COMPREHENSIVE EVALUATION OF THE ANTIOXIDANT ACTIVITY OF SIX WILD EDIBLE MUSHROOM SPECIES

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The present study describes antioxidant properties of six wild mushrooms (*Boletus edulis* Bull., *Boletus regius* Krombh., *Leccinum pseudoscaber* Kallenb., *Boletus impolitus* Fr., *Lactarius volemus* Fr., and *Lactarius deliciosus* (L.) Gray) collected in Serbia. The antioxidant properties were assessed through the evaluation of the reducing power and radical scavenging ability of the samples. The *Boletus regius* sample revealed the best results in almost all antioxidant activity assays, with the highest reducing power and the highest scavenging activity, 33.0 ± 0.1 mg TE/g dw in DPPH; 1.35 ± 0.02 mg AAE/g dw in TRP; 13.42 ± 0.08 mg Fe/g dw in FRAP; 37.5 ± 0.2 mg TE/g dw in CUPRAC; 243.6 ± 5.1 mg GAE/g dw in TPC. On the contrary, *Lactarius volemus* showed the lowest antioxidant activity. The results indicate that the analyzed wild mushroom species possess reductive capabilities, and they could be a potential source of antioxidants. The study was raised to the next level using a chemometric analysis, a principal component analysis to display the correlation between the antioxidant activity and different mushroom species.

Keywords: mushrooms, antioxidant activity, phenol content, chemometric analysis.

Introduction

Despite great progress in modern medicine in recent times, a natural source of antioxidants still plays an important role in a medical treatment. A huge number of medicinal plants have been investigated for their antioxidant and other activities. Antioxidants, both synthetic and natural, can be effective for helping the human body in reducing oxidative damage by reactive oxygen species (ROS) [1]. Today, in order to find a dietary source full of antioxidants that can help human body to reduce oxidative damage, different plant and mushroom species have been explored. The investigation of mushrooms and their extracts antioxidant activity and the content of antioxidant compounds is extremely current scientific subject.

The investigation of mushrooms and their extracts antioxidant activity and the content of antioxidant compounds is very actual scientific subject [2]. The antioxidant activity of some mushrooms extracts were also studied by other researchers. For example, Kosanić et al. [3] found a strong antioxidant activity for methanol extracts from *B. edulis* and *L. pseudoscaber*. *L. deliciosus* is one of the most popular mushrooms because they are an excellent source of antioxidant compounds like polyphenols [4].

Mushrooms have a long tradition of use in many countries. People usually use edible mushrooms for meals, consuming them fresh, prepared or even as spices. Edible mushrooms are food full of protein, crude fiber, minerals

and vitamins, with low fat and cholesterol contents, so they could be an ideal source of nutritional ingredients. Mushrooms have a low glycemic index which is especially beneficial for diabetics, a very low sodium (Na) concentration which is beneficial for hypertensive patients and a high content of potassium (K) and phosphorus (P) which is an important orthomolecular aspect [5]. Also, they are sources of beneficial bioactive substances that contribute to good health [3]. Edible mushrooms have got the attention as a commercial source of antioxidants. They can be used directly in enhancement of antioxidant defenses through dietary supplementation to reduce the level of oxidative stress. Mushrooms produce and store a wide range of secondary metabolites which includes phenolic compounds. Phenolic compounds can be classified as simple phenols and phenolic acids such as gallic acid, benzoic acid, syringic acid, chlorogenic acid and other associates, and polyphenols which are classified into many groups such as flavonoids, tannins, stilbenes, and so on [6].

The objective of this work was to evaluate the antioxidant properties of six mushroom species (*Boletus edulis* Bull., *Boletus regius* Krombh., *Boletus impolitus* Fr., *Leccinum pseudoscaber* Kallenb., *Lactarius volemus* Fr., and *Lactarius deliciosus* (L.) Gray). In Serbia, these mushroom species are in use as delicious food. For these mushrooms, the antioxidant activity was compre-

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hensively evaluated measuring free radical scavenging capacity (ABTS and DPPH), ferric reducing antioxidant capacity (FRAP), total reducing power (TRP), cupric reducing antioxidant capacity (CUPRAC) and total phenolic content (TP).

