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Journal:	<i>Analytical Methods</i>
Manuscript ID:	AY-ART-12-2014-003011.R1
Article Type:	Paper
Date Submitted by the Author:	19-Mar-2015
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ARTICLE

Screening of Antioxidant, Antimicrobial and Antiradical Activities of Twelve Selected Serbian Wild Mushrooms

Cite this: DOI: 10.1039/x0xx00000x

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Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

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

Introduction

Reactive radical species are unstable and very reactive molecules 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 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 case of excess of free radicals in human organism, 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 to inhibit oxidation of lipids, proteins, carbohydrates and DNA in human organism. Thus, taking foods rich in

antioxidants such as mushrooms help 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

1 content of fat is very low. The presence of certain enzymes and
2 fibers in mushrooms may lower cholesterol level in blood,
3 while production of antiviral and other proteins protect and
4 regenerate human cells.

5 A variety of mushrooms secondary metabolites allows the
6 possibility of their use for therapeutic purposes. Many edible
7 mushrooms (mostly *Basidiomycetes*) are good sources of
8 carbohydrates such as β -glucans; phenolics such as tocopherols;
9 B-vitamins such as niacin, flavin and pyridoxine; organic acids
10 such as ascorbate, shikimate, malate and fumarate;
11 monoterpenoid and diterpenoid; lipids; proteins such as
12 hydrophobins and trace elements such as selenium.⁴
13 Mushrooms are very good in prevention of fungal infections,
14 because they act as some sort of antibiotics. Some wild grown
15 mushrooms possess antioxidant activity, which is in correlation
16 with their total phenolic content. Antioxidant activity of
17 phenolic compounds is related to the capture of free radicals
18 and binding of metal ions, which are precursors in the
19 formation of free radicals.⁵

20 Environmental conditions in Serbia contribute to enabled high
21 versatility of wild edible fungi, some of which with great
22 gastronomic significance. Mushrooms as *Boletus edulis*,
23 *Cantharellus cibarius*, *Craterellus cornucopioides*, *Amanita*
24 *caesarea* and *Morchella esculenta*, are very popular in Serbian
25 cuisine. Mushrooms are mainly used as food, because of their
26 extraordinary flavor. In addition, their nutritional values are
27 often accompanied with delicious taste and medicinal
28 properties, such as, *Morchella esculenta* (morel), and it is
29 rightfully recognized as the gift of nature among people. It
30 contains high amounts of antioxidants and fibers, improves
31 liver function, has beneficial effect on the cardiovascular
32 system. *Cantharellus cibarius* except great taste also possesses
33 medicinal properties. It contains essential amino acids and
34 vitamin A, increases the body's resistance to infections of the
35 respiratory tract and strengthens the immune system. *Boletus*
36 has always been the most famous and most respected
37 mushrooms for food, and *Boletus edulis* is considered among
38 the most delicious boletus mushrooms that can be consumed in
39 many ways. Considering a great number *Boletus* species,
40 (poisonous and non-poisonous), majority of mushroom
41 collectors usually harvest several species (*Boletus edulis*,
42 *Boletus regius* and *Boletus appendiculatus*), recognizing them
43 as edible without differentiation on the species level.
44 *Craterellus cornucopioides* has a unique look, no toxic twins,
45 and it is almost impossible to misidentify it with a poisonous
46 one. It is a great treat, especially good when dried, ground into
47 powder and used as a spice. Interestingly, it was noticed that
48 last year's *Craterellus cornucopioides* was a great collector of
49 radioisotope released from a nuclear power plant in Chernobyl.
50 The mushroom *Amanita caesarea* was known in ancient Rome,
51 and was highly regarded. *Polyporus squamosus* and *Clitocybe*
52 *odora* are commonly used as a spice. The wild *Russula*
53 *virescens*'s distinct effects on the regulation of blood lipids, anti-
54 oxidation⁶ and antitumor activity⁷ was reported.

