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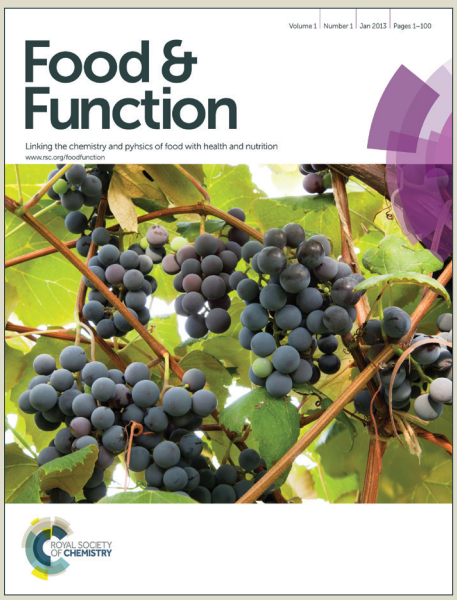

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Level of elements and antioxidant activity of commercial dietary supplement formulations based on edible mushrooms

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Abstract

Commercial preparations of *Cordyceps sinensis*, *Ganoderma lucidum* and *Coprinus comatus* mushroom marketed as healthy food supplements in Serbia were analyzed by atomic absorption spectrometry with a graphite furnace (GFAAS) for their elements content. Antioxidant activity potentials and total phenolics of the same mushrooms were determined. The elements content of mushroom samples ranged from 0.130-0.360 mg/kg for lead (Pb), <0.03-0.46 mg/kg for arsenic (As), 0.09-0.39 mg/kg for cadmium (Cd), 98.14-989.18 mg/kg for iron (Fe), 0.10-101.32 mg/kg for nickel (Ni), 5.06-26.50 mg/kg for copper (Cu), 0.20-0.70 mg/kg for cobalt (Co), 1.74-136.33 mg/kg for chromium (Cr) and 2.19-21.54 mg/kg for manganese (Mn). In the tests for measuring antioxidant activity, methanolic extract of *C. sinensis* showed the best properties. Same was for the analysis of selected phenolic compounds, *C. sinensis* found to have the highest content. Commercial preparations of *C. sinensis* and *C. comatus* can be considered to be safe and suitable food supplements included in well-balanced diets.

Keywords: *Cordyceps sinensis*, *Ganoderma lucidum*, *Coprinus comatus*, antioxidant activity, element concentration

1. Introduction

Interest in the safety of food supplements has been rapidly increasing as a consequence of high levels of some contaminants found recently in different natural products.[1-4] In addition, there is an increasing number of natural products, including food supplements, with perceived and real health benefits on the market.[5] As food supplements are one of the most easy to access complementary and integrative therapies, they are widely used in modern Western diets. For example, more than one half of the US population used them in 2003 to 2006 and similar trends were observed in Western European countries.[5,6] Their growing use is accompanied by an increasing concern because the safety of these preparations is not generally assessed before they enter the market. The traditional use of food supplements as a mushroom, herb or tea does not guarantee its safety when used as a supplement. They may contain compounds of concern, like elements, at levels far above those found in the regular diet, and therefore they can cause toxic effects to living organisms.[7- 9] Numerous data on elements content in mushrooms is indicating that they can accumulate large amounts of some toxic heavy elements, such as Pb, As and Cd.[10-12] A well documented example is the high accumulative capacity of *Coprinus comatus* for Pb. [13] As they are able to concentrate, higher fungi are also able to exclude specific metal ions, playing a key role in the cycling of trace elements with consequences on human health.[9,13,14]

Except trace elements, mushrooms can contain significant amounts of indigestible fibres, vitamins, and various physiologically active compounds. Synergy of these mushroom components is probably the main cause for their therapeutic properties in preventing some human diseases and disorders.[9,14,15] Among therapeutic properties, it was found that mushrooms can exhibit antioxidant activity also. Although almost all organisms are well-protected against

oxidative stress, supplements in the human diet are important to prevent or reduce free radical damage. Mushrooms are widely recognized as a functional food and they are an easily accessible source of natural antioxidants.[15-17]

