https://doi.org/10.31925/farmacia.2021.5.22 **ORIGINAL ARTICLE**

EVALUATION OF BIOLOGICAL ACTIVITIES OF ACETONE EXTRACT OF THE MUSHROOM *LECCINUM SCABRUM*

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Abstract

Bioactivity of acetone extract of *Leccinum scabrum* was evaluated. Antioxidant activity was evaluated by free radical scavenging activity, reducing power and determination of total phenolic content. The microdilution plate method was used to detect antimicrobial activity. Cytotoxic activity was tested by MTT assay. Genotoxic potential was evaluated by comet assay. Results showed a stronger free radical scavenging activity, but the reducing power was less pronounced. The total content of phenols in the extract was 14.91 µg PE/mg. The minimum inhibitory concentration related to the tested microorganisms fluctuated in the range 0.039 - 10 mg/mL. The most sensitive was *Bacillus cereus*. A strong cytotoxic activity was revealed with IC_{50} values ranging between 73.64 and 200.00 µg/mL, among which HeLa was the most sensitive. Results showed that the extract was not genotoxic and it decreased the H_2O_2-induced GDI values in comparison to the positive control. *L. scabrum* possesses bioactivities such as antioxidant, antimicrobial, cytotoxic, no genotoxic and protective against H_2O_2, and in the highest tested concentration it showed a synergistic activity.

Rezumat

Studiul prezintă evaluarea activității biologice a extractului în acetonă obținut din *Leccinum scabrum*. Activitatea antioxidantă a fost evaluată prin determinarea capacității de chelatare a radicalilor liberi, a capacității reducătoare și a conținutului total în fenoli. Activitatea antimicrobiană a fost testată prin metoda microdiluției, activitatea citotoxică prin metoda MTT, iar potențialul genotoxic a fost determinat prin calcularea indexului de deteriorare genetică. Conținutul total de fenoli a fost 14,91 µg PE/mg. Concentrația minimă inhibitorie a variat în intervalul 0,039 - 10 mg/mL. Activitatea citotoxică pronunțată a fost corelată cu valorile IC_{50} în domeniul 73,64 - 200,00 µg/mL. Rezultatele au arătat că extractul nu este genotoxic și au scăzut valorile indexului de deteriorare genetică-indus de H_2O_2, în comparație cu controlul pozitiv. *L. scabrum* posedă activități antioxidantă, antimicrobiană, citotoxică și protectoare împotriva H_2O_2.

Keywords: biological activities, mushroom *Leccinum scabrum*, acetone extract

Introduction

Edible mushrooms are nutritionally referred as functional food because of their beneficial components, including carbohydrates, proteins, vitamins and minerals, low levels of calories, fat and toxic metals [1, 2]. Moreover, their primary (e.g., polysaccharides and glycoproteins) and secondary (e.g., phenolic compounds) bioactive compounds have remarkable medicinal functions such as antiviral, anti-inflammatory, immunoregulatory, antioxidant and antitumor activities [3-7]. *Leccinum scabrum*, also known as "birch boletus" is an edible mushroom belonging to the *Boletaceae* family [8]. This species is a popular, wild-grown mushroom, used traditionally as gourmet in Scandinavia and in Central and Eastern Europe [1]. *L. scabrum* is a good source of minerals (Mg, K, Na, Ca, Zn) and phenolic acids (syringic and gallic) [9]. The study by Gąsecka *et al*. [10], based on chromatographic analysis revealed that *L. scabrum* is rich of flavonoids (catechin, kaempferol, apigenin and rutin) and phenolic acids (*t*-cynnamic, protocatechuic, vanillic, 2,5-dihydroxybenzoic, 4-hydroxybenzoic, syringic, salicylic, caffeic, chlorogenic and ferulic acids), as well as vitamins (ascorbic acid and ergosterol). The results of this study showed that the analysed *L. scabrum* is a potential source of some health-promoting substances. Studies have confirmed that *L. scabrum* possesses antioxidant [10-12], antiulcer and cytotoxic activities [9].