55 In general, detailed chemical composition of many wild edible
56 mushroom species from Serbia is not still elucidated as well as
57
58
59

their biological activities, though they constitute important
place in healthy diet, widespread appreciated among people.
Considering that mushrooms contain compounds with wide
range of antioxidant and antimicrobial activities, present study
is dedicated to collection of data on antioxidant and
antimicrobial activities of the ethanol extracts of the 12 edible
mushrooms species, widely used in diet to examine if there are
real reasons for their appreciation by folk. To the best of our
knowledge, present paper is first one about antioxidant and
antimicrobial potentials of the tested species from Serbia.

Also, as far as we know, there are no reports of 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. odora*, *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 ultrasonic four
times with 95% ethanol, evaporated to dryness and weighed to
obtain the yield. The same procedures were done for all
investigated mushrooms species and results are given as a
percentage of the original weight of crude sample (Table 3).
Yield of ethanol extract was the highest for *A. caesarea*
(6.90%) while 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 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
antioxidant activity. Phenolics content in the mushroom extract
is various among the analyzed species. The values measured
with 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: Folin–Ciocalteu reagent reacts not only with
phenolics but 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 Results of the present study showed in Table 2.
The highest total phenolic content (173.125 ± 0.475 μ g GAE/mg
dw) was found in mushroom *B. regius*, and the lowest was
found (9.362 ± 0.960 μ g GAE/mg dw) in *L. volemus*. According
Leal¹¹ *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/ g extract). Different phenolic compounds contribute to the antioxidant activity of mushrooms. Reviewing different HPLC studies for analysed species, we concluded that the 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. 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), vanilic 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 testing in DPPH and ABTS scavenging assays and the results are present in Figure 1. Effective concentration at 50 % inhibition (EC₅₀) (mg various extracts per ml) was the parameter used to compare radical scavenging activity. Effectiveness of antioxidant properties inversely correlated with their EC₅₀ values.¹⁷ A lower EC₅₀ value indicates higher activity. EC₅₀ values of all mushrooms extracts ranged from 10.997 – 51.159 mg/ml. *B. regius* has the highest ability to decrease the initial DPPH radical concentration by 50% (EC₅₀= 10.997±0.891 mg/ml, followed by *B. appendiculatus* (EC₅₀= 11.314±0.842 mg/ml), while the least ability was from *C. cibarius* (EC₅₀ = 51.159±1.012 mg/ml). It's important to note that the species of the same genus (*B. regius*, *B. appendiculatus* and *B. edulis*) show 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 Keleş¹⁷ who reported 43.30% of inhibition of DPPH radical for the same mushroom. Leal¹¹ reported that *B. regius* had the scavenging activity (EC₅₀ value = 2.06 mg/ml). Antioxidant activity of mushrooms, determined by ABTS assay, is shown in Figure 1. Results obtained by ABTS method showed deviation from results of other methods. Values for ABTS scavenging activity varied between (EC₅₀= 5.034±0.045 mg/ml), for *L. pseudoscabber* to (EC₅₀= 16.394±0.475 mg/ml) for *L. volemus*. From Figure 1 it is obvious that the highest amount of antioxidants was found in *L. pseudoscabber*. Although this type of mushroom belongs to another genus, it is still the same *Boletaceae* family like *B. regius*, *B. edulis* and *B. appendiculatus* so this result is expected. 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

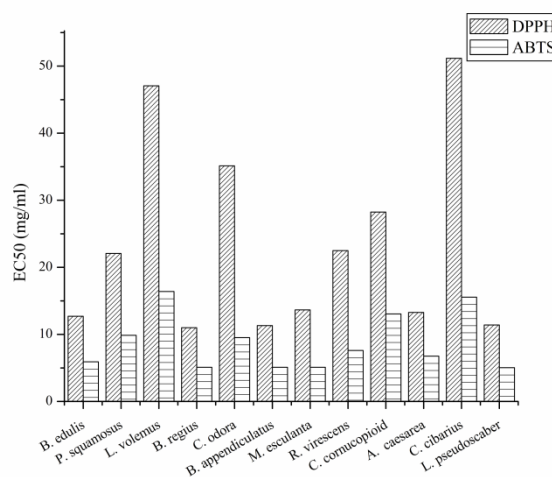


Fig.1. Free radical-scavenging capacities of the extract measured in DPPH and ABTS assay

