Edible mycorrhizal species *Lactarius controversus* Pers. 1800 as a source of antioxidant and cytotoxic agents

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Abstract
The aim of this work was to study chemical profile and antioxidant and cytotoxic activities of ethanol and water extracts of *Lactarius controversus* (Pers.) 1800 mushroom species growing in eastern Serbia. The chemical characterization of phenolic compounds performed by HPLC–MS/MS demonstrated the presence of quinic acid among others. Determination of antioxidant activity, including radical scavenging effects on DPPH$^{\bullet}$, NO$^{\bullet}$, $^{\bullet}$OH and SOA radicals and ferric reducing ability was investigated. The highest DPPH radical scavenging effect was obtained for water extract (LcAq) while ethanol extract (LcEtOH) demonstrated the highest FRAP activity. Hexane extract applied in antibacterial assay against three pathogenic strains demonstrated antibacterial effect only against *S. aureus* ATCC25922. Anti-proliferative properties against estrogen dependent MCF 7 breast cancer cell lines using MTT showed higher activity for ethanolic extract.

Keywords: *Lactarius controversus*, phenolic compounds, quinic acid, antioxidants, cytotoxic agents.

Available online at the Journal website: http://www.ache.org.rs/HI/

SCIENTIFIC PAPER

UDC 582.28(497.11-11):54:543.544

Hem. Ind. **70** (2) 113–122 (2016)

doi: 10.2298/HEMIND141229017N

For thousands of years, traditional oriental medicine has used natural products, including medicinal and toxic mushrooms, for curing and disease prevention. Most of medicinal mushroom preparations were used in a form of tonics, food or powder with unknown side-effects. Nowadays, wild-growing mushrooms still comprise a vast and largely untapped source of powerful new pharmaceutical products for the mankind in the future [1]. The number of mushroom species on Earth is estimated to approximately 140,000 species, while only 10% of them have been already determined. According to estimates that only 5% of the total known species, which is about 7000 species, are with detected and potential benefits for mankind, means that mushrooms are still insufficiently explored sources of bioactive compounds [2–5]. Mushrooms contain a variety of complex compounds derived from secondary metabolism such as phenolic compounds, polyketides, triterpenoids and steroids which are specific to each mushroom species and strain and have specific medicinal effects on humans, including antimicrobial, antitumor, antioxidant etc. [6,7]. Many such compounds have been used in the treatment of cancer [8,9]. Furthermore, evidence-based studies suggest that there is a relationship between the physiopathology of several chronic diseases (*e.g.*, cancer) and oxidative stress development. Therefore, the use of foods rich in antioxidants, such as phytochemical and mycochemical protectors, may be the most relevant factor in the prevention of oxidative stress related diseases [10]. Moreover, there is a continuous need for discovery of new molecules that are able to effectively reduce cancer which is the leading cause of death in Europe in 2006 [11].

Some literature data suggest that *L. controversus* is inedible species [12], but in Serbia this species is used as food, after obligatory cooking procedure before consumption.

In the present work we studied whether the autochthonous mushroom species *Lactarius controversus* Pers. 1800 possesses antioxidant, antibacterial and antiproliferative properties. To our knowledge, this is the first report dealing with the antioxidant growth inhibitory properties of *L. controversus* against MCF 7 breast cancer cell line.

EXPERIMENTAL

Standards and reagents

Folin–Ciocalteu (FC) reagent, anhydrous sodium carbonate, gallic acid, aluminium trichloride hexahydrate, sodium acetate trihydrate, quercetin hydrate, 2,2--diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), anhydrous iron(III)

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Paper received: 29 December, 2014
Paper accepted: 6 March, 2015

chloride, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), 2-thiobarbituric acid, disodium hydrogen phosphate, thiazolyl blue tetrazolium bromide, phenazine methosulfate (PMS), and β-nicotinamide adenine dinucleotide (NADH), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), sulforhodamine 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 purchased from PAA Laboratories (Pasching, Austria). Ascorbic acid, potassium dihydrogen phosphate, sodium nitroprusside dihidrate (SNP), naphthylethylenediamine dihydrochloride (NEDA), sulfanilamide (SA) 35% hydrogen peroxide, iron(II) sulfate heptahydrate, trichloracetic acid, and ethylenediaminetetraacetic acid (EDTA) were purchased from Lach-ner (Neratovice, Czech Republic). 2-Deoxy-D-ribose was obtained from Alfa Aesar (Karlsruhe, Germany). Reference standards of the phenolic compounds were obtained from Sigma–Aldrich Chem (Steinheim, Germany), Fluka Chemie GmbH (Buchs, Switzerland) or from Chromadex (Santa Ana, USA). HPLC gradient grade methanol was purchased from J. T. Baker (Deventer, The Netherlands), and p.a. formic acid and DMSO from Merck (Darmstadt, Germany). Deionized water was produced using a Millipore water purification system.

