



Research Journal of
Phytochemistry

ISSN 1819-3471



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Antioxidant Activity of Crude Methanolic Extracts from *Pleurotus ostreatus*

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ABSTRACT

The aim of our study was to evaluate the biological value of methanolic extracts obtained from *Pleurotus ostreatus* fruiting bodies cultivated in a traditional household. We used four substrate formulas containing wheat straw, borceag straw (borceag is a vetch and oat mixture used for animal nutrition) and beech shavings in order to determine the substrate influence on the content of antioxidant compounds and on the antioxidant potential of brute extracts. The antioxidant potential was determined using free radical scavenging activity (ABTS, DPPH and superoxide radicals), inhibition of lipid peroxidation, reducing power and chelating activity. Virtually, we observed that the presence of an increased lignin component (beech shavings) in substrates reduces the number of some biological active molecules. This fact was directly linked to a decreased biological activity. These results showed that, depending on how easily the substrate is decomposed by mycelium and the quantity on nutrients assimilated by it, the nutritional value of the obtained mushrooms will increase.

Key words: *Pleurotus ostreatus*, straw, beech, phenolic compounds, free radicals

INTRODUCTION

In Romania, *P. ostreatus* is the second largest mushroom species that are annually marketed, after *Agaricus bisporus*. This species is easily cultivated in rural households because it can be grown on various vegetal wastes (Vamanu, 2013a). Wheat straw is the most common substrate used due to its large distribution in all the regions of the country and to its relatively low price. In the past years, this low acquisition price of raw materials has paved the way for the appearance of a series of local producers who had marketplace even in regions considered less extended, such as Moldova region (Botosani County).

Increased production due to the extended demand in the market led to the use of supplementary materials as substrate, for example, different vegetable wastes such as corncobs (Sendi *et al.*, 2013). Generally, this material is commonly used in rustic households for heating. Although it has to be processed (chopped) in order to increase the productivity of the mushroom's mycelium, the acquisition does not require a financial effort. Likewise, there are other vegetal wastes in agriculture and other food industry sub-products that can be used (Zhang *et al.*, 2014). Another significant aspect is represented by the valorization of the mushroom substrate. It is a rich source of nutrients that can be used by plants (Medina *et al.*, 2009; Sendi *et al.*, 2013). It has no toxicity and is a way to enrich soil, in sustainable agriculture (Manjunath and Korikanthimath, 2009).

The great advantage of *P. ostreatus* mushroom is that it neither requires special cultivation parameters nor conditions for the cultivation. The culture adapts through many hybrids existing in the market to growing in different thermal regimes on the territory of our country. Thus, the aim of our study was to evaluate the total phenolic and flavonoid content as well as the antioxidant activity of *P. ostreatus* cultivated on different substrates in a rustic household in Botosani County, Romania.

MATERIALS AND METHODS

Biological material and cultivation conditions: In our study, we used *P. ostreatus* mycelium that was cultivated on wheat grains bought from SC Nutrimold SA Iasi. The substrate formulas were substrate P1: 80% wheat straws +20% beech shavings, substrate P2: 50% *Vicia pannonica* straws +50 beech shavings, substrate P3: 53% wheat straws +47% beech shavings, substrate P4: 23% wheat straws +67% beech shavings. Substrate humidity was 75%. The sterilization was carried out by immersion in a metallic tank where the substrate and water were combined at 85°C. After 24 h, the remaining water was eliminated and the substrate was pulled out. After chilling, we added 3% gypsum and 2-3% granulated mycelium. The obtained mixture was inserted in polyethylene bags (30 cm in diameter and 100 cm height) with holes in zigzag course of 15 cm spacing between them. Each bag was braced so that no empty spaces remained inside. All bags were incubated at 22-24°C for 20 days (Fernandes *et al.*, 2015).

Mushroom extraction: All samples were dried in steamer (Memmert U) at 50°C and chopped with a lab blend. About 10 g of mushroom dust (from each substrate separately) was extracted with methanol 30% and at room temperature for 24 h, 150 rpm in a LabTech shaking incubator. The extracts were obtained by filtration using Whatman no. 1 filter paper. All samples were introduced in brown bottles, labeled and preserved at 4°C (Elmastas *et al.*, 2007).

Determination of antioxidant compounds

Antioxidant composition: Total phenolic content was estimated by the Folin-Ciocalteu method and the total flavonoid content was estimated by an aluminum chloride colorimetric method (Vamanu and Nita, 2013). Total proanthocyanidin was estimated by vanillin and hydrochloric acid method (Aiyegoro and Okoh, 2010).

Determination of antioxidant potential

Free radical scavenging activity: The DPPH assay was performed as described by Hussain *et al.* (2011). The ABTS assay was performed as described by Aiyegoro and Okoh (2010). The superoxide scavenging assay was performed as described by Lobo *et al.* (2010). In all these assays, gallic acid was used for comparison (Vamanu, 2013a, b).

Inhibition of lipid peroxidation: This was assayed as described by Vamanu (2013a). Gallic acid was used for comparison.

Ferrous ion chelating assay: This was assayed as described by Vamanu (2013a). Gallic acid was used for comparison.

Cupric reducing capacity: This was assayed as described by Karakoca *et al.* (2013). Gallic acid was used for comparison.

Statistical analysis: All the parameters for antimicrobial and antioxidant activity were assessed in triplicate and the results were expressed as Mean±SD values of three observations. The mean values and standard deviation were calculated with the EXCEL program of Microsoft Office 2010 (Vamanu, 2013b).