There has been scarce data in the literature regarding the antioxidant properties and the level of heavy elements in mushrooms that has been used as food supplements while, there has not been reported data for *Cordyceps sinensis*, *Ganoderma lucidum* and *Coprinus comatus* mushroom marketed as healthy food supplements in Serbia. Thus, the aim of present work is to determine the level of elements and antioxidant potential of the methanol extracts of *Cordyceps sinensis* (Berk.) Sacc., *Ganoderma lucidum* (Curtis) P. Karst. and *Coprinus comatus* (O. F. Müll.) Pers. by four different antioxidant test systems namely; 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl (HO) and nitric oxide (NO) radical scavenging assays and ferric-reducing antioxidant power assay (FRAP) in addition to determination of their total phenolic contents and LC-MS analysis of the concentration of main phenolic compounds found in mushroom species. Therefore, to our best knowledge, this is the first report of *Cordyceps sinensis* (Berk.) Sacc., *Ganoderma lucidum* (Curtis) P. Karst. and *Coprinus comatus* (O. F. Müll.) mushroom commercial preparations with elements and antioxidant analysis.

2. Materials and methods

2.1. Reagents and solutions

Folin-Ciocalteu's reagent (FCR), methanol, p.a. formic acid, potassium ferricyanide, ferric chloride, N-(1-naphthyl)ethylenediamine dihydrochloride (NEDA) and trichloroacetic acid

(TCA) were obtained from E.Merck (Darmstadt, Germany). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2-deoxy-D-ribose, gallic acid, α-tocopherol as well reference standards of the phenolic compounds were obtained from Sigma Chemical Co. (Steinheim, Germany). 2-thiobarbituric acid and sulfanilamide were obtained from Fluka Chemie GmbH (Buchs, Switzerland) and sodium nitroprusside from Renal (Budapest, Hungary). Ultra-pure deionised water type Milli-Q (Simplicity, Millipore, France) with a specific resistivity of 18.2 MΩ/cm was used for preparation of standards and sample solutions. Concentrated 69% nitric acid (ccHNO3) ("for trace elements analysis" grade) and 30% hydrogen peroxide (H_2O_2) were purchased from J.T.Baker. All the plastic and glassware were cleaned by soaking in a 20% hydrochloric solution overnight then in 20% nitric acid overnight and finally rinsed with Milli-Q water. The As, Cd, Pb, Ni, Co, Cr, Cu, Fe and Mn stock standard solutions (1000 μg/mL) were supplied by J.T.Baker. The working standard solutions of 1 μg/mL for each element were obtained by diluting stock solutions in 3% nitric acid. The calibration curves were prepared using the so-called bulk solution prepared by mixing the standard solutions and the subsequent dilution. Automix option of the GFAAS was applied enabling automatic preparation of the calibration standards. All other reagents used in this study were of analytical grade.

2.2. Mushrooms and extraction procedure

Commercial preparations of mushrooms *Coprinus comatus*, *Cordyceps sinensis* and *Ganoderma lucidum* were used for the experiment. These commercial preparations can be found in Serbian pharmacies as food supplements and represent 100-percent, finely minced dried powder of whole mushroom. The mushroom samples (7 g) were extracted by using Soxhlet extractor for 5 hours with methanol and then filtered. After that, methanolic extracts were evaporated at 40 °C to dryness under reduced pressure and kept in the dark at 4 °C until tested.

121

122 *2.3. Microwave digestion*

123 Microwave (Ethos One, Milestone, Italy) with segmented rotor of high pressure (HPR-
124 1000/10S) and internal temperature sensor was used for digestion of the samples.

125 The method applied for heavy elements determination is previously used by Škrbić *et al*.[18]
126 Briefly, about 0.5 g of previously homogenized composite samples was weighted inside high-
127 pressure Teflon (TFM) vessels and 7 mL of ccHNO_3 (69%) and 1 mL of H_2O_2 (30%) were added.
128 The operational conditions and the heating program used were carried out according to the
129 conditions recommended by the manufacturer.

130 After cooling, digests were diluted with Milli-Q water to 25 mL in glass flask and finally,
131 transferred to previously acid-cleaned and labeled polypropylene vessel for further analysis.
132 From each kind of food samples three aliquots were digested and each sample solution was then
133 analyzed in triplicates.

134

135 *2.4. Instrumentation for elements analysis*

136 A Varian AA240/GTA120 model atomic absorption spectrometer (AAS) with deuterium
137 background correction, equipped with a graphite furnace (GF) for electrothermal atomization and
138 an automatic sampler was used in this study. The assembly was operated from an interfaced
139 computer running SpectrAA software. Varian hollow cathode lamps were used as line sources for
140 all analytes. Argon was used as the inert gas. The wavelengths used for determination of the
141 elements in dried mushroom samples were as follows: 193.7 nm for As; 228.8 nm for Cd; 283.3
142 nm for Pb; 240.7 nm for Co; 357.9 nm for Cr; 232 nm for Ni; 324.8 nm Cu; 372 nm for Fe; and
143 279.5 nm for Mn.

