International Journal of Medicinal Mushrooms, 22(4):347–357 (2020)

The Health Promoting Effects of the Fruiting Bodies Extract of the Peppery Milk Cap Mushroom *Lactarius piperatus* (Agaricomycetes) from Serbia

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ABSTRACT: Antioxidant, antimicrobial, genoprotective, anticancer, and neuroprotective potential of acetone extract of the fruiting bodies of the edible mushroom *Lactarius piperatus* was studied. The antioxidant activity was evaluated using different methods (DPPH radical scavenging, superoxide anion radicals scavenging, reducing power assay, and determination of total phenolic compounds). The microdilution method was used to reveal the antimicrobial potential. The genoprotective potential was determined by Comet assay. Cytotoxic activity was tested using MTT. The capacity of the extract to inhibit acetylcholinesterase was used for determining its neuroprotective potential. The received results show that *L. piperatus* extract possessed potent health enhancing effects. In the antioxidant activity, IC_{50} was 33.97 µg/mL for DPPH radicals scavenging and 22.52 µg/mL for superoxide anion radicals scavenging, whereas the absorbance for the reducing power was from 0.0510 to 0.1451. The total content of phenolic compounds in the extract was 5.08 µg PE/mg. The testing of the antimicrobial activity showed that MIC values were from 0.039 to 10 mg/mL. For Comet assay, all concentrations of extract increased the GDI values from 0.46 ± 0.05 to 0.99 ± 0.31. *L. piperatus* extract expressed relatively strong cytotoxic activity with IC_{50} values ranging from 37.83 to 65.94 µg/mL. Finally, the percentage of inhibition of acetylcholinesterase activity of tested extract was within the range 16.75–44.35%. Our results imply that the acetone extract of *L. piperatus* has rather strong antioxidant, antimicrobial, genoprotective, anticancer, and neuroprotective effects; thus this mushroom represents healthy food that could be used in the pharmaceutical industry and to prevent various diseases.

KEY WORDS: acetone extract, antimicrobial, antioxidant, cytotoxic, genoprotective, fruiting bodies, *Lactarius piperatus*, neuroprotective, medicinal mushrooms

ABBREVIATIONS: ABA, antibacterial activity; **AChE**, acetylcholinesterase; **AChI**, acetylcholine iodide; **AMA**, antimicrobial activity; **AMTA**, antimytagenic activity; **AOA**, antioxidant activity; **ATCC**, American Type Culture Collection; **CTA**, cytotoxic activity; **DMSO**, dimethyl sulfoxide; **DNA**, deoxyribonucleic acid; **DPPH**, 2,2-diphenyl-1-picryl-hydrazyl-hydrate; **DTNB**, 5,5′-dithiobis (2-nitrobenzoic acid); **EDTA**, ethylenediaminetetraacetic acid; **GDI**, genetic damage index; **GPA**, genoprotective activity; **IC_{50}**, concentration that reduces the effect by 50%; **MIC**, minimal inhibitory concentration; **MMC**, mitomycin C; **MTT test**, microculture tetrazolium test; **NADH**, nicotinamide adenine dinucleotide; **NBT**, nitroblue tetrazolium; **NCCLS**, National Committee for Clinical Laboratory Standards; **NPA**, neuroprotective activity; **PBS**, phosphate-buffered saline; **PE**, pyrocatechol equivalent; **PMS**, phenazine methosulfate

I. INTRODUCTION

Edible, wild mushrooms are collected and consumed around the world because of their remarkable taste and aroma. Mushrooms have higher protein content than most vegetables and are a rich source of vitamins, minerals, dietary fiber, and essential amino acids. The main carbohydrates that can be found in mushrooms are chitin, glycogen, trehalose, and mannitol. Mushrooms contain all the essential amino acids required by an adult. In addition to that, they are low in calories, fat, and cholesterol. The fats present in mushrooms are dominated by unsaturated fatty acids.[1] They are an excellent source of key dietary micronutrients including selenium, vitamin D, glutathione, and ergothioneine.[1,2]

Besides having important nutritional value, mushrooms also have beneficial effects on human health. Therefore, they are considered to be a functional food. Many species of mushrooms are a

considerable source of nutraceuticals, such as organic acids, terpenoids, alkaloids, steroids, and phenolic compounds.[3] They produce a wide range of secondary metabolites, which have high therapeutic values. Nowadays, various studies show that mushrooms have many important health benefits, including anticancer, immunomodulatory, anti-inflammatory, antidiabetic, antiviral, antioxidant, antimicrobial, and antineurodegenerative properties as well as antihypertensive and cholesterol-lowering properties.[3–5] Many cultures have used mushrooms both as food and as a medicine, but the utilization of mushrooms as a cure for different diseases is most prominent in Asian countries, where there is an abundance of evidence of the use of medicinal mushrooms. Modern medical studies combined with traditional medical knowledge have shown that it is possible to live healthily and to prevent many dangerous diseases with the right diet, and medicinal mushrooms may be the key to that. Hence, the consumption and usage of mushrooms in the Western Hemisphere started expanding over the past several decades.[6]