Mushroom samples

Lactarius controversus was collected from Sikola area in Serbia during the year 2012. After the identification of the species, a voucher specimen (12-00663) was deposited at the Herbarium Buns, Faculty of Sciences Department of Biology and Ecology, University of Novi Sad. The mushroom samples were pre-frozen at –20 °C and freeze-dried (Martin Christ GmbH, Germany). Freeze dried samples were ground to a fine powder, wrapped in plastic bags and stored in the dark at room temperature prior to analysis.

Extraction

The whole freeze dried and powdered sporocarps of *L. controversus* (10 g) were extracted with the following solvents: ethanol (EtOH), methanol (MeOH) or hexane (Hex) and distilled water (Aq) for 24 h on a shaker (Thermofisher Scientific, USA) at 120 rpm at room temperature (25 °C). The extracts were filtered through Whatman No.4 filter paper and the solvents were removed by rotary evaporation in vacuum at 40 °C (Bűchi, Switzerland). The extracts obtained (LcEtOH, LcMeOH, LcHex) were stored in refrigerator at 4 °C, while aqueous extract (LcAq) was stored at –20 °C prior to analysis. Dry residues were redissolved in DMSO to obtain LcAq, LcEtOH, for determination of antioxidant and anti-proliferative activities (5 mg/ml), and LcHex was used for evaluation of antibacterial activity.

HPLC–MS/MS screening of the phenolic compounds

For HPLC–MS/MS determination of the phenolic profile, method developed by Orčić *et al*. [13] was used. The Agilent 1200 series liquid chromatograph was used for separation of all analytes, using a Zorbax Eclipse XDB-C18 RR 4.6 mm×50 mm×1.8 mm (Agilent Technologies) reversed-phase column held at 40 °C. Detection was carried out by Agilent series 6410A triple-quadrupole mass spectrometer with electrospray ionization (ESI). MassHunter ver. B.03.01. software (Agilent Technologies) was used for instruments control and data analysis. The binary mobile phase consisted of 0.05% formic acid (A) and methanol (B) and was delivered at a flow rate of 1 ml/min. Gradient elution was performed using the following solvent gradient: starting with 70% A/30% B, reaching 30% A/70% B in 6.00 min, then 100% B at 9.00 min, holding until 12.00 min, with reequilibration time of 3 min. The injection volume for all samples was 5 µL. ESI parameters were: drying gas (N2) temperature, 350 °C; flow, 9 L/min; nebulizer gas pressure, 40 psi; capillary voltage, 4 kV, negative polarity. All compounds were quantified in dynamic MRM mode (multiple reaction monitoring mode). Compound-specific, optimized MS/ /MS parameters are given in Table 1. The mix of stock solutions was prepared, with concentration of each compound being 100 µg/ml, and then, subsequently serially diluted in methanol–water (1:1), giving working standard solutions with concentration ranging from 0.0015 do 25.0 µg/ml, which were used for construction of the calibration curves. Concentrations of standard compounds in extracts were determined from the peak areas by using the equation for linear regression obtained from the calibration curves (R2 gt; 0.995).

Total phenolic content

Total phenolic content (TP) in the ethanol and water extracts was determined according to method by Singleton *et al*. [14] adapted for plate reader (Multiskan Ascent, Thermo Electron Corporation). 125 µl of 0,1M Folin–Ciocalteu reagent was added to 25 µl of diluted extracts. After 10 min, 100 µl of 7.5% sodium carbonate was added and reaction mixture was incubated for 2 h. Absorbance was read at 690 nm after finished incubation period. In order to eliminate the interferences, correction was prepared by replacing the volume of reagents with the same volume of distilled water. Standard curve was prepared for gallic acid, and total phenolic content was expressed as mg gallic acid equivalents (GAE)/g of dry weight. Experiments were performed in triplicate.