144

145 *2.5. Quality assurance*

146 Analytical method used was accredited according to ISO 17025. Thus, appropriate quality
147 assurance procedures and precautions were carried out to ensure the reliability of the results. The
148 developed method was validated by in-house quality control procedure. Summary of validation
149 data of GFAAS method for analysis of the selected elements in samples digested by microwave
150 are given in Table 1.

151 [Insert Table 1 about here]

152 Calibration curves were obtained with acidified aqueous element standards by external
153 calibration procedure. The correlation coefficients obtained for calibration curves were all greater
154 than 0.9950. The limit of detection (LOD) and limit of quantification (LOQ) were calculated as
155 the mean signal of five blanks plus three or ten times the standard deviation, respectively.
156 Validation of the method accuracy was carried out by in house determination of the element
157 recoveries from dried mushroom samples.

158 Taking into consideration that the investigated elements are not regulated by the Serbian
159 and the European existing regulations for investigated samples, the arbitrary level for As (0.3
160 mg/kg), Cd (0.2 mg/kg), Pb (0.3 mg/kg), Ni (1 mg/kg), Co (0.1 mg/kg), Cr (1 mg/kg), Cu (2.5
161 mg/kg), Fe (1.5 mg/kg), and Mn(1.5 mg/kg) was chosen for the spiking. Recovery experiments
162 were performed in triplicates. The recoveries ranged from 60 to 132% (Table 1) while the
163 repeatability expressed as relative standard deviation of 3 spiked samples ranged from 1 to 19%
164 (Table 1).

165 All samples were analyzed in triplicate (n=3). Blank samples were included in every
166 batch of samples to check for possible contamination.

167

168 *2.6. Scavenging effect on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical*

The free radical scavenging activity of the mushroom extracts was measured from the bleaching of purple coloured methanol solution of DPPH according to Brand-Wiliams *et al.*[19] One millilitre of 90 μM methanolic DPPH solution was mixed with various volumes of mushroom samples (20-300 μL of 0.5% extract) and filled up to 4 ml with methanol. After a 60 min incubation period at room temperature the absorbance was read against a blank at 515 nm. Free radical scavenging capacity was calculated as follows:

$$RSC = 100 - 100 * A_{sample}/A_{blank},$$

where A_{blank} is the absorbance of diluted DPPH solution and A_{sample} is the absorbance of the test compund. Vitamin E (α-tocopherol) was used as a control.

The IC_{50} value, which represents the concentrations of the sample required to cause 50% inhibition of DPPH radical, was estimated by linear regression analysis from the obtained RSC values and was expressed in mg of mushroom extract per ml.

2.7. Hydroxyl-radical scavenger capacity

Hydroxyl-radical scavenger capacity was determined according to the method of Gutteridge.[20] A 0.1 mL of 2-deoxy-D-ribose solution (50 μmol/L) in phosphate buffer (pH 7.4) was mixed with 20 μL of extract (concentration range – 12.5 to 200 mg/mL) or solvent in control, 0.1 mL H_2O_2 (0.015%), 0.1 mL $FeSO_4$ (10 mmol/L) and subsequently diluted with 2.7 mL of phosphate buffer (pH 7.4). Amounts of 3 millilitres of phosphate buffer and 20 μL of extract were added in the blank probe. After incubation at 37 °C for 1 h, 0.2 mL of EDTA solution (0.1 mol/L) was added to all the samples. Thiobarbituric acid-reactivity was developed by adding 2 mL of aqueous mixture with TBA (3.75 mg/mL), $HClO_4$ (1.3%), and trichloroacetic acid (0.15 g/mL)) and afterwards heating at 100°C for 10 min. The absorbance of cooled mixtures was measured at 532

nm. Vitamin E was used as a positive control. All samples and the control were made in triplicate. IC_{50} values were determined.

