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REGULAR ARTICLE

Evaluation of metal contents and bioactivity of two edible mushrooms *Agaricus campestris* and *Boletus edulis*

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ABSTRACT

Here we determined metal concentrations, and antioxidant, antimicrobial and anticancer potential of two edible mushrooms *Agaricus campestris* and *Boletus edulis*. The concentrations of nine metals were determined and all metals are present in the allowable concentrations. Antioxidant activity was evaluated by free radical scavenging ability and reducing power. *B. edulis* had more potent free radical scavenging activity (IC_{50} = 266.32 μg/mL) than *A. campestris*. Moreover, the tested extracts had effective reducing power. The total content of phenol was examined using Folin-Ciocalteu reagent and the obtained values were expressed as pyrocatechol equivalents (PE). Furthermore, the antimicrobial potential was determined by a microdilution method. *A. campestris* showed a better antimicrobial activity with MIC values ranging from 2.5 to 20 mg/mL. Finally, the cytotoxic activity was tested using MTT method on the Hela, A549 and LS174 cells. *A. campestris* expressed stronger cytotoxic activity toward all cell lines with IC_{50} values ranging from 18.66 to 31.55 μg/mL.

Keywords: Anticancer activity; Antimicrobial activity; Antioxidant activity; Metal concentration; Mushrooms

INTRODUCTION

Contamination by heavy metals has been increasing with the increase of industrialization. This pollution accumulates in the soil and living organisms and thus directly affects ecosystem. Iron, copper, zinc and manganese are essential elements for the living organisms and they play an important role in the biological systems. However some non essential metals such as lead, cadmium, aluminum are toxic and their long-term accumulation in human bodies may cause serious diseases (Gebrelibanos et al., 2016).

Edible mushrooms are rich in protein, vitamins, iron, zinc, sodium and minerals and they are popular healthy food (Zengin et al., 2015; Boonsong et al., 2016). Mushrooms are capable to accumulate heavy metals from the environment and they play an important role in the decomposition of organic matter. On the accumulation and concentration of metals in mushrooms affects several factors. Metal contents in mushrooms are generally assumed to be species dependent, but substrate composition or pH of the soil also considered to be important factors (Garcia et al., 1998; Gebrelibanos et al., 2016; Lalotra et al., 2016).

Mushrooms have long been used for therapeutic purposes because they can produce a various secondary metabolites such as organic acids, alkaloids, terpenoids, steroids and phenolic compounds (Prasad et al., 2015). It has been published that some species of mushrooms have health promothing potential, such as antioxidant, antimicrobial, anticancer, cholesterol lowering and immunostimulatory activities (Ferreira et al., 2007; Mishra et al., 2015; Kosanić et al., 2016).

Very few studies describing the bioactive properties of mushrooms enriched with minerals could be found in the literature. Thus, the aim of present study is to investigate the metal contents in *Agaricus campestris* and *Boletus edulis* mushrooms. Since fungi are well-known natural antioxidants, our work evaluate antioxidant effect of methanol extracts of these mushrooms by DPPH method. This method is based on the reduction of alcoholic DPPH solution in the presence of hydrogen

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donating antioxidant (AH) due to the formation of non-radical form DPPH-H by the reaction DPPH + AH → DPPH-H + A. The reduction of ferrous ion (Fe3+) to ferric ion (Fe2+) which is measured by the intensity of the green-blue color of solution which absorbs at 700 nm was also employed. Furthermore, the present investigation was designed to show the antimicrobial activity of these two important edible mushrooms for their potential use in the prevention of various infections. Finally, mushroom can be used as natural agents in the treatment of cancer, due to the importance of mushrooms as anticancer agents has been confirmed in recent years. Therefore, the anticancer effect was also examined using MTT assay.

MATERIALS AND METHODS

Fungal materials

Fungal samples of *Agaricus campestris* L.:Fr., and *Boletus edulis* Bull. Fr., were collected from Kopaonik, Serbia, in June of 2014. The demonstration samples are preserved in facilities of the Department of Biology and Ecology of Kragujevac, Faculty of Science. The determination of mushrooms was done using standard keys (Uzelac, 2009).

Finely dry ground thalli of the examined mushrooms (100 g) were extracted using methanol (500 mL) in a Soxhlet extractor. The extracts were filtered and then concentrated under reduced pressure in a rotary evaporator. The dry extracts were stored at −18^{0}C until they were used in the tests. The extracts were dissolved in 5% dimethyl sulphoxide (DMSO) for the experiments (Kosanić et al., 2016). DMSO was dissolved in sterile distilled water to the desired concentration.

