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Original research paper

AN INSIGHT INTO *IN VITRO* BIOACTIVITY OF WILD-GROWING PUFFBALL SPECIES *LYCOPERDON PERLATUM* (PERS) 1796

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ABSTRACT: *Lycoperdon perlatum* (Pers) 1796 is saprobic puffball species with a global distribution. It is edible if young, when the gleba is still homogeneous and white. Since this species has a pleasant texture and taste, it has been used in soups as a substitute for dumplings. The aim of this work was to study bioactivity of crude extracts prepared from wild-growing sporocarps of *L. perlatum* collected from Eastern Serbia during 2012.

The bioactivity screens included antioxidant (DPPH$^{\bullet}$ and FRAP assays), antiproliferative (human breast MCF-7 cancer cell-line; MTT and SRB assays) and antibacterial (three referent ATCC strains; microdilution assay) effects. Polar extracts (aqueous - LycAq and ethanol – LycEtOH) and a nonpolar extract (hexane - LycHex) of the examined mushroom species were screened. In addition, LycAq and LycEtOH were primarily characterized by UV-VIS spectrophotometry, due to determination of chemical composition (total phenol and flavonoid contents).

The highest anti-DPPH radical activity was observed for LycAq (IC_{50} = 46.56 µg/ml). In comparison with LycAq, less polar LycEtOH showed slightly better ferric reducing antioxidant power (FRAP) (IC_{50} = 21.87 µg/ml and IC_{50} = 19.28 µg/ml, respectively). However, total phenol contents of both extracts were similar (≈ 2.0 mg GAE/g d.w.). Conversely, modest activities were found against *Staphylococcus aureus* ATCC 25922 (LycHex, MIC = 3.12 mg/ml) and MCF-7 cells (with the highest one obtained for LycEtOH after 72 h, IC_{50} = 367.54 µg/ml and IC_{50} = 390.03 µg/ml, MTT and SRB assays, respectively).

According to the obtained experimental data, *L. perlatum* can be considered as a good source of novel and potent natural antioxidants for use in regular diet.

Keywords: puffball, *Lycoperdon perlatum*, biological activity, natural antioxidants, medicinal food

INTRODUCTION

Since the ancient times, mushrooms have been valued by humankind as a culinary wonder and traditional medicine. Recently, it has been discovered that many fungal species, including puffballs, represent miniature pharmaceutical factories producing hundreds of natural products with potent and broad range bioactivities. Furthermore, these organisms have a long history of use in Oriental medicine, supported by contemporary studies (Miles and Chang, 2004).

Production of reactive oxygen and nitrogen species occurs during normal cell metabolism. The excess of free radicals leads to oxidative stress, resulting in oxidative DNA damage, implicated in the pathogenesis of numerous disorders, such

as cardiovascular, atherosclerosis, rheumatoid arthritis and cancer (De Silva et al, 2013).

On the other hand, antibiotic resistance has become a global concern (Westh et al., 2004). The clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug resistant pathogens (Bandow et al., 2003). The increasing failure of chemotherapeutics has led to the screening of a number of medicinal mushrooms for their antimicrobial potential (Karaman et al., 2009a; Karaman et al., 2014; Nwachukwu and Uzoeto, 2010; Ozen et al., 2011; Alves et al., 2013).

Mushrooms are believed to be a very good source of nutraceuticals, mainly with antioxidant, antiproliferative and antimicrobial properties (Wasser, 2002; Barros et al., 2008a; Karaman et al., 2009a; Ferreira et al., 2009; Cheung, 2010; Karaman et al., 2010; De Silva et al, 2012; Patel et al., 2012; De Silva et al, 2013; Heleno et el., 2014). *Lycoperdon perlatum* (Pers) 1796 is a species of puffball fungus from the family Agaricaceae. This saprobic organism grows solitarily, scattered, or in groups or clusters on the ground. It can also grow in fairy rings (Dickinson and Lucas, 1982). Several chemicals have been identified in its fruit bodies such as sterol derivatives ((*S*)-23-hydroxylanostrol, ergosterol α-endoperoxide, ergosterol 9,11-dehydroendoperoxide and (23*E*)-lanosta-8,23-dien-3β,25-diol), volatile compounds (3-octanone, 1-octen-3-ol and (*Z*)-3-octen-1-ol) and an unusual amino acid such as lycoperdic acid (Lamotte et al., 1978; Szumny et al., 2010). The puffball extracts are known for exhibiting antibacterial activity against human pathogens such as *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, with efficiency comparable to that of the antibiotic ampicillin (Ramesh and Pattar, 2010). Moreover, antifungal activities against *Candida albicans*, *C. tropicalis*, *Aspergillus fumigates* and *Alternaria solani* have been also reported (Pujolet et al., 1990).

