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Screening of antioxidant, antimicrobial and antiradical activities of twelve selected Serbian wild mushrooms

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This study was undertaken to evaluate and compare the antioxidant, antimicrobial and antiradical activities of twelve wild edible mushrooms from Serbia. The antioxidant activity was evaluated by five different methods: DPPH, ABTS, total reducing power (TRP), ferric reducing antioxidant power (FRAP), and cupric reducing antioxidant capacity (CUPRAC). Folin–Ciocalteu total phenolic compounds (TPC) were also determined. The present study shows that the ethanol extract of *Boletus regius* has the highest antioxidant values (10.997 ± 0.891 mg mL^{-1} in DPPH; 0.771 ± 0.004 mg AAE per mg dw in TRP; 56.924 ± 0.022 mmol Fe per mg dw in FRAP; 21.738 ± 0.108 mg TE per mg dw in CUPRAC; 173.125 ± 0.475 mg GAE per mg dw in TPC). This is the first report on the evaluation of the antioxidant activity of ethanol extracts of mushrooms by the CUPRAC method. The *in vitro* antimicrobial activity was investigated by the microdilution method. This is the first report on the antimicrobial activity for many analyzed mushroom species. The highest antiradical activity unit (EAU515) was from *Polyporus squamosus* (6.349 EAU515). We determined the antioxidant capacity and the antioxidant potency composite index (ACI) was calculated. Agglomerative hierarchical clustering (AHC) was applied to investigate the similarities between the used methods for antioxidant activities. PCA was used for determining the number of variables to explain the observed variances in the antioxidant activity data of the examined wild mushroom species.

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Introduction

Reactive radical species are unstable and very reactive molecule species because they have an unpaired electron in their structure. According to the chemical structure, they can be atoms, molecules and ions, and they all belong to the large family of free radicals. It is known that reactive oxygen species (ROS) and other pro-oxidant species are the by-products of very important biochemical processes of aerobic organisms. Normal cellular metabolism and body functions of aerobic organisms require a balance between free radical production and their elimination.[1] In the case of excess of free radicals in humans, they could cause smash-up of cells by chain reactions, such as, lipid peroxidation, DNA transformation, *etc.* possibly provoking a number of pathological states. Antioxidants are compounds that, in small quantities, are capable of inhibiting the oxidation of lipids, proteins, carbohydrates and DNA in humans. Thus, consuming foods rich in antioxidants such as mushrooms helps the endogenous defense system to reduce oxidative damage.[2]

Nowadays there has been increasing interest in discovering natural antioxidants, especially those of plant origin. Natural antioxidants derived from plants, chiefly phenolic, are of considerable interest as dietary supplements or food preservatives.[3]

Recent studies have shown that fungi are very significant sources of antioxidant components. We are witnesses of the golden age of mycology, the rapid raise of the science of mushrooms that began in the twenties of the last century. The reason for the great interest in this type of food in many countries, mainly in Europe and Asia, is their excellent taste and high nutritional value. However, awareness of mushrooms as an important source of biologically active substances with medicinal values has only recently emerged. Mushrooms contain large amounts of essential minerals necessary for human health, useful proteins, fibers and vitamins, while the fat content is very low. The presence of certain enzymes and fibers in mushrooms may lower the cholesterol level in blood, while the production of antiviral and other proteins protect and regenerate human cells.

A variety of mushroom secondary metabolites allows the possibility of their use for therapeutic purposes. Many edible

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mushrooms (mostly *Basidiomycetes*) are good sources of carbohydrates such as β-glucans; phenolic compounds such as tocopherols; B-vitamins such as niacin, flavin and pyridoxine; organic acids such as ascorbate, shikimate, malate and fumarate; monoterpenoid and diterpenoid; lipids; proteins such as hydrophobins and trace elements such as selenium.[4] Mushrooms are very good at preventing fungal infections, because they act as some sort of antibiotics. Some wild grown mushrooms possess antioxidant activity, which is in correlation with their total phenolic content. The antioxidant activity of phenolic compounds is related to the capture of free radicals and binding of metal ions, which are precursors in the formation of free radicals.[5]

Environmental conditions in Serbia contribute to the enabled high versatility of wild edible fungi, some of which with great gastronomic significance. Mushrooms such as *Boletus edulis*, *Cantharellus cibarius*, *Craterellus cornucopioide*, *Amanita caesarea* and *Morchella esculenta* are very popular in Serbian cuisine. Mushrooms are mainly used as food, because of their extraordinary flavor. In addition, their nutritional values are often accompanied by delicious taste and medicinal properties, such as *Morchella esculenta* (morel), and it is rightfully recognized as the gift of nature among people. It contains high amounts of antioxidants and fibers, improves liver functions, and has a beneficial effect on the cardiovascular system. In addition to its great taste, *Cantharellus cibarius* also possesses medicinal properties. It contains essential amino acids and vitamin A, increases the body's resistance to infections of the respiratory tract and strengthens the immune system. *Boletus* has always been the most famous and most respected mushrooms for food, and *Boletus edulis* is considered among the most delicious boletus mushrooms that can be consumed in many ways. Considering a great number of *Boletus* species, poisonous and non-poisonous, the majority of mushroom collectors usually harvest several species (*Boletus edulis*, *Boletus regius* and *Boletus appendiculatus*), recognizing them as edible without differentiation on the species level. *Craterellus cornucopioide* has a unique look, no toxic twins, and it is almost impossible to misidentify it with a poisonous one. It is a great treat, especially good when dried, ground into powder and used as a spice. Interestingly, it was noticed that last year's *Craterellus cornucopioide* was a great collector of radioisotope released from a nuclear power plant in Chernobyl. The mushroom *Amanita caesarea* was known in ancient Rome and was highly regarded. *Polyporus squamosus* and *Clitocybe odora* are commonly used as a spice. The wild *Russula virescens's* distinct effects on the regulation of blood lipids, anti-oxidation[6] and antitumor activity[7] were reported.

In general, detailed chemical composition of many wild edible mushroom species from Serbia is not still elucidated as well as their biological activities, though they constitute an important place in a healthy diet, widespread appreciated among people. Considering that mushrooms contain compounds with a wide range of antioxidant and antimicrobial activities, the present study is dedicated to the collection of data on antioxidant and antimicrobial activities of the ethanol extracts of the 12 edible mushroom species, widely used in diet to examine if there are real reasons for their appreciation by folk. To the best of our knowledge, the present paper is the first one about antioxidant and antimicrobial potential of the tested species from Serbia.

