

Antioxidant Properties and Chemical Compositions of Various Extracts of the Edible Commercial Mushroom, *Pleurotus Ostreatus*, in Romanian Markets

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The purpose of this study was to determine the antioxidant activities of four different extracts from the mushroom Pleurotus ostreatus available in Romanian markets. Antioxidant activity was calculated by the reducing power assay, the scavenging effect on free radicals, inhibition of lipid peroxidation and chelating effects on ferrous ions. Ethanolic extract presented the highest free radicals scavenging and metal chelating activities. Thus, significant positive correlations proved that the antioxidant effects of ethanolic extract were a result of the presence of the phenolic and flavonoidic compounds. However, the ability to inhibit lipid peroxidation depended on the solvent: methanol > ethanol > cold water > hot water. In the four extracts, different types of molecules with antioxidant properties were determined.

Key words: antioxidant; food property; freeze drying; functional properties; medicinal properties.

Pleurotus ostreatus is an edible mushroom with major medical and biotechnological applications. Fermentation biotechnologies today are using this mycelium for cultivation in liquid medium [1]. Mycelium, or mushroom extracts, are used to obtain functional products, or for isolating compounds with pharmaceutical effects [2]. *P. ostreatus* extract has a number of therapeutic qualities: stimulating the immune system; lowering blood cholesterol; preventing arterial hypertension; and atherosclerosis and hypoglycemic effect. Damages caused by free radicals, often leading to these diseases, cannot be entirely controlled by the human body's self-defense mechanisms. This type of product, based on extractive processes, compensates for these needs due to their diverse composition in antioxidant substances [3,4]. Our objective was to evaluate the antioxidant properties of the edible commercial mushroom *P. ostreatus* sold in Romanian markets, and its study including reducing power, scavenging effects on free radicals, inhibition on lipid peroxidation and chelating effects on ferrous ions. The potential antioxidant components of these medicinal mushrooms were also determined.

Experimental part

Chemicals. All chemicals used were of analytical grade and obtained from Sigma-Aldrich (Germany).

Preparation of samples

Mushrooms, *P. ostreatus* (trays weighing 500 g, collected in one day), were purchased from supermarkets in Bucharest. Undamaged mushrooms were selected and dried in a stream of dry air. The drying process took place at a constant temperature of 25°C in the oven for 15 days, until a constant weight was reached.

Obtaining of extracts.

The dried samples were subject to 70% ethanol extraction. 10 g of the dried mushroom sample was extracted using 100 mL solvent (ethanol and methanol), for 24 h, at 25°C and at 150 rpm. For hot water extract, 10 g of the sample was boiled in 500 mL of water for 30 min.

For cold water extraction, a sample of 10 g was extracted by stirring with 100 mL cold water at 4°C for 24 h (5). The extracts were filtered using a Whatman No. 4 filter paper [6]. The solvents used for extraction were removed using a rotary vacuum evaporator Buchi R215, with a vacuum controller V-850, and a heating bath for parallel evaporation Multivapor P-6 at 50°C under vacuum [7]. The resulting extracts was lyophilized in a freeze dryer ALPHA 1-2/LD plus (Martin Christ Gefriertrocknungsanlagen GmbH), at -55°C, for 48 hr

DPPH radical scavenging assay

The DPPH scavenging activity was measured using spectrophotometry. First, 0.05 mL of the samples dissolved in ethanol were added to an ethanolic solution of DPPH (200 µm) at different concentrations (varying from 2-10 mg/mL). An equal amount of ethanol was added to the control. After 20 min, the absorbance was read at 517 nm and the inhibition was calculated using the formula: DPPH scavenging effect (%) = $A_0 - A_p / A_0 \times 100$, where A_0 was the absorbance of the control and A_p was the absorbance in the presence of the sample [8, 9]. Ascorbic acid was used for comparison.

Reducing power

First, 200 µL of the samples were mixed with sodium phosphate buffer (pH 6.6), 1 mM FeSO₄ and 1% potassium ferricyanide. After incubation of the mixture for 20 min at 50°C, trichloroacetic acid was added and the mixtures were centrifuged. Next 2.5 mL of the resulting supernatant was mixed with an equal volume of water and 0.5 mL 0.1% FeCl₃. The absorbance was measured at 700 nm [9,10]. Ascorbic acid was used for comparison.

