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## Accepted Article

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# First Report about Mineral Content, Fatty Acids Composition and Biological Activities of Four Wild Edible Mushrooms

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## Abstract

The goal of this research was a comprehensive analysis of four wild edible mushroom species, *Cantharellus cinereus*, *Clavariadelphus pistillaris*, *Clitocybe nebularis* and *Hygrocybe punicea*, which have not been analyzed so far. Extracts of different polarities have been prepared and evaluated their antioxidant activities by DPPH, ABTS, FRAP, TRP and CUPRAC methods. Also, for all extracts total phenol content was determined. Based on the analysis, it was shown that solvent type has a significant effect on the antioxidant capacities of mushroom extracts, so water extracts show the highest activity. Furthermore, the analysis includes determination of mineral composition, fatty acid profiles, and antimicrobial activity. Unsaturated fatty acids, which are very important for human health, are dominant in the studied mushroom species. Linoleic and oleic acid consist of over 50% of the total fatty acid composition. Seventeen biologically important and toxic elements have been analyzed by ICP-OES and ICP-MS and results showed that the element concentrations were species-dependent. Also, it has been found that analyzed mushrooms didn't show antimicrobial activity. Chemometric analysis were used to understand the connection between the extracts of different polarities.


**Key words**: mushrooms, antioxidants, fatty acids, mineral and phenolic contents

## Introduction

Mushrooms are such a type of food staff that even in small quantities are able to improve the taste of victuals and, more importantly, contain nutritious and useful ingredients, adding only very modest quantities of fats or carbohydrates. Some people think of mushrooms as of vegetables, mostly because of their nutritional values.

Advantage of the wild edible mushrooms in comparison with vegetables is that they most commonly grow in uninhabited and unpolluted environment and therefore are considered to be "contaminant free" and therefore very healthy. The amount of mushrooms consumed by humans has increased dramatically in the last couple of decades, as people started using different mushrooms in their everyday cuisine.

Diplock et al.,[1] defined functional food as a food that improves health or wellbeing, or reduces disease risk, through beneficially targeting the body's functions. Because of these characteristics functional food represent one of the most interesting areas of research and innovation in the food industry.[2,3] It is well know that mushrooms help prevent occurrence of many diseases, although they cannot cure them. Mushrooms can be considered as a functional food because they have high nutritional and medicinal value. For example, under stress, our bodies produce more reactive oxygen species (ROS) (*e.g.*, superoxide anion radicals, hydroxyl radicals and hydrogen peroxide) than enzymatic (*e.g.*, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase) and non-enzymatic antioxidants (*e.g.*, glutathione).[4] This imbalance leads to cell damage and health problems.[5,6] By supporting the immune system, mushrooms help with general bodily stress and fatigue.

Mushrooms possess useful bioactive metabolites with a broad spectrum of pharmacological benefits. Reported bioactivities of mushrooms include antibacterial, antifungal, antioxidant, antiviral, antitumor, cytostatic, immunosuppressive, antiallergic, antiatherogenic, hypoglycemic, anti-inflammatory, and hepatoprotective activities.[7]

Lipids are the least studied components in mushrooms since their overall content in the known species is in range of 6–8% of the dry weight. While the low lipid content is one of the advantages of mushrooms as nutrients, the knowledge of lipids composition as primary metabolites is of interest because it represent addition to the general nutritional value and may characterize the special place of mushrooms in terrestrial life form.[8]

To better understand the hidden power of edible mushrooms, it is necessary to conduct a number of experiments. Three different types of extraction solvents (80% methanol, 80% ethanol and water) were used to determine their effects on antioxidant characteristics of selected mushrooms. According to Sultana et al.,[9] the nature of extraction solvent and varying chemical characteristics and polarities of antioxidant compounds can result in different extraction yields and antioxidant activities of the plant.

According to the authors' best knowledge present study is the first one for these four mushroom species, and the results from this research should be used for further research, as a baseline reference. The aims of this study were: to determine total phenolic content and antioxidant activity in 80% methanol, 80% ethanol and aqueous extracts; to determine fatty acids profiles; to determine and evaluate the amounts of toxic elements (Al, Ba, Cd, As, Hg and Pb) and biologically important elements (Ca, Co, Cu, Fe, K, Mn, Na, Ni, Sr, P and Zn) from *Cantharellus cinereus*, *Clavariadelphus pistillaris*, *Clitocybe nebularis* and *Hygrocybe punicea* mushrooms collected in the area that surrounds the city of Nis, Serbia. The *Cantharellus cinereus* is wild edible mushroom, with sweet taste and fruity smell. Belongs Cantharellaceae family. C*lavariadelphus pistillaris* is a rare mushroom specie which belongs

10.1002/cbdv.201800492

to the Gomphaceae family. It grows by itself under the beech tree, in summer. *Clitocybe nebularis* is edible and widely consumed mushroom. It belongs to the Tricholomataceae family and commonly known as the clouded funnel because it looks just like name suggests. *Hygrocybe punicea*, is a colourful member of the Hygrophoraceae family. It is known as edible in Europe with mild taste.