Also, as far as we know, there are no reports of the CUPRAC method for mushroom samples, so this is the first one. Also, it should be noted that *B. appendiculatus*, *B. regius*, *C. cornucopioides*, *R. virescens*, *C. odore*, *L. volemus* and *P. squamosus* were analyzed for the first time against tested pathogenic strains of bacteria.

Results and discussion

Crude extract % yield

3 g finely ground mushroom was extracted by ultrasonication four times with 95% ethanol, evaporated to dryness and weighed to obtain the yield. The same procedures were carried out for all investigated mushroom species and the results are given as a percentage of the original weight of the crude sample (Table 3). The yield of ethanol extracts was the highest for *A. caesarea* (6.90%) while the extract of *P. squamosus* had the smallest yield (2.11%). However, the extract yields and resulting antioxidant activities of the plant materials are strongly dependent on the nature of the extracting solvent, due to the presence of different antioxidant compounds of varied chemical characteristics and polarities that may or may not be soluble in a particular solvent.[8]

Total phenolic content (TPC)

Phenolic compounds are well known as secondary metabolites commonly found in plants and mushrooms and reported to have vital biological functions including antioxidant activity.[9] Based on the phenolic content in mushrooms, we can predict the antioxidant activity. The phenolic content in the mushroom extract varies among the analyzed species. The values measured with the Folin–Ciocalteu reagent and expressed in gallic acid or other phenolic compound equivalents are generally accepted as representation of the total phenolic content (TPC) although it is not fully correct: the Folin–Ciocalteu reagent reacts not only with phenolic compounds but also with other reducing ability possessing compounds in the reaction system.[10] This suggests that the antioxidant activity of the tested mushrooms is due to the entirety of different types of antioxidants they contain, not just one specific type.[1] The results of the present study are shown in Table 2. The highest total phenolic content (173.125 ± 0.475 μg GAE per mg dw) was found in mushroom *B. regius*, and the lowest was found (9.362 ± 0.960 μg GAE per mg dw) in *L. volemus*. Accordingly Leal[11] *B. regius* gave the best results in all the antioxidant activity assays, with the highest reducing power measured by Folin–Ciocalteu (30.21 mg GAE per g extract). Different phenolic compounds contribute to the antioxidant activity of mushrooms. Reviewing different HPLC studies for the analysed species, we concluded that mushrooms could contain the following phenolic acid: protocatechuic acid (*A. caesarea*,[12] *L. volemus*,[12] *C. odora*,[13] *B. edulis*,[14] *C. cibarius*,[14] *B. regius*,[15] *C. cornucopioides*,[15]), *p*-hydroxybenzoic acid (*A.*

Table 1 Species, common names, families and edibility of mushrooms

Species of mushrooms	Common name	Family	Edibility
Amanita caesarea	Caesar's mushroom	*Amanitaceae*	One of the most delicious mushrooms, collected at large for commercial purposes
Boletus appendiculatus	Butter bolete	*Boletaceae*	Edible and priced fungus
Boletus edulis	Porcin	*Boletaceae*	Edible and very aromatic fungus when dried, collected at large for commercial purposes
Boletus regius	Royal bolete	*Boletaceae*	Edible and priced fungus
Leccinum pseudoscaber	Hazel bolete	*Boletaceae*	Edible, difficult to digest when eaten raw
Cantharellus cibarius	Chatnarelle	*Cantharellaceae*	Edible and priced fungus, collected at large for commercial purposes
Craterellus cornucopioides	Black chanterelle	*Cantharellaceae*	Great treat, very good when dried, used as a spice
Morchella esculenta	Morel	*Morchellaceae*	Widely used fungus, rich in antioxidants
Polyporus squamosus	Dryad's saddle	*Polyporaceae*	Commonly used as a spice
Lactarius volemus	Lattariovolemo	*Russulaceae*	Edible but has tendency to develop a fishy odor
Russula virescens	Green brittlegili	*Russulaceae*	Edible fungi which shows anti-inflammatory activity
Clitocybe odora	Aniseed toadstool	*Tricholomataceae*	Commonly used as a spice

caesarea,[12] *L. volemus*,[12] *C. odora*,[13] *B. edulis*,[14] *C. cibarius*,[14] *B. regius*[15]), *p*-Coumaric acid (*A. caesarea*,[12] *L. volemus*,[12] *C. odora*[13]), cinnamic acid (*C. odora*,[13] *C. cibarius*[14]), homogentisic acid (*B. regius*,[15] *C. cornucopioides*[15]), ferullic acid (*R. virescens*[16]), gallic acid (*B. edulis*[15]), vanillic acid (*C. cibarius*,[14] *R. virescens*[16]), sinapic acid (*C. cibarius*[14]), ursolic acid (R. *virescens*[16]).

Free radical scavenging activity

Radical scavenging activities of twelve different ethanol extracts were tested in DPPH and ABTS scavenging assays and the results are presented in Fig. 1. The effective concentration at 50% inhibition (EC_{50}) (mg various extracts per mL) was the parameter used to compare the radical scavenging activity. The effectiveness of antioxidant properties is inversely correlated with their EC_{50} values.[17] A lower EC_{50} value indicates higher activity. EC_{50} values of all mushroom extracts ranged from 10.997–51.159 mg mL^{-1}. *B. regius* has the highest ability to decrease the initial DPPH radical concentration by 50% ($EC_{50} = 10.997 \pm 0.891$ mg mL^{-1}, followed by *B. appendiculatus* ($EC_{50} = 11.314 \pm 0.842$ mg mL^{-1}), while the least ability was from *C. cibarius* ($EC_{50} = 51.159 \pm 1.012$ mg mL^{-1}). It is important to note that the species of the same genus (*B. regius*, *B. appendiculatus* and *B. edulis*) show a similar radical scavenging ability, higher than other analyzed species (90.93%, 88.38% and 78.75%, respectively). Our RSC-DPPH value for *P. squamosus* (45.33%) was similar to those recorded by Keleş[17] who reported 43.30% of inhibition of the DPPH radical for the same mushroom. Leal[11] reported that *B. regius* had the scavenging activity (EC_{50} value = 2.06 mg mL^{-1}). The antioxidant activity of mushrooms, determined by ABTS assay, is shown in Fig. 1. The results obtained by the ABTS method showed deviation from the results of other methods. Values for ABTS scavenging activity varied between ($EC_{50} = 5.034 \pm 0.045$ mg mL^{-1}), for *L. pseudoscaber* to ($EC_{50} = 16.394 \pm 0.475$ mg mL^{-1}) for *L. volemus.* From Fig. 1 it is obvious that the highest amount of antioxidants was found in *L. pseudoscaber*. Although this type of mushroom belongs to another genus, it is still the same

