

POLYPHENOLS CONTENTS AND ANTIOXIDANT ACTIVITY OF SOME ROMANIAN WILD EDIBLE MUSHROOMS

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Abstract

Mushrooms are widely used for their nutritional value and pharmacological properties. The aim of our study was the determination of polyphenols contents and antioxidant activity of some Romanian wild edible mushrooms (*Boletus edulis*, *Armillaria mellea* and *Macrolepiota procera*), which were dried using different methods (sun-drying and freeze-drying). A spectrophotometric method was used for polyphenols determination and 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS^{•+}) reducing power for antioxidant activity. Lyophilisation is the optimum method for mushrooms drying, since it leads to a higher content of polyphenols and a stronger antioxidant activity. Freeze-dried *Boletus edulis* and *Macrolepiota procera* have the highest content of polyphenols (1.4010 g% and 1.2309 g%) and the best antioxidant activity. The negative correlation observed between total phenolic content and antioxidant activity involves the role of other active substances (polysaccharides, minerals) in the overall antioxidant capacity. Apart from their excellent nutritional value, the analysed mushrooms are a valuable source of natural antioxidants.

Rezumat

Ciupercile sunt utilizate în alimentație pentru valoarea nutritivă, dar și în terapeutică pentru numeroasele lor proprietăți farmacologice. Obiectivul cercetării a constat în determinarea conținutului în polifenoli și a activității antioxidante a unor bazidiomicete indigene, comestibile (*Boletus edulis*, *Armillaria mellea* și *Macrolepiota procera*), dependent de modalitatea de uscare (la soare sau prin liofilizare). Pentru determinarea conținutului în polifenoli s-a utilizat o metodă spectrofotometrică, iar activitatea antioxidantă a fost evaluată pe baza capacității de *scavenger* a radicalilor liberi DPPH (2,2-difenil-1-picrilhidrazil), ABTS^{•+} (acidul 2,2'-azinobis-3-etilbenzotiazolin-6-sulfonic) și pe baza capacității de reducere a ferului. Metoda de uscare influențează semnificativ conținutul în polifenoli totali și activitatea antioxidantă, cele mai mari valori obținându-se pentru ciupercile liofilizate. *Boletus edulis* și *Macrolepiota procera* uscate prin liofilizare au cel mai mare conținut de polifenoli totali (1,4010 g%, respectiv 1,2309 g%) și cea mai bună activitate antioxidantă, independent de metoda utilizată. Corelația negativă între activitatea antioxidantă și conținutul în polifenoli totali sugerează implicarea altor constituenți (polizaharide, minerale) în capacitatea antioxidantă. Pe lângă valoarea nutritivă, ciupercile analizate reprezintă o sursă excelentă de antioxidanți naturali.

Keywords: polyphenols, antioxidant capacity, mushrooms, *Boletus edulis*, *Armillaria mellea*, *Macrolepiota procera*

Introduction

Mushrooms are widely used for their nutritional value and pharmacological effects (antidiabetic, anticancer, immunomodulatory, antioxidant and antimicrobial properties) [7, 10, 15, 30]. Among Romanian wild edible mushrooms, *Boletus edulis* (porcini, king bolete), *Armillaria mellea* (honey fungus) and *Macrolepiota procera* (parasol mushroom) represent a highly appreciated food constituent. They are a rich source of poly-saccharides and proteins (36.91% for porcini and 24.22 % for parasol mushroom) [4]. *Boletus edulis* and *Macrolepiota procera* are also a source of amino acids (L-alanine, L-glutamic acid, L-lysine and L-threonine) [4]. All mentioned basidiomycetes contain fatty acids (oleic acid and linoleic acid) [11, 26] and mineral elements

(selenium for porcini [8]; magnesium, zinc, manganese for honey fungus [18, 21]). Recent studies regarding the chemical composition of *Armillaria mellea* revealed the presence of indole compounds (tryptamine, tryptophan, serotonin and melatonin) [6], sesquiterpenoids (armillaricin, armillarikin, armillol, judeol), ergosterol, ergosterol peroxide and sphingolipids (armillaramide) [18]. Parasol mushroom is a rich source of 5-hydroxytryptophan (10-22.9 mg/100 g d.w.) and α -tocopherol (4.5 μ g/100 g d.w.) [11]. Polyssaccharides from *Boletus edulis* have shown antioxidant, antitumor and immunomodulatory activities on renal cancer [2, 9], while armillarikin from honey fungus inhibited the viability of K562, U937, HL-60 leukemic human cells through caspase activation and increased intracellular

reactive species [32]. *Armillaria mellea* is well known for its antioxidant [12], antibacterial and immunomodulatory properties [18]. According to Arora S. *et al.*, an ethanolic extract of *Macrolepiota procera* has cytotoxic properties upon COLO-205 cancer cells [3].

