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ABSTRACT: *Ganoderma lucidum*, used in a traditional Chinese medicine, represents one of the most important medicinal mushrooms in the world, whose fruiting bodies and spores have been traditionally used because of a wide spectrum of biological activities such as antidiabetic, antioxidative, antiproliferative, cardioprotective, etc. Its ethnomedicinal importance in some parts of the Balkan region (Serbia and Croatia) is almost totally unknown and there should be more scientific investigations carried out. The aim of this work was to make a comparative study of antioxidative activities and total phenolic content of ethanol and hot water extracts of *G. lucidum*, collected from forests in Serbia (Morovićke šume, Fruška Gora) and Croatia (Donji Lapac, Plješevica). The present study was carried out to evaluate antioxidant potential of examined extracts via scavenging potential on ABTS, DPPH, OH and NO radicals, as well as of chelating effects via FRAP assay, together with determination of their total phenolic content. Results showed that both GLS extracts possessed better antiradical activities (IC50 = 0.23±0.01 for H2O and 2.75±0.01 μg/mL for EtOH for OH and DPPH assay, respectively) than in the ABTS assay (151.40±1.07 mg TE/g d.w. for EtOH), while the phenolic content was generally equal in extracts of Serbian and Croatian samples (60.74±0.57 mg GAE/g d.w. for EtOH and 77.10±0.27 mg GAE/g d.w. for H2O, respectively). Therefore, these extracts could be considered as a good source of natural antioxidants. These results showed that examined *G. lucidum* extracts (especially H2O) contain high amount of phenolic content which could significantly enhance the antiradical potential and reduce potential on iron ions. This is the first study reporting the comparison of antioxidant activities and phenolic contents of two different extracts between two *G. lucidum* strains from two different geographical origins from the Balkan region.

KEYWORDS: antioxidant capacity, *Ganoderma lucidum*, phenols, reducing power, radical reduction

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INTRODUCTION

In the last decades there has been a growing interest in the study of antioxidant activities of different foods possessing abundant free radicals scavengers and having an effect on many reactions in food systems (Klaus et al. 2011). It is also well documented that the implication of free radicals in oxidative stress represents one of the main causing agents in many diseases (Kozarski et al. 2015). Antioxidant compounds have the ability to trap free radicals and thus inhibit the oxidative mechanisms that lead to various degenerative disorders, including cancerogenesis, neurodegenerative and cardiovascular diseases (Ferreira et al. 2009; Janjušević et al. 2017; Nagaraj et al. 2014).

All organisms have endogenous antioxidant defences produced during normal cell aerobic respiration against free radicals, but there are various exogenous antioxidants (α-tocopherol, ascorbic acid, carotenoids, glutathione, and polyphenols) which can be found in whole grains, fruits, vegetables, teas, spices, and herbs (Nagaraj et al. 2014). Mushrooms are also organisms attractive as a source of antioxidants, which demonstrate antiradical activities and also reducing capacities (Ferreira et al. 2009; Karaman et al. 2014; Rašeta, 2016). Some advantages of using mushrooms in food as a good source of bioactive compounds are the following: the fruiting bodies can be produced in much less time, the mycelium may be rapidly produced in liquid culture, and the extracellular culture medium can be easily manipulated in order to produce optimal quantities of active products (Tešanović et al. 2017).

Mushrooms contain a variety of complex compounds that can be derived from both secondary and primary metabolism such as phenolics, polyketides, polysaccharides, triterpenoids, and steroids that characterize each type of fungal species and possess specific medicinal effects including antimicrobial, antioxidant, antiproliferative, etc. (Brakhage and Schroeckh, 2011; Klaus et al. 2011; Kozarski et al. 2015; Novaković et al. 2016; Rašeta, 2016; Rašeta et al. 2016; Zhang et al. 2016).

The presence and composition of all of these compounds in fungi mainly depend not only on genetic determinants, but also on ecological factors, including particular environmental conditions in specific habitats and their lifestyle (saprotrophic, parasitic and symbiotic/e.g. mycorrhizal) (Chaumenton et al. 1993).