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

Mushroom species	CUPRAC μg TE/1 mg dw	TRP mg AAE/1 mg dw	FRAP μmol Fe/1 mg dw	TPC μg GAE/1 mg dw
Amanita caesarea	9.573 ± 0.006	0.230 ± 0.001	10.690 ± 0.202	50.147 ± 0.703
Boletus appendiculatus	15.576 ± 0.012	0.595 ± 0.002	43.878 ± 0.042	122.788 ± 0.939
Boletus edulis	7.727 ± 0.024	0.291 ± 0.006	6.715 ± 0.085	73.805 ± 1.886
Boletus regius	21.738 ± 0.108	0.771 ± 0.004	56.924 ± 0.022	173.125 ± 0.475
Leccinum pseudoscaber	11.683 ± 0.032	0.235 ± 0.005	14.867 ± 0.021	70.404 ± 0.766
Cantharellus cibarius	10.137 ± 0.052	0.191 ± 0.012	5.744 ± 0.052	22.589 ± 1.032
Craterellus cornucopioides	10.569 ± 0.019	0.205 ± 0.005	11.958 ± 0.072	43.964 ± 0.926
Morchella esculenta	11.860 ± 0.072	0.449 ± 0.010	23.347 ± 0.006	100.484 ± 2.584
Polyporus squamosus	15.481 ± 0.132	0.249 ± 0.007	23.340 ± 0.046	49.745 ± 0.695
Lactarius volemus	4.164 ± 0.012	0.172 ± 0.001	3.147 ± 0.064	9.362 ± 0.960
Russula virescens	10.558 ± 0.018	0.236 ± 0.011	5.402 ± 0.021	36.849 ± 1.165
Clitocybe odora	5.911 ± 0.031	0.187 ± 0.009	3.614 ± 0.045	38.112 ± 0.251

Table 3 % Crude extract yield of the mushrooms and antiradical activity values

Mushroom species	Yield of extraction (Y%)	Antiradical activity (AU_{515})	The number of antiradical activity unites in 1 mg of extract (EAU_{515})	Number of antiradical activity units in the extract (PAU_{515})
Amanita caesarea	6.90	0.23	3.27	681.23
Boletus appendiculatus	4.16	0.06	1.54	187.31
Boletus edulis	5.88	0.20	3.49	618.59
Boletus regius	6.10	0.06	1.08	190.69
Leccinum pseudoscaber	6.80	0.12	1.81	370.72
Cantharellus cibarius	4.51	0.05	0.99	138.79
Craterellus cornucopioides	3.61	0.10	5.13	630.91
Morchella esculenta	2.59	0.23	5.49	1121.21
Polyporus squamosus	2.11	0.13	6.35	820.32
Lactarius volemus	4.12	0.04	0.89	114.23
Russula virescens	3.52	0.13	3.64	380.49
Clitocybe odora	5.65	0.13	2.24	390.00

Boletaceae family like *B. regius*, *B. edulis* and *B. appendiculatus* and so this result is expected. The high scavenging ability of ethanol extracts may be attributed to the high level of antioxidant components in the extract, which react rapidly with DPPH radicals and reduce most DPPH radical molecules.[18]

Ferric-reducing antioxidant power (FRAP) assay

Also, the antioxidant activity was measured by the FRAP method, which measures the capacity of an antioxidant to reduce a Fe^{3+} – TPTZ complex to Fe^{2+} – TPTZ complex[17] and the results are presented in Table 2. It is a simple and reproducible method which must be performed under acidic conditions (pH = 3.6). The obtained results confirmed the highest antioxidant activity for *B. regius* (56.924 ± 0.022 μmol Fe per mg dw). The FRAP value for *L. volemus* (3.147 ± 0.064 μmol Fe per mg dw) is nearly 20-times lower than the FRAP value for *B. regius* and this mushroom showed the lowest activity. When the antioxidant activity values of the wild mushrooms are compared with those of fruits, it was observed that mushrooms presented higher antioxidant activity than some fruits (aronia, raspberry, blackberry, cherry, and blackthorn) which ranged from 0.12 to 0.19 μmol Fe per mg dw.[19] When the values of antioxidant activity of *B. edulis* and *L. volemus* (6.723, and 3.141 μmol Fe per mg dw, respectively) determined by the FRAP method is compared with the values obtained by Keleş[17] (52957.14 and 3171.43 μmol g^{-1}) it can be concluded that they are similar.

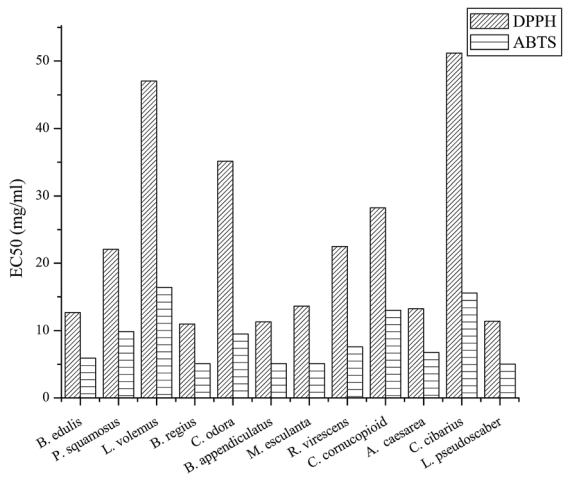

Fig. 1 Free radical-scavenging capacities of the extract measured in DPPH and ABTS assays.

Total reducing power (TRP) assay

The results of the reducing power assay were carried out in triplicate and are summarized in Table 2, expressed as mean values ± standard deviations. High absorbance indicates a high reducing power.[20] The reducing power ability of the ethanol extracts was determined using ascorbic acid as a standard and the results are given as mg ascorbic acid equivalents per mg dry extract weight (mg AAE per mg dw). Compounds with reducing power are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants.[21] Measurements of reducing power, based on measuring the formation of Pearl's Prussian blue at 700 nm, make it possible to determine the concentration of Fe^{3+} ions in the presence of extract. Among the tested mushroom samples, extracts of *B. regius* showed the highest reducing power (0.771 ± 0.004 mg AAE per mg dw), followed by extracts from *B. appendiculatus* (0.595 ± 0.002 mg AAE per mg dw) while other extracts showed a weaker reducing power. These two species have the highest amount of phenolic compounds; therefore show a high reducing power which confirms the TRP method. The reducing capacity may be a significant index of antioxidant activity.[18] Accordingly, the *Boletaceae* family, to which these two species belong, might contain higher amounts of antioxidants, which could react with free radicals to stabilize and block radical chain reactions. It was reported that the reducing power of mushrooms might be due to their hydrogen-donating ability.[22] Various authors prepared extracts in different ways in different concentrations, so it is difficult to compare the obtained results. Mau[23] analyzed *M. esculenta* and the reducing power of the extract, at a concentration of 25 mg mL^{-1}, is 0.97.