The peppery milk cap mushroom, *Lactarius piperatus* (L.) Pers. (Russulaceae, Agaricomycetes), is a well-known edible species, consumed widely across the world. Due to its acrid taste, it is usually used as a seasoning. It contains several marasmane sesquiterpenoids, as well as lactarorufin, furosardonin, blennin A and D, isolactarorufin, and also a natural liquid rubber latex.[6] Medicinal properties of *L. piperatus* have a wide range of application in traditional, alternative medicine. In traditional medicine, it is used in the treatment of urinary tract diseases and it is considered to have a positive effect on the regulation of urine formation. *L. piperatus* is also considered to have beneficial effects on joint and muscle relaxation.[7] There are several studies about its medicinal properties. Methanol extracts of *L. piperatus* have been investigated for its antimicrobial activity. Using agar disk diffusion assays, it was revealed that *L. piperatus* shows antimicrobial activity against *Escherichia coli, Proteus vulgaris*, and *Mycobacterium smegmatis*.[8] In another recent study,[9] *L. piperatus* was found to have high antibacterial and antifungal effects by using microdilution plate method. Similar to studies of its antimicrobial properties, several studies have found very strong antioxidant properties of *L. piperatus*.[10–12] A variety of biochemical assays were performed to screen antioxidant activity, including reducing power, 2,2-diphenyl-1-picrylhydrazyl radical, peroxide and H_2O_2 scavenging activity, inhibition of oxidative hemolysis in erythrocytes induced by 2,2'-azobis(2-amidinopropane) dihydrochloride, and inhibition of lipid peroxidation by β-carotene–linoleate system. The antioxidant compounds from *L. piperatus* can be used for different chronic diseases related to oxidative stress, such as cancer, Parkinson's disease, Alzheimer's disease, heart failure, myocardial infarction, schizophrenia, bipolar disorder, sickle cell disease, and chronic fatigue disease.[13]

Apart from antioxidant and antimicrobial properties, in this study we investigated genoprotective, anticancer, and neuroprotective potential of acetone extract from fruiting bodies of *L. piperatus.*

II. MATERIALS AND METHODS

A. Mushroom Material and Extraction

Mushroom samples of *L. piperatus* were collected in June 2018, in Kragujevac, Serbia. The taxonomic identification was done using standard literature.[14] Representative voucher specimens, issued with number 11, are deposited in facilities of the Department of Biology and Ecology, Faculty of Science University of Kragujevac (Serbia). Dried and then powdered basidiomes of *L. piperatus* were extracted in the Soxhlet extractor. Thereafter, the extract was filtered and concentrated under reduced pressure in the rotary evaporator. The dry extract was stored at −18°C prior to testing. For the experiments, the dry extract was dissolved in 5% dimethyl sulfoxide (DMSO), which was dissolved in sterile distilled water to the desired concentration.[3]

B. Antioxidant Activity

1. Scavenging DPPH Radicals

Previously reported modified DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical scavenging method was used to study antioxidant activity of mushroom extract.[15] Two milliliters of methanol solution of DPPH radical in the concentration of 0.05 mg/mL and 1 mL of mushroom extract (1000, 500, 250, 125, and 62.5 μg/mL) were placed in cuvettes. The mixture was shaken vigorously and left at room temperature for 30 min. Then the absorbance was measured at 517 nm in the spectrophotometer (Bibby Scientific Limited, Stone, UK). Ascorbic acid was used as positive control. The DPPH radical concentration was calculated using the following equation:

$$\text{DPPH scavenging ability } (\%) = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100 \quad (1)$$

where A_0 is the absorbance of the negative control and A_1 is the absorbance of the reaction mixture or standard. All the measurements were repeated three times, and the results are presented as the mean ± SD. The inhibition concentration at 50% inhibition (IC_{50}) was the parameter used to compare the radical scavenging activity. A lower IC_{50} meant better radical scavenging activity.

2. Superoxide Anion Radical Scavenging Activity

The superoxide anion radical scavenging activity of samples was detected according to the method of Nishikimi et al.[16] First, 0.1 mL of test samples (1000, 500, 250, 125, and 62.5 μg/mL) was mixed with 1 mL nitroblue tetrazolium (NBT) solution (156 μM in 0.1 M phosphate buffer, pH 7.4) and 1 mL nicotinamide adenine dinucleotide (NADH) solution (468 μM in 0.1 M phosphate buffer, pH 7.4). The reaction was started by adding 100 μl of phenazine methosulfate (PMS) solution (60 μM in 0.1 M phosphate buffer, pH 7.4). The mixture was incubated at room temperature for 5 min, and the absorbance was measured at 560 nm in the spectrophotometer (Bibby Scientific Limited, Stone, UK) against blank sample (phosphate buffer). Ascorbic acid was used as positive control. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$\text{Superoxide anion scavenging ability } (\%) = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100 \quad (2)$$

where A_0 is the absorbance of the negative control (consisting of all the reaction agents, except the extract) and A_1 is the absorbance of the reaction mixture or standard.