Superoxide radical scavenging assay

The reaction mixture contained the same volume of 120 µM PMS (phenazine methosulfate), 936 µM NADH, freeze-dried extract, and 300 µM NBT, in a total volume of 1 mL of 100 mM phosphate buffer (pH 7.4). After 5 min of incubation at ambient temperature, absorbance of the resulting solution was measured at 560 nm. The superoxide

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radical activity was calculated as: scavenging effect (%) = $(1 - \text{absorbance of sample} / \text{absorbance of control}) \times 100$. Ascorbic acid was used for comparison [11]. EC_{50} value (milligram extract/mL) is the effective concentration at which superoxide radicals were scavenged by 50% [12].

Hydroxyl radical scavenging assay

The assay is based on the quantification of the degradation product of 2-deoxyribose by condensation with thiobarbituric acid (TBA). Hydroxyl radicals were generated using the Fe^{3+} -ascorbate-EDTA- H_2O_2 system (the Fenton reaction). The reaction mixture contained, in a final volume of 1 mL: 2-deoxy-2-ribose (2.8 mM), KH_2PO_4 -KOH buffer (20 mM, pH 7.4), $FeCl_3$ (100 μ M), EDTA (100 μ M), H_2O_2 (1.0 mM), ascorbic acid (100 μ M), and various concentrations (0–200 μ g/mL) of the freeze-dried extracts or reference compound. After incubation for 1 h at 37°C, 0.5 mL of the reaction mixture was added to 1 mL 2.8% TCA, then 1 mL of 1% aqueous TBA was added and the mixture was incubated at 90°C for 15 min to develop the colour. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. All tests were performed 3 times. Percentage inhibition was evaluated by comparing the test and blank solutions. EC_{50} value (milligram extract/mL) is the effective concentration at which hydroxyl radicals were scavenged by 50% [12-15].

Nitric oxide scavenging assay

Nitric oxide scavenging activity was measured spectrophotometrically. Sodium nitroprusside (5 mmol/L) in phosphate buffered saline, pH 7.4, was mixed with different concentrations of the extract prepared in ethanol and incubated at 25°C for 30 min. A control without the test compound, but with an equivalent amount of ethanol, was also used. After 30 min, 1.5 mL of the incubated solution was removed and diluted with 1.5 mL of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% *N*-1-naphthylethylenediamine dihydrochloride). Absorbance formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with *N*-1-naphthylethylenediamine dihydrochloride was measured at 546 nm and the percentage scavenging activity was measured with reference to the standard (ascorbic acid). The EC_{50} value (milligram extract/mL) is the effective concentration at which nitric radicals were scavenged by 50% [12,16,17].

Hydrogen Peroxide scavenging assay

Samples with different concentration were added to 0.1 M phosphate buffer solution (pH 7.4, 3.4 mL), respectively, and mixed with 43 mM hydrogen peroxide solution (0.6 mL). After 10 min, the reaction mixture absorbance was determined at 230 nm. The reaction mixture without sample was used as the blank. Ascorbic acid was used as a reference compound. The percentage inhibition activity was calculated as: $[(\text{Abs. of control} - \text{Abs. of sample}) / \text{Abs. of control}] \times 100\%$ [18,19]. Ascorbic acid was used for comparison.

Ferrous ion Chelating Assay

To determine the ferrous ion-chelating ability, first, 1 mL of each extracts (2–10 mg/mL) was mixed with 3.7 mL of ultrapure water, then the mixture was reacted with ferrous chloride (2 mmol/L, 0.1 mL) and ferrozine (5 mmol/L, 0.2 mL) for 20 min. The absorbance at 562 nm was determined spectrophotometrically. EDTA was used as positive control. The chelating activity on the ferrous ion was calculated using the equation below: Chelating Activity (%) = $[(\text{Ab-}$

$\text{As}) / \text{Ab}] \times 100$, where Ab is the absorbance of the blank without the extract or ascorbic acid and As is the absorbance in the presence of the extract or ascorbic acid [9,20].

Lipid peroxidation assay

0.1 mL of different dilutions of each extract was added to a mixture that contained 1 mL fowl egg yolk emulsified with 0.1 M phosphate buffer, pH 7.4, to obtain a final concentration of 25 g/L, and 100 μ L of 1 mM Fe^{2+} . The mixture was incubated at 37°C for 1 hr, after which it was treated with 0.5 mL freshly prepared 15% trichloroacetic acid (TCA) and 1 mL 1% thiobarbituric acid (TBA). The reaction tubes were kept in a bath of boiling water for 10 min. Upon cooling with ice, the tubes were centrifuged at 3.500 rpm for 10 min to remove precipitated protein. The formation of TBARS was evaluated by measuring the absorbance at 532 nm. Ascorbic acid was used as a reference compound [18, 21, 22].