Ferric-reducing antioxidant power (FRAP) assay

Also, antioxidant activity was measured by the FRAP method, which measures the capacity of an antioxidant to reduce a Fe³⁺ - TPTZ complex to Fe²⁺ - TPTZ complex¹⁷ and results are present in Table 2. It's simple and reproducible method which must be performed in acidic conditions (pH = 3.6). The obtained results confirmed highest antioxidant activity for *B. regius* (56.924±0.022 μmol Fe/mg dw). FRAP value for *L. volemus* (3.147±0.064 μmol Fe/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 compared with fruits, it was observed that mushrooms presented higher antioxidant activity than some fruits (aronia, raspberry, blackberry, cherry, blackthorn) which ranged from 0.12 to 0.19 μmol Fe/mg dw).¹⁹ When values of antioxidant activity of *B. edulis* and *L. volemus* (6.723, 3.141 μmol Fe/mg dw, respectively) determined by FRAP method compared with values obtained by Keleş¹⁷ (52957.14, 3171.43 μmol/g) it can be concluded that they are similar.

Total reducing power (TRP) assay

The results of the reducing power assay were carried out in triplicate and summarized in Table 2, expressed as mean values ± standard deviations. High absorbance indicates high reducing power.²⁰ The reducing power ability of the ethanol extracts was determined using ascorbic acid as standard and results are given as mg ascorbic acid equivalents per mg dry extract weight (mg AAE/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 possible to determine the concentration of Fe³⁺

ion in presence of extract. Among tested mushroom samples, extract of *B. regius* showed the highest reducing power (0.771 ± 0.004 mg AAE/mg dw), followed by extract from *B. appendiculatus* (0.595 ± 0.002 mg AAE/mg dw) while other extracts showed weaker reducing power. This two species have the highest amount of phenolic compounds; therefore show a high reducing power which confirms TRP method. The reducing capacity may be a significant index of antioxidant activity.¹⁸ Accordingly, *Boletaceae* family, where belong these two species, 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's difficult to compare obtained results. Mau²³ analyzed *M. esculenta* and reducing power of extract, in concentration 25 (mg/ml), is 0.97.

CUPRAC

The cupric reducing antioxidant capacity (CUPRAC) of mushrooms extracts based on utilizing the copper(II)-neocuproine reagent as the chromogenic oxidizing agent.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 shows that the highest CUPRAC capacity is observed for *B. regius* (21.738 ± 0.108 μ g TE/mg dw), followed by *B. appendiculatus* (15.576 ± 0.012 μ g TE/mg dw) and the lowest activity showed *L. volemus* (4.164 ± 0.012 μ g TE/mg dw), just was expected according total phenolic content.

Antiradical activity

The numbers of antiradical activity units per 1 mg of extract (EAU₅₁₅) were calculated for all mushrooms species and are shown in Tables 3.

Results of antiradical activities show that a mushroom having high antiradical activity comparable to some plant extracts.²⁶ The highest EAU₅₁₅ units were calculated for extracts obtained from the *P. squamosus* (EAU₅₁₅ 6.35) and this extract could be considered as effective antiradical agent, while the lowest were from *L. volemus* (EAU₅₁₅ 0.89).

Antioxidant potency composite index-ACI

Every method for antioxidant activity has certain advantages and limitations and differs in terms of the assay principles. We used six methods to determined antioxidant capacity and in order to give an equal weight to these entire methods 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 as a solvent used 100.0 % DMSO. 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 activity. These differences in 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), but there was no bactericidal effect (except against *S. aureus* and *B. cereus* - MBC=6.3 mg/ml). Also, the samples *B. edulis*, *L. volemus*, *C. odora* and *M. esculenta* showed a good inhibitory activity (MIC=0.8-3.1 mg/ml), against *Kl. pneumoniae*. As significant result can be observed activity of samples *P. squamosus*, *L. volemus*, *B. regius*, *C. odora*, *B. appendiculatus*, *M. esculenta* and *R. virescens* against *S. aureus* and activity of samples *B. regius* and *B. appendiculatus* against *B. cereus* (in both cases the MIC/MBC=3.1-6.3/6.3mg/ml).Statistic analysis

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

Concerning that the results of antioxidant activity measured by different methods it is difficult to compared with each other, we're done all statistical tests with ACI values.