This study, focusing on *in vitro* bioactivity evaluation of autochthonous *L. perlatum*, included antioxidant, antiproliferative and antibacterial screens. To the best of our knowledge, this is the first report on bioactive properties of *L. perlatum* originating from Serbia.

MATERIAL AND METHODS

Chemicals

Folin-Ciocalteu (FC) reagent, anhydrous sodium carbonate, gallic acid (GA), aluminium trichloride hexahydrate, sodium acetate trihydrate, quercetin hydrate, 2,2-diphenyl-1-picrylhydrazyl (DPPH•), anhydrous iron(III) chloride, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), 2-thiobarbitu-ric acid, disodium hydrogen phosphate, thiazolyl blue tetrazolium bromide, phenazine methosulfate (PMS), β-nicotinamide adenine dinucleotide (NADH), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenylte-trazolium bromide (MTT), dimethyl sulfoxide (DMSO), sulfurhodamine B (SRB) and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich (Steinheim, Germany). Dulbecco's Modified Eagle's Medium with 4.5% of glucose (DMEM) and fetal calf serum (FCS) were obtained from PAA Laboratories (Pasching, Austria).

Puffball samples

Lycoperdon perlatum (Pers) 1796 was collected in Sikole area (a village near the Negotin town, Serbia) during 2012. After its identification, a voucher specimen (MYC12-00664) was deposited at the Herbarium BUNS, University of Novi Sad, Serbia. The puffball samples were frozen at –20 °C, prior to freeze-drying procedure (Bio alpha, Martin Christ GmbH, Germany). Freeze dried samples were ground to a fine powder, wrapped in plastic bags and stored in a dark place at room temperature, until further use.

Extraction

The processed sporocarps of *L. perlatum* (10 g) were extracted with distilled water (Aq), ethanol (EtOH) and hexane (Hex) for 24 h, on a shaker (Thermo Fisher Scientific, USA; 120 rpm) at room temperature (25 °C). The extracts were filtered through filter paper (Whatman No. 4). The organic solvents (EtOH and Hex) were removed by rotary evaporator at 40 °C (Bűchi, Switzerland), while the aqueous extract (LycAq) was freeze-dried. The obtained extracts (ethanol - LycEtOH and hexane –

LycHex) and water-LycAq were stored at +4 °C and –20 °C, respectively. The relevant dried residues were redissolved in 5% DMSO, prior to analysis.

Total phenol content

Total phenol (TP) content of LycEtOH and LycAq was determined according to method by Singleton et al. (1999), adapted for a 96-well plate reader (Multiskan Ascent, Thermo Electron Corporation). Folin-Ciocalteu reagent (125 µl, 0.1 M) was added to diluted extracts (25 µl). After 10 min, 100 µl of 7.5% w/v sodium carbonate was added and the reaction mixture was incubated for 2 h. Absorbance was read at 690 nm. TP is expressed as mg gallic acid equivalents (GAE)/g of dry weight (d.w.).

Total flavonoid content

Total flavonoid (TF) content of LycEtOH and LycAq was measured spectrophotometrically, in a 96-well plate reader, using a modified method by Chang et al. (2002). The relevant sample (30 µl) was mixed with methanol (90 µl), aluminium-trichloride (6 µl, 0.75 M), sodium acetate (6 µl, 1 M) and distilled aqua (170 µl). Absorbance was measured at 414 nm, after incubation of 30 min. The results are expressed as mg quercetin equivalents (QE)/g of dry weight (d.w.).

$DPPH^{\bullet}$ scavenging activity

$DPPH^{\bullet}$ scavenging activity was evaluated according to method by Espin et al. (2000). The reaction mixture consisted of sample (10 µl), $DPPH^{\bullet}$ solution (60 µl) and methanol (180 µl). After incubation of 60 min (dark place, at room temperature), absorbance was measured at 540 nm. Each sample was tested at five different concentrations (60 - 800 µg/ml, LycEtOH; 15–1000 µg/ml, LycAq). The results are expressed as IC_{50} values.

Ferric reducing antioxidant power (FRAP)

FRAP assay was performed according to a modified procedure of Benzie and Strain (1999). The FRAP reagent consisted of 300 mM acetate buffer (pH = 3.6), 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3$, in the ratio 10:1:1 (v:v:v). The sample (10 µl), FRAP reagent (225 µl) and distilled aqua (22.5 µl) were added in a 96-well plate. Absorbance was measured at 620 nm, after incubation of 6 min. The results are expressed as mg ascorbic acid equivalents (AAE)/g of dry weight (d.w.).