Metals quantitative analysis

Metals in mushrooms were analyzed using the method described in the recent literature (Kosanić et al., 2016). The collected samples of mushroom were cleaned with a plastic knife, air–dried for one week and further dried in an oven at 50°C until the samples reached a constant weight. Dried samples were ground to a powder, using an agate mortar and stored in polyethylene bottles until analysis. All solutions were prepared from analytical reagent grade and deionized water which was generated by a Milli-Q academic water purification system (Milford, MA, USA). Mineral acid (HNO_3) and oxidant (H_2O_2) of suprapure quality (Sigma–Aldrich, Germany and J.T. Baker, Netherlands, respectively) were used for sample digestion. For calibration, we used a series of standard solutions prepared by diluting stock solutions of 1000 mg/L of each element supplied by J.T. Baker, Netherlands.

Microwave digestion: Samples (0.5 g) of powdered mushrooms were transferred in TFM vessels and digested with 7 mL of HNO_3 (65%) and 1 mL of H_2O_2 (30% in microwave digestion system (Milestone ETHOS One)) for 25 min and finally diluted to 25 mL with deionized water. A blank digest was carried out in the same way. Digestion conditions for mushroom samples in the microwave digestion system, recommended by the manufacturer, are shown in Table 1. All sample solutions were clear. The digestion procedure was done in triplicate for each sample.

Metals Quantitative Analysis in the digested solutions was performed by inductively–coupled plasma optical emission spectrometry (ICP–OES) using a Thermo Scientific iCAP 6000 series spectrometer. The analytical parameters of investigated metals for ICP–OES are shown in Table 2.

Antioxidant activity

The free radical scavenging activity of lichen extracts was measured by1,1-diphenyl-2-picryl-hydrazil (DPPH) according to the Dorman et al's method (2004). The DPPH radical concentration was calculated using the following equation:

$$\text{DPPH scaveging ability (\%)} = \left[\frac{(A0 - A1)}{A0}\right] \times 100$$

where A0 is the absorbance of the negative control and A1 is the absorbance of reaction mixture or standard. For both extract and ascorbic acid, the inhibitory concentration (IC_{50}) at 50% was determined.

The reducing power of samples was determined according to the method described by Oyaizu (Oyaizu, 1986).

Total soluble phenolic compounds in the acetone extracts were determined with Folin-Ciocalteu reagent (Slinkard and Singleton, 1997) using pyrocatechol as a standard phenolic compound. The total concentration of phenolic compounds in the extract was determined as microgram of pyrocatechol equivalents (PE) per milligram of dry extract by using an equation that was obtained from a standard pyrocatechol graph as follows:

Absorbance = 0.0057× total phenols [μg PE/mg of dry extracts]–0.01646 (R2=0.9203)

Table 1: Digestion conditions for mushroom samples in the microwave digestion system

Step	Time (min)	Temperature (°C)	Power (W)
1	10	100	up to 500
2	7	200	up to 500
3	8	200	up to 500
Vent	10		

Antimicrobial activity

The sensitivity to extracts of the investigated species of microorganisms was tested by determining the minimal inhibitory concentration (MIC) by the broth microdilution method with using 96-well micro-titer plates (Sarker et al., 2007).

Cytotoxic activity

Human epithelial carcinoma Hela cells, human lung carcinoma A549 cells and human colon carcinoma LS174 cells were obtained from 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 56°C) FBS, 3 mM of L-glutamine, and antibiotics, at 37°C in humidified air atmosphere with 5% CO_2.

Stock solutions (50 mg/ml) of the extracts, made in DMSO, were dissolved in a corresponding medium to the required working concentrations. The final concentrations applied to the cells were 200, 100, 50, 25 and 12.5 μg/ml. In the control wells only the nutrient medium was added. The effect on cancer cell survival was determined 72 h after the addition of the extract, by the MTT test (Mosmann, 1983).

Data analyses

Data analyses were performed using the EXCEL and SPSS softwares package. To determine the statistical significance of tested activity, t-test was used. All values are expressed as mean ± SD of three parallel measurements.