A recent review on the chemical composition and biological properties of Ganoderma species has described this genus as a therapeutic biofactory (Paterson, 2006). A great deal of work on Ganoderma species has been carried out on one peculiar species, namely G. lucidum (Lingzhi, Munnertake, Sachitake, Reishi, and Youngzhi), which is the best known medicinal mushroom all over the world (Paterson, 2006). For hundreds of years, this species has been regarded as a traditional folk medicine using mushroom for the prevention and treatment of various human diseases, such as hepatitis, hypertension, chronic bronchitis, bronchial asthma, cancer, and others (Baby et al. 2015; Boh et al. 2007; Paterson, 2006; Rašeta, 2016).
In recent years, phytochemical studies of *G. lucidum* resulted in an impressively large number of more than 400 different bioactive compounds isolated (Baby *et al.* 2015): polysaccharides (Chiu *et al.* 2017; Kozarski *et al.* 2014), triterpenoids (Baby *et al.* 2015; Boh *et al.* 2007; Wang *et al.* 2006), and phenolic compounds (Rašeta, 2016; Rašeta *et al.* 2016; Stilinović *et al.* 2014; Yildiz *et al.* 2015). Based on literature data, many of these compounds have been already used as antioxidant agents, especially phenolic compounds that could be the major determinants of their antioxidant potentials (Balasundram *et al.* 2006; Ferreira *et al.* 2009). This was demonstrated in recent studies through the high correlation coefficient of analysed antioxidant activities and TP contents (Novaković *et al.* 2016; Rašeta *et al.* 2016).

The aim of the present study was to analyse antioxidative activities of the ethanol and hot water extracts, as well as their phenolic contents. Two different strains of wild medicinal mushrooms *G. lucidum* originating from Serbia and Croatia were compared using four radical scavengers and the reducing power assays *in vitro*.

**MATERIAL AND METHODS**

**Fungal material**

Fruiting bodies of *G. lucidum* were collected during the autumn of 2010 (Morovičke šume, Serbia) and the spring of 2016 (Donji Lapac, Croatia). After the identification of the species, voucher specimens were identified by MSc Eleonora Bošković at the Herbarium BUNS, Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad under number (12-00715 and 12-00724). Air-dried samples were ground to a fine powder and stored in dark bottles at room temperature prior to analysis.

**Preparations of extracts**

For the preparation of ethanolic extracts (EtOH), 20 g of powdered fungal material were extracted by maceration with 96% EtOH (JT Baker, Netherlands) during 72 h at room temperature (Sekljanik S400 W Chopper, Gorenje). For the preparation of the hot water extracts (H\textsubscript{2}O), 20 g of fungal material were mixed with 200 ml of boiling water and incubated at 80 °C for 60 min in water bath (Elektromedicine, Slovenia). After filtration (Whatman No. 4 Filter paper), EtOH was evaporated *in vacuo* (Büchi R-210; Büchi Labortechnik AG, Switzerland) at 35 °C and crude residue was dissolved in distilled water (10 mg/mL), whereas H\textsubscript{2}O was stored at -20 °C and then lyophilized (Christ Alpha 1-2 LD Freeze Dryer, Switzerland) for 72–96 h in ice condenser at -55 °C. Dried extracts were dissolved in distilled water to obtain 100 mg/mL stock solutions.
ABTS assay

The ABTS assay (Arnao et al. 2001) was used to measure radical scavenging activity of the extracts and standard compound, propyl gallate (PG). 10 ml of each extract or standard compound (trolox, vitamin E analogue) (except in a control probe when only the solvents were used) was added to 290 μl of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) or distilled water (corrections of work sample). Absorbance was measured spectrophotometrically at 515 nm after 30 min of reaction. The same procedure was repeated with PG as a positive control. The absorbance was measured at 734 nm after 5 min of reaction. All the samples were made in triplicate and mean values of ABTS scavenging activity were expressed as milligram trolox equivalents (TE) per gram of dry weight (mg TE/g d.w.), calculated according to the standard curve.