Antibacterial activity

LycHex was the only extract screened for antibacterial activity at *in vitro* conditions, after dissolving in 5% DMSO, to reach a final concentration of 0.5% (w/v) (CLSI procedure, 2008; slightly modified by Karaman et al., 2009b). Standard American Type Culture Collection (ATCC) strains of two Gram-positive bacteria, namely *S. aureus* ATCC 25922 and *B. subtilis* ATCC 6633, and one Gram-negative bacterium, namely *E. coli* ATCC 25923, were used. Two-fold microdilution assay in 96-well microplates (Spektar, Čačak, Serbia) was applied for determination of minimal inhibitory and bactericidal concentrations (MIC and MBC, respectively). Pure bacterial strains were subcultured on nutrient agar slants at 37 ºC for 24 h; their suspensions corresponding to McFarland 0.5 optical density, ≈ 1.5×10^8 CFU/ml. The extract concentration ranged from 0.78 to 25.00 mg/ml. After incubation at 35 ºC for 18-24 h, MIC and MBC were determined. Last two wells served as a growth control (positive control) and negative control (5% DMSO), respectively. The reference antibiotics (gentamicin and ampicillin) were applied as control standards.

Antiproliferative activity

Cells

Estrogen dependent MCF-7 cells were grown in DMEM (PAA Laboratories) supplemented with 10% FCS. The cells were seeded in a 96-well microplate (5000 cells per well). After incubation of 24 h, the growth medium was replaced with 100 µl of medium containing the examined samples (extracts) at four different concentrations (33.3 µg/ml, 100.0 µg/ml, 300.0 µg/ml and 900.0 µg/ml). The untreated cells served as the control, while pure DMSO was used as a positive control. The growth of MCF-7 cells was evaluated by standard colorimetric assays, MTT and SRB.

MTT Assay

After incubation of 24 h and 72 h respectively, the cell viability was determined by

the proliferation MTT assay (Mosmann, 1983). This assay is based on the color reaction of mitochondrial dehydrogenase in living cells with MTT reagent. Upon the incubation, MTT reagent was added to each well (50 µg/100 µl/well; at 37 °C, in 5% CO_2, for 3 h). The crystals of produced formazan were dissolved in 100 µl acidified isopropanol (0.04 M HCl in isopropanol). Absorbance was measured at 540 nm and 690 nm on a 96 well plate reader (Multiskan Ascent, Thermo Electron Corporation, USA). The results are expressed as IC_{50} values (sample concentration which inhibited 50% of the net cell growth).

SRB Assay

This assay estimates cell number indirectly, by staining cellular protein with the protein-binding dye SRB (adapted procedure, by Skehan et al., 1990). After incubation, the cells were fixed adding cold 50% TCA and incubated for 1 h at 4 °C. The wells were washed with deionised water and dried; SRB solution (0.4% in 1% acetic acid) was then added to each plate well and incubated for 30 min at room temperature. The unbound SRB was removed by washing with 1% acetic acid. The plates were air dried, the bound SRB was solubilized with 10 mM Tris (pH = 10.5), while absorbance was measured at 492 nm and 690 nm, using the microplate reader. DMSO was applied as a positive control. The results are expressed as IC_{50} values.

Data analysis

The absorbance was calculated from the difference of two absorbances: $A = A_{540} - A_{690}$ and $= A_{492} - A_{690}$ for MTT and SRB assays, respectively. Percentage of cytotoxicity was calculated as the ratio of the treated and the control group absorbance respectively, multiplied by 100. The obtained results are expressed as IC_{50}. These values were calculated from the cytotoxicity (%) - extract concentration plot (µg/ml) using the Origin v. 6.0 graphing and data analysis software (1999).

Statistical analysis

All the assays were carried out in triplicate. The data were statistically analyzed using the software Statistica (2013). The results of antioxidant and antiproliferative activities as well as total phenol (TP) and flavonoid (TF) contents are expressed as a mean value ± standard deviation (SD). The statistical significance was determined by analysis of variance (ANOVA), with Duncan's multiple range test as *post hoc* test. The Pearson correlation coefficients (r^2) were calculated for TP, TF, FRAP and IC_{50} values for anti-DPPH$^{\cdot}$ and antiproliferative activities.

RESULTS AND DISCUSSION

Antioxidant activity and total phenol and flavonoid contents

Among the examined extracts, IC_{50} value of LycAq stood out (46.56 µg/ml) in DPPH$^{\cdot}$ scavenging assay (Table 1). Interestingly, in both assays these extracts showed higher activities compared with the experimental data obtained for *L. perlatum* originating from Portugal and Turkey (Barros et al., 2008b; Sarikurkcu et al., 2015). While there was no significant difference in their TP contents, TF content of LycAq was more than three times higher (Table 1).