Table 4 Antioxidant potency composite index (ACI) for mushrooms by six different methods

Mushroom species	DPPH	ABTS	TPC	CUPRAC	TRP	FRAP	Average
Boletus edulis	88.39	85.52	42.50	35.56	38.09	11.81	50.31
Polyporus squamosus	56.55	51.94	28.72	71.20	32.40	41.02	46.97
Lactarius volemus	33.60	32.03	9.33	19.16	22.33	5.52	20.33
Boletus regius	100.00	98.85	100.00	100.00	100.00	100.00	99.81
Clitocybe odora	40.49	53.75	22.04	27.22	24.28	6.36	29.02
Boletus appendiculatus	97.57	98.68	70.85	71.69	76.93	77.08	82.13
Morchella esculenta	83.13	98.85	57.88	54.50	58.73	41.02	65.68
Russula virescens	55.74	66.92	21.24	48.60	30.45	9.49	38.74
Craterellus cornucopioides	47.10	39.76	25.38	48.65	26.23	20.97	34.68
Amanita caesarea	85.16	74.98	28.99	44.06	29.80	18.98	47.00
Cantharellus cibarius	31.98	33.67	13.21	46.71	24.28	10.08	26.66
Leccinum pseudoscaber	96.90	100.00	40.76	53.73	30.78	26.12	58.05

CUPRAC

The CUPRAC method is a simple and versatile antioxidant capacity assay useful for a wide variety of polyphenols, including phenolic acids, hydroxycinnamic acids, flavonoids, carotenoids, anthocyanins, as well as for thiols, synthetic antioxidants, and vitamins C and E.[24] The CUPRAC reagent is stable, easily accessible, low-cost, and is sensitive toward thiol-type antioxidants unlike FRAP. The reaction is carried out at nearly physiological pH as opposed to the acidic pH of FRAP or to the alkaline pH of Folin methods, constituting a basic advantage for the realistic assay of biological fluids.[25] Tests on antioxidants show that the highest CUPRAC capacity is observed for *B. regius* (21.738 ± 0.108 μg TE per mg dw), followed by *B. appendiculatus* (15.576 ± 0.012 μg TE per mg dw) and the lowest activity was shown by *L. volemus* (4.164 ± 0.012 μg TE per mg dw), just as expected according to the total phenolic content.

Antiradical activity

The numbers of antiradical activity units per 1 mg of extract (EAU_{515}) were calculated for all mushroom species and are shown in Table 3.

The results of antiradical activities show that a mushroom has high antiradical activity comparable to some plant extracts.[26] The highest EAU_{515} units were calculated for the extracts obtained from the *P. squamosus* (EAU_{515} 6.35) and this extract could be considered as an effective antiradical agent, while the lowest units were from *L. volemus* (EAU_{515} 0.89).

Antioxidant potency composite index-ACI

Every method for antioxidant activity has certain advantages and limitations and differs in terms of assay principles. We used six methods to determine antioxidant capacity and in order to give an equal weight to these entire methods the antioxidant potency composite index (ACI) was calculated. The ACI values are shown in Table 4. As it could be seen, *B. regius* showed the best antioxidant capacity, almost 100 (99.81) according to all six employed methods. *L. volemus* had ACI values (20.33), indicating that this mushroom has nearly five times lower antioxidant potency in comparison to *B. regius*.

Antimicrobial activity

The obtained mushroom extracts were screened for their *in vitro* antimicrobial activity against a panel of pathogenic bacterial strains (Table 5). The tested mushroom extracts were not soluble in 10.0% DMSO and this is the reason that 100.0% DMSO was used as a solvent. The negative control (dilutions of 100.0% DMSO) showed inhibitory activity (MIC) at a concentration of 25.0%, and bactericidal activity (MBC) at a concentration of 50.0%, against all tested strains. It was found that different species of mushrooms exhibit different antimicrobial activities. These differences in the antimicrobial activity of different species of mushrooms are probably a consequence of the presence of different components with antimicrobial activity. The inhibitory effect was observed in *B. regius* and *B. appendiculatus* against *Kl. pneumoniae*, *P. aeruginosa*, *S. aureus*, *E. faecalis* and *B. cereus* (MIC in the range of 0.8–6.3 mg mL^{-1}), but there was no bactericidal effect (except against *S. aureus* and *B. cereus* – MBC = 6.3 mg mL^{-1}). Also, the samples *B. edulis*, *L. volemus*, *C. odora* and *M. esculenta* showed a good inhibitory activity (MIC = 0.8–3.1 mg mL^{-1}), against *Kl. pneumoniae*. Significant results can be observed in the activity of samples *P. squamosus*, *L. volemus*, *B. regius*, *C. odora*, *B. appendiculatus*, *M. esculenta* and *R. virescens* against *S. aureus* and samples *B. regius* and *B. appendiculatus* against *B. cereus* (in both cases the MIC/MBC = 3.1–6.3/6.3 mg mL^{-1}).

Statistic analysis

The analysis of total phenolic content, DPPH, ABTS, CUPRAC, TRP, and FRAP of each mushroom species was based on three replications and the results are expressed as mean values ± standard error (SE).

Considering that the results of antioxidant activity measured by different methods are difficult to compare with each other; we have performed all statistical tests with ACI values.

Correlation

At the beginning of the statistical analysis we were calculating the correlation matrix, giving the correlation coefficients between each pair of variables. Each term of the matrix is a

Table 5 Antimicrobial activity of fungal extracts against pathogenic bacterial strains[a] (MIC/MBC in mg mL^{-1})