2.8. NO scavenger capacity

Nitric oxide radical scavenging capacity measurement was based on method of Lesjak *et al*.[21] The reaction mixture composed of sodium nitroprusside (10 mmol/L, 75 µL), phosphate buffer, pH 7.4 (75 µL) and extract (10 µL, concentration range – 2.5 to 100 mg/mL) or standard solution (α-tocopherol) was incubated for 90 min at 25 °C. Amounts of 10 µL of extract and 150 µL of buffer were added in the blank probe. After incubation, 150 µL of solution containing equal amounts of sulfanilamide (2% in 4% phosphoric acid) and N-(1-naphthyl)ethylenediamine dihydrochloride (0.2%) was added to the reaction mixture and was left to stand for 3 min. The absorbance of solutions was measured at 546 nm against appropriate blanks. All samples and control were made in triplicate. IC_{50} values were determined.

2.9. Reducing power

The reducing power was determined according to the method of Oyaizu.[22] Each of the extracts (1-10 mg/ml) in methanol (1 ml) was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. Reaction mixture was incubated at 50 ^{o}C for 20 min and then 2.5 ml of 10% trichloroacetic acid was added. The mixture was centrifuged at 1000 rpm for 10 minutes. The upper layer (2.5 ml) was mixed with 2.5 ml of deionised water and 0.5 ml of 0.1% ferric chloride, and the absorbance was measured at 700 nm against a blank. Vitamin E (α-tocopherol) was used as a control.

2.10. Determination of total phenolics

Phenolic content of mushroom extracts was assayed using the method given in the literature.[23] One hundred microlitre of 0.5% mushoom extracts were mixed with 500 µl of Folin-Ciocalteu reagent and 400 µl of 7.5% sodium carbonate solution in test tubes. After being vortexed and incubated in dark for 2 hours, absorbance was measured at 740 nm. The concentrations of phenolic compounds were calculated from the standard gallic acid graph and expressed as mg of gallic acid equivalents (GAE) per g of dry extract:

Absorbance = 0.0113 gallic acid (mg) + 0.0019 (R^2 : 0.9997)

All the assays for measuring of scavenging effect and reducing power, and determination of total phenolics were carried out in triplicate and the mean values were calculated.

2.11. LC–MS/MS analysis of the selected phenolics

LC–MS/MS analysis of the selected phenolics was done after Orcic *et al*.[24] Extracts were diluted with mixture of mobile phase solvents A (0.05% aqueous formic acid) and B (methanol), in 1:1 ratio, to obtain a final concentration of 2 mg/mL. Working standards, ranging from 1.53 ng/mL to $25.0*10^3$ ng/mL (15 standards), were prepared by serial 1:1 dilutions of standard mixture with solvent (A:B=1:1). Samples were analyzed with Agilent Technologies 1200 Series HPLC coupled with Agilent Technologies 6410A Triple Quad tandem mass spectrometer with electrospray ion source, controlled by Agilent Technologies MassHunter Workstation software – Data Acquisition (ver. B.03.01). Injection volume was 5 µL. Compounds were separated with Zorbax Eclipse XDB-C18 (50 mm x 4.6 mm, 1.8 µm) rapid resolution column heated at 50 °C. Mobile phase was delivered at flow rate of 1 mL/min in gradient mode (0 min 30% B, 6 min 70% B, 9 min 100% B, 12 min 100% B, re-equilibration time 3 min). Eluted components were detected by MS, using the ion source parameters as follows: nebulization gas (N2) pressure 40

psi, drying gas (N2) flow 9 L/min and temperature 350 °C, capillary voltage 4 kV, negative polarity. Data were acquired in dynamic MRM mode, using the optimised compound specific parameters. For all the compounds, peak areas were determined using Agilent MassHunter Workstation Software – Qualitative Analysis (ver. B.03.01). Limits of determination of the applied method for the studied compounds were in the range from 0.004 µg/mL to 0.04 µg/mL, while the limits of quantitation were from 0.01 µg/mL to 0.02 µg/mL. Calibration curves were plotted and samples' concentrations calculated using the OriginLabs Origin Pro (ver. 8.0) software.

3. Results and discussion

3.1. Concentration of elements

Elements concentrations of the investigated mushroom species were measured on a dry weight basis (d.w.). It should be stressed that there is a consensus that dry weight of mushrooms is 10% of their fresh weight.[14,25] In this study, concentrations of 9 trace elements (Pb, As, Cd, Fe, Ni, Cu, Co, Cr and Mn) have been determined. The conventionally adopted as heavy elements studied in the experiments are Pb, As and Cd.[9,11] The levels of trace elements in the analyzed samples have been shown in Table 2.

Concentrations of the elements in mushrooms are generally species-dependent. The trace elements contents of the species mainly depend on the ability of the species to extract elements from the substrate, and on the selective uptake and deposition of elements in their tissues. Furthermore, biochemical and chemical parameters of the substrate, the age of mycelium, substrate composition and the interval between fructification events can affect concentrations of the elements in mushrooms.[9,11,14,25].