Correlation

At the beginning of the statistical analysis we were calculating of correlation matrix, giving the correlation coefficients between each pair of variables. Each term of the matrix is a 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 present correlation between selected mushrooms with several meaningful correlations. The strongest positive correlations were between *L. pseudosclaber/A. caesarea* and *M. esculenta/C. odora* ($r = 0.98$, $p < 0.05$). Another group represented by *M. esculenta/B. edulis* also displayed a significant 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 exist 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*, *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 criterion²⁹ with eigenvalue higher than 1 (Figure 2). Eigenvalues give a measure of the variance accounted by the corresponding eigenvectors (components).³⁰ From scree plot graph of eigenvalues of the PCA (Figure 3) it can be seen that the first two PCs are enough to explain 90.67% of the pattern variation.³¹

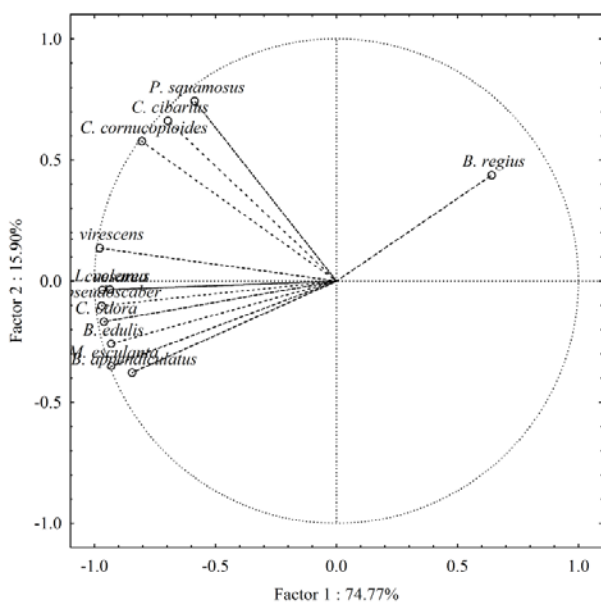


Fig2. PCA of mushrooms based on their antioxidant activity

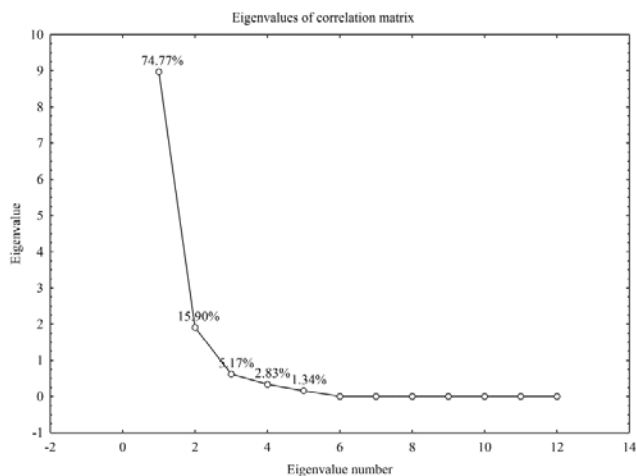


Fig3. Eigenvalues of correlation matrix

Two principal components (PC) were extracted applying the statistical analysis because they have eigenvalues higher than 1.0, as suggested by the Kaiser criterion. The first principal component (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 plot, because *B. regius* has highest positive loadings on PC1 (0.64). It's expected because this mushroom was proved high potential antioxidant activity. As opposite, *R. virescens* has highest negative loadings on PC1 (-0.98). With increasing positive score, antioxidant activity of variables increases along the PC axis, while negative score means that the antioxidant activity of variables decreases along the axis. When 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 highest positive loadings on same (0.74).

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 similarities between used methods.