Bacterial strains	Gram (−) bacteria					Gram (+) bacteria				
Tested samples (MIC/MBC in mg mL^{-1})	*E. coli* ATCC25922	*P. mirabilis* ATCC12453	*S. enteritidis* ATCC13076	*K. pneumoniae* ATCC10031	*P. aeruginosa* ATCC9027	*S. aureus* ATCC8538	*E. faecalis* ATCC19433	*L. monocytogenes* ATCC15313	*B. cereus* ATCC8739	*C. perfringens* ATCC19404
A	12.5/50.0	50.0/>50.0	25.0/25.0	3.1/>50.0	50.0/>50.0	12.5/25.0	50.0/>50.0	12.5/25.0	50.0/>50.0	50.0/>50.0
B	50.0/50.0	50.0/>50.0	25.0/50.0	25.0/>50.0	50.0/>50.0	6.3/6.3	50.0/>50.0	50.0/50.0	50.0/>50.0	50.0/>50.0
C	25.0/50.0	>50.0/>50.0	12.5/25.0	3.1/>50.0	>50.0/>50.0	6.3/6.3	>50.0/>50.0	50.0/50.0	>50.0/>50.0	>50.0/>50.0
D	25.0/50.0	25.0/25.0	12.5/25.0	3.1/25.0	3.1/50.0	3.1/6.3	6.3/12.5	12.5/25.0	3.1/6.3	12.5/25.0
E	50.0/50.0	50.0/>50.0	12.5/25.0	6.3/>50.0	50.0/>50.0	6.3/6.3	50.0/>50.0	50.0/50.0	>50.0/>50.0	>50.0/>50.0
F	12.5/50.0	25.0/50.0	50.0/50.0	0.8/25.0	0.8/25.0	6.3/6.3	6.3/25.0	12.5/25.0	6.3/6.3	12.5/25.0
G	50.0/50.0	50.0/>50.0	>50.0/>50.0	0.8/>50.0	50.0/>50.0	3.1/6.3	50.0/>50.0	50.0/50.0	>50.0/>50.0	25.0/25.0
H	50.0/50.0	50.0/>50.0	50.0/50.0	50.0/>50.0	50.0/>50.0	3.1/6.3	50.0/50.0	50.0/50.0	50.0/>50.0	50.0/>50.0
I	50.0/50.0	50.0/>50.0	50.0/50.0	50.0/50.0	50.0/>50.0	12.5/25.0	50.0/>50.0	50.0/50.0	>50.0/>50.0	50.0/>50.0
J	50.0/50.0	50.0/>50.0	12.5/50.0	25.0/>50.0	50.0/>50.0	12.5/25.0	50.0/50.0	50.0/50.0	50.0/>50.0	50.0/50.0
K	25.0/25.0	50.0/>50.0	12.5/50.0	12.5/>50.0	50.0/>50.0	12.5/25.0	50.0/50.0	50.0/50.0	50.0/>50.0	50.0/50.0
L	50.0/50.0	50.0/>50.0	12.5/50.0	50.0/50.0	50.0/>50.0	12.5/25.0	50.0/50.0	50.0/50.0	50.0/>50.0	25.0/50.0
DMSO (vol%)	12.5/50.0	12.5/50.0	12.5/50.0	12.5/50.0	12.5/50.0	12.5/50.0	12.5/50.0	12.5/50.0	12.5/12.5	12.5/12.5

[a] A – *B. edulis*; B – *P. squamosus*; C – *L. volemus*; D – *B. regius*; E – *C. odora*; F – *B. appendiculatus*; G – *M. esculenta*; H – *R. virescens*; I – *C. cornucopioides*; J – *A. caesarea*; K – *C. cibarius*; L – *L. pseudoscaber*.

number ranging from −1 to +1: the + or − sign indicates a positive or negative interdependence between variables (direction), and the absolute value indicates the strength of the interdependence.[27] In Table 6 is presented the correlation between selected mushrooms with several meaningful correlations. The strongest positive correlations were between *L. pseudoscaber*/*A. caesarea* and *M. esculenta*/*C. odora* ($r = 0.98$, $p < 0.05$). Another group represented by *M. esculenta*/*B. edulis* also displayed a significantly strong positive correlation ($r = 0.96$, $p < 0.05$). On the other hand, negative correlation was found between *B. regius* and all analyzed mushrooms. Negative correlation indicates that there exists an inverse relationship between the observed variables.

PCA

The PCA is a data reduction technique used in determining the number of variables to explain the observed variances in the data.[28] PCA was carried out on 12 mushroom species (*B. edulis*, *P. squamosus*, *L. volemus*, *B. regius*, *C. odora*, *B. appendiculatus*, *M. esculenta*, *R. virescens*, *C. cornucopioides*, *A. caesarea*, *C. cibarius*, and *L. pseudoscaber*) and applied to reduce the number of original variables.

The number of significant principal components was selected on the basis of the Kaiser criterion[29] with eigenvalues higher than 1 (Fig. 2). Eigenvalues give a measure of the variance accounted by the corresponding eigenvectors (components).[30] From the screen plot graph of eigenvalues of the PCA (Fig. 3) it can be seen that the first two PCs are enough to explain 90.67% of the pattern variation.[31]

Two principal components (PCs) were extracted by applying the statistical analysis because they have eigenvalues higher than 1.0, as suggested by the Kaiser criterion. The first principal compound (PC1) explained 74.77% of the variation, while the second principal component (PC2) contributed 15.90%. The PC score contains information on all of the constituents combined into a single number, while the loadings indicate the relative contribution each constituent makes to the score.

The first principal component distinctly separates *B. regius* species from all others on the positive side of the plot, because *B. regius* has the highest positive loadings on PC1 (0.64). It is expected because this mushroom was proved to have high potential antioxidant activity. In contrast, *R. virescens* has the highest negative loadings on PC1 (−0.98). With an increasing positive score, the antioxidant activity of variables increases along the PC axis, while a negative score means that the antioxidant activity of variables decreases along the axis. When the score is near 0 the antioxidant activity is poorly related to the PC axis. The direction of the variable arrows indicates the direction in which the antioxidant activity of the corresponding species increases most, and the length of the arrows equals the rate of change in that direction.[31] *C. cibarius*, *C. copioides* and *P. squamosus* are grouped in the positive side of PC2 and show similar properties. *M. esculenta* has the highest negative loadings on PC2 (−0.35), while *P. squamosus* has the highest positive loadings on the same (0.74).

Table 6 Correlation of twelve mushroom species[ab]

	A	B	C	D	E	F	G	H	I	J	K	L
A	1.00	0.30	**0.90**	−0.57	**0.94**	**0.87**	**0.96**	**0.86**	0.63	**0.95**	0.45	**0.95**
B		1.00	0.45	−0.15	0.40	0.28	0.27	0.66	**0.89**	0.54	**0.86**	0.53
C			1.00	−0.50	**0.90**	**0.82**	**0.88**	**0.91**	0.72	**0.91**	0.66	**0.86**
D				1.00	−0.74	−0.64	−0.77	−0.63	−0.21	−0.51	−0.25	−0.63
E					1.00	**0.80**	**0.98**	**0.95**	0.67	**0.90**	0.61	**0.92**
F						1.00	**0.88**	0.73	0.46	**0.89**	0.27	**0.89**
G							1.00	**0.88**	0.55	**0.90**	0.43	**0.93**
H								1.00	**0.85**	**0.90**	**0.81**	**0.91**
I									1.00	0.78	**0.92**	0.75
J										1.00	0.59	**0.98**
K											1.00	0.55
L												1.00

[a] A – *B. edulis*; B – *P. squamosus*; C – *L. volemus*; D – *B. regius*; E – *C. odora*; F – *B. appendiculatus*; G – *M. esculenta*; H – *R. virescens*; I – *C. cornucopioides*; J – *A. caesarea*; K – *C. cibarius*; L – *L. pseudoscaber*. [b] Bold correlations are significant at $p < 0.05$.

