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Selenium and Zinc content and radical scavenging capacity of edible mushrooms *Armilaria mellea* and *Lycoperdon saccatum*

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Summary

Armillaria mellea and *Lycoperdon saccatum* are two delicious mushrooms growing widely trough all Balkan region. Investigation of *A. mellea* and *L. saccatum* antioxidant properties includes preparation of mushrooms extracts, determination of Selenium and Zinc content and evaluation of theirs antioxidant activity involving scavenging activity of $^{\cdot}O_2^-$ radicals, DPPH and reducing power assay. Higher extraction yield of 24.48 % has been achieved for *L. saccatum*, but higher content of Selenium and Zinc was determined in *A. mellea* extract, 2.359 mg/kg and 50.380 mg/kg, respectively. The radical scavenging activity was found to exhibit 50 % of inhibition value (IC_{50} value) at the extracts concentration of 0.0161±0.0001 mg/ml for the *L. saccatum* extract and 0.0108±0.0002 mg/ml for *A. mallea* extract. The determined relative inhibition of $^{\cdot}O^{2-}$ radicals for *L. sacatum* extract is lower than for *A. malea.* It was determined that both mushrooms extract posses' reductive capabilities and thus were capable of reducing iron (III).

Keywords: Armilaria mellea, *Lycoperdon saccatum*, antioxidant activity, Zinc, Selenium

Introduction

Armillaria mellea and *Lycoperdon saccatum* are two delicious mushrooms growing widely trough all Balkan region. *Armillaria mellea*, also known as a "honey mushroom", belongs to class Agaricomycetes, order Agaricales, family Physalacriaceae and genus Armillaria. It is pathogenic organism that grows on living trees and on dead and decaying woody material. *Lycoperdon saccatum*, well know as "puffball" mushroom, also belong to class Agaricomycetes, order Agaricales, familiy Agariaceae, and genus Lycoperdon. This genus has a widespread distribution and contains about 50 delicious mushrooms species. Most of the time puffball grows on the dead wood, and if they grow through the ground they usually indicate that wood is buried. These two studied mushrooms species have been collected from Istrian peninsula in Croatia during late summer of year 2008. Considering the growing interest for the natural sources of antioxidants on one hand, and the unexplored and unused field of mushrooms from Balkan region on the other, this research took as its main objective the investigation of the antioxidant properties of these two edible species.

Reactive oxygen species can cause damages to proteins, lipids and nucleic acids and thereby compromise cell viability. This oxidative stress is associated with several human pathologies including cardiovascular diseases, cancer, arthritis, autoimmune diseases, Down's syndrome, etc. (Halliwel and Gutteridge, 2003). Antioxidant compounds are main components involved in active battle with reactive oxygen species. The antioxidant properties of mushrooms refer to different antioxidant compounds. Mushrooms are considered to be a good source of protein and phenolic antioxidants (Kasuga et al, 1995). Due to the presence of the conjugated ring structures and the number and arrangement of the polar hydroxyl groups (Cotelle et al, 1996), many phenolic compounds can function as antioxidants by scavenging superoxide anion (Robak and Gryglewski, 1988), singlet oxygen (Husain et al, 1987), and lipid peroxyl radicals (Torel et al, 1986), and by stabilizing free radicals that are involved in oxidative processes through hydrogenation or complexing with the oxidizing species (Shahidi et al, 1992). The antioxidant properties of mushrooms are also derived from the essential trace elements: Selenium and Zinc. Selenium is established as an essential trace mineral of fundamental importance to human health. It is essential for life, and no doubt exists that adequate amounts of this element are required for optimal human health (Vanda Papp et al, 2007). It is known primarily for its antioxidant activity and its therapeutic aspects, for its chemo-preventive, anti-inflammatory, and antiviral properties (Rayman, 2000). Selenium performs as an antioxidant through Selenium-proteins. Selenium-