One more reason to conduct this research is that antioxidant activities of some mushrooms have not been reported previously.

Methods and materials

Sample collection and preparation

Fruiting bodies were collected in the unpolluted region Ploče, near the town of Niš, the Republic of Serbia. To overcome variability of environmental conditions, mushroom samples were collected at four experimental points and from every experimental point three mushrooms of each examined species were taken. Mushrooms were cleaned, taxonomically identified and then cut and dried at room temperature. They were stored in paper bags and packed loosely with provision of aeration until the analysis started.

Chemicals and instruments

2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2'-azobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, Folin–Ciocalteu reagent, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and methanol were purchased from Sigma Co. St. Louis, Missouri, USA.

The following chemicals (p.a. grade): neocuproine (2, 9-dimethyl-1, 10-phenanthroline), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, Na_2CO_3 , HCl, 2, 4, 6-tris (2-pyridyl)-s-triazine (TPTZ), $\text{K}_3[\text{Fe}(\text{CN})_6]$, phosphate buffer (NaH_2PO_4 - Na_2HPO_4), ammonium acetate buffer, CCl_3COOH , $\text{K}_2\text{S}_2\text{O}_8$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, dimethyl sulphoxide (DMSO), were purchased from Merck, Darmstadt, Germany.

Spectrophotometric assays were performed on a double-beam UV–VIS spectrophotometer Perkin Elmer lambda 15 (Massachusetts, USA). Each of the mushroom samples was analyzed in triplicate.

Extraction and yield determination of the extracts

The preparation of methanol extracts of mushrooms was done as follows: a three g fine dried mushroom sample was ground in a mill and extracted with 30 mL of methanol. The extraction was performed four times in an ultrasonic bath for 15 minutes at 25 °C. The solvent extraction is the most frequently used technique for isolation of plant antioxidant compounds [7]. The extracts were filtered and then evaporated to dryness (35 °C) under reduced pressure in a rotary evaporator. For the entire analysis, compounds of the extract were dissolved in dimethyl sulfoxide (DMSO). The extracts were kept in the dark at 4 °C until the time of analysis.

The yields of concentrated dried extracts of mushrooms were calculated based on the dry weight as:

$$\text{Yield(\%)} = \frac{(W_1 \cdot 100)}{W_2}$$

Where:

W_1 is the weight of the evaporated extract

W_2 is the weight of the ground mushroom used for the extraction process

Determination of total phenolic content

The total phenolic content was determined using Folin–Ciocalteu reagent as originally described by Singleton and Orthofer [8]. The absorbance was measured at 750 nm using a spectrophotometer. Gallic acid solutions in methanol were used as standards and the measurement was compared to the calibration curve of gallic acid (GA). The results were expressed as mg gallic acid equivalents (GAE) per g of the dry weight (mg GAE/g dw).

Measurement of in vitro antioxidant activity

DPPH radical scavenging activity was determined according to the method of Mitic et al [9]. Stable DPPH radicals are widely used to evaluate the antioxidant activities of proton-donating substances according to the hydrogen donating ability [10]. Absorbance was measured at 515 nm and all determinations were performed in triplicate. The DPPH radical is long-lived organic nitrogen radical with a deep purple colour [11]. As opposed to other tests, higher absorbance of the reaction mixture indicates a lower antioxidant activity. The reason for this occurrence is the lack of antioxidants in the analyzed sample that reduced purple colour DPPH reagent and thus decreased the absorbance which would mean more activity. Radical scavenging activity was determined from the calibration curve, which was drawn by using Trolox as the positive control, in the concentration 1–16 mmol/L and expressed in mg of Trolox equivalents (TE) per g dry extract weight (mg TE/g dw).