HCA calculates the distances (or correlation) between all samples using a defined metric such as 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 done 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} . Euclidean distance is most common way to measure distance between objects.

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

Hierarchical cluster analysis (HCA) was applied to five methods (DPPH, ABTS, FRAP, CUPRAC, TRP) and they are grouped in clusters in terms of their similarity. The results obtained following HCA are shown as a dendrogram (Figure 4) and methods are grouped in two statistically significant clusters at $(D_{link}/D_{max}) \times 100 < 50$. Methods for determination radical scavenger capacity can be classified by the ability of the

donation of hydrogen atoms or electron transfer, so the tendencies to form groups arising from common analytical characteristics is clear in this data analysis procedure.

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

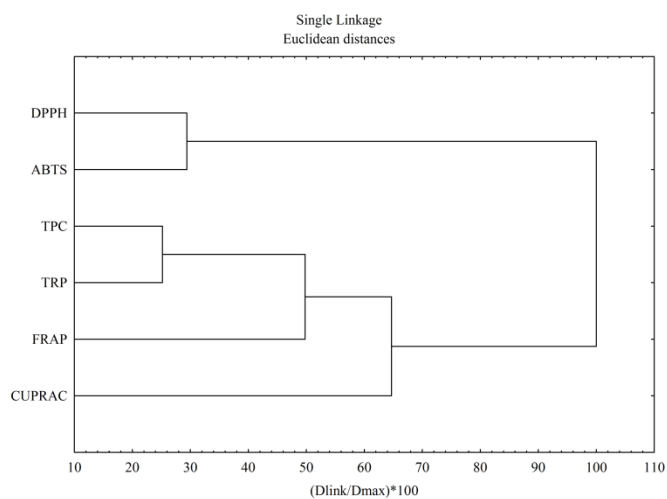


Fig4. Dendrogram showing clustering of five different methods

For comparison of data between two groups, 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 was taken 300 g of each examined mushroom species (total 1500 g for each mushroom species). Mushrooms were cleaned of all surface contamination by little brush. After collection and taxonomic identification, the mushrooms were cut and dried at room temperature. They were stored at polyethylene bags until the analysis started. Species, family and edibility of mushrooms used in this work have 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) chloride 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, NaCO₃, HCl, 2, 4, 2-tri (2-pyridyl)-s-triazine (TPTZ), K₃[Fe(CN)₆], phosphate buffer (NaH₂PO₄-Na₂HPO₄), ammonium acetate buffer, CCl₃COOH, K₂S₂O₈, FeSO₄*7H₂O, 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:

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

Where:

W₁ is weight of evaporated extract

W₂ is weight of 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 Mitic¹⁹ with some modifications. Stable DPPH radicals are widely used to evaluate the antioxidant activities of proton-donating substances according to hydrogen donating ability.¹⁸ 1.5 ml of methanol solution of DPPH radical, in the concentration of 100 μmol/L, 0.1 ml of extract, concentration of 20 mg/mL, and methanol to a total volume of 4 ml, were placed in test tube. The mixture was shaken and after 60 min spent in the dark, the absorbance measured at 515 nm with a spectrophotometer. All determinations were performed in triplicate. A fresh prepared DPPH solution was deep purple in colour which faded away as the DPPH free radicals were quenched and converted into a colourless product (2,2 diphenyl-1-hydrazine) resulting in absorbance decrease, thus the more rapidly the absorbance decreased the more effective was the antioxidant activity of the extracts.³⁵ Radical "scavenging" capacity (RSC) was determined from the calibration curve, which was drawn by using Trolox as positive control, in concentration 1 - 16 μmol/L, and expressed in μg of Trolox equivalents (TE) per mg

dry extract weight ($\mu\text{g TE/mg dw}$). DPPH radical "scavenging" capacity was expressed by applying the following equation:

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

Where:

A_0 - absorbance of blank solution

A_1 - absorbance of solution in the presence of active components

RSC values were used to obtain the mushrooms extract amount necessary to decrease the initial DPPH concentration by 50% (EC50) using an exponential curve.