Total flavonoid content

The flavonoid (TF) content of investigated extracts was measured spectrophotometrically in a 96-well

Table 1. Optimized dynamic MRM parameters

Compound	t_R / min	Precursor (*m*/*z*)	Product (*m*/*z*)	$V_{fragmentor}$ / V	$V_{collision}$ / V
Gallic acid	0.58	169	125	90	10
Catechin	0.74	289	245	150	10
Protocatechuic acid	0.79	153	109	105	9
5-*O*-Caffeoylquinic acid	0.80	353	191	100	10
Epigallocatechin gallate	0.81	457	169	165	16
Epicatechin	0.95	289	245	150	10
Gentisic acid	1.03	153	109	100	9
p-Hydroxybenzoic acid	1.08	137	93	80	10
Esculetin	1.13	177	133	105	15
Caffeic acid	1.18	179	135	100	10
Vanillic acid	1.24	167	108	100	15
Syringic acid	1.31	197	182	90	7
p-Coumaric acid	1.69	163	119	90	9
Umbelliferone	1.73	161	133	120	19
Scopoletin	1.77	191	176	80	8
Ferulic acid	1.90	193	134	90	11
Vitexin	1.90	431	311	200	22
Sinapic acid	1.92	223	193	100	17
Luteolin 7-*O*-glucoside	2.13	447	285	230	30
Hyperoside	2.16	463	300	200	30
Quercetin 3-*O*-glucoside	2.25	463	300	210	30
Rutin	2.33	609	300	135	42
Apiin	2.60	563	269	250	36
o-Coumaric acid	2.62	163	119	100	5
Myricetin	2.67	317	179	150	20
Quercitrin	2.75	447	300	190	27
Kaempferol 3-*O*-glucoside	2.80	447	284	190	30
Apigenin 7-*O*-glucoside	2.81	431	268	135	41
Secoisolariciresinol	2.90	361	165	130	26
3,4-Dimethoxycinnamic acid	2.99	207	103	110	7
Baicalein	3.40	445	269	140	22
Daidzein	3.43	253	208	145	31
Matairesinol	3.66	357	122	130	24
Quercetin	3.74	301	151	130	15
Naringenin	3.87	271	151	130	16
Cinnamic acid	3.91	147	103	100	5
Luteolin	4.03	285	133	135	25
Genistein	4.12	269	133	145	32
Kaepferol	4.55	285	285	130	0
Apigenin	4.71	269	117	130	25
Isorhamnetin	4.79	315	300	160	21
Chrysoeriol	4.82	299	284	125	20
Baicalein	5.15	269	269	165	0
Amentoflavone	5.78	537	375	220	35

plate reader using modified method by Chang *et al*. [15]. 30 µl of sample was mixed with 90 µl of methanol, 6 µl of 0.75 M aluminium trichloride, 6 µl of 1 M sodium acetate and 170 µl of distilled water. Absorbance was measured at 414 nm after 30 min of incubation. Correction was prepared in the way indicated above, with aluminium trichloride solution replaced with the equivalent volume of distilled water. Standard

curve was prepared using quercetin. Experiments were repeated three times, and results were expressed as mg quercetin equivalents (QE)/g of dry weight.

DPPH radical scavenging activity

Spectrophotometrical determination of free radical scavenging activity was based on the monitoring of DPPH radical transformation in the presence of antioxidants according to Espin *et al*. [16]. The reaction mixture in the wells consisted of 10 µl of sample, 60 µl of DPPH solution and 180 µl of methanol. Control contained ethanol instead of sample, and correction contained 10 µl of sample and 240 µl of methanol. After 60 min of incubation in the dark at room temperature, the absorbance was measured using plate reader at 540 nm. Each sample was tested at five different concentrations to obtain IC_{50}, and experiments were performed in triplicate.