RESULTS

Results of metals quantitative analysis of the tested mushroom species have been shown in Table 3. Average metal concentrations expressed as mg/kg (dry weight of mushroom) in fruiting bodies samples of mushrooms. According to the results, Fe was the most abundant element in the samples of *A. campestris* and *B. edulis*, with a values of 166.83 and 62.97 mg/kg dw, respectively. The levels of Zn, Cu, Mn, Ni, Cd, Cr and Co in *A. campestris* were 84.29, 50.32, 9.35, 1.74, 1.44, 1.33 and 0.14 mg/kg dw, while in *B. edulis* they were 38.05, 10.77, 7.90, 1.12, 0.27, 0.83, and 0.09 mg/kg dw, respectively. Pb content in *A. campestris* was 0.73 mg/kg dw, while in *B. edulis* Pb was not detected. Among the elements tested, Co had the lowest concentration value.

The scavenging DPPH radicals and reducing power of the studied extracts are shown in Table 4 and Table 5. Extract from *B. edulis* showed higher antioxidant activities than *A. campestris*. Difference between extracts and control was statistically significant ($P<0.05$). As shown in tables, ascorbic acid had stronger activity in comparison to the extracts. The total phenolics content in extracts of *A. campestris* and *B. edulis* were 46.01 and 81.33 μg PE/mg (Table 6).

The antimicrobial effect of the mushroom extracts is represented in Table 7. The both mushrooms acted selectively on the microorganisms tested. The extract from *A. campestris* inhibited four of the five bacteria and seven of the ten fungal species. The MIC fluctuated in a range 2.5–10 mg/mL. *B. edulis* extract showed slightly weaker activity. It inhibited three species of bacteria and six tested fungi with MIC values 2.5 – 20 mg/mL. The most sensitive bacteria was *B. cereus*, and the highest resistance was shown in *E. coli* and *S. aureus*. The most sensitive fungi were *C. albicans, F. oxysporum, A. alternata* and *C. cladosporioides,* while *A. flavus, A. niger* and *P. chrysogenum* were the most resistant. Streptomycin and ketoconazole as standards

Table 2: Instrumental parameters of investigated metals for ICP–OES

Power			1150 W		
Plasma position			Axial		
Pump rate			50 rpm		
Coolant gas flow			12 L min^{-1}		
Auxiliary gas flow			0.5 L min^{-1}		
Nebulizer gas flow			0.6 L min^{-1}		
Nebulizer pressure			2.9 bar		
Sample flow rate			2.5 mL min^{-1}		
Element wavelength (nm)	Cd 228.802	Co 228.616	Cr 267.716	Cu 324.754	Fe 238.204
	Mn 257.610	Ni 231.604	Pb 216.999	Zn 213.856	

Table 3: Metal concentrations (mg/kg, dry weight) of the mushroom samples

Mushroom	Fe	Zn	Cu	Mn	Ni	Cd	Pb	Cr	Co
Agaricus campestris	166.83±1.35	84.29±0.27	50.32±0.43	9.35±0.07	1.74±0.01	1.44±0.01[a]	0.73±0.07	1.33±0.03	0.14±0.00
Boletus edulis	62.97±0.30	38.05±0.09	10.77±0.07	7.90±0.10	1.12±0.01	0.27±0.00	nd	0.83±0.02	0.09±0.01

[a]mean±standard deviation, n=3, [b]nd: not detected. There is statistically significant difference between tested mushrooms ($p<0.05$)

Table 4: DPPH radical scavenging activity of methanol extracts of *Agaricus campestris* and *Boetus edulis*

Mushroom species	DPPH radical scavenging IC_{50} (µg/mL)
Agaricus campestris	416.12±2.35
Boletus edulis	266.32±1.28
Ascorbic acid	6.42±0.18

Values are expressed as mean±SD of three parallel measurements. There is statistically significant difference between tested mushrooms and control (p<0.05)

Table 5: Reducing power of methanol extracts of *Agaricus campestris* and *Boetus edulis*

	Absorbanca (700 nm)			
Mushroom species	1000 µg/mL	500 µg/mL	250 µg/mL	125 µg/mL
Agaricus campestris	0.433±0.031	0.355±0.025	0.105±0.008	0.039±0.004
Boletus edulis	0.905±0.043	0.623±0.030	0.468±0.012	0.252±0.009
Ascorbic acid	2.113±0.032	1.654±0.021	0.0957±0.008	0.0478±0.008

Values are expressed as mean±SD of three parallel measurements. There is statistically significant difference between tested mushrooms and control (p<0.05)