Determination of Antioxidant Component

Determination of total phenolic content

The extract sample (0.50 mL) was added to a 1:10 dilution of Folin-Ciocalteu reagent (2.5 mL). After 4 min, a saturated sodium carbonate solution (approximately 75 g/L, 2 mL) was added. The absorbance of the reaction mixture was measured at 760 nm after incubation for 2 h at room temperature. Gallic acid was used as a reference standard, and the results were expressed as milligram gallic acid equivalent (mg GAE)/g of extract [22].

Determination of total flavonoids

So μ L of each extract was mixed with 700 μ L of deionized water and 37 μ L of 5% $NaNO_2$. After a five minute incubation at room temperature, 75 μ L of 10% $AlCl_3$ was added, followed by 250 μ L of 1M NaOH for the next six minutes. After shaking, the mixture was centrifuged (5000 \times g, room temperature, 15 min), and the absorbance of the supernatant was read at 515 nm against a blank. Quercetin was used as a standard [23-25].

Determination of β -Carotene and lycopene

For β -carotene and lycopene determination, the dried extracts (100 mg) was vigorously shaken with 10 mL of an acetone-hexane mixture (4:6) for 1 min and filtered through Whatman No. 4 filter paper. The absorbance of the filtrate was measured at 453, 505, and 663 nm. β -carotene and lycopene content were calculated according to the following equations: lycopene (mg / 100 mL) = $-0.0458 \times A_{663} + 0.372 \times A_{505} - 0.0806 \times A_{453}$; β -carotene (mg/ 100 mL) = $0.216 \times A_{663} - 0.304 \times A_{505} + 0.452 \times A_{453}$. The results are expressed as mg of carotenoid/g of extract [24,26].

α -tocopherol content of the extracts from *P. ostreatus* was determined according to the method of Al-Naqeeb et al. (2009) [24,27].

Statistical analysis

All parameters for antimicrobial and antioxidant activity were assessed in triplicate, and the results were expressed as mean \pm SD values of 3 observations. The mean values and standard deviation were calculated with the EXCEL program from Microsoft Office 2010 package.

Results and discussions

From a study of the extracts, we see that the use of water as solvent leads to an extraction efficiency (g lyophilized extract/100 g dry mushroom) higher than those

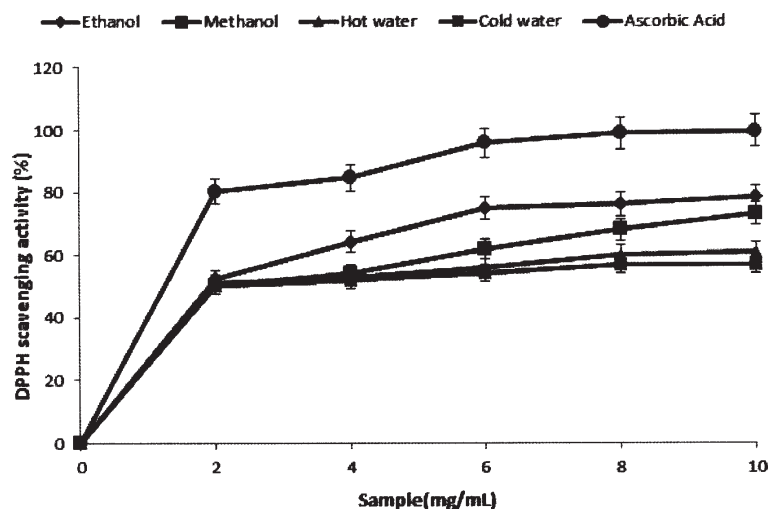


Fig. 1. Scavenging activity of *Pleurotus ostreatus* extracts on DPPH radicals. Each value is expressed as mean \pm SD (n = 3)

of the two types of alcohol. The order was as follows: cold water > hot water > methanol > ethanol. Ethanol, and especially methanol, were used because they have a greater capacity for extraction of phenols and flavonoids [25,28].