Ferric reducing antioxidant power (FRAP)

FRAP test was performed according to modified procedure of Benzie and Strain [17]. 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 volume ratio 10:1:1. 10 µl of sample, 225 µl of FRAP reagent and 22,5 µl of distilled water were added in 96-well plate. Extract was replaced by the same volume of ethanol in control, and correction contained distilled water instead of FRAP reagent. Absorbance was measured after 6 min at 620 nm. Ascorbic acid was used to construct the standard curve, and results were expressed as mg ascorbic acid equivalents (AAE)/g of dry weight. Each analysis was performed three times.

Nitric oxide radical scavenging capacity

Nitric oxide scavenging capacity was determined according to the procedure of Green *et al*. [18]. The reaction mixtures in the test tubes consisted of 30 µl tomato extract, 500 µl SNP, and 500 µl of phosphate buffer, pH 7.4. Control contained equivalent volume of ethanol, while the reagents were replaced by the phosphate buffer in the correction. Test tubes were incubated at room temperature for 90 min, under light exposure. After incubation, 1 ml of Griess reagent (0.2% solution of NEDA and 2% solution of SA in 4% phosphoric acid in the volume ratio 1:1) was added to samples, corrections, and control. Aliquots of 250 µl were transferred to the plate, and their absorbance was measured using plate reader at 540 nm. Samples were tested at five different concentrations to obtain IC_{50}, and experiments were performed in triplicate.

Superoxide anion radical scavenging capacity

Superoxide anion radical scavenging capacity of extracts was determined by measuring their ability to neutralize superoxide anion radicals generated during aerobic reduction of nitro blue tetrazolium by NADH mediated by PMS [19]. 100 µl of 677 µM NADH, 100 µl of 60 µM PMS, 200 µl of 144 µM NBT and 1,1 ml of phosphate buffer (pH 8.3) were mixed with 10 µl of extract in the test tube. Control contained ethanol instead of extract, and correction contained 10 µl of extract and 1,5 ml of phosphate buffer. After 5 minutes of incubation, aliquots of 250 µl were transferred to the plate, and their absorbance was measured at 540 nm. Five different concentrations of each sample were tested to obtain IC_{50}, and experiments were performed in triplicate.

Hydroxyl radical scavenging capacity

The content of OH radicals was determined from the degradation reaction of 2-deoxy d-ribose into fragments [20], while the malonyl dialdehyde (MDA) reaction with TBA reagent was determined spectrophotometrically at 532 nm. Each reaction was performed at five different concentrations using 10 µL of fungal extract (33.3–1000 µg/mL) to obtain IC_{50}, and experiments were performed in triplicate.

Antibacterial activity

In vitro antibacterial susceptibility assay was done for LcHex extract after dissolving in 5% DMSO to reach final extract concentration of 0,5%. Standard American Type Culture Collection (ATCC) strains of two Gram--positive bacteria, *Staphylococcus aureus* ATCC25922, *Bacillus subtilis* ATCC6633 and one Gram-negative bacteria, *Escherichia coli* ATCC25923were used.

Two-fold assay in 96-well microplates (Spektar, Čačak, Serbia) for determination of minimal inhibitory concentration (*MIC*) and minimal bactericidal concentration (*MBC*) values was employed. Pure bacterial strains were subcultured on nutrient agar slants at 37 °C 24 h, while suspensions of the tested strains were corresponding to McFarland 0.5 optical density $\approx 1.5\times10^8$ CFU/mL. 50 µl of extract was added to 50 µl of Műeller Hinton Broth (Torlak, Belgrade, Serbia) seeded with 1 µl bacterial suspensions. Evaluation of antibacterial activity was done according to the CLSI procedure (2008) modified by Karaman *et al.* [21] applying extract concentration in the final range from 0.78 to 25.0 mg/mL. After incubation at 3 °C for 18–24 h, *MIC* was determined as the lowest extract concentration preventing visible bacterial growth while the complete absence of growth was considered as *MBC*. It was confirmed by sub-culturing aliquots of 100 µL working solutions on Müeller Hinton agar plates and incubated at 35 °C overnight. Last two wells were free from tested extracts, and hence served as a growth control (positive control) and with 5% DMSO as a negative control. Reference antibiotics (ampicillin and gentamicin) were applied as control standards. All analysis were carried out in triplicate (n = 3).