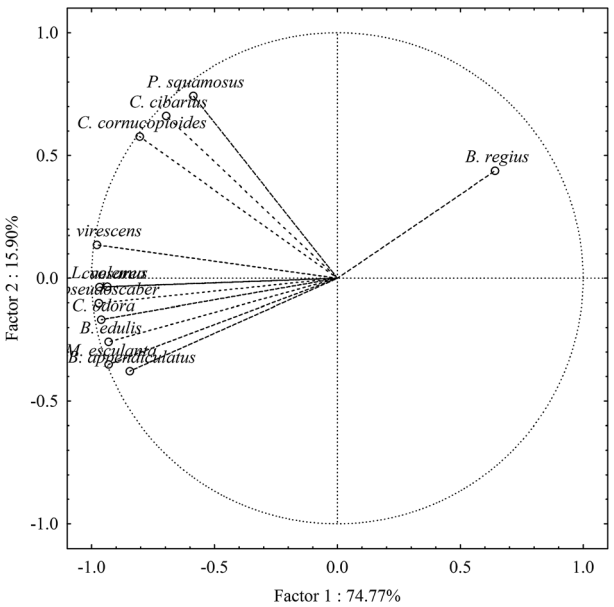

Fig. 2 PCA of mushrooms based on their antioxidant activity.

Agglomerative hierarchical clustering (AHC)

The primary purpose of cluster analysis is to group objects based on the characteristics which they possess. The most similar points are grouped forming one cluster and the process is repeated until all points belong to one cluster.[32] We applied HCA to investigate the similarities between used methods.

HCA calculates the distances (or correlation) between all samples using a defined metric such as the Euclidean distance and Manhattan distance.[33] The most similar methods are first grouped and they are merged according to their similarities. Eventually as the similarity decreases all subgroups are fused into a single cluster.[34] Cluster analysis was carried out by Ward's method using Euclidean distances as a measure of similarity. Ward's method minimizes the sum of squares of any two (hypothetical) clusters that can be formed at each step. The linkage distance is reported as D_{link}/D_{max}. The Euclidean distance is the most common way to measure the distance between objects.

$$\text{Distance}(x, y) = \left\{ \sum_i (x_i - y_i)^2 \right\}^{1/2}$$

Hierarchical cluster analysis (HCA) was applied to five methods (DPPH, ABTS, FRAP, CUPRAC, and TRP) and they are grouped in clusters based on their similarities. The results obtained following HCA are shown as a dendrogram (Fig. 4) and the methods are grouped in two statistically significant clusters at $(D_{link}/D_{max}) \times 100 < 50$. Methods for determining radical scavenger capacity can be classified by the ability to donate hydrogen atoms or electron transfer, so the tendency to form groups arising from common analytical characteristics is clear in this data analysis procedure.

The first cluster contains DPPH and ABTS methods while the second cluster contains TRP, FRAP and CUPRAC methods. The

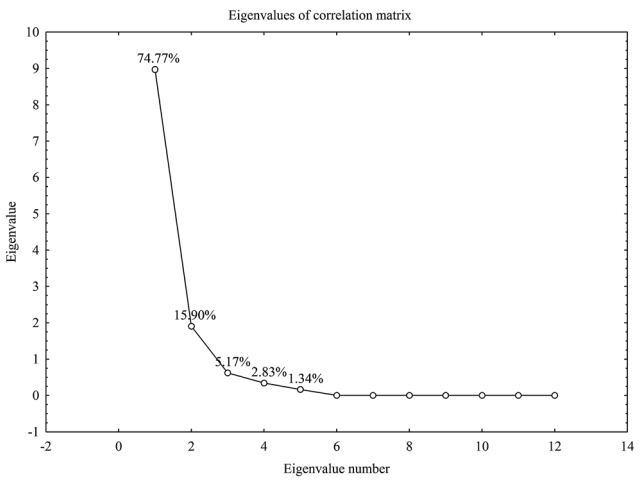

Fig. 3 Eigenvalues of the correlation matrix.

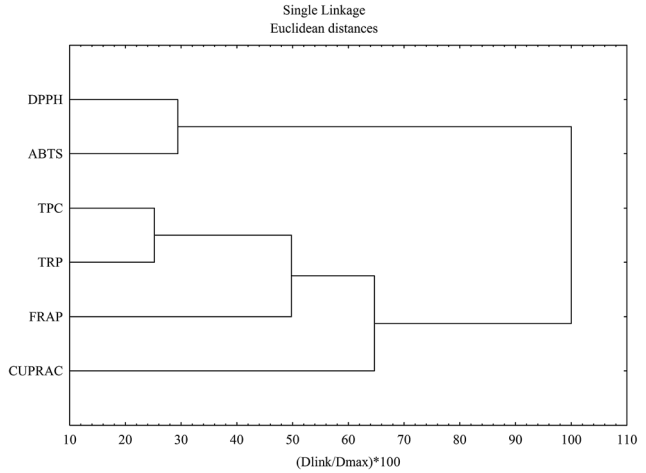

Fig. 4 Dendrogram showing clustering of five different methods.

strongest clustering is observed for ABTS and DPPH (27). The reaction mechanism of these methods is based on the hydrogen transfer reaction. The second cluster is divided in two sub clusters and in the second cluster the strongest subclustering is observed for TRP-FRAP (50).

For comparison of data between the two groups, the *t*-test was carried out to detect any significant differences ($p < 0.05$).

Materials and methods

Samples

The mushroom samples were collected in a rural unpolluted region near town Nis, Republic Serbia, during July and August of 2014. To overcome variability, such as the stage of development, soil texture and environmental conditions, the mushroom samples were collected at five experimental points and from every experimental point 300 g of each examined mushroom species (total 1500 g for each mushroom species) was taken. Mushrooms were cleaned of all surface contamination using a little brush. After collection and taxonomic identification, the mushrooms were cut and dried at room temperature. They were stored in polyethylene bags until the analysis started. Species, family and edibility of mushrooms used in this work are given in Table 1.