For both extracts and ascorbic acid, the inhibitory concentration (IC_{50}) at 50% was determined.

3. Reducing Power

The reducing power of extract was determined according to the method of Oyaizu.[17] First, 1 mL of extract (2000, 1000, 500, and 250 μg/mL) was mixed with 2.5 mL of phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [K_3-$Fe(CN)_6$] (2.5 mL, 1%). The mixtures were incubated at 50ºC for 20 min. Then,

trichloroacetic acid (10%, 2.5 mL) was added to the mixture and centrifuged. Finally, the upper layer was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL; 0.1% mL). The absorbance of the solution was measured at 700 nm in the spectrophotometer (Bibby Scientific Limited, Stone, UK). Ascorbic acid was used as positive control. Higher absorbance of the reaction mixture indicated that the reducing power is increased.

4. Determination of Total Phenolic Compounds

Total soluble phenolic compounds in the mushroom extract were determined with Folin–Ciocalteu reagent according to the method of Slinkard and Singleton,[18] using pyrocatechol as a standard phenolic compound. Briefly, 1 mL of the mushroom extract (1 mg/mL) was diluted with distilled water in a volumetric flask (46 mL). One milliliter of Folin–Ciocalteu reagent was added and the content of the flask was mixed thoroughly. After 3 min, 3 mL of sodium carbonate (2%) was added and then left to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm in the spectrophotometer (Bibby Scientific Limited, Stone, UK). The total concentration of phenolic compounds in the extract was determined as microgram of pyrocatechol equivalent (PE) per milligram of dry extract.

C. Antimicrobial Activity

The antimicrobial activity (AMA) of *L. piperatus* extract was tested against Gram-positive (*Staphylococcus aureus*, ATCC 25923; *Bacillus subtilis*, ATCC 6633; and *B. cereus*, ATCC 10987) and Gram-negative (*Escherichia coli*, ATCC 25922; *Proteus mirabilis*, ATCC 12453) bacteria as well as five test microfungi *Aspergillus niger* (ATCC 16888), *Candida albicans* (ATCC 10259), *Penicillium italicum* (ATCC 10454), *Mucor mucedo* (ATCC 20094), and *Trichoderma viride* (ATCC 13233).

Bacterial isolates were picked from overnight cultures in Mueller–Hinton agar and suspensions were prepared in sterile distilled water by adjusting the turbidity to match 0.5 McFarland standards to approximately 10^8 colony-forming units/mL. Fungal suspensions were prepared from 3- to 7-day-old cultures of fungi that grew on potato dextrose agar, except for *Candida albicans*, which was maintained on Sabouraud dextrose agar. The spores were suspended in sterile distilled water, the turbidity was determined spectrophotometrically at 530 nm, and then further diluted to approximately 10^6 colony-forming units/mL, according to the procedure recommended by NCCLS.[19] The 96-well microtiter plate assay, using resazurin as the indicator of cell growth,[20] was applied for the determination of the MIC of the active extract. DMSO, which shows no effect on test microbes, was used as a negative control.

D. Genoprotective Activity

The genoprotective activity (GPA) or antimytagenic activity (AMTA) was tested by single-cell gel electrophoresis (Comet assay). Lymphocyte suspension from three healthy donors was incubated for 30 min at 37ºC in phosphate-buffered saline (PBS) solution with different concentrations of the extract (50, 100, 150, and 200 μg/mL) separately and in treatment against known mutagens hydrogen peroxide (H_2O_2, final concentration of 10 μg/mL) or mitomycin C (MMC, final concentration of 0.5 μg/mL). The highest concentration was tested for cytotoxic activity and compared with the negative control (untreated cells) by trypan blue dye exclusion assay. After the incubation, cell suspension was mixed with 1% low melting point agarose (Sigma, St. Louis, MO) and spread onto the microscopic slides precoated with 1% normal melting point agarose. The slides were put in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, and 10% DMSO, pH 10) at 4ºC for 2 hrs. Next, they were immersed in fresh electrophoresis buffer (10 M NaOH, 200 mM EDTA, pH > 13) to allow the DNA to unwind (25 V, 300 mA, 30 min). The slides were neutralized by washing three times with Tris-HCl buffer (0.4 M,