## Results and discussion

Figure 1 shows total phenolic content (a) and antioxidant activity (b, c, d, e) of the samples extracted with different solvents.

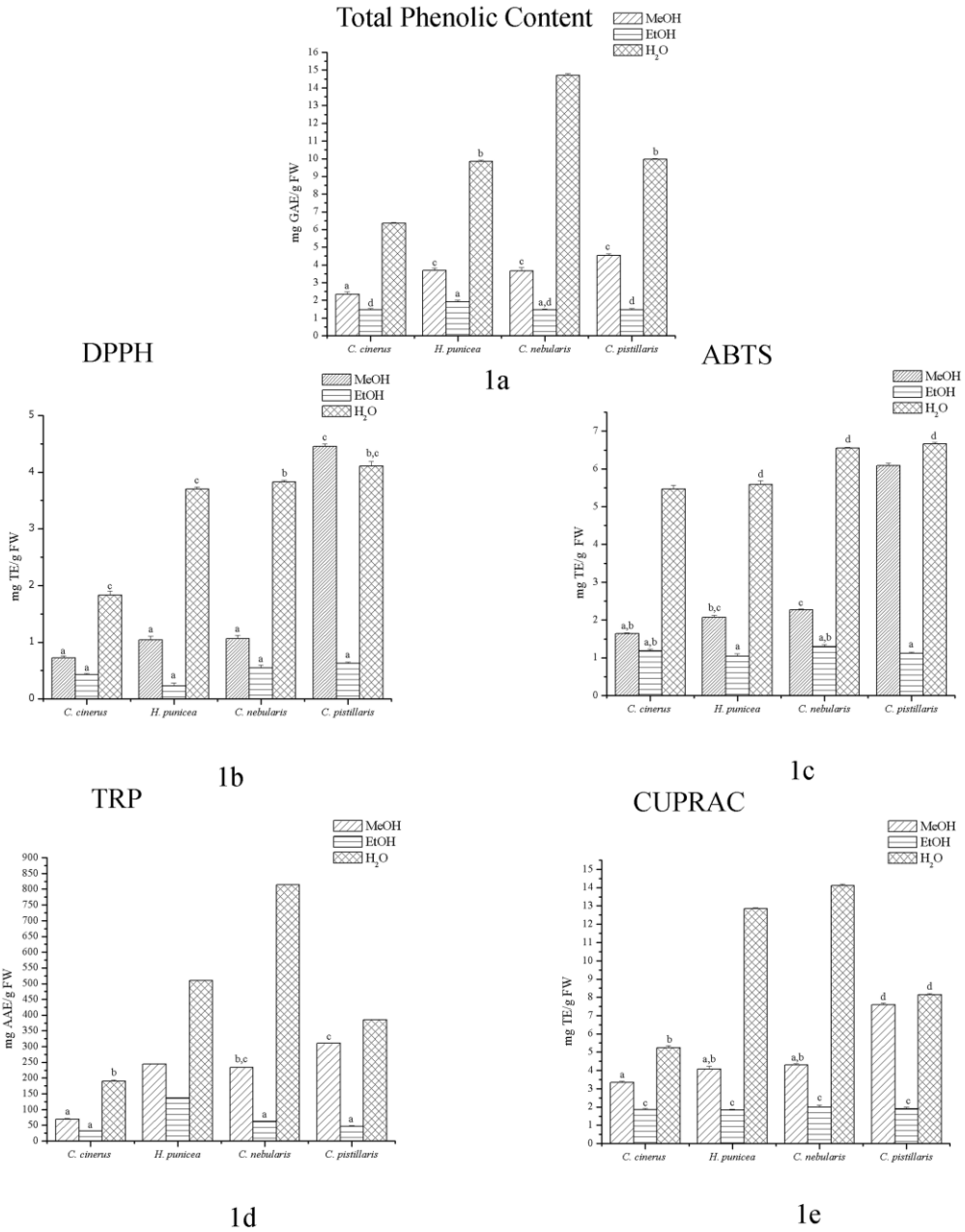


*Figure 1.* Antioxidant capacities of analyzed mushroom species determined by four different methods (b, c, d, e) and total phenolic content (a)
*Means sharing a same letter in their superscript are not significantly different according to a t- test ($P > 0.05$)

10.1002/cbdv.201800492

*Total Phenolic Content*

Results are shown in Fig 1a and indicate that the solvent extraction significantly influenced the amount of phenolic content.

Among the tree different extracts, water extracts contained the highest amount of phenolic compounds followed by 80% methanol and 80% ethanol extract. Total phenolic content varied between analyzed species, from 6.35±0.04 mg GAE/g FW in *C. cinereus* to 14.7±0.11 mg GAE/ g FW in the *C. nebularis*, for water extracts.