ABTS RADICAL "SCAVENGING" ACTIVITY

ABTS radical "scavenging" activity performed according to the method of Re. ³⁶ ABTS radical was produced by reaction of ABTS stock solution with potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12 - 16 h before use. The solution was then diluted by mixing 7 ml ABTS⁺ 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, was mixed with 1,8 mL of diluted ABTS solution in the concentration of 7 mmol/L 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 ($\mu\text{g TE/mg dw}$).

FERRIC-REDUCING ANTIOXIDANT POWER (FRAP) ASSAY

Ferric reducing antioxidant power assay was performed using method of Benzie and Strain ³⁷ and it is based on the reduction of ferric iron in tripyridyltriazine complex to the blue ferrous form at low pH. This reduction is monitored by measuring the change color in absorption at 595 nm. 1 ml of prepared FRAP reagent is mixed with 0.05ml of sample, concentration 20 mg/mL, and diluted with water to 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 ($\mu\text{mol Fe/mg dw}$), was obtained by comparing the absorption change in the test mixture with doses obtained from 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 a based on ability of antioxidants to reducing Fe (III) hexacyanate to Fe (II) hexacyanate which leads to increase in the absorbance of the reaction mixtures. 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 $\text{K}_3[\text{Fe}(\text{CN})_6]$, phosphate buffer (pH 6.6) and water. The mixtures were incubated at 50 °C for 30 minutes and then we added a 1ml 10% solution of trichloroacetic acid and 0.6 ml FeCl_3 . The absorbance was measured at 700 nm against blank sample and results were expressed as mg ascorbic acid equivalents per mg of dry extract weight (mg AAE/mg dw).

TOTAL PHENOLIC CONTENT (TPC)

Total phenolic content was determined using 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 kept 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 ($\mu\text{g GAE/mg dw}$).

CUPRIC REDUCING ANTIOXIDANT CAPACITY (CUPRAC) ASSAY

The CUPRAC assay was performed using method of Apak. ²⁵ Method is based on the reduction of a cupric neocuproine complex (Cu (II)-Nc) by antioxidants to the cuprous form (Cu(I)-Nc). CUPRAC method is capable to determine 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 30 min at room temperature and after that absorbance was measured at 450nm. Trolox was used as a standard and results were expressed as μg Trolox equivalents per mg of dry weight ($\mu\text{g TE/mg dw}$).

DETERMINATION OF ANTIRADICAL ACTIVITY

The anti-radical activity of the extracts was performed according to the method of Brand-Williamset ⁴¹, with some modification. Determination of antiradical activity was based on the principal behind that DPPH radical in its radical form has a characteristic absorbance at 515 nm which disappear after its reduction by an antiradical compound. ¹ 0.53ml mushrooms extract, concentration 50 mg/ml and 3.470 ml fresh prepared solution of DPPH radical, in concentration 100 $\mu\text{mol/L}$, were placed in test tube. The absorbance was measured at 515 nm at 0 and after 1 min of the reaction. The blank sample was prepared the same, only instead of the extract we added 0.53 ml of 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

A_{1C} - the absorbance of the blank sample after 1 min

Antiradical activity of the mushrooms extracts was described by antiradical activity unit which allow comparing antiradical activities. 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)

AU_{515} - antiradical activity of the extract

Total number of antiradical activity units in the extract was calculated:

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

Where:

Cle - total amount of extract (mg)

I_e - amount of extract in the measured sample

MICRO-WELL DILUTION ASSAY

The mushrooms extract samples were tested against a panel of pathogenic bacterial strains obtained from the American Type Culture Collection (ATCC), using a Micro-well Dilution Assay (NCCLS 2003, with some modifications). Bacterial suspensions were made 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) were prepared in 100.0% dimethylsulfoxide (DMSO) and serial dilutions tested in the range from 0.02 to 50.0 mg/ml in 96/well microtiter plate with inoculated Mueller-Hinton broth (MHB). The final volume was 100 μ l and the final bacterial suspension was 106 CFU/ml 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 is 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 investigated samples. To reduce the results of antioxidant activities obtained with 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 the best score for each test, and then calculating an index score for all other samples within the test as follows: $\text{antioxidant index score} / [(\text{sample score} / \text{best score}) \times 100]$; the average of all six tests for each samples 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 done by Grubb's test. All data were reported as mean \pm standard deviation of three replicates. For multifactorial comparison, principal component analyses (PCA) and agglomerative hierarchical clustering (AHC) were used to display the correlation between the various parameters and their relationship with the different mushroom species.⁴³