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proteins have a key role in a variety of biological processes and some of them are as well involved in antioxidant defence (Brenneisen et al, 2005). It was reported that Selenium supplementation in acute and chronic exercise increased antioxidant activity, and thereby preventing lipid peroxidation (Zamora et al, 1995). The deficiency of the second essential mineral Zinc leads to oxidative damage of lipids, protein, and DNA oxidation (Ho et al, 2003). Zinc can perform as antioxidant using different mechanisms including sulfhydryl protection against oxidation, the induction and binding redox active metals, Copper and Iron, and as a constituent of intracellular and extracellular antioxidant enzyme Cu-Zn superoxid dismutase (Powell, 2000). Zn acted jointly with other lipid-soluble (α-tocopherol) and water soluble (epicatechin) antioxidants in the prevention of Fe^{2+} induced lipid oxidation (Zago and Oteiza, 2001).
Investigation of *A. mellea* and *L. saccatum* includes preparation of mushrooms extracts, determination of Selenium and Zinc content, and evaluation of extracts antioxidant activity involving scavenging activity of $^{\cdot}O_2^-$ radicals, DPPH and reducing power assay.

Materials and methods

Chemicals and reagents

L-α-phosphatidylcholine, hypoxanthine and xanthine oxidase was purchased from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Potassium ferricyanide was purchased from Merck (Darmstadt, Germany). DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide) was purchased from Alexis Biochemical (Laursen, Switzerland). H_2O_2 was purchased from Renal (Budapest, Hungary). All other chemicals and reagents were of the analytical reagent grade.

Sample preparation

The mushroom samples of *A. mellea* and *L. saccatum* were collected from the mountain nature park Učka, at the east side of Istria in Croatia in summer 2008. The mushrooms fruiting bodies were gently cleaned of any residual compost. The fresh mushrooms were air-dried and then stored in an air-tight plastic bag at room temperature. The dried mushroom samples were milled in a blender before the extraction. The mushroom samples (10.0 g) were extracted by 50 % ethanol (50.0 ml). The extraction process was carried out using the ultrasonic bath (B-220, Branson and SmithKline Company, USA) at 45 °C for 40 minutes. After the filtration, the liquid extract (40.0 ml) was obtained and solvent was evaporated by a rotary evaporator (Devarot, Elektromedicina, Slovenia) under vacuum. The obtained extract was then dried at 60 °C to the constant mass. At the end, the dry extract was placed in glass bottles and stored at -4 °C to prevent oxidative damage until the analysis. All extractions were performed in triplicate and presented as means ± S.D.

The determination of Selenium and Zinc

For the analysis of trace element, Selenium and Zinc, the mushroom dry extract was digested in concentrated HNO_3 and afterwards the elements were quantified by inductively coupled plasma mass spectroscopy (ICP/MS Perkin Elmer 9000, Perkin Elmer, USA). All experiments were performed in triplicate and presented as means ± S.D.

DPPH assay

The free radical scavenging activity of mushroom dry extracts was determined as described by Espin (Espin et al, 2000). This assay was used for a preliminary free radical scavenging evaluation. It seemed simpler and faster method than any other method. Briefly, the mushroom dry extract was mixed with methanol (96 %) and 90 μM 2,2-diphenyl-1-picryl-hydrazyl (DPPH) to give final concentration of 0.01 and 0.02 mg/ml of dry extract. After 60 minutes at room temperature, the absorbance was measured at 517 nm and expressed as radical scavenging capacity. The decrease in the absorbance of $DPPH^{\cdot}$ radical was caused by the reaction between antioxidant molecules and the radical, which resulted in the scavenging of the radical by the hydrogen donation, which was visually noticeable as discoloration from purple to yellow. Radical scavenging capacity (*%RSC*) was calculated using the following equation:

$$RSC(\%) = 100 - \frac{A_{sample} \times 100}{A_{blank}}$$

where: A_{sample} is the absorbance of sample solution and A_{blank} is the absorbance of a blank sample.

This activity was also expressed as the inhibition concentration at 50 % (IC_{50}), the concentration of the solution tested was required to obtain 50 % of radical scavenging capacity. All experiments were performed in triplicate and presented as means ± S.D.