Available literature contains very few data about the biological activities of this species. Thus, the aim of this study was to determine the biological (antioxidant, antimicrobial, cytotoxic and genotoxic) activities of the acetone extract from the mushroom *L. scabrum*.

Materials and Methods

Fungal materials

Fungal samples of *L. scabrum* (Bull.) Gray were collected from Priboj, Serbia, in September 2017. The voucher sample is preserved in the facilities of the Department of Biology and Ecology of Kragujevac, Serbia, Faculty of Science. The determination of mushrooms was performed using standard methods [8].

Extraction

Dry finely ground thalli of the examined mushrooms were extracted using in a Soxhlet apparatus. The extract was filtered and then concentrated under reduced pressure in a rotary evaporator. The dry extract was stored at -18ºC until it was used in the tests. The extract was dissolved in phosphate buffer saline (PBS: NaCl, KH_2PO_4, Na_2HPO_4 x 2 H_2O, KCl) for the experiments.

Antioxidant activity

The antioxidant activity was evaluated by free radical (DPPH and superoxide anion radical) scavenging and reducing power. The free radical scavenging activity of the mushroom extract was measured by 1,1-di-phenyl-2-picryl-hydrazil (DPPH) technique according to the Dorman *et al.*'s method [13]. The superoxide anion radical scavenging activity was detected according to the Nishimiki *et al.*'s method [14]. The Oyaizu method [15] was used to determine the reducing power. Total phenolic compounds in the mushroom extract were determined with Folin-Ciocalteu reagent according to the method of Slinkard and Singleton [16] using pyrocatechol as a standard phenolic compound.

Antimicrobial activity

The antimicrobial activity was evaluated against 10 microorganisms, including five strains of bacteria: *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Bacillus cereus* (ATCC 10987), *Escherichia coli* (ATCC 25922) and *Proteus mirabilis* (ATCC 12453) and five species of fungi: *Aspergillus niger* (ATCC 16888), *Candida albicans* (ATCC 10259), *Penicillium italicum* (ATCC 10454), *Mucor mucedo* (ATCC 20094), *Trichoderma viride* (ATCC 13233) obtained from the ATCC (American Type Culture Collection).

The bacteria isolates were picked from over-night cultures in Müller-Hinton agar and suspensions were prepared in sterile distilled water by adjusting the turbidity to match 0.5 McFarland standards to approximately 10^8 CFU/mL. Fungal suspensions were prepared from 3- to 7-day-old cultures that grew on a potato dextrose agar except for *C. albicans* that was maintained on Sabouraud dextrose agar. The spores were rinsed with sterile distilled water, used to determine the turbidity spectrophotometrically at 530 nm, and then further diluted to approximately 10^6 CFU/mL according to the procedure recommended by NCCLS [17]. The 96-well micro-titre assay using resazurin as the indicator of cell growth [18] was employed for the determination of the minimum inhibitory concentration (MIC) of the active extract.

Cytotoxic activity

Human epithelial carcinoma HeLa cells, human colon carcinoma LS174 cells, human lung carcinoma A549 and human lung fibroblast MRC-5 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). All cancer cell lines were cultured as a monolayer in the RPMI 1640 nutrient medium, with 10% (inactivated at 56ºC) FBS, 3 mM of L-glutamine, and antibiotics, at 37ºC in humidified air atmosphere with 5% CO_2. The effect on cancer cell survival was determined 72 h after the addition of the extract, by the MTT assay [19].

Genotoxic potential

Lymphocytes were isolated from peripheral whole blood of healthy donors using Histopague-1077 (Sigma-Andrich Co., United Kingdom) and subjected to the alkaline comet assay. Human peripheral blood was collected by venepuncture from healthy, non-smoking donors who had not been exposed to known mutagens. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee. The obtained lymphocytes were incubated for 30 min at 37ºC in PBS solution with various concentrations of the *Leccinum. scabrum* mushroom acetone extract (50, 100, 150 and 200 µg/mL) separately and in treatment against the known mutagen, hydrogen peroxide (H_2O_2).