This study was supported by Gan et al.,[10] in which their findings showed that water contributed to the highest yield of total phenolic content in mushrooms under the same extraction conditions.
It's important to note that antioxidant activity of the extract is related to amount of phenolic compounds.
Since these four mushrooms have not been analyzed, obtained values were compared with the values of other edible mushrooms. González-Palma et al.,[11] reported 4.9 mg GAE/g in the methanol extract of *Pleurotus ostreatus* and 5.05 mg GAE/g in the aquatic extract.

*Determination of free radical scavenging (FRS) activity*

With the progress of reaction among radicals and antioxidants, the purple color of the DPPH radical turns yellow. The literature search reveals that some bioactive organic compounds such as gallic acid, glutathione, ascorbic acid, tocopherol, flavonoids, phenols, amines are decolorizing DPPH gradually by the hydrogen donating capability.[12] The rate of discoloration of extract is proportional to the level of antioxidants in the sample.

The antioxidant activity of different extracts was determined through DPPH assay and the experimental results are presented in the Fig 1b. Antioxidant activity depended, on the solvent used for extraction and type of analyzed species. The antioxidant activity of the analyzed species were determined to be in the order of water>80% methanol>80% ethanol extracts. Extraction with water showed the highest scavenging activity (1.83±0.07, 3.70±0.04, 3.83±0.03 and 4.11±0.08 mg TE/ g FW for *C. cinereus*, *H. punicea*, *C. nebularis* and *C. pistillaris*, respectively) followed by methanol extraction (0.72±0.04, 1.04±0.07, 1.06±0.06 and 4.46±0.04 mg TE/g FW for *C. cinereus*, *C. nebularis*, *H. punicea*, and *C. pistillaris*, respectively) and ethanol extraction (0.23±0.05, 0.43±0.02, 0.55±0.05 and 0.63±0.02 mg TE/g FW for *H. punicea*, *C. cinereus*, *C. nebularis* and *C. pistillaris*, respectively).

It was noted that the results obtained using ABTS method are consistently higher than results obtained by DPPH assay but showed the same pattern as for the DPPH method. In general, the ABTS assay is recognized as more suitable for the antioxidant capacity estimation compared to DPPH method for plant foods containing hydrophilic, lipophilic, and highly pigmented antioxidants.[13] Among the three different solvent extracts, water extracts showed the highest antioxidant capacity, followed by methanol and water.

The ABTS assay is based on the disappearance of color originating from a blue/green ABTS radical, which is inhibited by presence of antioxidants. The scavenging effects of different extracts on the ABTS radical were evaluated and the results are shown in Fig 1c. Indeed, the extraction with water showed the highest scavenging activity (5.47±0.1, 5.59±0.1, 6.55±0.02 and 6.66±0.05 mg TE/g FW for *C. cinereus*, *H. punicea*, *C. nebularis* and *C. pistillaris*, respectively).

10.1002/cbdv.201800492

*Cupric reducing antioxidant capacity (CUPRAC) assay*

The advantage of this reagent compared to other chromogenic total antioxidant capacity (TAC) reagents is that CUPRAC reagent has been shown to be much less dependent on phenolic lipophilicity, steric effects, pH, dissolved oxygen, humidity, and daylight.[14,15] The experimental findings showed that extracts of different polarities exhibited significant antioxidant activity (Fig 1). Among the tested mushroom samples, water extracts of *C. nebularis* showed the highest reducing power (14.12±0.08 mg TE/g FW), followed by other water extracts while 80% methanol and 80% ethanol extracts showed a weaker reducing power.

*Total reducing power (TRP) assay*

A lot of factors, such as temperature or the type of solvent extraction can affect antioxidant activity and because of that all three types of solvent were chosen. Same pattern also occurred in this assay, water extracts have showed the highest antioxidant properties.

Higher values indicate higher antioxidant capacity because values are based on reduction of ferric ions, where antioxidants are the reducing agent. Antioxidants are donating a single electron or hydrogen atom for reduction.

With regard to aqueous extracts, it shows that *C. nebularis* has the strongest antioxidant effect (813.55±2.5 mg AAE/g FW) among the four mushroom species studied, followed by *H. punicea* (510.18±1.01 mg AAE/g FW), *C. pistillaris* (384.97±0.51 mg AAE/g FW) and *C. cinereus* (190±4.06 mg AAE/g FW) (Fig 1d).

The different antioxidant levels obtained from the assays may reflect a relative difference in the ability of antioxidant compounds in the extracts to quench aqueous peroxyl radicals and to reduce ABTS, DPPH free radicals and ferric iron in in vitro systems.

*C. nebularis* and *C. pistillaris* were stood out as the mushrooms with the best antioxidant activities among the analyzed mushrooms.

Antioxidant activity of the four mushroom species were compared by t-test ($p < 0.05$). Values with different letters within columns are statistically different.