Wild mushrooms have a delicious taste, but they can be collected only in certain periods of the year (porcini in May-September, honey fungus in August-September and parasol mushroom in September-October). Mushrooms are known as one of the most perishable food products, so it is important to find a method for preserving them, in order to maintain a high content of active substances during storage.

The aim of our study was to determine the total phenolic content and antioxidant activity of some Romanian wild edible mushrooms, which were dried by means of different methods (sun-drying - traditional method and freeze-drying).

Materials and Methods

Materials

Boletus edulis = BE (whole fruiting body – Figure 1a.), *Armillaria mellea* = AM (cap and 1 - 2 cm of the stalk – Figure 1b) and *Macrolepiota procera* = MP (cap – Figure 1c) were collected in September 2014, from the northern part of Cotmeana platform, Săpunari village, Argeş district (44°58' North, 24°34' East), Romania. The mushrooms were sun-dried (SD) and freeze-dried (FD) (Christ Alpha 1-2/B Braun, Biotech-International lyophilizer).

Reagents, solvents and apparatus

All chemicals were purchased from Roth. (Germany), unless otherwise stated. 2,2-diphenyl-1-picrylhydrazyl and diammonium salt of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) were from Sigma-Aldrich (Germany) and potassium persulphate, trichloroacetic acid were acquired from Merck (Germany). For all spectrophotometric determinations a Jasco V-530 (Jasco, Japan) spectrophotometer was used.

Preparation of samples

For the spectrophotometric and antioxidant assays, 5 g of each mushroom (sun-dried or freeze-dried) was heated with water, on a reflux condenser for 30 min. The extractive solutions were encoded BE_{SD}, AM_{SD}, MP_{SD} (for sun-dried mushrooms) and BE_{FD}, AM_{FD}, MP_{FD} (for freeze-dried mushrooms).

Spectrophotometric determination of polyphenols

Total polyphenols were determined with Folin-Ciocalteu reagent according to Singleton V.L. [29] method modified by Makkar H.P.S. [17]. Results were expressed as g% tannic acid, based on a calibration curve (2.04 - 9.18 µg/mL, R² = 0.9994, n = 8) [13].

The antioxidant capacity was assessed by means of well-known methods (common for both herbal products and mushrooms): the scavenging capacity of ABTS^{•+}, DPPH free radicals and the reducing power [5, 27].

DPPH assay

DPPH free radical scavenging capacity was determined according to Ohnishi M. [20]. Briefly, 0.5 mL of BE_{SD}, AM_{SD}, MP_{SD}, BE_{FD}, AM_{FD}, MP_{FD} extractive solutions (0.99 - 9.96 mg/mL) were mixed with 3 mL of 0.1 mM ethanolic solution of DPPH. The mixture was kept in the dark, at room temperature for 30 min. and the absorbance of the DPPH solution was measured at λ = 517 nm before (A_{start}) and 30 min. after adding the extractive solutions (A_{end}). Ethanol was used as a blank. Vitamin C (0.005-0.5 mg/mL) was used as a positive control. The ability to scavenge the DPPH free radical was calculated according to the following formula: DPPH radical scavenging activity:

$$(\%) = (A_{\text{start}} - A_{\text{end}}) / A_{\text{start}} \times 100 \text{ (formula 1).}$$

The concentration of mushrooms extractive solutions/vitamin C that inhibited 50% of the DPPH free radical activity (EC₅₀, mg/mL) was determined graphically from the linear regression curve plotted between percent (%) of inhibition and extractive solutions/positive control concentrations (mg/mL).

ABTS^{•+} radical cation scavenging assay

This assay was performed according to Re R. method [25]. The ABTS^{•+} radical cation was generated by incubation of ABTS diammonium salt (7 mM) with potassium persulphate (2.45 mM) in the dark, at room temperature for 16 h. The absorbance of the ABTS^{•+} radical solution was equilibrated to a value of 0.700 ± 0.02 at λ = 734 nm after dilution with ethanol. Briefly, 100 µL of BE_{SD}, AM_{SD}, MP_{SD}, BE_{FD}, AM_{FD}, MP_{FD} solutions (0.99 - 9.96 mg/mL) were treated with 3 mL of ABTS^{•+} free radical and the mixture was kept in the dark. The absorbance of the free radical cation was measured at λ = 734 nm before (A_{start}) and 6 min. after adding the extractive solutions (A_{end}). Ethanol was used as a blank. The ability to scavenge the ABTS free radical was calculated according to formula (1). Vitamin C (0.005-0.5 mg/mL) was used as a positive control. The concentration of mushrooms extractive solutions/positive control that inhibited 50% of the ABTS^{•+} free radical activity (EC₅₀, mg/mL) was determined graphically from the linear regression curve plotted between percent (%) of inhibition and extractive solutions/vitamin C concentrations (mg/mL).