Evaluation of anti-proliferative activity

Cells

MCF-7 cells were grown in Dulbecco's modified Eagle's medium with 4.5% of glucose (DMEM, PAA Laboratories) supplemented with 10% fetal calf serum (FCS). For the experiment, the cells were seeded in a 96-well microplate (5000 cells per well). After 24 h incubation, the growth medium was replaced with 100 µl of medium containing samples at four different concentrations (33.3, 100, 300 and 900 µg/mL). Untreated cells served as the control, and DMSO was used as a positive control. The effects of the extracts on the growth of human tumor cell line were evaluated according to the two procedures.

MTT Assay

After 24 and 72 h, the cell viability was determined by the proliferation test MTT assay [22], which is based on the colour reaction of mitochondrial dehydrogenase in living cells with MTT reagent. At the end of the treatment period, MTT was added to each well (50 µg/100 µl /well), which was then incubated at 37 °C in 5% CO_2 for 3 h. The coloured crystals of produced formazan were dissolved in 100 µl acidified isopropanol (0.04 M HCl in isopropanol). The absorbance was measured at 540 nm and 690 nm on plate reader (Multiskan Ascent, Thermo Electron Corporation, USA).

SRB (sulforhodamine B) Assay

This colorimetric assay estimates cell number indirectly, by staining cellular protein with the protein--binding dye SRB, in adapted procedure by Skehan *et al*. [23]. After incubation period, the cells were fixed adding cold 50% trichloroacetic acid (TCA) and incubated for 1 h at 4 °C. Wells were washed with deionized water and dried; sulforhodamine solution (0.4% in 1% acetic acid) was then added to each plate well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air dried, the bound SRB was solubilised with 10 mM Tris (pH 10.5) and absorbance was measured at 492 and 690 nm in the microplate reader. The results were expressed in EC_{50} values (sample concentration that inhibited 50% of the net cell growth). DMSO was used as positive control.

Percentage of cytotoxicity was calculated as the ratio of treated group absorbance and the control group absorbance, multiplied by 100. Experiments were performed twice in triplicate, and the obtained results were expressed as IC_{50} values (sample concentration that inhibited 50% of the net cell growth). IC_{50} values were calculated from the cytotoxicity (%) – extract concentration (µg/mL) plot using the Origin v. 6.0 graphing and data analysis software (1999).

Statistical analysis

Results were expressed as mean ± standard deviation (*SD*). Statistical analysis was performed using Statistica software system (StatSoft, Inc. (2013), version 12.0 (www.statsoft.com)). Significant differences between two groups were determined by Student's *t*-test. Pearson correlation coefficients were calculated between content of antioxidant compounds in extracts and their antioxidant and antiproliferative activity in different assays. Cluster analysis was performed using Mahalanobius distances.

RESULTS AND DISCUSSION

LC–MS/MS determination of phenolic compounds

Forty-five phenolic compounds were recorded using HPLC–MS/MS technique (Table 1), and only quinic acid was identified and quantified (8.9 µg/g d.w.), while concentrations of other detected compounds were under the limits of quantification (*LOQ*) of the method. According to the obtained results we cannot claim that other detected compounds, which could not be quantified in this work, are not present in examined extracts, but we could expect them to be present in amount lower than listed *LOQ*.

Quinic acid is a crystalline acid that is usually obtained from plants and it is a versatile starting material for the synthesis of new pharmaceuticals [25]. It was found in some species from genus *Lactarius*, *L. volemus* in previous studies [26]. Previous research [25,27,28] showed that quinic acid is a common constituent of human diet, capable of conversion into tryptophan and nicotinamide *via* the micro flora of the gastro intestinal (GI) tract, thus providing *in situ* physiological source of these essential metabolic ingredients to humans [25,27,29].

Antioxidant activity

Antioxidant activity is manifested in a wide variety of actions, such as inhibition of oxidizing enzymes, chelating of transition metals, transfer of hydrogen or a single electron to radicals, singlet oxygen deactivation, or enzymatic detoxification of reactive oxygen species [30,31].

The overall antioxidant activity should be evaluated by different methods in order to extensively characterize the antioxidant potential of pure compounds or extracts [32]. Therefore, water and ethanol extracts of *L. controversus* were examined with regard to scavenging capacity towards, $DPPH^{\bullet}$, $NO^{\bullet}$, $^{\bullet}OH$ and SOA radicals and ferric reducing power.