263 [Insert Table 2 about here]

264 Results from a great number of original papers, dealing with heavy elements in edible
265 mushrooms show that cadmium, arsenic and lead are the elements of toxicological importance.[10-
266 12] In the present study, the contents of Pb and Cd did not exceed the statutory limits of 0.3 and
267 0.2 mg/kg fresh weight, respectively, for edible mushrooms (corresponding to 2.0 and 3.0 mg/kg
268 dry weight), set by the EU.[26] In the study of Garcia *et al*[13] *C. comatus* had the highest Pb content
269 among 28 species of edible mushrooms collected in the northern parts of Spain. According to
270 them, *C. comatus* is the mushroom with high capacity of Pb accumulation. They have measured
271 mean value of 3.6 mg/kg dry weight which is under maximum allowed concentration. On the
272 contrary Cocchi *et al*[27] in the study of 60 mushroom species, showed that *C. comatus* had Pb
273 level below allowed concentration which is also observed in the present study.

274 There is presently no legislation for arsenic in mushrooms within the EU. There are only
275 recommendations on intake limitations with provisional tolerable weekly intake (PTWI) value of
276 0.003 mg/kg body weight or 0.18 mg for an average consumer (considering 60 kg of body
277 weight).[28] PTWI value for Cd is 0.006 mg/kg body weight or 0.36 mg for an average consumer.[29]
278 According to EFSA[30] two toxicological reference dietary intake values of Pb are identified for
279 adults: 0.00063 mg/kg body weight/day (or 0.04 mg) for nephrotoxic effects and 0.0015 mg/kg
280 body weight/day (or 0.09 mg) for cardiovascular effects. These values correspond to 0.04 and
281 0.09 mg for Pb, 0.05 mg for Cd and 0.03 mg for As, on a daily basis. In addition, for intake
282 calculations, 30 g portion of dry weight mushrooms per meal is assumed.[9,11,31] The element
283 intakes as mg per serving are presented in Table 3. By comparison of these three mushrooms it
284 can be noticed that *G. lucidum* has highest concentration of Pb and Cd, and *C. comatus* has
285 highest concentration of As, but these values are within safe limits being for G. *sinensis* 10% (for
286 nephrotoxic effects) and 4% (for cardiovascular effects), 6% and 3% of toxicological references

for Pb, Cd and As, respectively.[26,28-31] Therefore, the intake of elements of toxicological importance (Pb, Cd, As) by consumption of 30 g portion of dry weight mushrooms daily poses no risk.

We could not reach any record for element content of commercial, cultivated or wild-growing *G. lucidum* and *C. sinesis* in the literature. As far as the literature survey could as certain, elements levels of only wild-growing *C.comatus* has previously been evaluated.[13,27,32,33]

Among investigated trace elements iron was the element with the highest concentration in all three studied mushrooms. The highest Fe content was found in the samples of *G. lucidum* (989.18 ± 56.38) whereas the lowest was in *C. sinensis* (98.14 ± 1.16). In the study of Yamac *et al*[32] the range of Fe concentrations was between 110 and 11460 mg/kg in 15 different mushroom species. Among these investigated species the second highest Fe concentration was determined as 3640 mg/kg in the sample of *C. comatus*. Wide variations in iron content are evident from data of numerous studies which confirms our findings.[11,14,25,33,34]

Fe is followed with Cr and Ni in the samples of *G. lucidum* and with Cu in the samples of *C. sinensis* and *C. comatus*. From the data for numerous species, usual chromium contents were between 0.5 and 5 mg/kg d.w., less frequently between 5 and 10 mg/kg d.w. and in only several reports above 10 mg/kg d.w.[9,11,14,32-34] The highest Cr concentration determined here is 136.33 ± 15.27 mg/kg d.w. in *G. lucidum*. However, the Cr levels in *C. sinensis* and *C. comatus* are 1.74 ± 0.48 and 3.90 ± 0.11 mg/kg d.w.