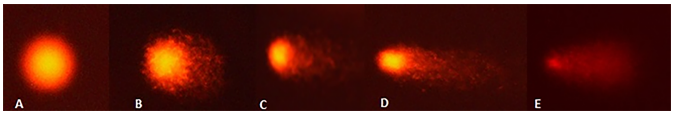

FIG. 1: Different degrees of DNA damage in PBLs: class A – undamaged cells with no tail; class B – low level damage; class C – medium level damage; class D – high level damage and class E – total destruction

pH 7.5) and stained with ethidium bromide.[21] Cells were analyzed using fluorescent microscope (Nikon E50i) and classified into five categories (A–E) depending on the degree of DNA damage: A – undamaged cells with no tail (< 5% damaged DNA); B – low-level damage (5%–20%); C – medium level damage (20–40%); D – high-level damage (4–95%), and E – total destruction (> 95%) (Fig. 1). A total of 300 cells per concentration were analyzed (100 per donor in three independent experiments). The Genetic Damage Index (GDI) was visually determined, based on the size and intensity of the comet tails, using the following formula:

$$GDI = B + 2 \times C + 3 \times D + 4 \times E / A + B + C + D + E.^{22} \quad (3)$$

E. Cytotoxic Activity

Human epithelial carcinoma HeLa cells, human colon carcinoma LS174 cells, human lung carcinoma A549 cells, and normal MRC5 human embryonic lung fibroblast cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cancer cell lines were cultured as a monolayer in the RPMI 1640 nutrient medium, with 10% (inactivated at 56ºC) fetal bovine serum, 3 mM of L-glutamine, and antibiotics, at 37ºC in humidified air atmosphere with 5% CO_2. The cytotoxic activity (CTA) on cancer cells survival was determined 72 hr after the addition of mushroom extract, by the MTT test (microculture tetrazolium test).[23] First, 20 µl of MTT solution (5 mg/mL PBS) was added to each well and further incubated for 4 hr, at 37°C, in 5% CO_2 and humidified air. Subsequently, 100 µl of sodium dodecyl sulfate (10%) was added to solubilize the formazan crystals formed from MTT after the conversion by mitochondrial dehydrogenases of viable cells. Absorbencies, proportional to the number of viable cells, were measured using a microplate reader (Multiskan EX, Thermo Scientific, Finland) at 570 nm. Each experiment was performed in triplicate and independently repeated at least four times.

F. Neuroprotective Activity

The capacity of the extract to inhibit acetylcholinesterase (AChE) was used for testing its neuroprotective activity (NPA). The rate of inhibition of acetylcholinesterase activity was determined spectrophotometrically using 96-well microtiter plates.[24] Acetylcholine iodide (AChI) was used as substrate for the enzyme AChE, which degrades this compound to acetate and thiocholine. In the next step, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) was transformed with thiocholine to the yellow colored 5-thio-2-nitrobenzoate anion and the change in absorbance was recorded at 412 nm wavelength, 25°C. The reaction mixture contained 140.0 µl of 0.1 mM sodium phosphate buffer (pH 8.0), 20.0 µl of DTNB, 20.0 µl of extract (1000, 500, 250, and 125 µg/mL), 20.0 µl of AChE, and 10.0 µl of AChI. A mixture of 5% (v/v) DMSO and sodium phosphate buffer (pH 8.0) was used as a blank. The rate of inhibition of AChE activity was determined according to the formula:

$$\text{Rate of inhibition of AChE } (\%) = \left[\frac{(E - S)}{E}\right] \times 100 \quad (4)$$

where E is the enzyme activity without the extract and S is the enzyme activity with the extract.

The obtained values were compared with the commercial inhibitor of AChE, i.e., galantamine.

G. Statistical Analysis

The statistical analysis was evaluated by one-way analysis of variance (ANOVA) assay, with Tukey's *post hoc* test for the comparison of different treatments versus the respective controls. The relationship between the tested concentrations of extract and GDI values was determined by Pearson correlation coefficient. All values were expressed as mean ± SD. A difference at $p < 0.05$ was considered statistically significant.

III. RESULTS AND DISCUSSION

A. Antioxidant Activity

The results of the DPPH radical scavenging assay, reducing power assay, and superoxide anion radical scavenging activity of the studied extract are presented in Table 1. The IC_{50} values were 33.97 µg/mL and 25.52 µg/mL for DPPH assay and superoxide anion radicals scavenging activity, respectively. The reducing power was concentration-dependent with absorbance values from 0.0510 to 0.1451. The total phenolic content in the extract was 5.08 µg PE/mg (Table 1). The obtained data of antioxidant activity (AOA) of acetone extract revealed difference between the AOA of acetone extract and the control ($p < 0.05$).

The results showed that the acetone extract of *L. piperatus* possesses very strong DPPH radical and superoxide anion radical scavenging activities with IC_{50} values 33.97 and 25.52 µg/mL, while the reducing power was less pronounced with values of absorbance 0.1451, 0.0934, 0.0773, and 0.0510 at concentrations of 2000, 1000, 500, and 250 µg/mL. These activities of the tested extract were based on its ability to take part in formation of the nonradical form of DPPH-H, as well as on its ability to destroy superoxide radicals produced in the PMS/NADH reaction, which is a weak oxidative agent, but induces the formation of highly reactive free radicals.