*Correlations among antioxidant activity assays and total phenolic contents*

For the determination of antioxidant activity, different assays were applied and often produced different results. To improve understanding of the correlation between antioxidant activity evaluation indices and phenolic compound contents, all extracts were used in an analysis of the correlation between TPC and reducing power and free radical scavenging activity.
After correlation between all pairs of antioxidant assays was done, we noticed positively high correlation, regardless whether they are based on SET (single-electron transfer) or HAT (hydrogen atom transfer) reaction mechanisms, which indicates that all extracts had comparable activities in all five assays.

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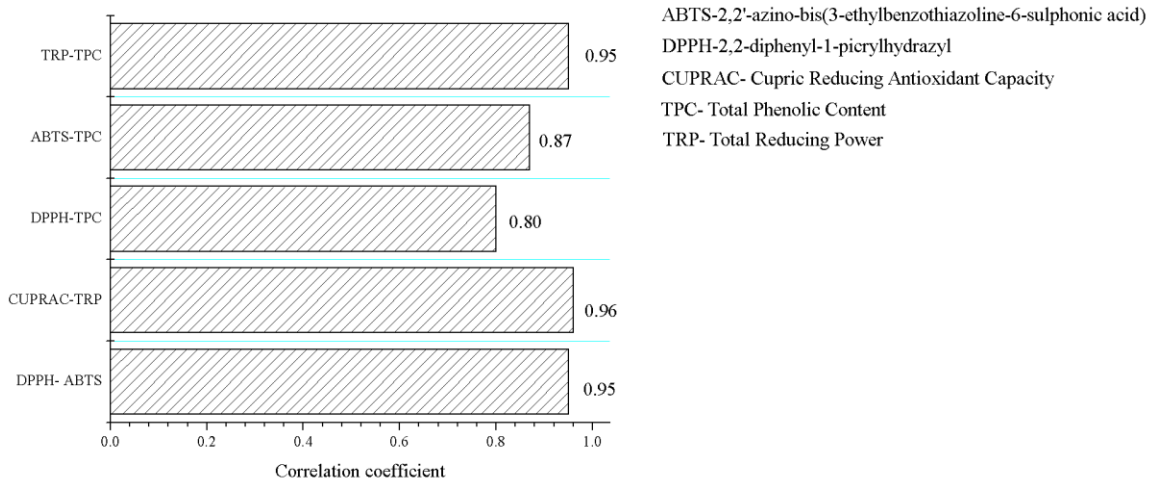


*Figure 2.* Correlations among antioxidant activity parameters and total phenolic contents

As indicated in Fig 2, the correlation coefficients obtained from a comparison of the results of antioxidant activity using different methods (DPPH, ABTS, TRP, CUPRAC and TPC methods) revealed that the CUPRAC and TRP assays had the highest, positive correlation coefficient of 0.96 followed by the DPPH and ABTS methods and with correlation coefficients of 0.95. These correlations were expected since they were based on the same reaction mechanisms.

Strong correlation of total phenolic and reducing power assay (0.95) is in accordance with the study of Hassas-Roudsari et al.[16]

The good correlations obtained between antioxidant capacity determined by the DPPH (0.80) and ABTS (0.87) assays and phenolic concentration, suggest that phenolic content could be used as an indicator of antioxidant properties of the examined mushroom species.

**Accumulation of elements in mushrooms**

Mineral content in mushrooms depends on several factors, some of which are substrate where each fungus grows, the place of origin, environment pollution, and also of mushroom species.

All examined elements' contents were determined on a dry weight of fruiting body.

In order to evaluate the association between the quality of the mushrooms and mineral content, examined elements in this study can be divided into two groups: toxic elements (Al, Ba, Cd, As, Hg and Pb) and biologically important elements (Ca, Co, Cu, Fe, K, P, Mn, Na, Ni, Sr and Zn).