The results for antioxidant activity of analyzed extracts are shown in Table 2. Although both extracts possessed antioxidant properties, water extract provides higher antioxidant activity (IC_{50} = 219.37 µg/ml) than

*Table 2. Antioxidant activity of L. controversus extracts and their total phenolic and total flavonoid content; *,**: significant differences between two groups were determined by student's t-test (p < 0.001)*

Parameter	LCEtOH	LCAq
Antiradical assays[a], IC_{50} / µg ml^{-1}		
DPPH$^{\bullet}$	355.64*±41.5	219.37**±5.7
NO$^{\bullet}$	52.61*±5.7	90.2*±24.4
SOA	128*±6.6	4.41**±4.4
OH$^{\bullet}$	12.05*±3.9	12.80*±1.1
Antioxidant assay[b]		
FRAP	10.93*±0.9	3.0**±0.8
Total content[c]		
TP	45.84*±0.9	3.50**±0.9
TF	25.05*±0.31	1.20**±0.3

[a]Results are expressed as IC_{50} (µg/ml) – concentration of extracts that caused 50% (25%) of activity in assays (in NO assay); [b]ferric reducing antioxidant power (*FRAP*) is expressed as mg ascorbic acid equivalents/g extract dry weight (mg AAE/g d.w.); [c]total phenol content (*TP*) was expressed as mg gallic acid equivalents/g extract dry weight (mg GAE /g d.w.) and total flavonoid content (*TF*) was expressed in mg quercetine equivalents/g extract dry weight (mg QE/g d.w.)

ethanol extract for the DPPH and SOA assay, while ethanolic extract showed higher activity than water extract according to reducing power capacity (*FRAP*). Moreover, both analyzed extracts did not differ significantly only in $^{\bullet}$OH and NO$^{\bullet}$ assay according to IC_{50} values. Investigated extracts showed higher activities for DPPH assay than *L. piperatus* and methanol extract of *L. deliciosus* [30].

Results showed that ethanol extract contained more total phenols than water extract (Table 2).These results showed higher values than in the previous studies for the genus *Lactarius* [33]. The content of flavonoids between extracts did not differ significantly. Phenols are expected to be the key components accounting for the demonstrated results that are statistically determined *via* correlations (Table 3).

Antibacterial activity

Hexane extract provides antibacterial activity against only one strain *S. aureus*at concentration of 3.12 mg/ml for *MIC* and 6.25 mg/ml for *MBC*. Analyzed extract showed lower activities than antibiotics (gentamicin and ampicillin: *MIC*, 2.5 and 8 µg/ml and *MBC*, 10 and 128 µg/ml, respectively). These results are in agreement with previous data for chloroformic extracts [21].

Antiproliferative activity

Antiproliferative activity of water and ethanol extracts of *L.controversus* against MCF 7 human breast cancer cell line evaluated by MTT and SRB assays are presented in Table 4. Under the experimental conditions both extracts showed cytotoxic activity in two different assays applied at the highest concentration (900 µg/mg), percentage of cytotoxic inhibition was in the range from 53.12%-LcAq to 95.35%-LcEtOH after 24 h and from 36.15%-LcAq to 79.96-LcEtOH after 72 h. In particular, LcEtOH displayed the strongest growth inhibitory activity after 24 h acute phase (IC_{50} = 166.42 µg/ml). Differences in IC_{50} values obtained for the same samples by the two assays can probably be attributed to the difference in sensitivity of targets they reflect, since they measure distinct biological parameters in living cells. While SRB assay does not depend on enzymatic activity but on protein content of the cells, in MTT assay the results reflect the activity of mitochondrial dehydrogenase which is more sensitive parameter, and therefore in many cases with changes detectable in lower concentrations. However, the effects observed for each sample also strongly depend on the specific mixture of compounds present in the sample, their interaction and action of their metabolites that induce specific and often unexpected cellular responses.

*Table 3. Cytotoxic activities of L. controversus extracts on MCF7 (IC_{50}; µg/ml); *: correlations are significant at p < 0.05; **: correlations are significant at p < 0.01; ***: correlations are significant at p < 0.001*

Extract	MTT assay		SRB assay	
	24 h	72 h	24 h	72 h
LCAq	306.17*±15.5	<900*	623.80*±49.81	249.02**±44.8
LCEtOH	166.42**±3.1	302.74**±9.6	526.98*±35.4	696.37*±8.4

[a]Total flavonoid content (*TF*) was expressed in mg quercetine equivalents/g extract dry weight (mg QE/g d.w.); [b]total phenol content (*TP*) was expressed as mg gallic acid equivalents/g extract dry weight (mg GAE/g d.w.)