Scavenging activity on $O_2^{\cdot-}$

The ability of the investigated mushroom extract to remove $O_2^{\cdot-}$ radicals was evaluated by the difference between the amplitudes of the EPR (Electron Paramagnetic Resonant) signals of trapped radicals in radical-generating systems, with and without the addition of the investigated extract. Electron Paramagnetic Resonant (EPR) spectroscopy is the only employed technique used for direct detection and identification of free radicals (Lundqvist et al, 2008), with sensitivity as high as 10^{-10} M. EPR spectra were recorded at room temperature using Varian E104-A EPR spectrometer operating at X-band (9.51 GHz) with the following settings: the modulation amplitude, 2 G; the modulation frequency, 100 KHz; the microwave power, 10 mW; the time constant, 0.032 s; the field centre, 3,410 G; the scan range, 200 G. The spectra were recorded using EW software (Scientific Software, Bloomington, USA). The samples were drawn into 10 cm long gas-permeable Teflon tubes (wall thickness 0.025 mm and internal diameter 0.6 mm; Zeus industries, Raritan, USA). The measurements were performed using quartz capillaries, in which Teflon tubes were placed. The recording started 2 minutes after the beginning of reaction, and lasted for 4 minutes. Hypoxanthine/xanthineoxidase system (HX/XO) was used as a generating system. HX/XO consists of hypoxanthine, 1.6 mM, and xanthine oxidase, 1.6 IU/ml, dissolved in bidestillated water. 28 mM DEPMPO was used as a spin trap. DEPMPO reacts with $^{\cdot}O_2^-$ to form DEPMPO/OOH adduct. The final concentration of the extracts was 0.2 mg/ml. The incubation time was 2 minutes. The sample with no extract added was used as a blank sample. The antioxidant activity of ascorbate at final concentration of 0.2 mg/ml was estimated to compare the effects of the extracts with the effects of this frequently used antioxidant.

The scavenging activity on $O_2^{\cdot-}$ radicals are here presented as Relative Inhibition (%), which presents the relative decrease of radical production:

$$RI(\%) = \frac{PA_{rgs} - PA_{rgs+e}}{PI_{rgs}} \times 100$$

where: PA_{rgs} is the second peak amplitude (radical-generating system)
PA_{rgs+e} is the second peak amplitude (radical-generating system + extract)
PI_{rgs} is the second peak intensity (radical-generating system).

All experiments were performed in triplicate and presented as means ± S.D.

The determination of reducing power

The reducing power of the mushroom extracts and ascorbic acid, the standard antioxidant, were determined by the Oyaizu method (Oyaizu, 1986). Various concentration of the mushroom extracts (0.2, 0.5 and 1.0 mg/ml) and ascorbate (0.01, 0.02, 0.05, 0.1, 0.2, 0.5 and 1.0 mg/ml) were mixed with the phosphate buffer (2.5 ml, 0.2M, pH 6.6) and 2.5 ml of 1 % potassium ferricyanide ($K_3Fe(CN)_6$). The mixture was incubated for 20 minutes at 50 °C. After the incubation, 2.5 ml of 10 % trichloroacetic acid solution was added to the mixture, and the mixture was centrifuged for 10 minutes (3,000 rpm). The obtained supernatant (2.5 ml) was mixed with bidestillated water (2.5 ml) and 0.1 % $FeCl_3$ solution (0.5 ml). The absorbance was measured at 700 nm. The higher absorbance indicates a higher reducing power and higher antioxidant activity. All experiments were performed in triplicate and presented as means ± S.D.

Results and Discussion

Extraction yield and content of Selenium and Zinc

The extraction yield, obtained by 50 % ethanol as extraction solvent, and content of Selenium and Zinc in obtained *A. mallea* and *L. saccatum* dry extracts are presented in Table 1.