Reducing power

The reducing power assay was determined according to M. Oyaizu [23] method. Briefly, 2.5 mL of BE_{SD}, AM_{SD}, MP_{SD}, BE_{FD}, AM_{FD}, MP_{FD} solutions (0.99 - 9.96 mg/mL) were mixed with 2.5 mL of

0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. Samples were kept at 50°C in a water bath (Raypa, Spain) for 20 min. After, 2.5 mL of 10% trichloroacetic acid was added and the mixture was centrifuged at 2500 rpm for 5 min. (Universal 16 centrifuge). The upper layer (2.5 mL) was mixed with 2.5 mL water and 0.5 mL of a 0.1% ferric chloride solution. The absorbance was measured at $\lambda = 700$ nm, after 10 min., against a blank that contained all reagents except the mushroom samples/positive control. A higher absorbance indicates a stronger reducing power. Vitamin C (0.005 - 0.5 mg/mL) was used as a positive control. The mushrooms extractive solutions/positive control concentration providing 0.5 of absorbance (EC_{50} mg/mL) was determined graphically from the linear regression curve plotted between absorbance and extractive solutions/positive control concentrations (mg/mL).

Statistical analysis

For each of the mushroom species, three samples were analysed and all assays were carried out in triplicate ($n = 3$). The results are expressed as mean \pm standard deviation. The effect of drying method (freeze-drying or sun-drying) upon total phenolic content and antioxidant activity was analysed using one way analysis of variance (ANOVA), followed by Tukey's test with $\alpha = 0.05$. The correlation between antioxidant assays and total phenolic content was determined using Pearson coefficient (r). Results were considered statistically significant if $p < 0.05$. Statistical analysis was carried out using GraphPad Prism v.5 for Windows.

Results and Discussion

The macroscopic exam (Figure 1) confirmed the mushrooms identity.

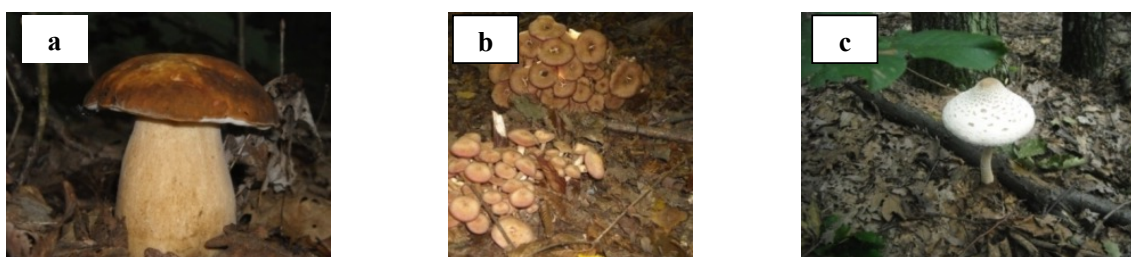


Figure 1.

Macroscopic exam of:

a. *Boletus edulis* (porcini), b. *Armillaria mellea* (honey fungus), c. *Macrolepiota procera* (parasol)

Our results pointed out that there are significant differences ($p < 0.05$) between all analysed mushrooms, which were dried by the same method. Moreover freeze-drying significantly and positively influenced *Boletus edulis* and *Macrolepiota procera* polyphenols contents ($p < 0.05$) (Table I). It is well known that lyophilisation is an excellent drying method due to the absence of liquid water and low temperatures required. It can be applied for herbal products/mushrooms that contain heat

sensitive compounds (polyphenols, carotenoids) and gives a final product of excellent quality, for which most deterioration and microbiological reactions are stopped [1].

For freeze-dried *Armillaria mellea*, there is a slight increase of polyphenols content, but without significant differences ($p > 0.05$), since we have obtained similar results for both drying methods (0.4918 g% tannic acid and 0.5719 g% tannic acid respectively) (Table I).