*Table 4.Correlation between antiproliferative assays and antioxidant assays and total phenolicand total flavonoid content in crude extracts; *: correlations are significant at p < 0.05; **: correlations are significant at p < 0.01; ***: correlations are significant at p < 0.001*

Parameter	MTT assay		*TF*[a]	*TP*[b]
	24 h	72 h		
DPPH	–0.8031	–0.8387	0.8839*	0.9515**
FRAP	–0.9549**	–0.9486**	0.9623**	0.9038*
$NO^{\bullet}$	0.4338	0.5974	–0.6044	–0.3017
$OH^{\cdot}$	0.1122	0.0736	–0.0490	–0.0672
SOA	–0.9767***	–0.9951***	0.9789***	0.7105
TF	–0.9621**	–0.9939***	–	–
TP	–0.7568	–0.7546	–	–

[a]Total flavonoid content (*TF*) was expressed in mg quercetine equivalents/g extract dry weight (mg QE/g d.w.); [b]total phenol content (*TP*) was expressed as mg gallic acid equivalents/g extract dry weight (mg GAE/g d.w.)

Cluster analysis was done in order to classify extracts with different examined concentrations (33.3, 100, 300 and 900 µg/ml) on the basis of percentage of cytotoxic inhibition, depending on the treatments applied, *e.g.*, incubation time exposure (24h and 72h). In this study, we calculated Mahalanobis distance between samples, which were used for cluster analysis (Fig. 1). All samples were clustered into two major groups which separated LcEtOH 900 µg/ml (57% CI to 91% CI at both incubation times) from others which were divided in two main groups, (IIb – water extract except LcEtOH at 300 µg/ml , and IIa – ethanolic extract except LcAq at 33.3 µg/ml). In previous studies, *L. controversus* collected in Portugal was described in the literature as an ergosterol producer (58.6 mg/100 gf.w.) [12]. Therefore, it could be assumed that sterols and similar compounds (triterpenoids) may be a major class of active constituents contributing to *in vitro* cytotoxicity against cancer cells [9].

Correlation analysis between obtained IC_{50} values in antioxidant assays, and total phenolic and flavonoid content and cytotoxic activity are presented in Table 4. The highest significant positive correlation was observed for DPPH, FRAP and SOA assays with *TP* and *TF* contents. The observed correlations are in agreement with the data of Kalogeropoulos *et al.* [34], who reported that antioxidant activity of wild growing mushrooms correlated well with total phenolic acids and flavonoid content. SOA assay showed the strongest significant negative correlation with cytotoxic activity for both incubation times and FRAP assay.