Table 1.
Antioxidant activity and total phenol and flavonoid contents of *Lycoperdon perlatum* extracts

Antioxidant Assay	LycEtOH	LycAq
DPPH$^{\cdot}$*	176.04 ± 23.58^{a}	46.56 ± 15.78^{b}
FRAP**	21.87 ± 0.90^{a}	19.28 ± 0.80^{a}
Total content* **		
TP	1.92 ± 0.01^{a}	1.96 ± 0.05^{a}
TF	0.17 ± 0.06^{b}	0.58± 0.13^{a}

**Expressed as concentration of the extracts that caused 50% activity - IC_{50} (µg/ml). **Expressed as mg ascorbic acid equivalents/g extract of dry weight (mg AAE/g d.w.). ***Total phenol (TP) content is expressed as mg gallic acid equivalents/g extract of dry weight (mg GAE/g d.w.), while total flavonoid (TF) content is expressed as mg quercetin equivalents/g extract of dry weight (mg QE/g d.w.)*

[a,b]The results are expressed as a mean value ± SD. The means with different superscript within the same row are statistically different (p<0.05)

Table 2.
Antiproliferative activity of *Lycoperdon perlatum* extracts on MCF-7 cells

Extracts	MTT Assay*		SRB Assay*	
	24 h	72 h	24 h	72 h
LycAq	>900[a]	417.31± 17.17[a]	327.86±10.54[b]	707.05±16.29[a]
LycEtOH	691.64±36.45[b]	367.54± 22.34[a]	854.849± 22.62[a]	390.03±21.27[b]
Positive control (DMSO)	78.24	78.01	261.10	179.10

**Expressed as concentration of the extracts that caused 50% activity - IC_{50} (µg/ml)*
[a,b]The results are expressed as a mean value ± SD. The means with different superscript within the same column are statistically different (p<0.05)

Table 3.
The Pearson correlation coefficients (r^2) between TP/TF contents and observed bioactivity in anti-proliferative (MTT and SRB) and antioxidant (DPPH and FRAP) assays

	MTT Assay				SRB Assay			
	24 h		72 h		24 h		72 h	
	Aq	EtOH	Aq	EtOH	Aq	EtOH	Aq	EtOH
TP[a]	–	0.18	0.96	0.64	0.61	0.39	0.01	0.29
TF[b]	–	0.82	0.90	**0.99***	0.98	0.96	0.90	0.91
	DPPH Assay				**FRAP Assay**			
TP[a]	**0.92***	**0.92***			0.12	0.32		
TF[b]	**0.94***	0.83			0.02	0.01		

[a]Total phenol (TP) content is expressed as mg gallic acid equivalents/g extract of dry weight (mg GAE/g d.w.); [b]Total flavonoid (TF) content is expressed as mg quercetin equivalents/g extract of dry weight (mg QE/g d.w.). The correlations are significant at p<0.05

However, both extracts contained much lower TP and TF contents than ethanol/methanol extracts of the same fungal species collected in Slovenia and India, respectively (TP = 34.56 mg/g and TF = 3.01 mg/g; TP = 6.25 mg/ml and TF = 2.52 mg/ml; respectively) (Vidović, 2011; Ramesh and Pattar, 2010).

Taking into account both parameters (antioxidant activity and chemical composition), it may be assumed that phenolic and flavonoid compounds are not the only ones responsible for the observed activities.

Antiproliferative activity

The antiproliferative activity against human breast MCF-7 cancer cell-line was evaluated by MTT and SRB assays. The examined extracts (LycAq and LycEtOH) exhibited different activity in the aforementioned bioassays (Table 2). Generally, it was modest with LycAq (IC_{50} = 327.86 µg/ml) being the most potent one (SRB assay, 24 h). As other bioactivities, this one also directly depends on the chemical composition (Table 2) (Houghton et al., 2007; Kaišarević, 2011).

Cinnamic acid, previously found in some puffball species, has been recently linked to growth inhibition of a lung cancer cell-line (Barros et al., 2009; Vaz et al., 2012). In addition, the current literature data suggest phenolic acids, steroids and triterpenoids as the key cytotoxic principles of the puffballs (Waser, 2002; Ferreira et al., 2010; De Silva et al., 2013).