ABTS radical “scavenging” activity

ABTS radical scavenging activity was performed according to the method of Re et al. [12]. This method is similar to DPPH assay. Antioxidant molecules react with stable ABTS radical, inhibit harmful reactions and suppress the colour development. After incubation at room temperature, the reduction in absorbance was measured at 734 nm. The results are expressed as mg of Trolox equivalents (TE) per g dry extract weight (mg TE/g dw).

Ferric-reducing antioxidant power (FRAP) assay

Ferric reducing antioxidant power assay was performed using the method of Benzie and Strain [13] and it is based on the reduction of ferric iron in the tripyridyltriazine complex to the blue ferrous form at low pH. This reduction is monitored by measuring the colour change during absorption at 595 nm. The final results are expressed as mg of Fe (II) equivalents per g dry weight (mg Fe/g dw) obtained by comparing the absorption change in the test mixture with the doses obtained from the Fe (II) standard calibration curve in the con-

centration 2.5-25 mmol/l.

Total reducing power (TRP) assay

The reducing power of the extracts was determined by the method of Oyaizu [14]. This method is based on the ability of antioxidants to reduce Fe (III) hexacyanate to Fe (II) hexacyanate which leads to the increase in the absorbance of the reaction mixtures. The absorbance was measured at 700 nm against the blank sample and the results were expressed as mg ascorbic acid equivalents per g of dry extract weight (mg AAE/g dw).

Cupric reducing antioxidant capacity (CUPRAC) assay

The CUPRAC assay was performed using the method of Apak et al. [15]. The method is based on the reduction of a cupric neocuproine complex (Cu(II)-Nc) by antioxidants to the cuprous form (Cu(I)-Nc). The CUPRAC reagent is much more stable and easily accessible than chromogenic radical reagents (e.g. ABTS and DPPH) [16]. After 30 min incubation at room temperature, the absorbance was measured at 450 nm. Trolox was used as a standard and the results were expressed as mg Trolox equivalents per g of dry weight (mg TE/g dw).

Antioxidant Composite Index - ACI

The results of the antioxidant activity measured by different methods is difficult to compare with each other, primarily because of different mechanisms of action, different units used to express the results and very different techniques for the preparation of the investigated samples. To reduce the results of antioxidant activities obtained with different methods at comparable values, we calculated the total antioxidant index (ACI - Antioxidant Composite Index). An overall antioxidant potency composite index was determined by assigning all assays an equal weight, assigning an index value of 100 to the best score for each test, and then calculating an index score for all other samples within the test as follows:

antioxidant index score = [(sample score/best score) × 100]; the average of all six tests for each samples was then taken for the antioxidant potency composite index [17].

Statistical analysis

Statistical analyses were performed with Statistica 8 (StatSoft, Tulsa) software packages. All chemical analyses were carried out in triplicate and the results expressed as mean ± standard deviation (SD). The significance of differences was defined at $p < 0.05$. Principal component analysis (PCA) was carried out by the same software in order to connect antioxidant activity assays and the analysed mushrooms.

Results and discussion

Crude extract % yield

Extract yields and resulting antioxidant activities of the mushroom materials strongly depend on the nature of the solvent (used for extraction) due to the presence of different antioxidant compounds with varied chemical characteristics and polarities that may or may not be soluble in a particular solvent [5]. All mushroom species were carried out at the same procedures and the results are given as a percentage of the original weight of the crude sample (Table 1). The best yield was obtained for *B. impolitus* extract (19.08%) while *L. deliciosus* extract had the least one (8.78%). Polar solvents were used for the extraction of phenolic compounds. The most suitable solvents are aqueous mixtures containing ethanol, methanol, acetone, and ethyl acetate (hot or cold) [18]. If we compare these results with the previous research, Dimitrijevic et al. [19] where ethanol is used as a solvent, it could be concluded that methanol is a better solvent and the extracts obtained in this way exhibit a better antioxidant activity.