Scavenging ability on DPPH radicals

The scavenging activity on DPPH free radicals represents a widespread and accepted method for assessing the antioxidant capacity of an extract. The four extracts from the commercial mushroom *P. ostreatus* were evaluated in parallel with ascorbic acid. This method relies on the ability to donate hydrogen radicals to a DPPH synthetic compound. As a result, there is a color change from violet to yellow, and the monitoring of absorbance decrease at 517 nm [29]. DPPH scavenging effect was amplified by increasing the concentration of the extract. According to figure 1, the best results are obtained by using ethanol and methanol as solvents. Ethanolic extract at a concentration of 10 mg/mL had a scavenging effect on DPPH radicals at 78.28%, being 6.74% higher than the methanolic one, about 22% higher than the hot water extract, and with 27.18% than the cold water extract. Compared to the standard, the scavenging effect was 21.48% lower. Compared with ethanolic extracts of *Agaricus bisporus* and *P. citrinopileatus*, the obtained values correspond to a maximum of 70% DPPH scavenging effect for 5 mg/mL, also for the ethanolic extract. The other three

extracts had lower values of \approx 60% for a similar concentration.

Reducing power

The reducing power values are determined not only by the presence of reductones, but also of other antioxidant molecules. The reductones work by breaking the chain of free radicals and by donating hydrogen atoms, but can, in addition, bind some precursors of peroxide, thus preventing the formation of this radical. Depending on the amount of antioxidants in an extract, the reduction of Fe^{3+} to Fe^{2+} continues to take place [30]. Ethanolic extract showed the highest reducing power when compared with the other three types of extracts (fig. 2). The reducing power was 0.72, for a concentration of 10 mg/mL. The methanolic extract showed a reduction power lower by 16.66%, while for hot and cold water the decrease was 33.33% and 38.85%, respectively. Differences were recorded at a maximum concentration of 10 mg/mL. However, ascorbic acid had a value of 0.86. Thus, the reducing power of the four extracts from the edible fungus *P. ostreatus* was directly proportional to the increase of the extract concentration.

Metal chelating activity

The transition metal ion, Fe^{2+} , possesses the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions

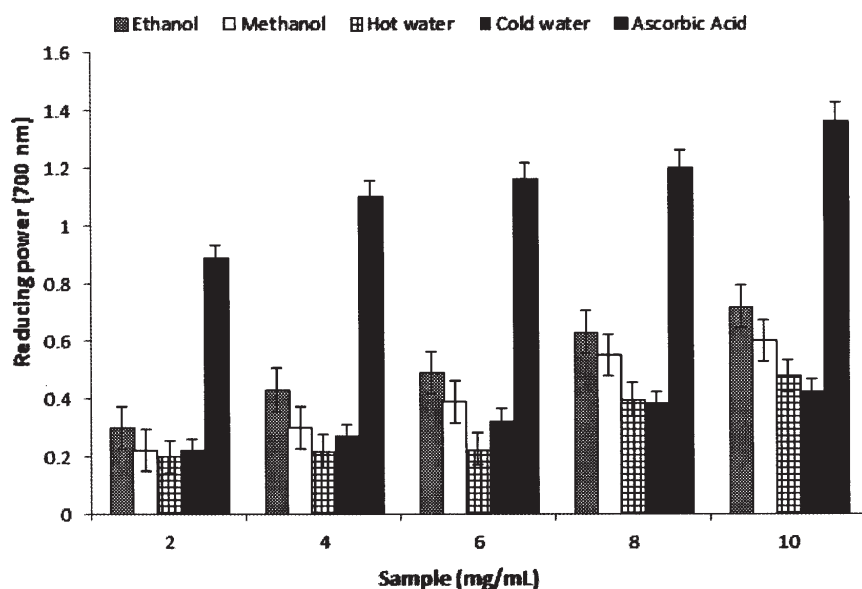


Fig. 2. Reducing power of *Pleurotus ostreatus* extracts. Each value is expressed as mean \pm SD (n = 3)