DPPH assay

The DPPH assay was performed as described before (Espin et al. 2000), following the transformation of the 1,1-diphenyl-2-picryl-hidrazyl (DPPH) radical to its reduced, neutral form (DPPH-H). 10 μl of each extract or standard compound (except in a control probe when only the solvents were used) was added to 60 μl of DPPH reagent and mixed with 180 μl of methanol. Absorbance was measured spectrophotometrically at 515 nm after 30 min of reaction. The same procedure was repeated with PG as a positive control. The antioxidant activity was expressed as a concentration of the sample that inhibited the DPPH radical formation by 50% (IC$_{50}$), and the experiment was performed in triplicate.

OH· assay

The hydroxyl radical scavenging activity was determined according to the method of Halliwell et al. (1987). 10 μl of each extract or standard compound (except in a control probe when only the solvents were used), was mixed with 100 μl of 0.015% H$_2$O$_2$, 100 μl of 10 mmol/L FeSO$_4$, 100 μl of 0.05 mol/L 2-deoxy-D-ribose, and 2.7 ml of phosphate buffer (pH=7.4). After incubation at room temperature for 60 min, 200 μl of 0.1 M EDTA and 2 ml TBA reagent were added to the mixture and heated at 100 °C for 10 min. After cooling, aliquots of 250 ml were transferred to the plate, and absorbance was measured at 532 nm. The antioxidant activity was expressed as IC$_{50}$ (μg/mL), and the experiment was performed in triplicate.

NO· assay

Nitric oxide scavenging capacity was determined according to the method of Green et al. (1982). 30 μl of each extract and PG (except in a control probe when only the solvents were used) was added to 500 μl SNP and 500 μl of phosphate buffer (pH=7.4). Test tubes were incubated at room temperature for 90 min under light exposure. After the incubation, 1 ml of Griess reagent was added to all samples. Aliquots of 250 μl were transferred to the plate and absorbance was measured at 546 nm. The antioxidant activity was expressed as IC$_{50}$ (μg/mL), and the experiment was performed in triplicate.
FRAP assay

The Ferric reducing antioxidant power (FRAP) assay was done according to Benzie and Strain (1999). A volume of 10 μl of the sample was transferred to a 96 microwell plate with 225 μl of FRAP reagent and 22.5 μl of dH₂O. The absorbance was determined at 593 nm after incubation of 6 min. All the samples were made in triplicate and mean values were expressed as milligrams of ascorbic acid equivalents (AAE) per gram of dry weight (mg AAE/g d.w.), calculated according to the standard calibration curve.

Determination of total phenolic content

Total phenolic content in the analysed extracts was determined according to the method by Singleton et al. (1999). 25 μl of each extract or standard compound (gallic acid) (except in a control probe when only the solvents were used) was added to 125 μl of Folin-Ciocalteu (FC) reagent and mixed after 10 min with 100 μl of sodium carbonate. Absorbance was measured spectrophotometrically (Thermo Scientific Multiskan Spectrum Microplate Photometers – Photometer with Curvette Holder, Model 51118650) at 760 nm after 2h of reaction.

Statistical Analysis

The results were expressed as mean values ± standard deviation (SD). IC₅₀ values (μg/mL) were determined by the linear regression analysis of RSC (Microsoft Excel for Windows, v. 2010 and Origin 8). Statistical analysis was performed using one-way analysis of variance ANOVA with post hoc Turkey’s test (STATISTICA; version 10.0; www.statsoft.com). Correlation coefficient (Microsoft Excel for Windows, v. 2010) was used to estimate the relationship between the antioxidant activity of extracts and TP contents.