Table 1. % Crude extract yield of the mushrooms and antioxidant potency composite index (ACI) for six different methods

Mushroom species	Yield (%)	DPPH	ABTS	TRP	CUPRAC	FRAP	TPC	Average
<i>B. edulis</i>	11.86	67.60	37.29	69.04	86.81	60.87	70.72	65.39
<i>B. regius</i>	10.59	100	53.74	100	100	100	100	92.29
<i>L. pseudoscaber</i>	14.49	33.15	14.29	34.73	34.22	37.22	39.06	32.11
<i>B. impolitus</i>	19.08	36.33	17.16	28.29	34.40	52.75	28.03	32.83
<i>L. volemus</i>	9.14	5.56	77.09	13.01	12.12	0	17.19	20.83
<i>L. deliciosus</i>	8.78	7.98	100	29.31	35.86	0	24.09	32.87

Table 2. Antioxidant capacities of selected mushrooms determined by six different methods (mean ± standard deviation, n = 3)

	<i>B. edulis</i>	<i>B. regius</i>	<i>L. pseudoscaber</i>	<i>B. impolitus</i>	<i>L. volemus</i>	<i>L. deliciosus</i>
DPPH	22.3	33.0	10.9	11.9	1.84	2.63
(mg TE/g dw)	±0.1	±0.1	±0.1	±0.1	±0.08	±0.08
ABTS	3.06	4.41	1.17	1.41	6.33	8.21
(mg TE/g dw)	±0.06	±0.07	±0.08	±0.06	±0.08	±0.09
TRP	0.93	1.35	0.467	0.381	0.175	0.394
(mg AAE/g dw)	±0.01	±0.02	±0.006	±0.007	±0.008	±0.004
CUPRAC	32.5	37.5	12.8	12.9	4.54	13.4
(mg TE/g dw)	±0.2	±0.2	±0.1	±0.1	±0.05	±0.1
FRAP	8.17	13.42	4.99	7.08	0	0
(mg Fe/g dw)	±0.05	±0.08	±0.06	±0.08	±0	±0
TPC	172.26	243.6	95.14	68.3	41.9	58.7
(mg GAE/g dw)	±4.01	±5.1	±2.01	±3.9	±1.1	±1.1

Total phenolic content (TPC)

It has been reported that the antioxidant activity was well correlated with the content of phenolic compounds [20]. Phenolic compounds are secondary metabolites that can act as antioxidants due to their ability to donate hydrogen, quench singlet oxygen and act as metal chelators [21]. Therefore, it was important to quantify their contents in mushrooms and the results (expressed as milligram equivalent of gallic acid per gram of dry extract) are presented in Table 2. The highest amount of the phenolic content was found in *B. regius* extract (243.6 mg GAE/g dw) followed by *B. edulis* (172.26 mg GAE/g dw), *L. pseudoscaber* (95.14 mg GAE/g dw), *B. impolitus* (68.3 mg GAE/g dw), *L. deliciosus* (58.7 mg GAE/g dw) and *L. volemus* (41.9 mg GAE/g dw) extracts.

The obtained results confirmed the connection between TPC and the antioxidant activity. Therefore, *B. regius* extract showed the best antioxidant activity in four of the five applied methods. The results for *B. regius* extract achieved in this study were in an agreement with the results of Leal et al. [22].

Antioxidant activities

There is a large number of antioxidant assays which can be used to evaluate the antioxidant activity. The methods based on the reducing power and the ability of "scavenging" are most commonly used for the evaluation of antioxidant activities of extracts. The principal tests include: the Folin–Ciocalteu assay, which is specific to determine phenolic compounds content; the ABTS and DPPH assays, which evaluate the antioxidant power toward inhibition of free radicals, and the ferric reducing antioxidant power (FRAP) assay, based on a redox reaction occurring between the substrate (electron donor) and Fe^{3+} ions (electron acceptor), producing Fe^{2+} ions [23]. To obtain comparable values, the results of ABTS, DPPH and CUPRAC assays were expressed as Trolox equivalents.