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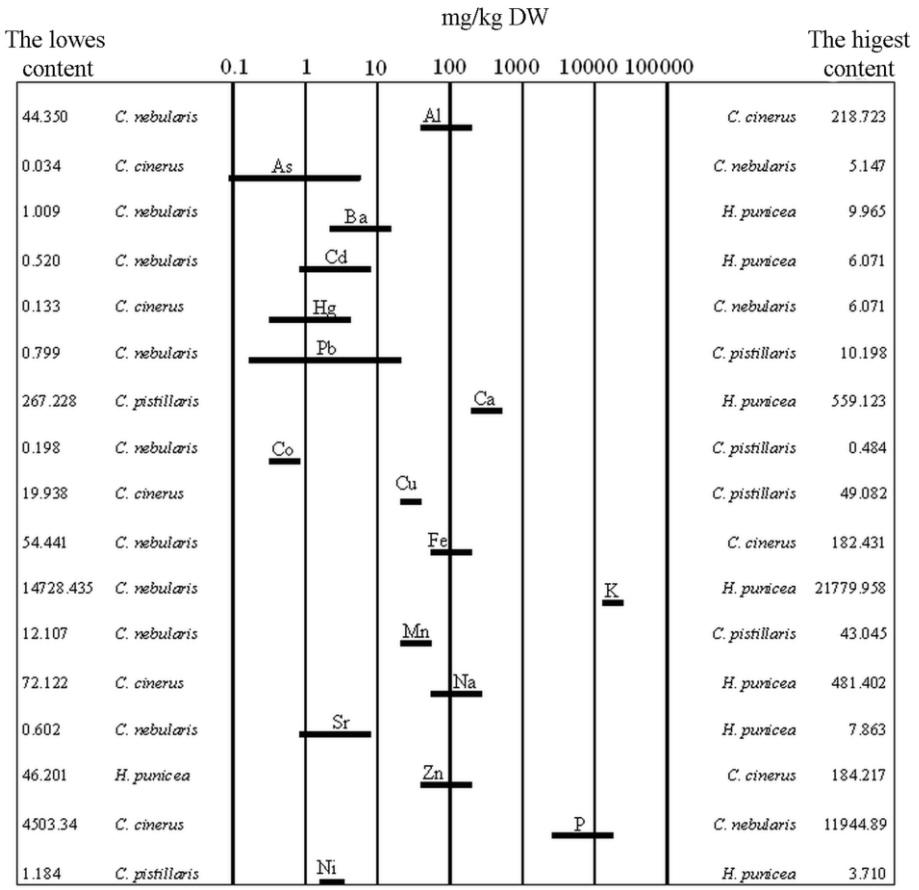


*Figure 3*. Elements contents (mg/kg DW) in the different species of mushrooms

The concentrations of toxic elements in mushrooms could be considered as a marker for the level of pollution of the environment. The highest Al level was found as 218.723 mg/kg for the species *C. cinereus*, whereas the lowest Al level was 44.350 mg/kg in *C. nebularis*. Among the wild mushrooms, the highest Ba and Cd levels were 9.965 and 6.071 mg/kg, respectively, for species of *H. punicea* and the lowest Ba and Cd levels were 1.009 and 0.520 mg/kg, respectively, for species *C. nebularis*. The highest Hg and As level were determined as 2.784 and 5147mg/kg, respectively, for specie of *C. nebularis*, the lowest were 0.133 and 0.034 mg/kg respectively, for the species of *C. cinereus*. The highest Pb level (10.198 mg/kg) was determined for the species *C. pistillaris*. They are naturally present in environment and aren't biodegradable, so in higher amount can be very dangerous.

Metals such as mercury, cadmium, arsenic and lead enter the environment as a consequence of anthropogenic emissions or due to the presence of the same in the dust suspended in the ground layer of air. The toxicity of these metals is reflected in the fact that since they have no biological function in the body, they attack cells and disrupt cellular functions. Such influence leading to toxicity in a number of organs. What makes them even more dangerous is process known as bioaccumulation.

Some mushroom species, owing to their specific capability to accumulate heavy metals (e.g. lead and cadmium) may contain multiple times higher levels of metals than the substratum they had grown on.[17]

Benbrahim et al.,[18] analyzed level of cadmium in mushrooms which were collected in unpolluted areas (control plots) and concentration varied between 1-12 mg/kg DW. Higher contents of cadmium in mushrooms from unpolluted areas may possibly be results of high

cumulative properties of same mushroom species, which was the evidence for *Boletus chrysenteron*.[19]

The provisional tolerable weekly intake (PTWI) for cadmium is 0.42 mg/week.[20] Meal consisting of 300-g portion fresh mushrooms will provide 0.03-0.182 mg of Cd, which is on a weekly basis much lower than allowed. Crucially important are the biologically available forms of metal present in the soil. The absorption of cadmium proceeds exceptionally easy, proportionally to its concentration in soil or air.[21]

According to other reports, concentration of lead in mushrooms from unpolluted areas varied between 0.5 mg/kg and 5 mg/kg. Obtained values for Pb from unpolluted area are relatively higher (0.799-10.198 mg/kg) in comparison with the literature for other species. Since the provisional tolerable weekly intake for lead is 1.5-1.7 mg/week, all four analyzed species do not exceed this limit when converted to a portion of 300g of fresh mushrooms (0.02-0.3 mg/week).

Second group composed of biologically important elements play an essential role in the human organism. When they get into the body, the human organism immediately starts to develop a specific response- defense mechanisms.