There are several studies on the AOA of *L. piperatus*,[10–12] but the extraction was done using methanol as a solvent. In this study, we used acetone as a solvent, to confirm the AOA of this fungus. The use of different solvents in extraction, because of their polarity, might lead to the extraction of various compounds, which

TABLE 1: Antioxidative Potential of *Lactarius piperatus* Acetone Extract and Commercial Antioxidant, Ascorbic Acid

Antioxidant	DPPH• scavenging activity [IC50 (µg/mL)]	Superoxide anion scavenging activity [IC50 (µg/mL)]	Reducing power absorbance 700 nm			
			2000 µg/mL	1000 µg/mL	500 µg/mL	250 µg/mL
Lactarius piperatus extract	33.97 ± 1.22	25.52 ± 1.03	0.1451 ± .037	0.0934 ± .005	0.0773 ± .004	0.0510 ± .003
Ascorbic acid	6.42 ± 0.18	115.21 ± 1.49	2.113 ± .032	1.654 ± .021	0.0957 ± .008	0.0478 ± .004

Ascorbic acid was used as the standard.
Values are expressed as mean ± SD of three parallel measurements.

may synergistically interact and thus the AOA of the mushroom extract might be even more prominent. Several researchers found that acetone is a very effective solvent for the extraction of bioactive secondary metabolites.[25–27] Therefore, in this study acetone was used as a solvent for extracting bioactive compounds from *L. piperatus* and by the obtained results we confirmed its efficiency as a solvent.

B. Antimicrobial Activity

The AMA of the mushroom extract against the tested microorganisms is shown in Table 2. The results showed that the extract of *L. piperatus* affected all of the test microbes (Table 2). The MIC values varied from 0.039 to 0.156 mg/mL for bacteria and from 2.5 to 10 mg/mL for fungi. The most susceptible microorganism was *Staphylococcus aureus* (MIC = 0.039 mg/mL). The AMA of the mushroom extract was compared with standard antibiotics, streptomycin (for bacteria) and ketoconazole (for fungi). The results showed that *L. piperatus* had similar antibacterial activity (ABA) as streptomycin, while ketoconazole was more active than the tested mushroom extract.

The probable mechanisms of AMA are inhibition of cell wall, protein synthesis, or nucleic acid synthesis, similar to antibiotics. The level of AMA activity of *L. piperatus* depends on test microbes and the concentration of mushroom extract. ABA of *L. piperatus* was observed against both Gram-positive and Gram-negative bacteria. Among these the Gram-negative bacteria were more resistant due to their cell wall structure. Test fungi were more resistant to *L. piperatus* extract than bacteria due to more complex structure of fungal cell wall.[3,28] Strong AMA of *L. piperatus* was reported previously.[10–12]

C. Genoprotective Activity

Detection of GPA or AMTA of *L. piperatus* extract was evaluated using comet assay in PBLs *in vitro*. The advantages this assay has are the requirement of small number of cells, high sensitivity, and uncomplicated application. The most reliable parameter is the percentage of DNA in the comet tail (% GDI). This parameter is widely used because it is linear. The tested concentrations of *L. piperatus* extract increased the GDI values ($0.46 \pm 0.05 – 0.99 \pm 0.31$) compared with negative control (0.16 ± 0.04; $p > 0.05$) (Table 3 and Fig. 1). Pearson correlation coefficient demonstrated that *L. piperatus* extract increased GDI values in a dose-dependent manner ($r = 0.810$, $p < 0.005$). In cotreatment against H_2O_2, the extract significantly decreased the GDI values in all tested concentrations (from 2.52 ± 0.19 to 1.17 ± 0.42; $p < 0.05$) in comparison to the positive control (3.45 ± 0.10). In cotreatment against MMC, the extract significantly decreased

TABLE 2: Minimum Inhibitory Concentration (MIC) of Acetone Extract of *Lactarius piperatus*

Bacteria and microfungi	*Lactarius piperatus* extract	Streptomycin	Ketoconazole
		MIC (mg/mL)	
Bacillus cereus	0.078	0.031	—
Bacillus subtilis	0.156	0.016	—
Escherichia coli	0.078	0.016	—
Proteus mirabilis	0.156	0.062	—
Staphylococcus aureus	0.039	0.062	—
Aspergillus niger	10	—	0.078
Candida albicans	2.5	—	0.039
Mucor mucedo	10	—	0.156
Penicillium italicum	10	—	0.156
Trichoderma viride	5	—	0.078

TABLE 3: Genotoxic and Antimutagenic Activities of *Lactarius piperatus* Acetone Extract against H_2O_2 and MMC in Cultured Human Lymphocytes