Table 1. Extraction yield, Zinc and Selenium content (mg/kg) in *A. mellea* and *L. saccatum* dry extracts

Sample	Extraction yield (%)	Selenium (mg/kg)	Zinc (mg/kg)
A. mellea	18.86±0.08	2.359±0.005	50.380±0.02
L. saccatum	24.48±0.03	0.458±0.002	46.890±0.04

Higher extraction yield (24.48±0.03 %) has been achieved for *L. saccatum*, but higher content of Selenium and Zinc was determined in obtained *A. mallea* dry extracts (2.359±0.005 mg/kg and 50.380±0.02 mg/kg, respectively). Higher contents of these two essential trace elements could contribute to

A. mallea higher scavenging activity of some radical species and by this, higher antioxidant activity.

DPPH assay

The scavenging effect of the mushroom extracts on DPPH˙ radicals increased with the increase of the concentrations. At concentration of 0.02 mg/ml, the radical scavenging capacity (RSC) of the investigated extract was 61.96±1 % for *L. saccatum* and 92.16±2 % for *A. mallea*. The radical scavenging activity was found to exhibit 50 % of inhibition value (IC_{50} value) at the extract concentration of 0.0161±0.0001 mg/ml for *L. saccatum* dry extract and 0.0108±0.0002 mg/ml for *A. mallea* dry extract (Table 2). Lower IC_{50} value of *A. mallea* extract, indicating higher antioxidant activity than that of *L. saccatum* dry extract.

Table 2. IC_{50} and RSC (%) value of *A. mellea* and *L. saccatum* dry extract

Sample	IC_{50} (mg/ml)	RSC at the concentration of 0.01 mg/ml (%)
A. mellea	0.0108±0.0002	57±3 %
L. saccamut	0.0161±0.0001	40±5 %

IC_{50} of *A. mallea* is lower than that reported IC_{50} value of *Boletus edulis* ethanolic extract (0.016±0.003 mg/ml), *Boletus aurantiacus* ethanolic extract (0.024±0.004 mg/ml) (Vidovic et al., 2010), the chloroform extracts of *Ganoderma applantum*, *Ganoderma lucidum* and *Meripilus giganteus* (0.019±0.0015 mg/ml, 0.016±0.0008 mg/ml and 0.062±0.0018 mg/ml), and much lower than that of the methanolic extract of *Meripilus giganteus* (0.25±0.0016 mg/ml) (Karaman et al, 2009).

Scavenging activity on $^{\cdot}O^{2-}$ radicals

The scavenging activity on $^{\cdot}O^{2-}$ radicals were presented as Relative Inhibition, which signifies the relative decrease of radical production. The superoxide anion is a weak oxidant, yet it gives rise to the generation of powerful and dangerous hydroxyl radicals, as well as the singlet oxygen, both of which contribute to the oxidative stress (Meyer and Isaksen, 1995). The determined relative inhibition of $^{\cdot}O^{2-}$ radicals for *L. sacatum* dry extract was 40±5 % and for *A. malea* 57±3 % (Table 2), this indicating that *A. mallea* could be considered as $^{\cdot}O^{2-}$ radical scavenger. Relative inhibition of $^{\cdot}O^{2-}$ radicals for *L. saccatum* dry extract showing not so significant antioxidant action against this radical species. Relative inhibition of the well-known antioxidant compound ascorbate is 85±5 %.

Determination of reducing power

Ferrous iron has the capacity to reduce oxygen to superoxide radical, it can catalyze the decomposition of peroxide and yielding hydroxyl radical from hydrogen peroxide (Fraga and Oteiza, 2002). For the measurements of the reductive ability, $Fe^{3+} \rightarrow Fe^{2+}$ transformation, in the presence of the mushroom extract, was investigated. The reducing capacity of analyzed mushroom dry extract may serve as the indicator of its potential antioxidant activity. Fig. 1 indicates the reductive capabilities of the analyzed mushroom extract and standard antioxidant compound ascorbic acid.

Both mushroom dry extracts posses' reductive capabilities and thus were capable of reducing iron (III). This characteristic could belong to chemical compounds, phenols and Zinc, that are present in investigated extracts. The phenolic compounds can chelate pro-oxidant metal ions, thus preventing free radical formation from these pro-oxidant species (Kris-Etherton et al, 2002). Zinc, the metal that does not have redox activity, has a capacity to compete with iron for multiple cellular binding sites and that replacement of Iron with Zinc can prevent the redox-cycling of Iron, thus minimizing the rate of oxidizing of chemical groups (Fraga and Oteiza, 2002). At the extract concentration of 0.2 mg/ml, measured absorbances of investigated extracts were almost equal, for *A. mellea* was 0.135±0.001, while for *L. saccatum* was 0.133±0.002. At the higher concentrations *A. mellea* showed some better reductive capabilities. For example at the extract concentration of 1 mg/ml absorbance for *A. mellea* was 0.750±0.002, while for *L. saccatum* extract was 0.652±0.002.