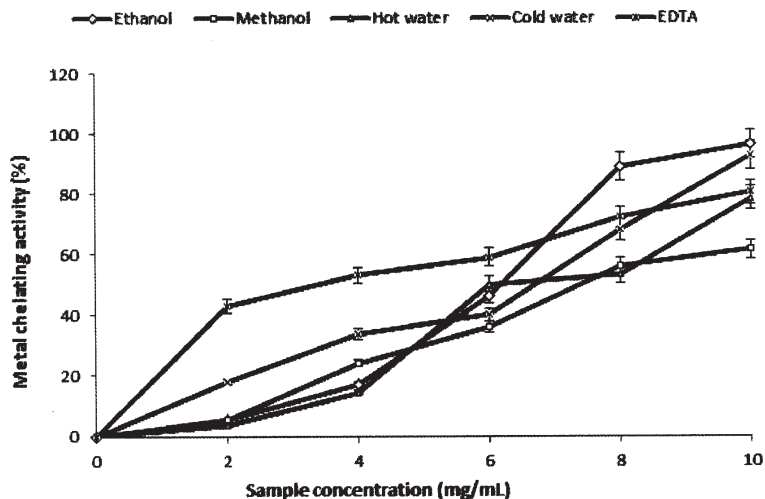


Fig. 3. Chelating effect of *Pleurotus ostreatus* extracts on ferrous ions. Each value is expressed as mean \pm SD (n=3)

[31,32]. Ferrous ions can initiate lipid peroxidation by the Fenton reaction, as well as accelerating peroxidation. Ferrozine can make complexes with ferrous ions. In the presence of chelating agents, a complex (colored in reddish-pink) formation is interrupted and as a result, the colour of the complex is decreased [32,33]. According to figure 3, the chelating effects of mushroom extracts increase with sample concentration, as well as EDTA, used as standard. At a concentration of maximum 10 mg/mL, the ethanolic and cold water extracts showed higher values than the standard, of $96.29 \pm 0.13\%$ and $92.59 \pm 0.33\%$, respectively (fig. 3.). A value close to the standard had the hot water extract, the chelating activity being $H \approx 2.7 \pm 0.06\%$ lower. The results are confirmed by other previous researches in terms of methanol extract from *P. ostreatus* [34]. The results obtained with cold water extract are comparable to those achieved with acetone extract of the same species [35]. However, the superior results with ethanolic extract show the higher power of penetration of this solvent, and also that lyophilization represents the most effective way to preserve biologically active molecules at a maximum quantity.

Inhibition of lipid peroxidation

This process is prevalent in the brain and liver due to the presence of polyunsaturated lipids that promote the free radicals attack. Following lipid peroxidation, a number of toxic products are released, that cause damage to the DNA molecule [36]. Also, the changes which can occur in the low density lipoproteins represent a major cause of atherosclerosis appearance. The mushroom extract is considered to be a good inhibitor of lipid peroxidation

through its phenolic components [25,37]. The inhibition of lipid peroxidation in the case of the four extracts and of the ascorbic acid, was appropriate to the concentration (fig. 4). Depending on the solvent, the sequence of the extracts was: methanol > ethanol > cold water > hot water. Thus, at the maximum concentration of 10 mg/mL, the standard and the methanolic extracts showed similar values, i.e. $70.54 \pm 0.04\%$ and $70.42 \pm 0.38\%$, respectively. These results confirm the high capability of methanolic extract to inhibit lipid peroxidation, as also evidenced by alternative studies [35].

Antioxidant components

Phenolic compounds are the main antioxidant component, which were identified in aqueous and alcoholic extracts (table 1). They inhibit the oxidative stress exerted by different types of free radicals [35]. Ethanolic extract (70.2 ± 0.54 mg gallic acid/g extract) had the highest amount of phenols, while the lowest one was found in the hot water extract (57.2 ± 0.52 mg gallic acid/g extract). These quantities are higher than those found in other studies performed with the same species, both for the aqueous extract (29.30 ± 1.60 mg gallic acid/g extract) and for the ethanolic one (37.98 ± 1.88 mg gallic acid/g extract) [38]. Instead, in the extracts lyophilized in hot and cold water a similar amount as that of *Ganoderma lucidum* (55.96 mg gallic acid/g extract) was identified [39].