RESULTS AND DISCUSSION

Antioxidant activity and TP content

Antioxidant activity is manifested in a wide variety of actions, such as inhibition of oxidizing form of enzymes, chelating of metal ions, transfer of H-atom or electrons to radicals, singlet oxygen deactivation, etc. (Halliwell, 1996). Antioxidant compounds found in fruiting bodies, mycelium and extracellular broth so far have been confirmed to be mainly phenolic compounds (phenolic acids and flavonoids), followed by other compounds such as ergothioneines, glycosides, polysaccharides, and vitamins (tocopherols, carotenoids, and ascorbic acid) (Ferreira et al. 2009; Kozarski et al. 2015). Mushroom antioxidants can exhibit their protective properties at different stages of the oxidation process and by different mechanisms: primary (scavenging of free radicals and chain breaking)
and secondary (regeneration of primary antioxidants, inhibition of lipid peroxidation and quenching of singlet oxygen) (Brewer, 2011; Kozarski et al. 2015).

A whole range of scientific data reported high antioxidant potentials of different types of *G. lucidum* extracts (Deepalakshmi et al. 2013; Ferreira et al. 2009; Rašeta et al. 2016; Saltarelli et al. 2009; Stilinović et al. 2014; Tel et al. 2015; XiaoPing et al. 2009; Yildiz et al. 2015; Zhang et al. 2016), but still there is no data for the strain from Croatia. The obtained results for the TP content and antioxidant activity of analysed extracts are shown in Table 1. Although both types of extracts possessed antioxidant properties, H$_2$O extracts demonstrated higher antioxidant activity than EtOH extracts, mainly for GLS in OH, DPPH, and FRAP assays ($IC_{50}=0.23\pm0.01$ μg/mL, $IC_{50}=2.75\pm0.01$ μg/mL and $696.38\pm1.33$ mg AAE/g d.w., respectively) and NO assay for GLC ($IC_{50}=62.16\pm1.14$ μg/mL). We compared activities of analysed extracts using standard compound PG and all obtained results are statistically significant with the exception of OH$^-$ assay ($IC_{50}=0.66\pm0.01$ μg/mL for PG and $IC_{50}=0.23\pm0.01$ μg/mL for H$_2$O of Croatian samples, respectively) (Table 1).

The ABTS radical reactions involve electron transfer and the process take place at a faster rate when compared to DPPH radicals (Arnao et al. 2001). In the present study, EtOH extracts of *G. lucidum* strain from Serbia (GLS) and Croatia (GLC) showed weaker activity in ABTS reduction (151.40±1.07 mg TE/g d.w. and 107.55±1.11 mg TE/g d.w., respectively) in comparison with PG (711.53±1.94 mg TE/g d.w.). In summary, the ABTS radical scavenging activity was more active in EtOH extracts, which is in high correlation with TP content ($R^2=0.93–0.98$) (Table 1).

In the present study, analyzed extracts showed concentration-dependent activity in DPPH and FRAP assays, which may be attributed to its hydrogen-donating ability via high reducing power ability confirmed for both H$_2$O extracts GLS and GLC ($696.38\pm1.33$ mg AAE/g d.w. and 127.00±0.29 mg AAE/g d.w., respectively). The reducing properties are generally high for H$_2$O extract ($696.38\pm1.33$ mg AAE/g d.w for GLS and 127.00±0.29 mg AAE/g d.w for GLC, respectively) and associated with the TP content with the exception of EtOH extract of GLS (Table 1).

Hydroxyl radical is the most reactive among free radical species and obtained results of antiradical activities were the highest for the GLS ($IC_{50}=0.23\pm0.01$ μg/mL for H$_2$O and $IC_{50}=05.35\pm0.01$ μg/mL for EtOH extract) which was in the range of activity for PG (Table 1).

In the present study, scavenging activity on NO radicals was relatively low ($IC_{50}=765.28\pm0.71–1108.30\pm2.82$ μg/mL) with the exception of H$_2$O extract for GLC ($IC_{50}=62.16\pm1.14$ μg/mL) (Table 1).