Untreated lymphocytes were used as negative control, while lymphocytes treated with H_2O_2 alone (final concentration of 10 µg/mL) were used as positive control. After the incubation, cell suspension was suspended in 1% low melting-point agarose (Sigma, St. Louis, MO) for embedding on slides. Slides were kept about 3 minutes on ice. After removing the coverslips, cells were lysed with freshly prepared lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100 and 10% dimethyl sulfoxide, pH 10) for 2 h, in the dark, at 4ºC. Subsequently, alkaline denaturation was performed in an electro-phoresis buffer solution (10 M NaOH, 200 mM EDTA, pH > 13) and slides were electrophoresed for 30 min at 25 V and 300 mA. Then, the slides were washed in neutralizing Tris-HCl buffer for 15 min (0.4 M Tris, pH 7.5) and rinsed in distilled water. The slides were stained with ethidium bromide (50 µg/mL). Experiments were performed in dark to minimize the occurrence of additional DNA damage [20].

One hundred randomly selected cells from each slide were viewed under Nikon E50i fluorescent microscope at 400x magnification. Selected cells were analysed based on the criteria defined by Collins [21]: class 0 – no damage; class 1 – low damage; class 2 – medium damage; class 3 – high damage and class 4 – total destruction. The Genetic Damage Index (GDI) for each sample was calculated using the following formula:

GDI = (Class1 + 2 x Class2 + 3 x Class3 + 4 x Class4)/ (Class0 + Class1 + Class2 + Class3 + Class4).

Statistical analysis

The SPSS (version 20) software package was used for statistical analysis. The statistical analysis was performed by one-way analysis of variance (ANOVA). The comparisons between groups were made using Dunnett T3 post hoc test. Results are expressed as mean ± standard deviation (SD). In all comparisons, $p < 0.05$ was considered as indicating statistical significance.

Results and Discussion

The free radical scavenging activity and reducing power of the tested extract are presented in Table I. The IC_{50} values were 52.16 and 14.90 µg/mL for DPPH radicals and superoxide anion radicals scavenging activity, respectively. As shown in Table I, the reducing power was concentration dependent. The measured values of absorbance for the reducing power varied from 0.0632 to 0.0199. The total content of phenols in the extract was 14.91 ± 0.92 µg PE/mg (Table I).

Table I

Free radical scavenging activity, reducing power and phenolic content of the acetone extract of the edible mushroom *Leccinum scabrum*

Tested mushroom	DPPH radical scavenging activity IC_{50} (µg/mL)			
Leccinum scabrum	52.16 ± 1.76			
Ascorbic acid	6.42 ± 0.18			
Tested mushroom	Superoxide anion scavenging activity IC_{50} (µg/mL)			
Leccinum scabrum	14.90 ± 1.01			
Ascorbic acid	115.21 ± 1.49			
Tested mushroom	Reducing power Absorbance (700 nm)			
	2000 µg/mL	1000 µg/mL	500 µg/mL	250 µg/mL
Leccinum scabrum	0.0632 ± 0.021	0.0449 ± 0.007	0.0263 ± 0.005	0.0199 ± 0.002
Ascorbic acid	2.1130 ± 0.032	1.6540 ± 0.021	0.0957 ± 0.008	0.0478 ± 0.004
Tested mushroom	Phenolic content (µg PE/mg of extract)			
Leccinum scabrum	14.91 ± 0.92			

Ascorbic acid was used as standard. Values are expressed as mean ± SD of three parallel measurements. PE - pyrocatechol equivalents

Presented results indicate that the acetone extract of *L. scabrum* showed very strong free radical scavenging activity, while the reducing power was less pronounced. This activity of the tested extract was based on the its ability to accelerate the formation of the non-radical form of DPPH-H as well as on its ability to destroy the superoxide radical produced from the phenazine methosulfate (PMS)/NADH reaction which is a weak oxidative agent but induces the formation of highly reactive free radicals.