Treatment (μg/mL)	No. of analyzed cells	Class of damage degree in cells					GDI (mean ± SD)
		0	1	2	3	4	
Negative control	300	86.66	10.67	2.67	—	—	0.16 ± 0.04
50 μg/mL of *L. piperatus* extract	300	68.00	20.00	9.67	2.33	—	0.46 ± 0.05
100 μg/mL of *L. piperatus* extract	300	61.33	22.67	11.00	5.00	—	0.60 ± 0.14
150 μg/mL of *L. piperatus* extract	300	50.00	22.33	17.67	9.00	1.00	0.89 ± 0.09
200 μg/mL of *L. piperatus* extract	300	55.00	11.67	17.00	12.00	4.33	0.99 ± 0.31
Positive control – H_2O_2 (10 μg/mL)	300	—	—	7.00	41.33	51.67	3.45 ± 0.10
50 μg/mL of *L. piperatus* extract + H_2O_2	300	8.00	14.33	21.33	30.00	26.33	2.52 ± 0.19*
100 μg/mL of *L. piperatus* extract + H_2O_2	300	11.67	14.00	25.00	29.67	19.67	2.32 ± 0.48*
150 μg/mL of *L. piperatus* extract + H_2O_2	300	34.00	22.00	21.33	16.33	6.33	1.39 ± 0.61*
200 μg/mL of *L. piperatus* extract + H_2O_2	300	44.33	21.33	13.00	15.66	5.67	1.17 ± 0.42*
Positive control – MMC (5 μg/mL)	300	2.33	13.67	16.67	35.67	31.66	2.81 ± 0.35
50 μg/mL of *L. piperatus* extract + MMC	300	13.00	9.33	19.00	37.33	21.33	2.45 ± 0.08
100 μg/mL of *L. piperatus* extract + MMC	300	32.00	18.67	30.00	23.67	5.67	1.52 ± 0.13*
150 μg/mL of *L. piperatus* extract + MMC	300	56.67	13.33	14.00	10.00	7.00	0.99 ± 0.35*
200 μg/mL of *L. piperatus* extract + MMC	300	61.67	17.00	12.33	8.33	0.67	0.70 ± 0.09*

*Statistically significant difference in comparison to positive control cells (ANOVA, *$p < 0.05$).

GDI values (from 1.52 ± 0.13 to 0.70 ± 0.09) in higher concentrations (100, 150, and 200 μg/mL; $p < 0.05$) compared with the control (2.81 ± 0.35). Correlation between extract concentrations and GDI values was negative ($r = -0.811$, $p < 0.0005$ for H_2O_2 and $r = -0.945$, $p < 0.0005$ for MMC).

To our knowledge, no studies exist regarding the GPA or AMTA of *L. piperatus* on PBLs. In this study, tested concentrations of the extract did not show genotoxic activity, i.e., the number of undamaged cells with no tail was just slightly reduced compared with the negative control. Our results agree with other studies on different mushroom species,[29–31] such as *Agaricus brasiliensis*. On the other hand, in treatment against known mutagens, tested concentrations of extract showed AMTA. The number of undamaged cells with no tail increased compared with the positive controls (H_2O_2 or MMC alone). It was revealed that methanolic extract of *Lactarius vellereus* possesses GPA at the highest concentration,[32] i.e., caused 70% reduction in DNA damage induced by mutagen 2-amino-3-methylimidazo(4,5-f) quinoline in human hepatoma HepG2 cells, while ethanol extract of *Inonotus obliquus* acted protectively against oxidative damage induced by H_2O_2 in PBLs.[33]

D. Cytotoxic Activity

The obtained data for CTA of *L. piperatus* showed that the IC_{50} against HeLa, LS174, and A549 cell lines was 37.83, 65.94, and 48.15 μg/mL, respectively (Table 4). Tested mushroom extract possesses weaker activity compared with *cis*-DDP as a positive control. *L. piperatus* extract expressed relatively strong CTA on the used cancer cells, among which HeLa cells were the most sensitive. Data concerning the anticancer capacity of the tested mushroom is very scarce. Before our research, it was found that hot water extract of *L. piperatus* inhibits Lewis pulmonary adenoma in white mice, with an inhibition rate of 80% against sarcoma 180, and 70% against Ehrlich carcinoma.[34]

TABLE 4: Cytotoxic Effect of *Lactarius piperatus* Acetone Extract on Tested Cell Lines Expressed by IC_{50} (μg/mL)

Treatment	Cell lines			
	HeLa	LS174	A549	MRC-5
Lactarius piperatus extract	37.83 ± 0.46	65.94 ± 0.37	48.15 ± 1.22	< 200
cis-DDP	2.36 ± 0.28	20.38 ± 0.44	17.93 ± 0.88	10.52 ± 0.22

IC_{50} values are expressed as the mean ± SD determined from the results of MTT assay in two independent experiments.

TABLE 5: Neuroprotective Activity of *Lactarius piperatus* Acetone Extract

AChE inhibitors	Concentration (μg/mL)	Level of AChE inhibition (%)
Lactarius piperatus extract	1000	44.35 ± 0.82
	500	38.43 ± 0.77
	250	27.66 ± 0.81
	125	16.75 ± 0.68
Galantamine	1000	92.23 ± 0.56
	500	87.08 ± 0.51
	250	74.31 ± 0.44
	125	68.12 ± 0.32

In our study, the cytotoxic effect of *L. piperatus* on normal MRC5 human embryonic lung fibroblast cells was not detected, so it is important to emphasize that this mushroom has acted selectively when it comes to cytotoxic potential. Further research will be necessary to identify compounds responsible for the observed CTA.