For all analyzed elements, the lowest and the highest concentration values (mg/kg) were determined : **Ca:** 266.445 (*C. nebularis*) – 559.123 (*H. punicea*), **K**: 14728.435 (S. luteus (L.) – 21779.958 (*H. punicea*), **Na:** 72.122 (*C. cinereus*) – 481.402 (*H. punicea*), **P**: 4503.34 (*C. cinereus*) – 11944.89 (*C. nebularis*), **Co**: 0.198 (*C. nebularis*) – 0.484 (*C. pistillaris*), **Cu:** 19.938 (*C. cinereus*) – 49.082 (*C. pistillaris*), **Fe:** 54.441 (*C. nebularis*) – 182.431 (*C. cinereus*), **Ni:** 1.184 (*C. pistillaris*) – 3.710 (*H. punicea*), **Sr:** 0.602 (*C. nebularis*) – 7.863 (*H. punicea*), **Mn**: 12.107 (*C. nebularis*) – 43.045 (*C. pistillaris*) and **Zn:** 46.201 (*H. punicea*) – 184.217 (*C. cinereus*).

Considering that this is the first report on the mineral composition of the analyzed mushrooms, it is impossible to compare the obtained values with the literature but it could be compared with the mineral composition of other mushroom species. The determined values for iron are in accordance with the reported Fe content (30-150 mg/kg).[22] Copper levels in the studied mushrooms (19.938-49.082 mg/kg) are in agreement with those reported earlier (18-54 mg/kg DW) and copper content in mushrooms is higher than in vegetables and it should be considered as a nutritional source of this element.[23]

The WHO permissible limit of zinc in foods is 60 mg/kg.[24] The values for zinc in *C. cinereus* was above the WHO's values while the remaining three species are within the allowed limits.

Toxicity limits of manganese for plants are high (400–1,000 mg/kg). Obtained values are under toxicity limits.[25]

Mushrooms are often compared with meat and can serve as a substitute for it. However, regarding mineral composition is mushrooms are better. Cullere et al.[26] analyzed rabbit meat, as one of the healthiest, and reported that average concentrations for K - 4300 mg/kg, for Na - 420 mg/kg and 2280 mg/kg for P. Mushrooms are richer with potassium and phosphorus which is good, and contain less sodium, making them healthier for human consumption.

10.1002/cbdv.201800492

*Fatty acids*

Fatty acid composition including saturated fatty acids (SFAs), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFAs), of the studied wild edible mushrooms has been presented in Fig 4. According to the Fig 4, it is clearly seen that the mushroom species consists of palmitic (C16:0), stearic (C18:0), oleic (C18:1) and linoleic (C18:2) acids in high amounts. Fatty acid composition of mushroom samples shows differences between species.

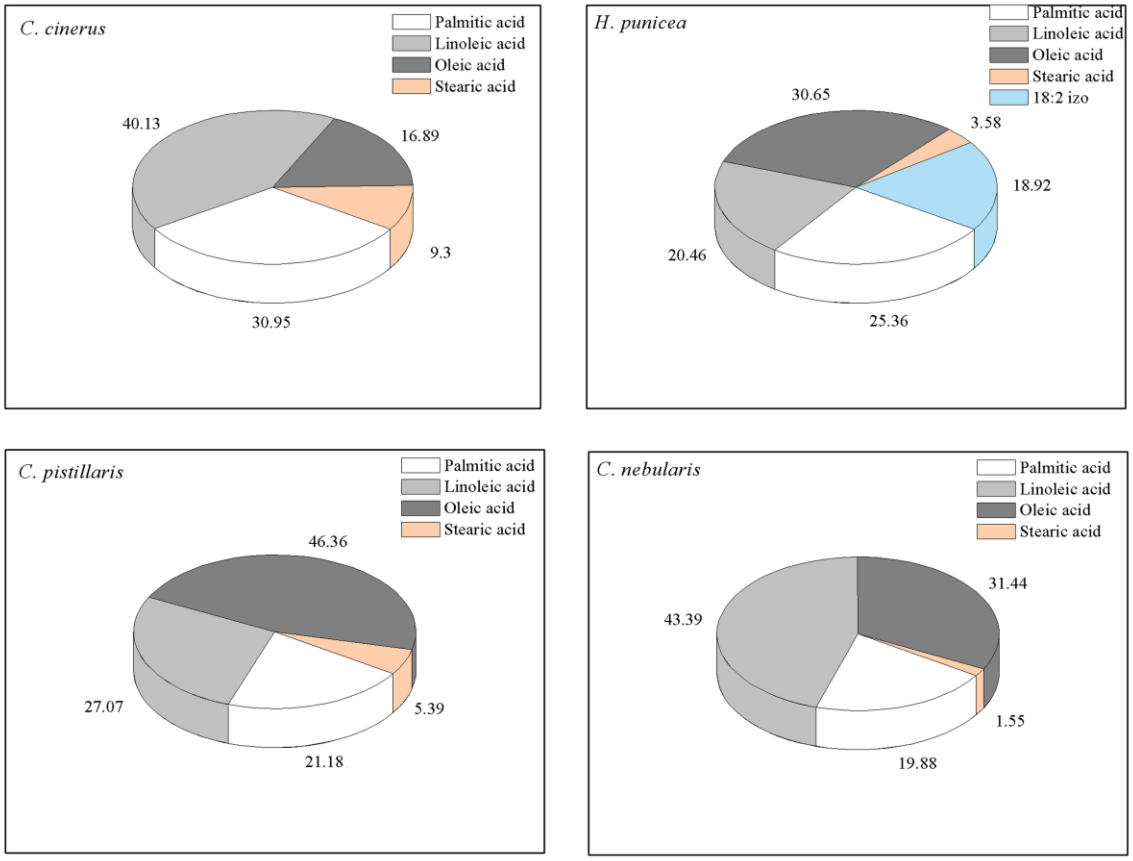


Figure 4. Fatty acids composition of studied mushroom species (%).