In the literature there are several data regarding the antioxidant activity of *L. scabrum* [10-12]. It was determined the antioxidant activity for this species, but using other extraction solvents and high antioxidant activities were registered in all cases. In this study, the antioxidant activity of the mushrooms was confirmed by acetone extract. Different solvents, according to their polarity, may extract various compounds which results in a great antioxidant activity. This means that synergistic activities may occur between these constituents leading to the pronounced antioxidant activity of the mushroom extract (containing the anti-oxidant active components).

The antimicrobial activity of the mushrooms extract against the test microorganisms is shown in Table II. Extract from *L. scabrum* acted on all tested micro-organisms. The MIC fluctuated in the range 0.039 - 0.156 mg/mL for bacteria and 5 - 10 mg/mL for fungi. The most sensitive, among the microorganisms, was *B. cereus* (MIC value 0.039 mg/mL). The antimicrobial activity was compared with the standard antibiotics, streptomycin (for bacteria) and ketoconazole (for fungi). The results showed that the tested sample had similar antibacterial activity to streptomycin, while ketoconazole was more active than the tested mushroom extract. In the negative control, DMSO had no inhibitory activity on the tested organisms.

There is no available information about the anti-microbial potential of *L. scabrum* species, but some researchers have examined the antimicrobial potential of other related mushrooms. For example, Kosanić *et al*. [22] showed that acetone and methanol extracts from *L. carpini* exhibited antibacterial activity in lower concentrations (1.25 - 10 mg/mL) than our tested ones, while the antifungal activity was approximate as in our experiment. The intensity of the examined anti-microbial activity of *L. scabrum* depended on the tested microorganisms and the used concentration of extract. In this experiment, Gram negative bacteria were more resilient than Gram positive. This resistance

is likely due to the difference in the structure of the cell wall. Gram negative bacteria have a wall associated with an outer complex membrane, which slows down the passage of hydrophobic compounds. Gram positive bacteria are more susceptible to the antibiotic agents due to the absence of an outer membrane. Fungi were more resistant than bacteria due to the more complex structure of the cell wall [22].

Table II

Minimum inhibitory concentrations (MIC) of the acetone extract of *Leccinum scabrum*

Bacteria and microfungi	*Leccinum scabrum*	Streptomycin	Ketoconazole
	MIC (mg/mL)		
Bacillus cereus	0.039	0.019	-
Bacillus subtilis	0.078	0.019	-
Escherichia coli	0.156	0.039	-
Proteus mirabilis	0.156	0.078	-
Staphylococcus aureus	0.078	0.039	-
Aspergillus niger	10	-	0.078
Candida albicans	5	-	0.039
Mucor mucedo	5	-	0.156
Penicillium italicum	5	-	0.156
Trichoderma viride	5	-	0.078

The cytotoxic activity of *L. scabrum* extract is shown in Table III. The IC_{50} against HeLa, LS174 and A549 cell lines were 73.64, 130.63 and 76.82 µg/mL respectively. As shown in Table III, the tested mushroom extract showed a weaker activity compared to cis-DDP as a positive control. The extract expressed relatively strong cytotoxic activity on the used cancer cells, among which HeLa cells were the most sensitive. Cytotoxic activities on normal cells were not detected, so it is important to emphasize that this mushroom acted selectively when it comes to its cytotoxic potential. There is no available information about the cytotoxic potential of *L. scabrum* species, but some researchers have examined the cytotoxic potential of other related mushrooms. For example, Kosanić *et al.* [23] showed that methanolic extracts from *Agaricus campestris* and *Boletus edulis* exhibited significant cytotoxic activities. Also, it has been proven that other edible mushrooms possess anticancer activities [24, 25]. Further research will be necessary in order to identify compounds responsible for the observed antitumor activities.