E. Neuroprotective Activity

In spite of the fact that the etiology of neurodegenerative disorders, primarily Alzheimer's disease, has not been fully explained, it is known that at the base of these diseases is a decreased level of acetylcholine, i.e., dopamine. Enzyme AChE hydrolyses the neurotransmitter acetylcholine, thereby stopping the synaptic transmission. Therefore, AChE inhibitors are considered to be the most effective agents in the treatment of these disorders; by reducing the activity of this enzyme, they help restore the level of acetylcholine in cholinergic synapses. Since synthetic inhibitors of AChE are expensive and have different side effects more attention is paid to finding natural alternative sources.

As shown in Table 5, concentration-dependent inhibition values of AChE activity of tested extract of *L. piperatus* were 44.35%, 38.43%, 27.66%, and 16.75%.

Previous research has shown that the extracts of *L. piperatus* can contain compounds that inhibit AChE activity.[35–37] This activity is attributed to terpenoids and alkaloids that inactivate AChE by binding for the active center or peripheral binding sites.[35,38] In comparison with AChE activity inhibition rate in other mushrooms, *L. piperatus* extract can be considered as a good neuroprotective agent.

IV. CONCLUSION

This investigation proves that the studied edible mushroom *L. piperatus* possesses relatively strong antioxidant, antimicrobial, genoprotective, anticancer, and neuroprotective activities. Since this species has a

large distribution, its consumption is highly recommended to prevent various diseases. Furthermore, it is crucial to conduct more detailed studies to identify the active constituents of this mushroom, to enable its application in the pharmaceutical industry and medicine.

ACKNOWLEDGMENT

This study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant Nos. III41010, 173032, 175011).

REFERENCES

1. Kalač P. A review of chemical composition and nutritional value of wild-growing and cultivated mushrooms. J Sci Food Agric. 2013;93(2):209–18.
2. Kalaras MD, Richie JP, Calcagnotto A, Beelman RB. Mushrooms: A rich source of the antioxidants ergothioneine and glutathione. Food Chem. 2017;233:429–33.
3. Kosanić M, Ranković B, Rančić A, Stanojković T. Evaluation of metal concentration and antioxidant, antimicrobial, and anticancer potentials of two edible mushrooms Lactarius deliciosus and Macrolepiota procera. J Food Drug Anal. 2016;24(3):477–84.
4. Yin C, Fan X, Liu C, Fan Z, Shi D, Yao F, Cheng W, Gao H. The antioxidant properties, tyrosinase and α-glucosidase inhibitory activities of phenolic compounds in different extracts from the golden oyster mushroom, Pleurotus citrinopileatus (Agaricomycetes). Int J Med Mushrooms. 2019;21:865–74.
5. Shomali N, Onar O, Karaca B, Demirtas N, Cihan AC, Akata I, Yildirim O. Antioxidant, anticancer, antimicrobial, and antibiofilm properties of the culinary-medicinal fairy ring mushroom, Marasmius oreades (Agaricomycetes). Int J Med Mushrooms. 2019;21(6):571–82.
6. Rogers R. The fungal pharmacy: The complete guide to medicinal mushrooms and lichens of North America. Berkeley, CA: North Atlantic Books; 2012.
7. Zeitlmayr L, Caspari C, Stangl J. Knaurs pilzbuch. München (Germany): Droemer Knaur; 1976.
8. Dulger B, Yilmaz F, Gucin F. Antimicrobial activity of some Lactarius species. Pharm Biol. 2002;40(4):304–6.
9. Kosanic M, Rankovic B, Dasic M. Antioxidant and antimicrobial properties of mushrooms. Bulgarian J Agric Sci. 2013;19(5):1040–6.
10. Barros L, Baptista P, Ferreira IC. Effect of Lactarius piperatus fruiting body maturity stage on antioxidant activity measured by several biochemical assays. Food Chem Toxicol. 2007;45:1731–7.
11. Keles A, Koca I, Genccelep H. Antioxidant properties of wild edible mushrooms. J Food Process Technol. 2011;2:130–5.
12. Ozen T, Darcan C, Aktop O, Turkekul I. Screening of antioxidant, antimicrobial activities and chemical contents of edible mushrooms wildly grown in the Black Sea region of Turkey. Comb Chem High Throughput Screen. 2011;14:72–84.
13. Joshi S, Vishwakarma MP, Mahar R, Bhatt RP. Medicinally important and edible species of genus Lactarius from Garhwal Himalaya, India. Mycosphere. 2013;4(4):714–20.
14. Hagara, L. Ottova encyklopédia hùb. Prague (Czech Republic): Europrint; 2014.
15. Kosanić M, Ranković B, Rančić A, Stanojković T. Evaluation of metal contents and bioactivity of two edible mushrooms Agaricus campestris and Boletus edulis. Emir J Food Agric. 2017;29:98–103.
16. Nishikimi M, Rao NA, Yagi K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem Biophys Res Commun. 1972;46(2):849–54.
17. Oyaizu M. Studies on products of browning reaction. J Hum Nutr Diet. 1986;44(6):307–15.
18. Slinkard K, Singleton VL. Total phenol analysis: Automation and comparison with manual methods. Am J Enol Vitic. 1977;28(1):49–55.
19. NCCLS (National Committee for Clinical Laboratory Standards). Reference method for broth dilution antifungal susceptibility testing of conidium-forming filamentous fungi, Proposed Standard M38-P. NCCLS: Wayne, PA; 1998.
20. Sarker SD, Nahar L, Kumarasamy Y. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. Methods. 2007;42:321–4.
21. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res. 1988;175:184–91.
22. Collins AR. The comet assay for DNA damage and repair: Principles, applications, and limitations. Mol Biotechnol. 2004;26:249–61.

23. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65:55–63.
24. Ellman GL, Courtney KD, Andres V, Featherstone RM. A new and rapid colourimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961;7:88–95.
25. Kamiyama M, Horiuchi M, Umano K, Kondo K, Otsuka Y, Shibamoto T. Antioxidant/anti-inflammatory activities and chemical composition of extracts from the mushroom Trametes versicolor. Int J Nutr Food Sci. 2013;2:85–91.
26. Kosanić M, Ranković B, Stanojković T, Radović-Jakovljević M, Ćirić A, Grujičić D, Milošević-Djordjević O. Craterellus cornucopioides edible mushroom as source of biologically active compounds. Nat Prod Commun. 2019;14(5). doi: 10.1177/1934578X19843610.
27. Ukaegbu CI, Shah SR, Hazrulrizawati AH, Alara OR. Acetone extract of Flammulina velutipes caps: A promising source of antioxidant and anticancer agents. Beni-Suef Univ J Basic Appl Sci. 2018;7:675–82.
28. Nowacka N, Nowak R, Drozd M, Olech M, Los R, Malm A. Antibacterial, antiradical potential and phenolic compounds of thirty-one Polish mushrooms. PLoS One. 2015;10:e0140355.
29. Gameiro PH, Nascimento JS, Rocha BH, Piana CF, Santos RA, Takahashi CS. Antimutagenic effect of aqueous extract from Agaricus brasiliensis on culture of human lymphocytes. J Med Food. 2013;16:180–3.
30. Radaković M, Djelić N, Stevanović J, Soković M, Radović D, Van Griensven LJ, Stanimirović Z. Evaluation of the antigenotoxic effects of the Royal Sun mushroom, Agaricus brasiliensis (higher Basidiomycetes) in human lymphocytes treated with thymol in the comet assay. Int J Med Mushrooms. 2015;17:321–30.
31. Živković L, Borozan S, Čabarkapa A, Topalović D, Ciptasari U, Bajić V, Spremo-Potparević B. Antigenotoxic properties of Agaricus blazei against hydrogen peroxide in human peripheral blood cells. Oxid Med Cell Longev. 2017;2017:8759764.
32. Mlinaric A, Kac J, Fatur T, Filipic M. Anti-genotoxic activity of the mushroom Lactarius vellereus extract in bacteria and in mammalian cells in vitro. Pharmazie. 2004;59:217–21.
33. Park E, Jeon KI, Byun BH. Ethanol extract of Inonotus obliquus shows antigenotoxic effect on hydrogen peroxide induced DNA damage in human lymphocytes. Cancer Prev Res. 2005;10:54–9.
34. Jayko LG, Baker TI, Stubblefield RD, Anderson RF. Nutrition and metabolic products of Lactarius species. Can J Microbiol. 1974;8:361–71.
35. Patocka J. Natural cholinesterase inhibitors from mushrooms. Military Med Sci Lett. 2012;81:40–4.
36. Janjušević Lj, Karaman M, Šibul F, Tommonaro G, Iodice C, Jakovljević D, Pejin B. The lignicolous fungus Trametes versicolor (L.) Lloyd (1920): A promising natural source of antiradical and AChE inhibitory agents. J Enzyme Inhib Med Chem. 2017;32:355–62.
37. Ćilerdzić J, Galić M, Vukojević J, Stajić M. Pleurotus ostreatus and Laetiporus sulphureus (Agaricomycetes): Possible agents against Alzheimer and Parkinson diseases. Int J Med Mushrooms. 2019;21:275–89.
38. Cör D, Knez Ž, Hrnčič, MK. Antitumour, antimicrobial, antioxidant and antiacetylcholinesterase effect of Ganoderma lucidum terpenoids and polysaccharides: A review. Molecules. 2018;23:649.