Linoleic acid (C18:2) is very important for human health and it is an essential fatty acid. It is known that linoleic acid (C18:2) is the precursor of eight carbon volatiles in fungi, such as 1-octen-3-ol, 3-octanol, 1-octen-3-one and 3-octanone,[27] which are the principal aromatic compounds in most species,[28] contributing also to the flavor of most of the mushroom species analysed in the present study.[29] Higher amount of linoleic acid were observed in all examined species with the maximum value of 43.39% in *C. nebularis.* In a slightly smaller amount (40.13%) can be found in the *C. cinereus*. *H. punicea* and C. *pistillaris* contain linoleic acid in almost twice smaller amount (20.46% and 26.71%), respectively.

Concerning monounsaturated fatty acid (MUFA), oleic acid was found in higher percentages in *C. pistillaris* (45.75%). *H. punicea* and *C. nebularis* have the similar amount of oleic acid (30.65% and 31.44%) respectively.

The values of unsaturated fatty acids obtained for the analyzed species, indicate that the mushrooms are very healthy food that contain over 50% of linoleic and oleic acids. In analyzed samples *trans*-isomers of unsaturated fatty acids were not detected. It is known that

increased intake of *trans* fatty acids has been associated with risk factors for cardiovascular disease.

Palmitic and stearic acids are obtained in smaller amounts but not negligible. Stearic acid is reported not to have a detrimental effect on human health and it has a neutral effect on serum total cholesterol.[30] However, it was also observed that in contrast with other saturated fatty acids, stearic acid apparently does not raise serum low-density-lipoprotein cholesterol.[31] Saturated fatty content was higher in *C. cinereus* due to palmitic acid (30.95%) and 9.30% due to stearic acid and the lowest in *C. nebularis* as result of the small levels of both palmitic and stearic acids (19.88% and 1.55%) respectively.

It should be noted that *H. punicea*, in addition to all of the above mentioned fatty acids, contains fatty acid C18: 2 in the amount of 18.92%. However the exact position and stereochemistry of double bonds was not determined.

Obtained fatty acid profile of all examined mushrooms, with the most dominant unsaturated linoleic and oleic acid suggested that consummation of these mushrooms could have beneficial effects on blood lipid profiles.

*Antimicrobial activity*

None of the analyzed sample did not show the antimicrobial activity.

## CONCLUSION

Present study deals with mushroom species *Cantharellus cinereus, Clavariadelphus pistillaris, Clitocybe nebularis* and *Hygrocybe punicea* which served as a source of phenolic compounds, exerting significant antioxidant activities as well. Usage of solvents with different polarity for extraction gave better insight in mushrooms antioxidant properties because natural antioxidants are multifunctional. This proved through the presented results which showed that all aqueous extracts had better antioxidant capacities than ethanol and methanol ones. Because of high antioxidant activities, mushrooms can be classified in functional foods or even drugs. Concerning mineral contents, examined mushrooms have proven to be a very good source of biologically important elements and also can be used as indicator organisms to monitor environmental pollution. Although values for toxic elements in examined mushrooms seem high, when calculated on a provisional tolerable weekly intake, safe level of consummation was not exceeded. Investigated mushrooms species did not show antimicrobial activity. To the best of authors' knowledge, the fatty acid profiles, antioxidant activity and metal content of these mushrooms were described for the first time and clearly indicate that they provide key nutrients, such as unsaturated fatty acids.

## EXPERIMENTAL SECTION

*Generals*

All used solvents (chloroform, methanol, hexane and dimethyl sulphoxide) were purchased from Sigma Aldrich, such as Folin–Ciocalteu reagent, gallic and ascorbic acids, iron(III) hloride hexahydrate, 2,2-Diphenyl-1-picrylhydrazyl, 2,20-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), 6-hydroxy-2,5,7,8-tetramethylchroman-2- carboxylic acid and 12% methanolic solution of boron trifluoride.

Chemical substances of analytical grade obtained from Merck were: neocuproine, copper(II) chloride dihydrate, sodium carbonate, hydrochloric acid, 2,4,2-tri(2-pyridyl)-s-triazine,

potassium ferricyanide, phosphate buffer, ammonium acetate buffer, trichloroacetic acid, potassium peroxodisulfate, ferrous sulfate heptahydrate, anhydrous magnesium sulphate and potassium hydroxide.

