against it.

Puffballs, which have been used in their mature stadium as wound dressings and for the treatment of some skin conditions, are indeed a promising candidates for wound healing and various skin care products. The autolysis they undergo slightly increasesthe availability of some biologically active compounds involved in skin care and skin regeneration, such are *N*-acetylglucosamine, ergosterol (provitamin D_2), α-tocopherol (vitamin E), linoleic acid and phenolic compounds. Optimization of the extraction conditions could improve their yields, since these are compounds with different polarity. Nevertheless, the extracts proved very active in biological assays. Autolysis affected positively the antioxidant capacity of the extracts and vastly improved their ability to inhibit tyrosinase, the enzyme responsible for the production of melanin, which is sometimes undesired, as for example in cases of postinflammatory hyperpigmentation. Although autolysis may negatively affect the antimicrobial activity of the extracts, they still possess relatively good activity, including inhibitory activity on MRSA, which mostly causes skin infections. The extracts also contain trehalose, a sugar thatis used in cosmetic products as a very effective humectant. The findings of this and previous studies not only justify the traditional use of puffballs, but they also open a new chapter in the potential exploitation of these unique mushrooms.

Author contributions: PP participated in all experiments and wrote the draft version of the manuscript. KI and MK were both involved in the experiments concerning antioxidant and tyrosinase inhibitory activities, the literature search and review of the manuscript. MS and MP were involved in sugar analysis. VM performed HPLC analysis of α-tocopherol and phenolic acids. AC performed GC-MS analysis of ergosterol and free fatty acids. AJ participated in literature search and writing. The whole process was supervised by BB and AK, who also reviewed the manuscript. All authors approved the final manuscript.

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