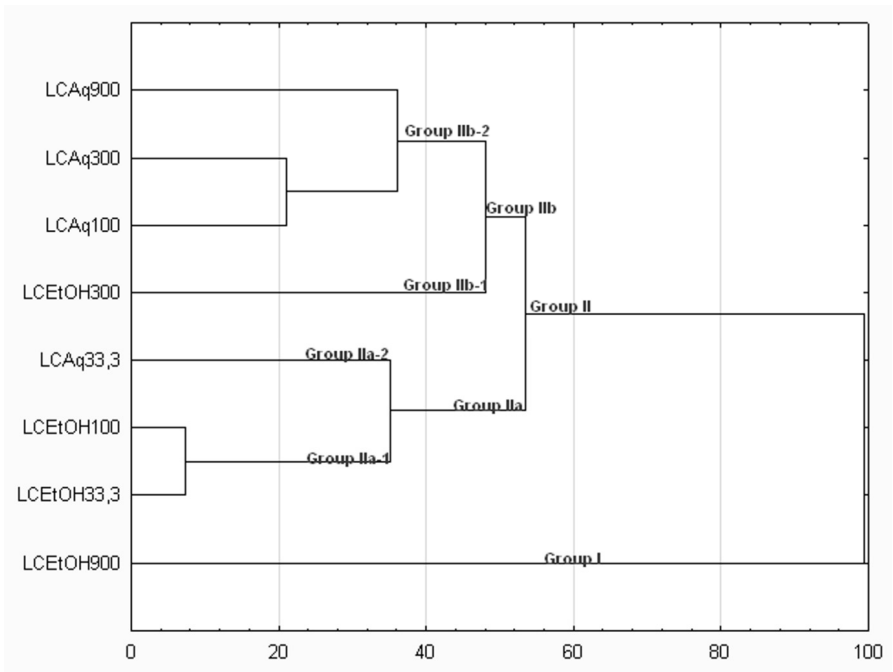

Figure 1. Diagram of cluster analysis based on cytotoxic activities.

MTT assay results significantly correlated negatively with TF, especially after 72h (–0.9939).These negative linear correlations proved that the highest antioxidant (lowest IC_{50}) activities showed higher cytotoxic effects.

CONCLUSION

In summary, quinic acid (8.9 µg/g d.w.) was identified and quantified by HPLC-MS/MS in methanol extract of *L. controversus* which confirms that this species is a potentially good source of nutraceuticals. Both ethanol and water extracts of *L. controversus* possessed antioxidant activity, with water extract being the most potent in DPPH and SOA assays and ethanol extract in FRAP assay. In antiproliferative assay both extracts showed activity against MCF 7 cell line, although ethanol extract was more potent. To our knowledge, this is the first report describing antioxidant, antibacterial activity, and growth inhibitory properties of *L. controversus*. Our results revealed that the ethanol and water extracts *L. controversus* showed anticancer, antioxidant, and low antibiotic capacities. Together, these activities indicate that these mushrooms are promising sources of bioactive compounds.

Acknowledgement

This paper is the result of research within the projects III 46001 and 172058, financed by the Ministry of Education, Science and Technological Development, Republic of Serbia. The authors would like to thank Prof. Goran Anačkov, PhD, for his valuable support in statistical analysis performance.

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IZVOD

JESTIVA MIKORIZNA VRSTA *Lactarius controversus* PERS. 1800 KAO IZVOR ANTIOKSIDATIVNIH I CITOTOKSIČNIH AGENASA

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Lactarius controversus Pers. 1800 je vrsta gljive sa veoma oštrim ukusom, ali se može koristiti u ljudskoj ishrani nakon kuvanja. Cilj ovog rada je bio da se prouči hemijski profil i antioksidativna i citotoksična aktivnost etanolnih i vodenih ekstrakata ove samonikle vrste sa lokacije iz istočne Srbije. Sadržaj ukupnih fenola i flavonoida je imao više vrednosti za etanolne ekstrakte (45,84 mg GAE/g d.w. i 3,50 mg QE/g d.w).Takođe je ispitana i antioksidativna aktivnost, uključujući aktivnost protiv $DPPH^{\bullet}$, $NO^{\bullet}$, $^{\bullet}OH$ i SOA radikala i sposobnost redukcije gvožđa. Najveću aktivnost protiv $DPPH^{\bullet}$ je imao vodeni ekstrakt (IC_{50} = 219,37 µg/ml) dok je etanolni ekstrakt demonstrirao najveću FRAP aktivnost od 10,93 mg AAE/g. Hemijska karakterizacija fenolnih jedinjenja izvedena pomoću HPLC–MS/MS je pokazala prisustvo hininske kiseline (8,9 µg/g d.w.). Heksanski ekstrakt primenjen u antibakterijskom testu duplog razblaženja, protiv tri patogena soja, je pokazao antibakterijsko dejstvo samo protiv *S. aureus* ATCC25922, postižući *MIC* i *MBC* od 3,12 i 6,25 mg/ml, redom. Antiproliferativno dejstvo na estrogen zavisnu MCF 7 ćelijsku liniju raka dojke određeno pomoću MTT testa je bilo jače za etanolne ekstrakte (166,42±3,1 µg/ml). Podaci dobijeni u ovi testovima ukazuju da je ova vrsta gljive obećavajući izvor bioaktivnih jedinjenja sa antioksidativnim i citotoksičnim dejstvom.

Ključne reči: *Lactarius controversus* • Fenolna jedinjenja • Hinska kiselina • Antioksidanti • Citotoksični agensi