The reported Ni values for wild-growing mushrooms were usually from traces to 15 mg/kg d.w.[9,14,32,33] The highest Ni level was reported by Demirbas.[35] This author determined value of 145 mg/kg d.w. in *Pleurotus ostreatus*. In the present study Ni concentration in *C. comatus* is 0.10 ± 0.02, in *C. sinensis* is 2.18 ± 0.25 and in *G. lucidum* is 101.32 mg/kg d.w. It is

important to point out that Ni is an essential element for many organisms, but nutritional requirements or recommended dietary allowances have not been established.[9,14]

G. lucidum has also highest values of Mn (21.54 ± 1.582 mg/kg d.w) and Co (0.70 ± 0.04 mg/kg d.w) and in the case of Cu, *C. comatus* has the highest concentration (26.50 ± 2.49 mg/kg d.w). Data obtained in this study about levels of Cu, Co and Mn in commercial mushroom preparations is highly in agreement with those presented in other reports dealing with wild-growing species.[9,12,14,32,33]

3.2. Antioxidant activity

Antioxidant activity can be explained with different mechanisms of action, such as inhibition of oxidizing enzymes, chelation of transition metals, transfer of hydrogen or single electron to radicals, singlet oxygen deactivation, or enzymatic detoxification of reactive oxygen species. Therefore, in order to extensively characterize the antioxidant potential of extracts there is need for combining several different methods.[21] Thus, methanolic solutions of DPPH, hydroxyl, and nitric oxide radicals were used for testing of the radical scavenging ability of mushroom extracts. Determined scavenging capacities expressed as IC_{50} values are shown in Table 4.

The advantage of DPPH is its stability in the radical form and being unaffected by certain side reactions. [36] All the assessed extracts were able to reduce DPPH in dose dependent manner with the IC_{50} values ranging from 172 to 483 μg/ml. All the extracts proved to have free radical scavenging activity but to different extent (Table 4). Comparing results of the present study with those of other authors it can be noticed that the scavenging activity of here investigated extracts is significant. As shown in Table 4 methanolic extract of *C. sinensis* has the highest scavenging activity with measured IC_{50} value of 172 μg/ml. In the study of Dong and Yao[37] aqueous extract

from cultural mycelia of *C. sinensis* demonstrated similar antioxidant action. IC_{50} value of *C. comatus* extract is 236 µg/ml which is considerably lower than the values from studies of Li *et al*[38] (786 µg/ml of ethanolic extract) and Vaz *et al*[39] (256 µg/ml of ethanolic extract). The highest IC_{50} value is obtained for *G. lucidum* extract but that result is in accordance with other published data on the same mushroom.[40,41]

Unlike DPPH test, the scavenging effect of the *C. comatus* extract on the highly reactive hydroxyl radical was higher than the two other mushroom extracts and α-tocopherol also. The IC_{50} value was not determined for *G.lucidum* extract and α-tocopherol because they reached no more than 27% of inhibition in the investigated concentration range. The activity regarding neutralization of the nitric oxide radical was practically the same for α-tocopherol. In this test, *C. sinensis* extract showed highest IC_{50} value and *C. comatu*s extract did not reach 50% inhibition under the same conditions. The result of well-known liposoluble antioxidant, α-tocopherol, can be explained by poor solubility of it in aqueous buffers, while there is need of further investigation for the explanation of the results of *G.lucidum* extract in hydroxyl radical and *C. comatu*s extract in nitric oxide radical test.[21]

[Insert Table 4 about here]

The assay of reducing activity was based on the reduction of Fe^{3+}/ferricyanide complex. Presence of reducers (antioxidants) causes the reduction of Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, ferrous ion concentration was then monitored by measuring the formation of Perl's Prussian blue at 700 nm.[15,42] Table 5 shows the reducing power of mushroom methanolic extracts as a function of their concentration. The reducing power of the mushroom methanolic extracts increased with concentration. At 10 mg/ml concentration, the absorbance values were higher than 1.0 for the all extracts. According to the results, the most active

mushroom is *C. sinensis* with an absorbance value of 1.392 ± 0.009. At maximal concentration value, this mushroom is followed by *C. comatus* and *G. lucidum*, respectively. This is highly in agreement with those found in similar studies.[37,39-41] Reducing power test confirmed DPPH test, or vice versa, because same results have been achieved in both with *C. sinensis* as the most active and *G. lucidum* as the least active mushroom.

[Insert Table 5 about here]

Phenolic substances have received special attention in the past 20 years because of their putative role in the prevention of oxidative stress. The antioxidant potential of various dietary phenolic compounds have been described as exerting a variety of biological actions such as free-radical scavenging, chelation of metals and modulation of enzyme activity. Traditionally, plant extracts, including essential oils, are considered to be a main source of phenolic compounds. There are numerous studies in different experimental models showing positive antioxidant activity of plants thanks to their high content of phenolic compounds.[43,44] Recently, it has been found that mushrooms contain low molecular weight compounds, such as phenols, which are very efficient scavengers of peroxy radicals.[37,39,41] Thus, the total phenolic contents of the mushrooms have been also evaluated.