Table I

Influence of drying method upon polyphenols content of mushrooms

Mushroom	Drying method – Polyphenols content (g% tannic acid)	
	sun-drying (SD)	freeze-drying (FD)
<i>Boletus edulis</i>	0.4902 \pm 0.0494	1.401 \pm 0.0729
<i>Armillaria mellea</i>	0.4918 \pm 0.0322	0.5719 \pm 0.0341
<i>Macrolepiota procera</i>	0.7658 \pm 0.04366	1.2329 \pm 0.0556

Results are expressed as mean \pm standard deviation ($n = 3$)

We assume that honey fungus lower content of reducing compounds and polyphenols might be responsible for this behaviour. Our hypothesis is supported by Nowacka N. *et al.* [19] that found a total polyphenolic and protocatechuic acid contents of 4.23 mg gallic acid/g and 2.25 mg/kg d.w. for honey fungus and 8.21 mg gallic acid/g, 5.19 mg/kg

d.w. for *Boletus edulis*. Palacios I. *et al.*, reported that porcini is the best source of polyphenols among other mushrooms collected from Spain (*Agaricus bisporus*, *Cantharellus cibalius*, *Lactarius deliciosus*) [24]. *Boletus edulis* is a rich source of phenol-carboxylic acids (caffeic, chlorogenic, *p*-coumaric, gallic, *p*-hydroxybenzoic, homogentisic, protocatechuic)

and flavonoids (myricetin, naringenin, kaempferol, apigenin, hesperidin), that can be easily oxidised by sun heat [22, 24].

The antioxidant activity is also influenced by the drying method, since lyophilisation involves a better scavenging capacity and reducing power.

Our results pointed out that DPPH and ABTS^{•+} free radicals inhibition had higher values for freeze-dried mushrooms compared to sun-dried ones in the same concentration range as follows: at 4.98 mg/mL the scavenging capacity of DPPH radical

was 67.96% (for sun-dried porcini) compared to 83.91% (for freeze-dried porcini), whilst for sun-dried *Macrolepiota procera* the inhibition was 52.31% compared to 76.56% (for freeze-dried parasol) (Fig. 2A). As for the scavenging capacity upon ABTS^{•+} free radical (Fig. 2B), at 4.98 mg/mL the inhibition was 34.96% for porcini (SD) and 54.98% (FD) and 26.90% for *Macrolepiota procera* (SD) and 55.21% (FD). Concerning the reducing power (Fig. 2C), higher absorbances were observed for all freeze-dried mushrooms.

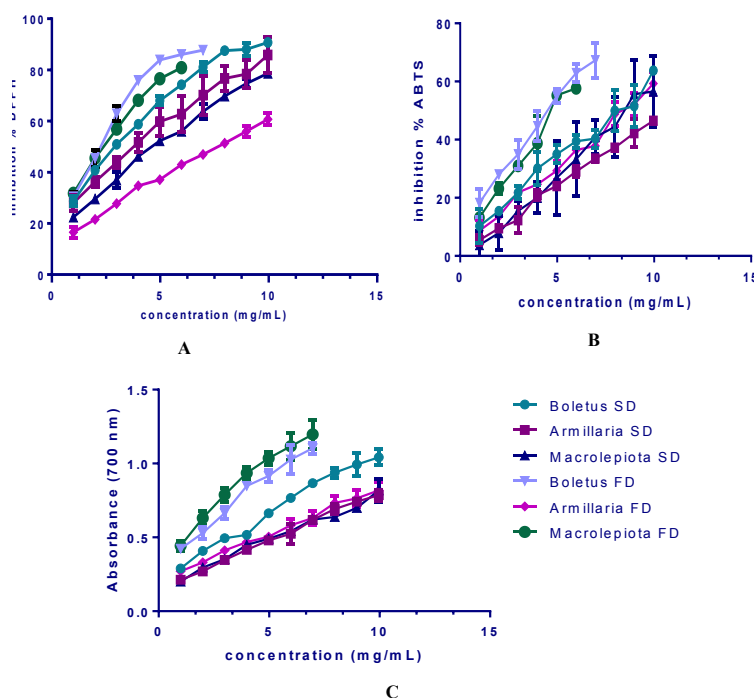


Figure 2.

Antioxidant capacity of analysed mushrooms

A – DPPH scavenging activity, B – ABTS^{•+} scavenging activity, C – reducing power

FD: freeze-dried, SD: sun-dried

Our statistical analysis emphasized that porcini, honey fungus and parasol freeze-dried mushrooms have a higher antioxidant activity compared to sun-dried ones ($p < 0.05$), since they have lower EC₅₀ (mg/mL) values (Table II).