Flavonoids and flavonols (represented primarily by chrysin) [35] are antioxidant compounds widely prevalent in plant material, being mainly water soluble compounds. Flavonoids reduce the incidence of cardiovascular

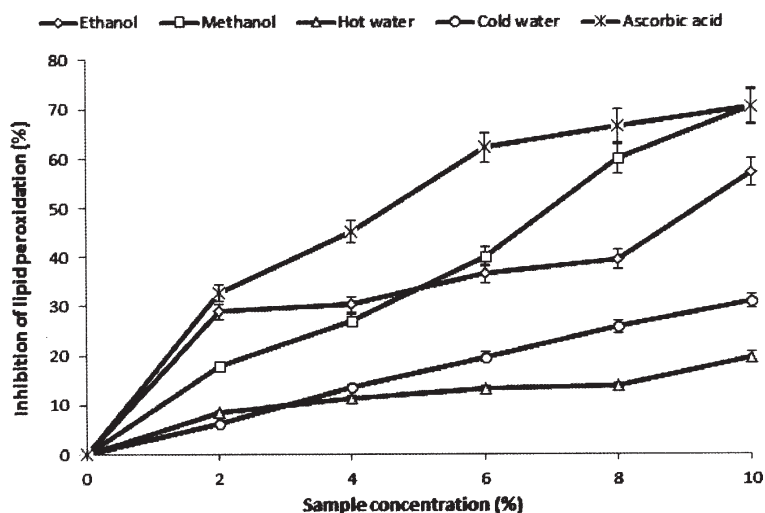


Fig. 4. Inhibitory effect of *Pleurotus ostreatus* extracts on lipid peroxidation. Each value is expressed as mean \pm SD (n=3)

diseases, of cancer and of degenerative diseases, which, today, occur in an increasing number of elderly people. Depending on the type of the solvent used, the order was: ethanol > methanol > cold water > hot water. Thus, the amount of flavonoids ranged from 23.1 ± 0.41 to 212.25 ± 0.36 mg quercetin/g extract. The measurements were in agreement with other studies on the same species, displaying a significant amount of flavonoids, of over 200 mg quercetin/g extract, as was shown by the phytochemical analysis [40].

Ascorbic acid is a natural antioxidant, widespread in the mushroom extract. Ascorbic acid serves as a reducing agent, by transferring a hydrogen atom to oxygen. The determined amount ranged from 6.72 ± 0.27 mg/g extract (cold water extract) to 23.36 ± 0.15 (ethanolic extract). The amount was higher than in other species of fungi, like *Leucopaxillus giganteus* (0.13 ± 0.0069 mg/g extract), *Sarcodon imbricatus* (0.16 ± 0.0072 mg/g extract), and *A. arvensis* (0.35 ± 0.0015 mg/g extract) [41].

α -tocopherol is a classical and important antioxidant, well known as a scavenger of free radicals. It possesses

anti- and pro-oxidant properties that act against low density lipoproteins, ensuring protection of the blood vessels and prevention of cardiovascular diseases [28]. In this study, α -tocopherol concentration ranged from 6.44 ± 1.91 to 29.15 ± 0.53 mg/100 g extract. The maximum amount was identified in the methanolic extract, thus demonstrating the role of the solvent in the extraction capacity of compounds with antioxidant effect.

Carotenoids are efficient antioxidants, scavenging singlet molecular oxygen and peroxy radicals. In the human body, carotenoids are part of the antioxidant defense mechanism. They interact with other antioxidants. A combination of carotenoids is more effective than single compounds [42]. In this study, β -carotene was found only in the two alcoholic extracts. The amount of lycopene ranged between 0.001 ± 0.0006 and 0.01 ± 0.009 mg/100 g extract and was not identified in the cold water extract.

EC₅₀ values for the antioxidant properties

According to the data presented in table 2, the lyophilized extracts from the mushroom *P. ostreatus*

Antioxidant components	Extract			
	Ethanolic	Methanolic	Hot water	Cold water
Total phenols (mg gallic acid/g extract)	70.2±0.54	67.6±0.33	57.2±0.52	58.8±1.32
Flavonoids (mg quercetina/g extract)	212.25±0.36	118.05±0.27	23.1±0.41	24±0.52
Flavonols (mg quercetina/g extract)	11.87±0.07	9.85±0.49	3.87±0.32	5.75±0.37
Ascorbic acid (mg/g extract)	23.36±0.15	19.89±0.31	10.69±0.65	6.72±0.27
β -carotene (mg/100 g extract)	0.01±0.008	0.006±0.0006	-	-
Lycopene (mg/100 g extract)	0.006±0.0006	0.01±0.009	0.001±0.0006	-
α -tocopherol (mg/100 g extract)	25.54±2.07	29.15±0.53	13.74±0.75	6.44±1.91

Each value is expressed as mean \pm SD (n=3).