Once again, *C. sinensis* found to be the best among the investigated mushroom species. The *C. sinensis* sample presented the highest content of phenolic compounds (211.667 mg GAE/g of dry extract). This value is followed by *C. comatus* (151.017 mg GAE/g of dry extract) and *G. lucidum* (79.109 mg GAE/g of dry extract). As shown previously, *G. lucidum* has highest concentration of Ni and this can be explanation for the weakest antioxidant activity. This is supported by an experiment with *Macrolepiota procera* exposed to high Ni concentrations. The

exposure to nickel induced oxidative stress, which has initiated an efficient antioxidant defense system of mushroom.[45]

It must be noted that Folin-Ciocalteu method, employed here for measuring total phenolic content, has several analytical interferences. In this method reagent mixture of phosphotungstic and phosphomolibdic acid also reacts with other non-phenolic reducing compounds leading to an overestimation of the phenolic content. Nevertheless, this method is widely used prior to liquid chromatography quantification of phenolic compounds.[39,41,46] Same steps were carried out in this study, after Folin-Ciocalteu method, LC-MS/MS technique was used for accurate measuring of the selected phenolics.

As can be found in the literature, the phenolic composition of the mushrooms seems to be characterized by mainly the presence of phenolic acids. This confirms our finding that myricetin, phenolic compound which is not a phenolic acid, was not present in the investigated mushroom samples. In general, it is assumed that only plants, not mushrooms or fungi, possess the ability to synthetize flavonoids, such as myricetin.[46,47] Further, phenolic acids can be divided into two major subgroups, hydroxybenzoic acids and hydroxycinnamic acids. Hydroxybenzoic acids are commonly present as a component of complex polymers like lignins, tannins and sugar derivatives and hydroxycinnamic acids are mainly bound to cell-wall structural components.[46] In this study *p*-hydroxybenzoic acid was found in all three mushroom extracts, being the major compound in the case of *C. sinensis* and *C. comatus*. In the case of *G. lucidum*, protocatechuic acid, which also belongs to hydroxybenzoic acids, was the most prominent phenolic. Gallic acid, phenolic compound from the same group, was present only in the extract of *C. sinensis* in the concentration similar to *p*-hydroxybenzoic acid. Phenolic compound from the hydroxycinnamic acids group found here was *p*-coumaric acid, which was not the case for caffeic acid. *P*-coumaric acid was detected in the samples of *C. sinensis* and *C. comatus* (Table 6). In the literature can be

found that wild growing *C. comatus* has excellent phenolic profile and antioxidant activity. In our study, concentration of *p*-hydroxybenzoic, *p*-coumaric and protocatechuic acid in *C. comatus* sample was higher from that found in the literature.[39]

Taking into the consideration a sum of investigated phenolics, *C. sinensis* showed the highest concentration (Table 6). Therefore, the pronounced antioxidant activity of the methanolic extract of *C. sinensis*, manifested as free radical scavenging and reducing power, is possibly due to its high phenolic content. To our knowledge this is the first report of LC-MS analysis of phenolic compounds from *C. sinensis* mushroom.

[Insert Table 6 about here]

4. Conclusion

Commercial preparations of *C. sinensis* and *C. comatus* can be considered to be safe and suitable food supplements included in well-balanced diets due to their favorable trace elements content and as a rich source of antioxidants. On the other hand, commercial preparation of *G. lucidum* needs to be further studied. At the end, from this study can be concluded that other dietary supplements, not only plants, can reduce the amount of free radicals and be potent and safe antioxidants.