Chemicals and instruments

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), iron(III) hloride hexahydrate, Folin–Ciocalteu reagent, gallic acid (3,4,5-trihydroxybenzoic acid), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ascorbic acid and methanol were purchased from Sigma Co. St. Louis, Missouri, USA.

The following chemical substances of analytical grade were supplied from the corresponding sources: neocuproine (2,9-dimethyl-1,10-phenanthroline), copper(II) chloride dihydrate, $NaCO_3$, HCl, 2,4,2-tri(2-pyridyl)-s-triazine (TPTZ), $K_3[Fe(CN)_6]$, phosphate buffer (NaH_2PO_4–Na_2HPO_4), ammonium acetate buffer, CCl_3COOH, $K_2S_2O_8$, $FeSO_4 \cdot 7H_2O$, and DMSO (dimethyl sulphoxide) 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

A fine dried mushroom (3 g) was ground to powder and extracted with 95% of ethanol. Extraction was performed four times in an ultrasonic bath for 15 minutes. The temperature was maintained at 25 °C. Extracts were filtered and then evaporated to dryness (35 °C) under reduced pressure in a rotary evaporator. Extracts were dissolved in dimethyl sulphoxide (DMSO).

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

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

where W_1 is the weight of the evaporated extract and W_2 is the weight of the grinded mushroom powder.

Antioxidant activity assays

DPPH "scavenging" radical capacity. The quantitative assays of the ethanol extracts on DPPH radicals were performed according to the method of Mitic[19] with some modifications. Stable DPPH radicals are widely used to evaluate the antioxidant activities of proton-donating substances according to the hydrogen donating ability.[18] 1.5 mL of methanol solution of the DPPH radical, in the concentration of 100 μmol L^{-1}, 0.1 mL of extract, concentration of 20 mg mL^{-1}, and methanol to a total volume of 4 mL, were placed in a test tube. The mixture was shaken and after 60 min in the dark, the absorbance was measured at 515 nm with a spectrophotometer. All determinations were performed in triplicate. The DPPH radical is a long-lived organic nitrogen radical with a deep purple color.[35] Radical "scavenging" capacity (RSC) was determined from the calibration curve, which was drawn by using Trolox as the positive control, in concentration 1–16 μmol L^{-1}, and expressed in μg of Trolox equivalents (TE) per mg dry extract weight (μg TE per mg dw). DPPH radical "scavenging" capacity was expressed by applying the following equation:

$$\text{DPPH} - \text{RSC}(\%) = 100 \times \left(\frac{A_0 - A_1}{A_0}\right)$$

where A_0 – absorbance of blank solution and A_1 – absorbance of solution in the presence of active components.

RSC values were used to obtain the mushroom extract amount necessary to decrease the initial DPPH concentration by 50% (EC_{50}) using an exponential curve.

ABTS radical "scavenging" activity. ABTS radical "scavenging" activity was performed according to the method of Re.[36] The ABTS radical was produced by the reaction of ABTS stock solution with potassium persulfate and the mixture was allowed to stand in the dark at room temperature for 12–16 h before use.

The solution was then diluted by mixing 7 mL $ABTS^{\bullet+}$ solution with 120 mL methanol to obtain an absorbance of 0.7 ± 0.02 units at 734 nm. An aliquot of each extract, concentration 20 mg mL^{-1}, was mixed with 1.8 mL of diluted ABTS solution in the concentration of 7 mmol L^{-1} and diluted with methanol to a total volume of 4 mL. After 6 min at room temperature, the reduction in absorbance was measured at 734 nm. Results are expressed as μg of Trolox equivalents (TE) per mg dry extract weight (μg TE per mg dw).

Ferric-reducing antioxidant power (FRAP) assay. Ferric reducing antioxidant power assay was performed using the method of Benzie and Strain[37] 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 color change during absorption at 595 nm. 1 mL of prepared FRAP reagent is mixed with 0.05 mL of sample, concentration 20 mg mL^{-1}, and diluted with water to make up a volume of 4 mL. After 5 min incubation at 37 °C, the absorbance is recorded at 595 nm. FRAP values expressed as μmol of Fe(II) equivalents per mg dry weight (μmol Fe per mg dw) were obtained by comparing the absorption change in the test mixture with doses obtained from the Fe(II) standard calibration curve.

Total reducing power (TRP) assay. The reducing power of extracts was determined by the method of Oyaizu.[38] This method is based on the ability of antioxidants to reduce Fe(III) hexacyanate to Fe(II) hexacyanate which leads to an increase in the absorbance of the reaction mixtures. The increase in the absorbance indicates an increase in the antioxidant activity.[39] Reaction mixtures were prepared by mixing 0.01 mL of extract, 1 mL of 1% solution $K_3[Fe(CN)_6]$, phosphate buffer (pH 6.6) and water. The mixtures were incubated at 50 °C for 30 minutes and then we added 1 mL 10% solution of trichloroacetic acid and 0.6 mL $FeCl_3$. The absorbance was measured at 700 nm against the blank sample and the results were expressed as mg ascorbic acid equivalents per mg of dry extract weight (mg AAE per mg dw).

Total phenolic content (TPC). The total phenolic content was determined using the Folin–Ciocalteu reagent as originally described by Singleton and Orthofer.[40] Briefly, 0.05 mL of the extract was mixed with 0.5 mL of Folin–Ciocalteu reagents, 2 mL sodium carbonate solution and diluted with water to a total volume of 7.55 mL. The reaction was carried out in the dark for 30 min, and then the absorbance was measured at 750 nm. Gallic acid was used to calculate the standard curve and the results were expressed as μg gallic acid equivalents (GAE) per mg of dry weight (μg GAE per mg dw).

Cupric reducing antioxidant capacity (CUPRAC) assay. The CUPRAC assay was performed using the method of Apak.[25] 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 method is capable of determining hydrophilic and lipophilic antioxidants. To a test tube were added 0.05 mL of extract, 1 mL of phosphate buffer (pH 7.0), neocuproine, copper(II) chloride and diluted with water to a total volume of 4.1 mL. The mixture was left for 30 min at room temperature and after that absorbance was measured at 450 nm. Trolox was used as a standard and results were expressed as μg Trolox equivalents per mg of dry weight (μg TE per mg dw).