Table 1
ANTIOXIDANT CONTENTS
OF VARIOUS EXTRACTS
FROM *PLEUROTUS*
OSTREATUS

	EC ₅₀ (mg/mL)					
	Correlation Coefficient (r ²)					
	Ethanolic	Methanolic	Hot water	Cold water	Ascorbic acid	EDTA
Scavenging effect on DPPH radicals	3.98±0.11	5.22±0.29	5.96±0.15	6.21±0.07	0.09±0.03	-
	0.5196	0.9984	0.8733	0.9753	-	-
Reducing power	12.08±0.27	17.71±0.41	43.52±0.21	27.07±0.3	0.86±0.05	-
	0.8174	0.9808	0.9957	0.984	-	-
Scavenging ability on superoxide anion	12.49±0.43	4.86±0.17	1.77±0.23	1.61±0.37	0.6±0.02	-
	0.6369	0.9771	0.0833	0.9526	-	-
Scavenging ability on hydroxyl radical	4.31±0.58	7.81±0.47	6.62±0.54	6.89±0.7	0.07±0.02	-
	0.9858	0.9591	0.8374	0.8392	-	-
Scavenging ability on nitric oxide	15.31±0.26	16.06±0.41	22.41±0.21	23.19±0.24	0.08±0.01	-
	0.7396	0.6196	0.9813	0.7482	-	-
Scavenging ability on hydrogen peroxide	6.41±0.12	8.95±0.52	19.42±0.51	47.01±0.41	1.08±0.13	-
	0.9387	0.948	0.9257	0.4289	-	-
Metal chelating effect	6.35±0.41	1.73±0.56	1.52±0.79	8.28±0.65	-	0.05±0.06
	0.2597	0.7256	0.1673	0.177	-	-
Inhibition on lipid peroxidation	5.81±0.11	6.74±0.25	18.43±0.64	12.31±0.42	5.78±0.25	-
	0.9825	0.983	0.8231	0.9211	-	-

Each value is expressed as mean \pm SD (n=3).

Table 2
EC₅₀ VALUES AND CORRELATION
COEFFICIENT (R²) BETWEEN
DIFFERENT BIOLOGIC ACTIVITIES
AND TOTAL PHENOLIC CONTENT OF
VARIOUS EXTRACTS FROM
PLEUROTUS OSTREATUS.

showed a significant inhibitory effect of different types of free radicals, reducing power, as well as the inhibition capacity of lipid peroxidation and metal chelation. The ethanolic extract had the most powerful antioxidant effect, demonstrated also by the R^2 coefficient, related to the total content of phenolic compounds, ranging from 0.5196 to 0.9858. The lower values (for DPPH, superoxide anion, nitric oxide scavenging activity and metal chelating effect) are compensated by the value of R^2 , which had a very good linear correlation with the amount of flavonoids, ascorbic acid and carotenoids, which ranged between 0.9184 and 0.9965. In this case, ethanol, compared with the other three solvents, had a higher capacity of extraction both of the phenols and of the flavonoids, ascorbic acid and β -carotene (table 1). In the case of hot and cold water extracts, the low values of EC_{50} were due to the presence of intracellular polysaccharides (IPS). The antioxidant activity of the IPS included the scavenging activity against free radicals as well the chelating ability. A good correlation between these scavenging activities was determined ($R^2 = 0.934-0.9639$). Evaluation of the antioxidant effect of the polysaccharides leads to a better understanding of the mechanism that enables these fungi to exert a functional effect on the human body. Another important aspect that emerges from such a study is that of optimizing the extractive process, and of the final processing, in order to maximize the amount of antioxidant molecules [9]. From all the above findings, results show that lyophilization is the most effective extract, which is superior to maintaining a biologically active extract in dissolved form (different concentrations) in a solvent (ethanol, methanol), at 4°C [43].

Conclusions

The different freeze-dried extracts of *P. ostreatus* obtained from Romanian markets exhibited strong radical scavenging activities *in vitro*, a metal chelating effect and capacity to inhibit the lipid peroxidation. These biological activities depended on the quantity of the compounds with antioxidant effect, and on the extractive capacity of the four solvents, as was demonstrated by the values of $R^2 > 0.9$. Contents of ascorbic acid, α -tocopherols, carotenoids, flavonoids and total phenols were associated with EC_{50} value of *in vitro* antioxidant properties. The present study confirms that the *P. ostreatus* mushroom obtained from Romanian markets is a significant source of natural antioxidant compounds and bestows great benefits on human health by its regular consumption.

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