The Pearson correlation coefficient (r^2) between TP/TF contents and this activity in both applied assays (Table 3) pointed out much higher values for TF (in a narrow range, from 0.82 to 0.99, with the only one being statistically significant - LycEtOH in MTT assay, 72h) than TP content (in a wider range, from 0.18 to 0.96, with no statistical significance). The same correlations for antioxidant activity (Table 3) indicated TP/TF content significance only for anti-DPPH radical activity. The other phenolics including phenolic acids could be responsible for demonstrated antioxidant activities according to previous literature data (Karaman et al., 2010; Karaman et al., 2014; Ferreira et al., 2009). The observed chelating ability is likely to be linked to other classes of organic compounds (found in some mushroom species) such as polysaccharides, proteins and terpenic acids. Actually, the further research is expected to clarify this particular issue (Kalogeropoulos et al., 2013).

Table 4.
Antibacterial activity of *Lycoperdon perlatum* hexane extract

Bacterial Strain	**MIC***	**MBC***
Escherichia coli ATCC 25923	>25	>25
Staphylococcus aureus ATCC 25922	3.12	6.25
Bacillus subtilis ATCC 6633	>25	>25
Antibiotics		
Gentamicin** (μg/ml)	2.50	10.00
Ampicillin** (μg/ml)	8.00	128.00

**MIC – Minimal Inhibitory Concentration (mg/ml); MBC – Minimal Bactericidal Concentration (mg/ml).*
***The obtained values against the strain* Staphylococcus aureus *ATCC 25922*

Antibacterial activity

LycHex practically displayed a modest antibacterial activity only against the strain *S. aureus* ATCC 25922 (MIC = 3.12 mg/ml, MBC = 6.25 mg/ml) (Table 4). These results are in good accordance with recently published data for the same puffball species collected in India (Ramesh and Pattar, 2010).

CONCLUSIONS

L. perlatum, the examined fungal species in this study, may be considered as a promising source of novel natural antioxidants with potential significance for regular diet. If its autochthonous origin is taken into account, this fact is even more important. Puffball species in Serbia have not been examined enough so far. To our knowledge, this is the first report on bioactivity of the autochthonous species *L. perlatum*. The future research work should be primarily directed towards elucidation of chemical profiles of LycAq and LycEtOH and their mechanism(s) of action.

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УВИД У *IN VITRO* БИОАКТИВНОСТ САМОНИКЛЕ ПУХАРЕ *LYCOPERDON PERLATUM* (PERS) 1796

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Сажетак: *Lycoperdon perlatum* (Pers) 1796 је сапробна пухара са широком распрострањеношћу. Јестива је као млада, када је глеба још целовита и бела. Због пријатне текстуре и укуса, користи се у супама, као замена за кнедле. Циљ овог рада био је да се проучи биоактивност сирових екстраката врсте *L. perlatum* припремљених од самониклих спорокарпа пореклом из источне Србије (Сиколе, село близу Неготина) током 2012. године.
Скрининг биоактивности обухватио је антиоксидативну (тестови DPPH• и FRAP), антипролиферативну (ћелијска линија хуманог аденокарцинома дојке, MCF-7; тестови MTT и SRB) и антибактеријску активност (три референтна ATCC соја; микродилуциони тест). Поларнији екстракти испитиване гљиве (водени - LycAq и етанолни - LycEtOH) тестирани су на антиоксидативном и антитуморском нивоу, док је неполаран екстракт (LycHex) тестиран на антибактеријском нивоу. Осим тога, LycAq и LycEtOH хемијски су прелиминарно окарактерисани (садржај укупних фенола и флавоноида) помоћу UV-VIS спектрофотометрије.
Највећа анти-DPPH радикалска активност уочена је за LycAq (IC_{50} = 46.56 μg/ml). У поређењу са LycAq, мање поларни екстракт LycEtOH показао је незнатно бољи потенцијал за редукцију гвожђа (IC_{50} = 21.87 μg/ml и IC_{50} = 19.28 μg/ml). Међутим, садржај укупних фенола у оба екстракта био је сличан (≈ 2.0 mg GAE/g.d.w.). С друге стране, крајње блага антибактеријска активност уочена је на соју *Staphylococcus aureus* ATCC 25922 (LycHex, MIC = 3.12 mg/ml) и ћелије MCF-7 (уз најзначајнију активност за LycEtOH након 72 h, IC_{50} = 367.54 μg/ml и IC_{50} = 390.03 μg/ml, на тестовима MTT и SRB).
Према добијеним експерименталним подацима, *L. perlatum* може се сматрати добрим извором нових и потентних природних антиоксиданаса са потенцијалном применом у редовној исхрани.

Кључне речи: пухара, *Lycoperdon perlatum*, биолошка активност, природни антиоксиданси, лековита храна

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