Free radical scavenging activity

Free radical scavenging assay has mechanisms based on inhibition of lipid oxidation commonly used to estimate the antioxidant activity. In this study, the radical scavenging of mushroom extracts was tested using a methanol solution of the 'stable' free radical, DPPH [24] and ABTS. The results are shown in Table 2. It can be seen that scavenging activity of six different methanol extracts is significantly different. *Boletus regius* extract showed the strongest DPPH radical scavenging effect (33.0 mg TE/g dw) while the lowest activity of DPPH radical-scavenging was obtained from *L. volemus* extract (1.84 mg TE/g dw). The results obtained by ABTS method showed deviation from the results obtained by DPPH method. Among the tested mushroom samples, the extracts of *L. deliciosus* showed the highest scavenging activity (8.21 mg TE/g dw), followed by the extracts obtained from *L. volemus* (6.33 mg TE/g dw). Pre-

sent ABTS value for *L. deliciosus* was also comparable with the previous report [4].

Total reducing power (TRP) assay

The reducing capacity may be a significant index of the antioxidant activity [10]. The yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of each compound [25]. The results of this assay are presented in Table 2.

By comparing the reducing capacity of six different mushrooms, the methanol extract of *B. regius* showed the highest total reducing power (1.35 mg AAE/g dw), while *L. volemus* showed a much lower value (0.175 mg AAE/g dw).

CUPRAC

In the present study, the antioxidant activity was measured by the CUPRAC method. In this method, the capacity of an antioxidant was evaluated by measuring the produced colour at nearly physiological pH (pH of ammonium-acetate buffer) which is opposite to the acidic conditions (pH 3.6) needed for the FRAP method, or basic conditions (pH 10) necessary for Folin assay [16]. Among methanol extracts of six mushrooms, *B. regius* and *B. edulis* showed the highest value 37.5 and 32.5 mg TE/g dw, respectively (Table 2), just as expected taking into consideration the total phenolic content.

FRAP

The antioxidant activity (FRAP) was evaluated for methanol extracts of *B. regius*, *B. edulis*, *B. impolitus* and *L. pseudoscaber* while it could not be measured for the extracts of *L. volemus* and *L. deliciosus* (Table 2). During mixing the extract with FRAP, the reagent residue appeared. The highest values for *B. regius* extract (13.42 mg Fe/g dw) can be explained by their high level of phenolic compounds in comparison with other analysed mushrooms. Jaworska et al. [26] determined the antioxidant activity of edible mushrooms, including *B. edulis*. Our result for *B. edulis* is in accordance with the value obtained in the mentioned paper.

Antioxidant potency composite index-ACI

Bearing in mind that the antioxidant activity of the analysed mushrooms is expressed in different units, it is hard to compare the same methods; the ACI value equates all units and gives the values that are comparable. The ACI values are shown in Table 1. As it could be seen, *B. regius* showed the best antioxidant capacity, 92.29 according to all six applied methods. *L. volemus* showed the lowest ACI value (20.83).

The correlation coefficients (R^2) between the estimations of the antioxidant capacity made by different methods (Table 1) and the concentrations of total phenols should be presented.

Statistic analysis

PCA involves a mathematical procedure that identi-

fies patterns in data, and expresses them in such a way as to highlight similarities and differences [27]. So, in this manuscript, PCA was applied to the data set of six different mushroom species (*B. edulis*, *B. regius*, *B. impolitus*, *L. pseudoscaber*, *L. volemus* and *L. deliciosus*) to separate the samples according to similarity of the antioxidant activity. This technique is very useful because it reduces original variables (DPPH, ABTS, CUPRAC, TRP, FRAP and TPC) based on Principal Component and gives the relationship between analyzed mushroom species and applied methods. The results obtained for each method were adopted as columns and the mushroom species as rows. For interpretation of PCA results, the Kaiser criterion [28] has been useful to determine the number of Principal Components.

Two principal components were extracted because they have eigenvalues higher than 1.0, as suggested by the Kaiser criterion. Eigenvalues give the measure of the variance accounted by the corresponding eigenvectors (components) [29]. Principal component 1 (PC1) explained up to 81.32% of total variance and PC2 explained 17.09% (Figure 1a and 1b).