Determination of antiradical activity. The anti-radical activity of the extracts was performed according to the method of Brand-Williams,[41] with some modifications. The determination of antiradical activity was based on the principle behind that the DPPH radical in its radical form has a characteristic absorbance at 515 nm which disappears after its reduction by an antiradical compound.[1] 0.53 mL mushroom extract, concentration 50 mg mL^{-1} and 3.470 mL freshly prepared solution of DPPH radical, in concentration 100 μmol L^{-1}, were placed in a test tube. The absorbance was measured at 515 nm at 0 and after 1 min of the reaction. The blank sample was prepared in the same way, only instead of the extract we added 0.53 mL DMSO and the absorbance was measured at 515 nm at 0 and after 1 min of the reaction. The antiradical activity of each extract was calculated as:

$$AU_{515} = (A_0 - A_1) - (A_{0C} - A_{1C})$$

where AU_{515} – antiradical activity of the extract, A_0 – the absorbance of the sample at 0 min, A_1 – the absorbance of the sample after 1 min of the reaction, A_{0C} – the absorbance of blank sample at 0 min, and A_{1C} – the absorbance of the blank sample after 1 min.

The antiradical activity of the mushroom extracts was described by the antiradical activity unit which allows the antiradical activities to be compared. The antiradical activity units were calculated per 1 mg of extract.

$$EAU_{515} = \frac{AU_{515}}{I_e}$$

where EAU_{515} – number of antiradical activity units, I_e – amount of extract in the sample (mg), and AU_{515} – antiradical activity of the extract.

The total number of antiradical activity units in the extract was calculated as follows:

$$PAU_{515} = \frac{Cle \times AU_{515}}{I_e}$$

where Cle – total amount of extract (mg), and I_e – amount of extract in the measured sample.

Micro-well dilution assay. The mushroom extract samples were tested against a panel of pathogenic bacterial strains obtained from the American Type Culture Collection (ATCC), using a micro-well dilution dssay (NCCLS 2003, with some modifications). Bacterial suspensions were prepared from overnight culture in sterile saline (0.9% NaCl) and their optical density was standardized to 0.5 McFarland. Stock solutions of fungal extracts (100.0 mg mL^{-1}) were prepared in 100.0% dimethylsulfoxide (DMSO) and serial dilutions tested in the range of 0.02 to 50.0 mg mL^{-1} in a 96/well microtiter plate with inoculated Mueller–Hinton broth (MHB). The final volume was 100 μL and the final bacterial suspension was 106 CFU mL^{-1} in each well. Serial dilutions of DMSO (100.0%) served as a negative control. The plates were cultivated at 37 °C for 24 h and after that the broth was taken from each well without visible growth

of bacteria, inoculated in Mueller Hinton agar (MHA) and incubated at 37 °C for 24 h. Minimum inhibitory concentration – MIC (concentration of samples without visible growth) and minimal bactericidal concentration – MBC (concentration of samples that killed 99.9% of bacterial cells on MHA agar plate) determinations were carried out in triplicate.

Antioxidant potency composite index ACI. The results of antioxidant activity measured by different methods are difficult to compare with each other, primarily because of the different mechanisms of action, different units used to express the results and the very different techniques for the preparation of the investigated samples. To reduce the results of antioxidant activities obtained by different methods at comparable values, we calculated the total antioxidant index (ACI – Eng. 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 be 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 sample was then taken for the antioxidant potency composite index.[42]

Statistic analysis

The evaluation of the obtained analytical data was performed by statistical means. The elimination of outliers was carried out by Grubb's test. All data were reported as the mean ± standard deviation of three replicates. PCA is a linear, unsupervised and pattern recognition technique used for analyzing, classifying and reducing the dimensionality of numerical datasets in a multivariate problem.[43]

Cluster analysis (CA), an unsupervised classification, was performed to measure the similarity between objects. Principal component analysis provides the weights needed to get the new variable that best explains the variation in the whole dataset in a certain sense.[44,45] PCA is a statistical tool which transforms a set of original variables into new uncorrelated variables. PCA results in a smaller number of newly derived variables, called principal components (PC), that explain the maximum variance in data used, allowing us to recognize cluster trends in the data by the distribution of PC scores and to identify the variables responsible for the discrimination pattern through PC loadings.[46] The first Principal Component (PC) always describes the statistical relationship that accounts for the greatest amount of sample variation and the following PC successively explains smaller parts of the original variance.[47] Cluster analysis (CA) is a multivariate technique with the purpose of classifying the objects of the system into categories or clusters based on their similarities.[34] The results obtained by cluster analysis are typically shown by a dendrogram. Cluster analysis was carried out by Ward's method using Euclidean distances as a measure of similarity.

Multivariate analysis including principal component analysis (PCA) and cluster analyses (CA) was performed using a statistical package running on a computer (Statistica 8.0, StatSoft, Tulsa, Oklahoma, USA). A probability level of $p < 0.05$ was considered statistically significant.[48] Correlation between mushrooms was established using regression analysis at a 95% significance level ($p \leq 0.05$).

Conclusion

According to the results of this study, it is clearly indicated that the ethanolic extracts of tested mushroom species have significant antioxidant activity against various antioxidant systems *in vitro*. The tested mushroom extracts have a strong antioxidant activity against various oxidative systems *in vitro*. The intensity of the antioxidant activity depended on the tested mushroom species. According to this study, the obtained results reveal that wild mushrooms appear to be good and safe natural sources of antioxidants with high level of polyphenols, a possible food supplement or in the pharmaceutical industry and an important source for low caloric, low fat functional foods. Antioxidant assays used in this study proved that *B. regius* possess the highest antioxidant activity, while the results for other mushroom species varied depending on the method that is used. *In vitro* antimicrobial activity against a panel of pathogenic bacterial strains was investigated by the microdilution method and the minimum inhibitory concentration (MIC) was determined. As far as we know, this is the first antimicrobial study for *B. appendiculatus*, *B. regius*, *C. cornucopioides*, *R. virescens*, *C. odore*, *L. volemus* and *P. squamosus* against the tested pathogenic strains of bacteria. *B. regius* and *B. appendiculatus* are species which show significant results against stain *B. cereus.* With an increasing number of bacteria that have developed resistance to commercial antibiotics, extracts and derivatives from wild mushrooms present great promise for novel medicines. The antiradical activities of each extract and antioxidant potency composite index were calculated. Agglomerative hierarchical clustering (AHC) was applied to investigate the similarities between used methods for antioxidant activities. Principal component analysis (PCA) was used for determining the number of variables to explain the observed variances in the data of antioxidant activities of the examined wild mushroom species. From the wild mushrooms studied, "*Boletus*" may be an interesting group due to their high total phenol concentration, antioxidant and antimicrobial activity, as well as its high relative abundance in the region. These mushrooms might serve as possible protective agents in human diets to help humans reduce oxidative damage.

Conflict of interest

There is no conflict of interest between authors.

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