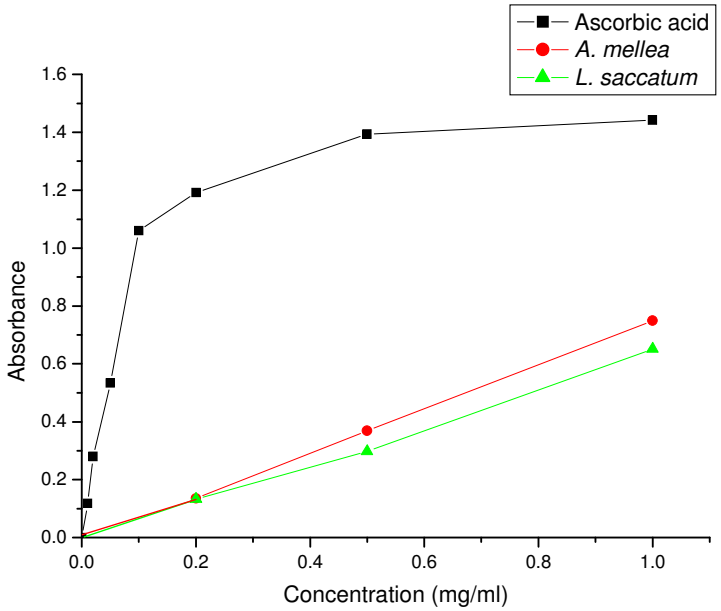

Fig. 1. Reducing power of *A. mellea* and *L. saccatum* dry extract and ascorbic acid, measured by spectrophotometric detection of $Fe^{3+} \rightarrow Fe^{2+}$ transformation at different concentration

The reducing power of the investigated extracts was compared to reducing power of standard antioxidant compound ascorbic acid. It is clear that ascorbic acid has few times better reductive capability than both extracts. At the concentration of 0.5 mg/ml absorbance of ascorbic acid was 1.394±0.001, while for *A. mellea* and *L saccatum* it was 0.369±0.004 and 0.299±0.003, respectively.

Conclusions

Finding new antioxidant source could be important for health benefit considering many diseases that reactive oxygen species induce in biological systems. Discovering a natural source of antioxidants could be significant also for the replacement of the artificial toxic antioxidants in food industry. The results of this study clearly indicate that extracts obtained from two delicious mushrooms, *A. mellea* and *L. saccatum*, that growing widely on Istra peninsula in Croatia, posses antioxidant activity against various radical producing systems. These extracts are effective scavengers of many radicals' species what was researched by applying different methods of antioxidant activity investigation. Both have the ability to chelate ferrous ion what indicating possible application for prevention of lipid peroxidation. These mushrooms are a source of network antioxidant compounds, among them Selenium and Zinc are very important. These trace mineral elements are rather relevant considering their vital role in human health. Many European countries still have dietary Selenium and Zinc intake below recommended, so, apart from their use as antioxidant products, the investigated extracts can as well be utilized in food and pharmaceutical industry, which would steadily increase the consumption of these minerals. If we compare antioxidant activity of these two mushrooms extracts, than we can say that *A. mellea* extract posses' stronger antioxidant activity than *L. saccatum*, what is in accordance with higher content of both trace elements, Selenium and Zinc.

Abbreviations:

DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide);
DPPH (1,1-diphenyl-2-picryl-hydrazyl-hydrate);
EPR (Electron Paramagnetic Resonant) spectroscopy;
IC_{50} - 50 % of inhibition value;
ICP/MS - inductively coupled plasma mass spectroscopy;
RI - relative inhibition;
RSC - radical scavenging capacity;
UV-VIS - ultraviolet-visible spectroscopy

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