Figure 1a. Score plot obtained from PCA of the antioxidant activity and the phenolic content of methanol extracts of six wild mushroom species.

DPPH, FRAP, CUPRAC, TRP and TPC are strongly positive correlated and grouped on the left side of the plot (Figure 1a). ABTS is found in opposition to DPPH, FRAP, CUPRAC, TRP and TPC. *B. regius* is on the negative side of the plot from all others because it has the highest negative loadings on PC1 (-3.46). It is expected because this mushroom was proved to have the highest antioxidant activity and therefore stands out from other mushrooms. In contrast, *L. deliciosus* has the highest negative loadings on PC2 (-1.31). *B. regius* and *B. edulis* are located on the third quadrants (Figure 1a) and this suggests that these mushrooms have the highest antioxidant activity (Table 2). In contrast, *B. regius* and *B. edulis* have low values for ABTS, which is collocated diametrically opposite to DPPH, FRAP, CUPRAC, TRP and

TPC. The location of ABTS in the loading plot confirmed no significant correlation with other assays. *L. deliciosus* and *L. volemus* are located diametrically opposite to *B. regius* and *B. edulis* indicating a low antioxidant activity but a high value of ABTS assay. The use of score (Figure 1a) and loading plots (Figure 1b) from the principal component analysis allowed characterization of the location sampling of mushrooms based on their antioxidant activity and total phenolic concentration.

Figure 1b. Loading plot obtained from PCA of five antioxidant tests of methanol extracts of six wild mushroom species.

Conclusion

As far as our literature survey could be certain, there is no report on the antioxidant activity of *B. impolitus* in the literature. Therefore, the data given for the mushroom here could be assumed as the first report on this topic.

In conclusion, the results reveal that the investigated wild mushroom extracts possess the antioxidant activity against various antioxidant systems. The highest total phenols content, the highest scavenging activity and the highest reductive capabilities have been determined for the *B. regius* mushroom extract. Studies thus provide the precise antioxidant status of six mushroom species (*Boletus edulis* Bull., *Boletus regius* Krombh., *Leccinum pseudoscaber* Kallenb., *Boletus impolitus* Fr., *Lactarius volemus* Fr., and *Lactarius deliciosus* (L.) Gray) and can serve as a useful database for the selection of mushrooms as a natural antioxidant.

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Izvod

PROVERA ANTIOKSIDATIVNE AKTIVNOSTI ŠEST VRSTA DIVLJIH PEČURAKA

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U ovom radu prikazani su rezultati ispitivanja antioksidativne aktivnosti šest vrsta divljih pečuraka (*Boletus edulis* Bull., *Boletus regius* Krombh., *Leccinum pseudoscaber* Kallenb., *Boletus impolitus* Fr., *Lactarius volemus* Fr., and *Lactarius deliciosus* (L.) Gray) poreklom iz Srbije. *Boletus regius* je pečurka koja je pokazala najbolje rezultate u skoro svim primenjenim testovima, 33.0 ± 0.1 mg TE/g za DPPH test; 1.35 ± 0.02 mg AAE/g za TRP; 13.42 ± 0.08 mg Fe/g za FRAP; 37.5 ± 0.2 mg TE/g za CUPRAC; 243.1 ± 5.1 mg GAE/g za TPC). Sa druge strane, *Lactarius volemus* je vrsta koja pokazuje najmanju antioksidativnu aktivnosti. Na osnovu dobijenih vrednosti može se zaključiti da analizirane vrste pečuraka poseduju redukcijske sposobnosti što ih čini dobrim izvorom prirodnih antioksidanasa. Rad je upotpunjen hemometrijskom analizom kako bi se bolje sagledala veza između antioksidativne aktivnosti i analiziranih vrsta pečuraka.

Ključne reči: pečurke, antioksidativna aktivnost, sadržaj fenola, hemometrijska analiza.