10 % $HNO_3$ was used for soaking all glassware which were rinsed well with distilled water.

*Sample collection*

Four wild edible mushrooms (*C. cinereus*, *H. punicea*, *C. pistillaris* and *C. nebularis*) which were analyzed were collected in, Gradac, near town Nis, during 2015. All samples of mushrooms were cleaned, lyophilized and stored in laboratory.

*Sample preparation for element analysis*

0.25 g dried mushrooms were digested with $HNO_3/H_2O_2$ (7:1) acid mixtures in a microwave digestion system. After digestion all samples were quantitatively transferred into volumetric flask (25 ml) and diluted with distilled water. The accuracy of applied analytical method was determined using the European Reference Materials "ERM-CD281: As, Cd, Cr, Cu, Hg, Mn, Ni, Pb and Zn."

*Extracts preparation for antioxidant activities*

All samples were lyophilized in order to remove water. Portions of 0.5000g lyophilized powdered mushrooms were extracted with appropriate solvent. Three different solvents were used (80% of ethanol, 80% of methanol and distilled water) for extraction.
Eighty percent ethanol and methanol were used instead of absolute ethanol and methanol because extraction yield is increased significantly using mixture of organic solvent with water.[32]
Sonication was done four times in an ultrasonic bath for 15 minutes. Extracts were filtered and diluted with solvent to 10 ml.

*Extracts preparation for antibacterial activities*

For antibacterial activities, extracts of mushrooms were done by sonication, 15 min at 25°C. Extracts were filtered, evaporated to dryness and dissolved in dimethyl sulfoxide at concentration of 50 mg/ml

*Extraction and derivatization samples for fatty acids*

Extraction and derivatization of all samples was done according to the method described by Dimitrijevic et al.[33]

*Determination of total phenolic content*

Folin-Ciocalteu reagent was used to estimate total phenolic content of mushrooms following a method by Singleton and Orthofer.[34] When assessing phenolic compound extraction, solvent type has been considered as one of the most important factor; water or organic solvent (ethanol, methanol, acetone, and diethyl ether) can be used.[35]

10.1002/cbdv.201800492

## Methods for estimating antioxidant activity

The methods for determining antioxidant activity used in this study can be divided into two groups based on the mechanism of action: based on reducing power and ability of "scavenging".

*Determination of free radical scavenging (FRS) activity*

The interaction between free radicals and antioxidants can show directly evidence for antioxidants to scavenging free radicals and has been widely used to evaluate the radical scavenging ability of antioxidants.[36]

The free radical scavenging capacity of mushroom extracts was determined according to the previously reported procedure using the stable DPPH radicals.[37]

ABTS radical scavenging activity was performed according to the method of Re et al.[38]

Trolox was used as a standard antioxidant in both methods.

*Reducing power*

Antioxidants are also known as reductants. The redox potential or reducing power of antioxidants is an important indicator of their antioxidant efficacy, and is measured through redox reaction with various metal ions, such as iron, copper, chromium, and cerium, among others and this group of methods can be classified as ET-based methods.[39] The methods based on this mechanism are FRAP, CUPRAC and TRP.

*Ferric reducing antioxidant power (FRAP) assay*

Ferric reducing antioxidant power assay was performed using the method of Benzie and Strain,[40] and it is based on the reduction of $Fe^{3+}$ to the blue $Fe^{2+}$ in acidic media.

*Cupric reducing antioxidant capacity (CUPRAC) assay*

The CUPRAC assay was performed using the method of Apak et al.[41]

*Total reducing power (TRP) assay*

The reducing power of extracts was determined by the method of Oyaizu.[42]

## Antimicrobial activity

The disk diffusion assay is used to evaluate antimicrobial activity of the extracts. Analysis was performed according to Faleiro et al.[43] Antimicrobial activity was evaluated against two Gram-positive bacteria (*Bacillus spizizenii* ATCC 6633 and *Staphylococcus aureus* ATCC 6538) and three Gram-negative bacteria (*Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027 and *Salmonella abony* NCTC 6017). Antifungal activity was tested against *Candida albicans* ATCC 10231 and *Aspergillus brasiliensis* ATCC 16404. Antimicrobial activity was done in triplicate.

10.1002/cbdv.201800492

**Statistical analysis**

STATISTICA 8 was used for chemometric analysis. The statistical differences between the methanol, ethanol and water extracts were analyzed using Student's t-test.

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**Author Contribution Statement:**

Marija V Dimitrijevic - did antioxidant activity and wrote manuscript

Violeta D Mitic - did chemometric analysis

Jelena S Nikolic - did antioxidant activity

Aleksandra S Djordjevic-did antimicrobial activity

Jelena J Mutic- analyzed mineral content by ICP-OES and ICP-MS

Vesna P Stankov Jovanovic - did proof reading

Gordana S Stojanovic- analysed chromatographs

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Graphical Abstract