Table 6: Total phenolic content of methanol extracts of *Agaricus campestris* and *Boletus edulis*

Mushroom species	Phenolics content (µg PE/mg of extract)
Agaricus campestris	46.01+1.024
Boletus edulis	81.33+1.125

Values are expressed as mean±SD of three parallel measurements. PE - pyrocatechol equivalents. There is statistically significant difference between tested mushrooms (p<0.05)

Table 7: Minimum inhibitory concentration (MIC) of methanol extracts of *Agaricus campestris* and *Boletus edulis*

Mushroom species	*A. campestris*	*B. edulis*	S	K
Bacillus cereus	2.5	2.5	0.016	-
Bacillus subtilis	2.5	5	0.016	-
Eschrichia coli	/	/	0.062	-
Proteus mirabilis	10	10	0.062	-
Staphylococcus aureus	10	/	0.031	-
Aspergillus flavus	/	/	-	0.312
Aspergillus niger	/	/	-	0.078
Candida albicans	5	5	-	0.039
Penicillium expansum	10	20	-	0.156
Penicillium chrysogenum	/	/	-	0.078
Alternaria alternata	5	10	-	0.078
Trichoderma viride	10	10	-	0.078
Cladosporium cladosporioides	5	10	-	0.039
Fusarium oxysporum	5	10	-	0.078
Mucor mucedo	10	/	-	0.156

Values given as mg/mL. Antibiotics: K – ketoconazole, S – streptomycin. There is statistically significant difference between tested mushrooms and control (p<0.05)

Table 8: Growth inhibitory effects of methanol extracts of *Agaricus campestris* and *Boletus edulis* on Hela, A549 and LS174 cell lines

Mushroom species	IC_{50} (µg/mL)		
Cell lines	Hela	A549	LS174
Agaricus campestris	18.66±0.38	31.55±1.61	30.22±2.04
Boletus edulis	35.7±0.52	34.91±1.42	48.09±1.53
Cis-DDP	0.86±0.33	4.91±0.42	3.18±0.29

There is statistically significant difference between tested mushrooms and control (p<0.05)

had stronger antimicrobial effect compared to the tested samples.

The results obtained for anticancer potential *of A. campestris* and *B. edulis* extracts are shown in Table 8. The IC_{50} value for both mushrooms against the tested cells ranged from 18.66 to 48.09 µg/mL. The IC_{50} for *A. campestris* extract against Hela, A549 and LS174 cells was 18.66, 31.55 and 30.22 µg/mL, respectively. *B. edulis* manifested a slightly lower cytotoxic activity. The IC_{50} value was 35.7 µg/mL related to Hela cell, 34.91 related to A549 cells and 48.09 µg/mL related to LS174 cell line. Furthermore, both extracts showed less activity compared to cis-DDP.

DISCUSSION

The contents of nine metals were evaluated in this study. The analysis of the metal concentration of these and related mushrooms was previously published (García et al., 2009; Çayır et al., 2010; Kalač, 2010; Ayaz et al., 2011; Sarikurkcu et al., 2011). Compared to those studies, the results of mineral content in our mushroom samples are in accordance with previous results, with small differences in the content of Zn, Mn and Cd.

According to Food and Agriculture Organization and World Health Organization (FAO/WHO) standards for toxic metals (Cd and Pb) the acceptable daily intake (ADI) levels for Cd and Pb for an adult (of 60 kg body weight) are 0.06 and 0.214 mg, respectively. Also, for an adult the provisional tolerable daily intake (PTDI) levels for metals Fe, Zn and Cu are 48, 60 and 3 mg, respectively (FAO/WHO, 1993, 1999). For the calculations, we used the fact that 300 g portion of fresh mushrooms per meal contains 30 g of dry matter (Kalač and Svoboda, 2000). The metals concentrations in the analyzed mushrooms in our experiment were low and within the legal limit suggested by the FAO/WHO standards.

The extracts of tested mushrooms showed *moderate* antioxidant activity in DPPH test. Also, the reducing power may indicate potential antioxidant properties of tested mushrooms. The reducing features are mainly related with the presence of reductones which antioxidant activity is based on the destruction of the free radical chain by

donating a hydrogen atom (Gordan, 1990). Extracts used in this research contain high level of phenols for which it has been found that can act in a similar way as reductones, aborting free radical chain reactions (Sasikumar et al., 2010).