EtOH extracts of GLS had high amount of TP ($60.74\pm0.57$ mg GAE/g d.w.) and our previous study showed high correlation between TP content and obtained phenolic profile via LC-MS/MS, where the content of protocatechuic, p-hydroxybenzoic, p-coumaric, and quinic acids dominated (Rašeta et al. 2016), while H$_2$O extracts of GLC had the highest TP content ($77.10\pm0.27$ mg GAE/g d.w.) and its phenolic profile has been unknown so far.
Obtained results showed lower activities for analyzed EtOH extracts for ABTS assay than MeOH extracts of *G. lucidum* from Turkey (Tel *et al.* 2015), EtOH extracts of the same species from India and Italy (Deepalakshmi *et al.* 2013; Saltarelli *et al.* 2009) and ~ 5 times higher activity than MeOH extracts (Rani *et al.* 2015). Products which scavenge DPPH *in vitro* may scavenge polycyclic aromatic hydrocarbon cations *in vivo* (Deepalakshmi *et al.* 2013).

Obtained results for DPPH and FRAP assays showed mostly higher values than the values obtained from the literature data (Deepalakshmi *et al.* 2013; Rani *et al.* 2015; Rašeta *et al.* 2016; Saltarelli *et al.* 2009; Tel *et al.* 2015). Our results are in accordance with data presented in previous studies for OH' assay (Deepalakshmi *et al.* 2013; Mohsin *et al.* 2011).

Generally, obtained results showed that all analyzed extracts, except H$_2$O/GLS, contained high TP content (Table 1). These results showed higher values than in previous studies for the same fungal species from Korea and Serbia (Hasnat *et al.* 2013; Kim *et al.* 2014; Veljović *et al.* 2017). Phenolic compounds may be the key components accounting for the demonstrated results and manifested antioxidant activities, statistically determined through correlations (Table 1) (Novaković *et al.* 2016).

**Table 1.** Antioxidant activity, TP content and correlations between TP and antioxidant activities of two *G. lucidum* fungal strains

<table>
<thead>
<tr>
<th>Assay</th>
<th>GLS</th>
<th>GLC</th>
<th>PG</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>EtOH</td>
<td>H$_2$O</td>
<td>EtOH</td>
</tr>
<tr>
<td>ABTS (mg TE/g d.w.)</td>
<td>151.40±1.07$^a$</td>
<td>23.30±2.15$^d$</td>
<td>107.55±1.11$^b$</td>
</tr>
<tr>
<td>DPPH (μg/mL)</td>
<td>3.64±0.15$^b$</td>
<td>2.75±0.01$^b$</td>
<td>56.51±2.80$^d$</td>
</tr>
<tr>
<td>OH (μg/mL)</td>
<td>5.35±0.01$^b$</td>
<td>0.23±0.01$^a$</td>
<td>95.68±2.15$^a$</td>
</tr>
<tr>
<td>NO (μg/mL)</td>
<td>765.28±0.71$^c$</td>
<td>1108.30±2.82$^d$</td>
<td>1014.65±0.01$^e$</td>
</tr>
<tr>
<td>FRAP (mg AAE/g d.w.)</td>
<td>111.52±0.94$^b$</td>
<td>696.38±1.33$^a$</td>
<td>76.46±0.73$^d$</td>
</tr>
<tr>
<td>TP (mg GAE/g d.w.)</td>
<td>60.74±0.57$^b$</td>
<td>11.55±0.30$^d$</td>
<td>42.78±0.32$^c$</td>
</tr>
</tbody>
</table>

R$^2_*$

Legend: GLS – *G. lucidum* (Serbia); GLC – *G. lucidum* (Croatia); PG – propyl gallate (synthetic antioxidant); TP – total phenol content

Data are reported as mean ± standard deviation of triplicates.

$^a,b,c,d,e$ – different letters in the same row indicate significant difference between extracts (p<0.01)

R$^2$ – correlation coefficient between antioxidant activity and TP content, $^*$ – all values are statistically significant (p<0.05)
The correlation between the TP and antioxidant capacity was significant (specifically for GLC extracts), and these results suggested that a significant amount of phenolic compounds was strongly linked to antioxidant and reducing power of analyzed fungal extracts. Furthermore, the chemical characterizations and determinations of biopotentials of autochthonous species from different geographical regions could have a great importance for future use of mushrooms as potential medicinal supplements.