However, no significant differences ($p > 0.05$) were found between freeze-dried and sun-dried *Armillaria mellea*, regarding the scavenging activity upon ABTS^{•+} free radical (Table II).

Table II

EC₅₀ (mg/mL) values for the analysed mushrooms

Method	BOLETUS		ARMILLARIA		MACROLEPIOTA		Vitamin C
	SD	FD	SD	FD	SD	FD	
DPPH	3.0594 ± 0.1082	2.2204 ± 0.1984	4.0302 ± 0.4760	7.6304 ± 0.2523	5.0019 ± 0.0821	2.4962 ± 0.0198	0.0215 ± 0.0008
ABTS ^{•+}	8.1191 ± 0.8214	4.6536 ± 0.3378	10.2900 ± 0.7919	8.5088 ± 0.3450	8.7461 ± 1.8188	4.9284 ± 0.1447	0.0536 ± 0.0033
Reducing power	3.1970 ± 0.0412	1.5460 ± 0.0305	5.3744 ± 0.3923	4.6721 ± 0.3302	5.3204 ± 0.1202	1.0867 ± 0.1320	0.0213 ± 0.0015

Results are presented as mean ± standard deviation (n = 3); SD: sun-dried, FD: freeze-dried

We have also found significant differences ($p < 0.05$) regarding the antioxidant activity (determined by all methods) of analysed mushrooms, dried by the

same method, except for *Boletus* SD vs *Armillaria* SD, *Boletus* SD vs. *Macrolepiota* SD, *Armillaria* SD vs. *Macrolepiota* SD, *Boletus* FD vs. *Macro* FD

(ABTS method; $p > 0.05$) and *Armillaria* SD vs. *Macrolepiota* SD, *Boletus* FD vs. *Macro* FD (for reducing power method; $p > 0.05$). The antioxidant activity of all analysed mushrooms (sun-dried or

freeze-dried) was significantly ($p < 0.05$) lower compared to ascorbic acid (Table II).

Our results revealed a strong negative correlation between antioxidant activity and polyphenols contents (Table III).

Table III
Pearson correlation coefficients (r)

	Total polyphenols	DPPH	ABTS	Reducing power
Total polyphenols	–	-0.5754 ($p = 0.2322$) ^{ns}	-0.9274 ($p = 0.0077$) ^{**}	-0.8077 ($p = 0.0519$) ^{ns}
DPPH	–	–	0.5840 ($p = 0.2236$) ^{ns}	0.7054 ($p = 0.1174$) ^{ns}
ABTS ⁺⁺	–	–	–	0.9483 ($p = 0.0039$) ^{**}

ns – not significant ($p > 0.05$), ** significant ($p < 0.01$)

These results aren't unexpected, since polyphenols are not the main compounds found in mushrooms. We assume that other active substances (polysaccharides, mineral elements) might be involved in the overall antioxidant capacity. Our results are also supported by other authors, who found a negative correlation between polyphenols contents and antioxidant activity [19]. Recently, many reports on *Inonotus obliquus* and *Agaricus blazei Murrill* polysaccharides showed that freeze-drying was a good choice for better antioxidant abilities on DPPH radical scavenging, reducing power and lipid peroxidation inhibition activity compared to hot or vacuum-drying effects upon physicochemical and antioxidant properties of these compounds [16, 31]. According to Ka-Chai S. *et al.*, polysaccharides from *L. edodes*, *G. Frondosa* and *Trametes* showed cytoprotective effects in PC12 cell culture against hydrogen peroxide induced oxidative stress and injury [14]. Porcini and parasol mushrooms have a stronger antioxidant capacity compared to honey fungus, probably because of their mineral elements (zinc, manganese, selenium) contents, which are involved in the endogenous antioxidant defence system. Among analysed mushrooms, *Boletus edulis* is the only source of selenium (48.5 mg/kg d.w.) [8], while *Macrolepiota procera* has a high content of chromium (5 mg/kg d.w.) [28]; however both honey fungus and parasol have similar zinc and manganese contents (88.4 mg/kg d.w.; 86 mg/kg d.w. and 53.6 mg/kg d.w.; 55.59 mg/kg d.w.) [18, 28].

Pearson coefficients showed a good and positive correlation between antioxidant methods (Table III). A significant correlation was found between ABTS⁺⁺ radical cation scavenging assay and reducing power ($p < 0.05$).

Conclusions

Freeze-dried mushrooms have a higher antioxidant capacity compared to traditionally sun-dried ones.

Apart from their excellent nutritional value, the analysed mushrooms are a valuable source of natural antioxidants. Further pharmacological studies are needed, in order to determine their therapeutic properties.

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