The intensity of antioxidant activity depended on both the tested mushroom species and the extraction solvent used. The variation in the antioxidant power of different solvents depends on their ability to extract bioactive substances. It is known that antioxidative nature of the extracts is a result of their phenolics. In this research tested extracts with the higher amount of phenolics exhibited more potent antioxidant capacity. In many studies has been found a high correlation between phenolic content and antioxidative activities (Alvarez Parrilla et al., 2007; Ferreira et al., 2007; Kosanić et al., 2012). Phenolics are potential antioxidants which can donate hydrogen to free radicals and thereby stop the chain reaction of lipid oxidation at the initial stage, due to the presence of their phenolic hydroxyl groups (Kosanić et al., 2012).

There are several reports on antioxidant activity of *B. edulis* and *A. campestris* (Vidović et al., 2010; Kosanić et al., 2012; Woldegiorgis et al., 2014) by using other extraction solvents. In this study, the antioxidant capacity of selected mushrooms was confirmed by methanol extracts. Depending on the polarity, different extraction solvents may extract different substances which contribute to the powerful antioxidant activity, which means that between individual antioxidant substances in the extracts there is a synergistic interaction, which resulting in prominent antioxidant effect of mushrooms.

Similar to our obtained data, numerous investigators found relatively high antimicrobial activity for *A. campestris* and *B. edulis* (Giri et al., 2012; Kosanić et al., 2012; Ranadive et al., 2013). In our experiments, the intensity of the antimicrobial effect depended on the species of the mushroom, its concentration and the tested organism. Presence of various antimicrobial substances in the extracts affects on the overall antimicrobial potential of extracts (Kosanić et al., 2012). However, need have in mind the fact that extracts are mixtures of compounds, and their antimicrobial potential may be the result of their interactions, which can have various effects on the overall activity of extracts.

In our study, the investigated mushrooms in the same concentrations showed a weaker antifungal than antibacterial activity. This difference between the fungi and bacteria probably is the result of different permeability of their cell walls. Gram-positive bacteria have wall consisting mainly of mureins and teichoic acids, while the gram-negative bacteria have a more complex wall consisting of lipopolysaccharides and lipopoliproteins. Fungi cell wall consists primarily of hitchin, glucan, mannan and diaminopimelic acid (Farkaš, 2003).

A. campestris and *B. edulis* were previously tested on cytotoxic activity. For instance, Lemieszek et al. (2013) found that fractions from *B. edulis* not exhibited toxicity against normal colon epithelial cells and in the same concentration range caused a very powerful antiproliferative effect in colon cancer cells. Also, lectin from the *B. edulis* has anticancer activity (Bovi et al., 2011). Different solvent extracts of A. campestris were also *found* to possess good *antitumor activities* (Li et al., 2005). The mechanism of action of the selected mushrooms remains to be investigated. For further more detailed investigations is necessary to determine compounds which are responsible for the observed anticancer effect, as well as to find ways to *increase* the selectivity.

CONCLUSION

In conclusion, it can be stated that tested mushroom extracts have allowed metals concentrations and they also showed the strongest bioactivity *in vitro* in all the assays. Since the synthetic antioxidants have been suspected to exhibit toxic and carcinogenic effects, the development and utilization of more effective antioxidants of natural origins are required. Our results showed that *A. campestris* and *B. edulis* are promising mushrooms regarding alternative antioxidants which should replace the synthetic ones. Furthermore, the results obtained showed that the selected mushrooms had shown a significant antimicrobial effect relative to the tested microorganisms. That can be useful in treatment of numerous diseases caused by bacteria and fungi. Nowadays, there is a huge problem in the treatment of infectious diseases because the microorganisms had developed resistance to numerous antibiotics, so the tested mushrooms could have an important role in their therapy. Finally, many studies have proven that mushrooms have shown excellent cytotoxic activities and their dietary consumption is believed to be chemo-preventive against many cancer types. The extracts of *A. campestris* and *B. edulis* showed promising results *in vitro* for the anticancer activity and therefore these two mushroom species should be further investigated as the potential new anticancer drugs.

Based on these results, the tested mushrooms appear to be good natural antioxidant, antimicrobial and anticancer agents. The identification of the active antioxidant, antimicrobial and anticancer compounds of these mushroom species can lead to their potential commercial usage in medicine, food production and the cosmetic industry.

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Authors' contributions

All authors contributed equally in conducting the research and in preparing this manuscript.

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