CONCLUSION

In the present study, two types of *Ganoderma lucidum* extracts (EtOH, H$_2$O) from different localities (Serbia-GLS and Croatia-GLC) were investigated for their antioxidant, reducing power potential and total phenolic content.

Our results suggest that *G. lucidum* and their extracts could be very good sources of naturally-derived antioxidants. The obtained assays showed that H$_2$O extracts in analyzed GLS and GLC extracts had mainly better antioxidant activities than EtOH, with the exception of ABTS assay for both strains (151.40±1.07 mg TE/g d.w. for GLS and 107.55±1.11 mg TE/g d.w. for GLC, respectively), OH assay for GLS (IC$_{50}$=0.23±0.01 μg/mL), and NO assay for GLS (IC$_{50}$=765.28±0.71 μg/mL). PG, as a derivate of gallic acid, is essential phenol which is used in foodstuffs to protect fats against oxidative rancidity and showed the most powerful antioxidant activity with the exception of scavenging of OH radical (IC$_{50}$=0.66 μg/mL and IC$_{50}$=0.23 μg/mL for GLS H$_2$O extract, respectively).

The antioxidant activity of analyzed extracts had high correlation with the content of their TP, therefore the fungal phenols may act as antioxidants.

This study suggests that H$_2$O extract of *G. lucidum* generally possesses better antioxidant activity, which firmly supports further studies with an aim to promote consumption of H$_2$O extracts in the form of tea or soups of *G. lucidum* in the Balkan region, which represent food supplements rich in phenolics with valuable health benefits for humans.

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ПОРЕЂЕЊЕ АНТИОКСИДАНТНИХ КАПАЦИТЕТА ДВА СОЈА *Ganoderma lucidum* РАЗЛИЧИТОГ ГЕОГРАФСКОГ ПОРЕКЛА

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РЕЗИМЕ: *Ganoderma lucidum* представља једну од најзначајних медицинских гљива на свету, чије се плодно тело и споре традиционално употребљавају у кинеској медицини због широког спектра биолошких активности: антидијабетске, антиоксидативне, антипролиферативне, кардиопротективне. Међутим, још је непознат етномедицински значај у појединим деловима Балкана, као што су Србија и Хрватска. Циљ овог рада је упоредна студија антиоксидантних активности и садржаја укупних фенола етанолних и водених екстраката *G. lucidum* из Србије (Моровићке шуме, Фрушка гора) и Хрватске (Доњи Лапац, Пљешевица). Одређена је антиоксидантна активност екстраката праћењем потенцијала редукције ABTS, DPPH и OH радикала, хватања NO као и редукције 

Fe$^{2+}$ у Fe$^{3+}$ FRAP тестом, као и носиоци те активности, тј. садржај укупних фенола. Резултати указују да су екстракти узорака из Србије добро редуковали OH∙ и DPPH∙ (IC$_{50}$=0.23±0.01 за H$_2$O односно 2.75±0.01 μg/mL за EtOH, потом ABTS$^{+}$ (151.40±1.07 mg TE/g с.м. за EtOH), док је садржај фенола био скоро исти у Српским и хрватским узорцима (77.10 ±0.27 mg GAE/g d.w. за H$_2$O односно 60.74±0.57 mg GAE/g d.w. за EtOH) те се ови екстракти могу сматрати значајним изворима природних антиоксиданата. Ови резултати показују да испитивани екстракти (нарочито H$_2$O) садрже висок садржај фенола, који су главни носиоци антиоксидативне активности. Ово је прва студија у којој је саопштено поређење антиоксидативне активности и садржаја фенола екстраката два соја *G. lucidum* различитог географског порекла са територије Балкана.

КЉУЧНЕ РЕЧИ: антиоксидантни капацитет, *Ganoderma lucidum*, феноли, редукција радикала