Table III

Growth inhibitory activity of *Leccinum scabrum* on HeLa, LS174, A549 and MRC5 cell survival

Cell lines	HeLa	LS174	A549	MRC-5
Tested mushroom		IC_{50} (µg/mL)		
Leccinum scabrum	73.64 ± 1.35	130.63 ± 1.85	76.82 ± 0.49	< 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 IV

Protective activity of acetone extracts of *Leccinum scabrum* against H_2O_2 in cultured human lymphocytes using comet assay

Treatment (µg/mL)	No. of analysed cells	Classes of damage degree in cells					GDI (mean ± SD)
		0	1	2	3	4	
Negative control	300	85.67	9.33	4.00	0.33	0.67	0.21 ± 0.04
Positive control	300	/	/	7.00	41.33	51.67	3.45 ± 0.10
Separate treatment							
50	300	76.33	8.67	7.33	5.67	2.00	0.48 ± 0.09
100	300	64.33	10.00	12.00	8.67	5.00	0.80 ± 0.13
150	300	37.00	13.33	17.33	15.00	17.33	1.62 ± 0.43
200	300	30.00	16.33	16.33	19.00	18.33	1.79 ± 0.64
Treatment against H_2O_2							
50 + H_2O_2	300	17.33	11.00	17.33	21.00	33.33	2.42 ± 0.16*
100 + H_2O_2	300	20.67	18.00	18.00	20.33	23.00	2.07 ± 0.23*
150 + H_2O_2	300	19.67	23.33	11.33	19.00	26.67	2.09 ± 0.45
200 + H_2O_2	300	7.00	19.67	15.00	24.00	34.67	2.60 ± 0.29

*statistically significant difference in comparison to positive control (ANOVA, $^*p < 0.05$)

The results of the genotoxic potential are shown in Table IV. All tested concentrations of extract caused no significant increase of the GDI values (from 0.48 ± 0.09 to 1.79 ± 0.64) compared to the negative control (0.21 ± 0.04), which clearly shows that the extract was not genotoxic. Having determined that the mushroom itself did not induce DNA damage, the next step was to determine how it works against the

H_2O_2 toxic effect. H_2O_2 is a known mutagen used as a positive control, which induced a significant increase in the number of cells with tail (comets) compared to the negative control ($p < 0.001$). The extract decreased the H_2O_2 induced GDI values ($p < 0.05$) in all tested concentrations but significantly in concentrations of 50 (2.42 ± 0.16) and 100 µg/mL (2.07 ± 0.23) in comparison to the positive control (3.45 ± 0.10). Thus, our results suggest that the extract showed clear protective activity against H_2O_2 in lower concentrations, while at the highest tested concentration it showed synergistic activity.

Similarly, previous studies have reported that related mushrooms did not produce genotoxic activity on human lymphocytes *in vitro* [26-28]. Also, Park *et al*. [29] and Živković *et al*. [28] have showed that extracts of *Inonotus obliquus* and *Agaricus blazei* offer protection against H_2O_2 induced DNA damage in healthy human lymphocytes at levels similar to those provided by other known antioxidants (ascorbic acid, trolox, α-tocopherol). Ascorbic acid displayed a decrease of over 50% in the level of DNA damage in human lymphocytes [29]. However, Vickers [30] has shown that the combination of different compounds of a mushroom extract with the mutagen agents may have synergistic activity, which reinforces the influence of both individual factors. For these reasons, there is an increasing need to investigate the effects of mushroom extracts not only on healthy, but also on cells that have previously been exposed to mutagenicity.

Conclusions

The present study has shown that the mushroom *L. scabrum* has antioxidant and antimicrobial effects, but also a good cytotoxic and protective activity. It can be used as a good source of natural antioxidants and for pharmaceutical purposes in the prevention and treatment of various diseases.

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Conflict of interest

The authors declare no conflict of interest.

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