PCA and CA are the most common multivariate statistical methods used in environmental and foodstuff studies.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 new derived variables, called principal components (PC), that explain the maximum variance in data used, allowing to recognize cluster trends in the data by the distribution of PC scores and to identify the variables responsible for the discrimination pattern through the 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 explain 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 Results obtained by cluster analysis are typically are shown by a dendrogram. Cluster analysis was done by Ward's method using Euclidean distances as a measure of similarity.

Multivariate analysis included principal component analysis (PCA) and cluster analyses (CA) were 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 mushrooms species have significant antioxidant activity against various antioxidant systems *in vitro*. The tested mushrooms extracts have a strong antioxidant activity against various oxidative systems *in vitro*. The intensity of antioxidant activity depended on the tested mushroom species. According to this study, 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 pharmaceutical industry and 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 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 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 result against *B. cereus*. With an increasing number of bacteria that have developed resistance to commercial antibiotics, extracts and derivatives from wild mushrooms present a 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 similarities between used methods for Antioxidant activities. Principal component analysis (PCA) was used in determining the number of variables to explain the observed variances in the data of antioxidant activities of examined wild mushrooms 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 human reduce oxidative damage.

Conflict of interest

There is no conflict of interest between authors.

Acknowledgements

The research was supported by Ministry of Education, Science and Technological Development of the Republic of Serbia [172047]. The authors wish to acknowledge the support of providing and determining mushrooms samples. The authors wish to acknowledge to Marjan Kustera and Mycological

Society of Nis for the sampling and determining mushrooms samples.

Notes and references

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Journal Name

Tables

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

Table 1. Species, common name, families and edibility of mushrooms

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

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

Journal Name

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)
<i>Amanita caesarea</i>	6.90	0.23	3.27	681.23
<i>Boletus appendiculatus</i>	4.16	0.06	1.54	187.31
<i>Boletus edulis</i>	5.88	0.20	3.49	618.59
<i>Boletus regius</i>	6.10	0.06	1.08	190.69
<i>Leccinum pseudoscaber</i>	6.80	0.12	1.81	370.72
<i>Cantharellus cibarius</i>	4.51	0.05	0.99	138.79
<i>Craterellus cornu copioides</i>	3.61	0.10	5.13	630.91
<i>Morchella esculenta</i>	2.59	0.23	5.49	1121.21
<i>Polyporus squamosus</i>	2.11	0.13	6.35	820.32
<i>Lactarius volemus</i>	4.12	0.04	0.89	114.23
<i>Russula virescens</i>	3.52	0.13	3.64	380.49
<i>Clitocybe odora</i>	5.65	0.13	2.24	390

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

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

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

Journal Name

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Bacterial strains	Gram (-) bacteria					Gram (+) bacteria				
	<i>E. coli</i> ATCC25922	<i>P. mirabilis</i> ATCC12453	<i>S. enteritidis</i> ATCC13076	<i>K. pneumoniae</i> ATCC10031	<i>P. aeruginosa</i> ATCC9027	<i>S. aureus</i> ATCC8538	<i>E. faecalis</i> ATCC19433	<i>L. monocytogenes</i> ATCC15313	<i>B. cereus</i> ATCC8739	<i>C. perfringens</i> ATCC19404
15 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
16 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
17 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
18 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
19 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
20 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
21 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
22 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
23 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
24 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
25 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
26 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
27 DMSO (sol %)	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-B. edulis; B-P. squamosus; C-L. volenus; 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

Table 5. Antimicrobial activity of fungal extracts against pathogenic bacterial strains (MIC/MBC in mg/mL)