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528 **Table 1**. Summary of the validation data of GFAAS method for heavy elements analysis determination

	Pb	As	Cd	Ni	Co	Cr	Cu	Fe	Mn
LOD (mg/kg)	0.003	0.04	0.001	0.02	0.02	0.02	0.02	0.30	0.002
LOQ (mg/kg)	0.003	0.13	0.001	0.03	0.05	0.05	0.03	0.61	0.010
Recovery (%)	132	74	89	60	60	90	115	125	123
RSD (%)	1	19	7	7	1	19	12	14	7

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530 **Table 2**. Mean value ± standard deviation (as mg/kg, dry weight basis) of investigated elements in studied dried mushroom samples

531 corrected for in house determined recoveries

Samples	Pb	As	Cd	Fe	Ni	Cu	Co	Cr	Mn
C. sinensis	0.130 ± 0.097	<0.03	0.09 ± 0.01	98.14 ± 1.16	2.18 ± 0.25	5.06 ± 0.24	0.20 ± 0.01	1.74 ± 0.48	2.19 ± 1.018
G. lucidum	0.360 ± 0.045	0.12 ± 0.03	0.39 ± 0.02	989.18 ± 56.38	101.32 ± 2.82	13.09 ± 2.40	0.70 ± 0.04	136.33 ± 15.27	21.54 ±1.582
C. comatus	0.27 ± 0.025	0.46 ± 0.01	0.35 ± 0.01	452.97 ± 9.13	0.10 ± 0.02	26.50 ± 2.49	0.27 ± 0.01	3.90 ± 0.11	14.03 ± 2.033

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535 **Table 3**. Daily intakes of elements as mg per serving

Samples	Pb	As	Cd	Fe	Ni	Cu	Co	Cr	Mn
C. sinensis	0.004 ± 0.003	<0.001	0.003 ± 0.000	2.944 ± 0.035	0.065 ± 0.008	0.152 ± 0.007	0.006 ± 0.000	0.052 ± 0.014	0.066 ± 0.031
G. lucidum	0.011 ± 0.001	0.004 ± 0.001	0.012 ± 0.001	29.675 ± 1.691	3.040 ± 0.085	0.393 ± 0.072	0.021 ± 0.001	4.090 ± 0.458	0.646 ± 0.047
C. comatus	0.008 ± 0.001	0.014 ± 0.000	0.011 ± 0.000	13.590 ± 0.274	0.003 ± 0.001	0.795 ± 0.075	0.008 ± 0.000	0.117 ± 0.003	0.421 ± 0.061

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540 **Table 4**. IC_{50} values for evaluated antioxidant assays of examined mushroom species and α-tocopherol[a]

Samples	IC_{50} values for scavenging activity (μg/ml) Radical species		
	DPPH	HO	NO
C. sinensis	172.2 ± 6.3	383.7 ± 16.2	299.1 ± 12.9
G. lucidum	482.7 ± 10.1	n.a.[b]	314.2 ± 8.3
C. comatus	235.7 ± 8.9	307.9 ± 6.2	n.a.
α-tocopherol	2.5 ± 0.1	n.a.	n.a.

541 [a] Values are expressed as means ± SD of three parallel measurements

542 [b] n.a., 50% inhibition not achieved

543 **Table 5**. Reducing power of mushroom species expressed as absorbance of 700 nm[a]

Samples	Concentration (mg/ml)				
	0.1	**1**	**2**	**5**	**10**
C. sinensis	**-**	**0.142 ± 0.004**	**0.252 ± 0.004**	**0.647 ± 0.008**	**1.392 ± 0.009**
G. lucidum	**-**	**0.107 ± 0.003**	**0.236 ± 0.002**	**0.583 ± 0.004**	**1.018 ± 0.002**
C. comatus	**-**	**0.084 ± 0.003**	**0.196 ± 0.004**	**0.525 ± 0.008**	**1.090 ± 0.007**
α-tocopherol	**0.179 ± 0.001**	**-**	**-**	**-**	**-**

544 [a] Values are expressed as means ± SD of three parallel measurements

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546 **Table 6.** Concentrations of main phenolic compounds found in mushroom species (expressed as μg of phenolics per gram of

547 mushroom dry extracts).

Compound	Extract		
	C. sinensis	*G. lucidum*	*C. comatus*
p-Hydroxybenzoic acid	185.6	28.1	78.1
Protocatechuic acid	17.1	172.8	6.6
Gallic acid	140.5	< LOD[a]	< LOD
p-Coumaric acid	11.9	< LOD	32.4
Caffeic acid	< LOD	< LOD	< LOD
Myricetin	< LOD	< LOD	< LOD
Total phenolic compounds	355.1	200.9	117.7

548 [a] < LOD– peak not observed, concentration is lower than the LOD

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551 Highlights

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553 - Dietary supplements based on edible mushrooms from Serbian pharmacies were investigated.

554 - *C. sinensis* mushroom showed best antioxidant properties.

555 - *C. sinensis* and *C. comatus* elements concentrations are within safe limits.

556 - *G. lucidum* has very high level of nickel and the weakest antioxidant properties.

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