RESULTS AND DISCUSSION

Total phenolic content: Phenolic component contains the main bioactive compounds that can be determined using extracts obtained from fruiting bodies (Vamanu, 2013a) or mycelium of edible mushrooms (Vamanu *et al.*, 2011). Moreover, carotenoid component such as β -carotene or lycopene, vitamin C (ascorbic acid) and tocopherols (especially α -tocopherol) can be determined (Vamanu, 2012; Kortei *et al.*, 2014). The phenolic component stabilizes the reactions leading to generate free radicals by donating an electron (Tenkerian *et al.*, 2015). As shown in Table 1, total phenolic content in substrates P1 and P3 is almost similar. Substrate P2 determined fruiting bodies with a three times smaller total phenolic content of 43.6 mg g⁻¹ gallic acid equivalent. An intermediate value was obtained after cultivation on substrate P4, with maximum quantity of beech shavings. Based on our results, we can conclude that there is no directly proportional interrelation between the total phenolic content and the quantity of wheat straw used for substrate. On the contrary, other supplements or the straw rate added to the substrate composition with another material containing more lignin has a significant influence even if it cannot establish a connection between these elements and metabolites accumulation.

Data obtained on substrates P1 and P3 match with previous studies conducted on the same species bought at local store. When we used *V. pannonica* straws, the phenolic content had lower values compared even with warm or cold water extracts. The observed differences were about 25% for *V. pannonica* straws and 13% for the substrate with a majority of wood shavings (substrate P4) (Vamanu, 2013a). Also, the results obtained on substrate P2 are similar to methanolic samples extracted from *P. ostreatus* species bought at local stores from rural area Addis Ababa, Kaffa zone (site Bonga) and Benishangul Gumuz region (site Asosa), Ethiopia (Woldegiorgis *et al.*, 2014). These mushrooms were also grown on wood wastes obtained from the aforementioned areas demonstrating that substrate has a direct influence on antioxidant compounds accumulation.

Total flavonoid content: Flavonoid content is directly related to the analyzed species capacity to combat oxidative stress effects (Unekwu *et al.*, 2014). Thereby, from Table 1, we can conclude that flavonoid accumulation is not similar to the previous studies regarding total phenolic content. On substrate P2, we registered a maximum amount of flavonoids (71.05 μ g mL⁻¹ quercetin equivalent) with almost 70% higher than in mushrooms cultivated on substrate P3 (equal quantities of wheat straws and beech shavings). The maximum usage of beech shavings in substrate (substrate P4) determined a minimum quantity of 19.45 μ g mL⁻¹ quercetin equivalent, value that can be interpreted as a reduced lignicola raw material usage, linked to a reduced

Table 1: Medium quantity of the antioxidant compounds

Substrates	Total phenolic content (mg mL ⁻¹ gallic acid equivalent)	Total flavonoid content (μ g mL ⁻¹ quercetin equivalent)	Proanthocyanidin content (mg mL ⁻¹ gallic acid equivalent)
P1	61.20±2.50	64.50±0.74	7.67±0.05
P2	43.60±0.71	71.05±3.65	8.62±0.02
P3	68.00±1.09	22.20±0.20	5.34±0.75
P4	50.40±0.15	19.45±1.77	5.16±0.05

digestion and weak biological efficiency. An contradictory result was observed on substrate P1, where the majority presence of wheat straws had a positive effect on flavonoidic compound accumulation. The quantity was similar to the one obtained on substrate P2.

Compared to the marketed species in Romania, our methanolic extracts were reduced proving that both species and substrate formula have an enormous influence on flavonoid content. Another reason for these differences can be the cultivation conditions, which were not always controlled like in a classic mushroom farm (Vamanu, 2013a). Flavonoid content in obtained mushrooms (e.g., rutin or chrysin) represents a direct indicator of extracts' antioxidant potential. This effect can be translated by scavenging capacity against free radicals, inhibition of lipid peroxidation, or a chelating activity as high as possible (Jayakumar *et al.*, 2011).

We determined high quantities of proanthocyanidins of 7.67 and 8.62 mg mL⁻¹ gallic acid equivalent. The determined quantities are directly linked to flavonoidic compounds proving an accumulation of catechin and epicatechin (wikipedia), to the detriment of other classic flavonoids like quercetin (Jayakumar *et al.*, 2011). The obtained results are comparable with previous data regarding the compounds' content in species of *Flammulina* or *Psalliota* (Udu-Ibiam *et al.*, 2014).

Chelating activity: Several prooxidizing metals like iron or copper act as a promoter of oxidizing reactions at cell level causing free radical formation. The presence of some compounds with chelating activity has the effect of stabilizing the oxidized form of the metal along with forming a characteristic complex (Koncic *et al.*, 2011). The extracts obtained as a result of cultivation on *V. pannonica* straws and on the substrate with a majority of beech shavings had a similar activity of approximately 79% (Fig. 1a). The other two extracts had similar activities, too but were around 74%, around 5% lower than control sample (gallic acid, 1 mg mL⁻¹).

The obtained results are compared with lyophilized ethanolic extracts at 8 mg mL⁻¹ concentration and also with methanolic extracts from marketed *P. ostreatus* at concentrations higher than 10 mg mL⁻¹ (Vamanu, 2013a). In return, in comparison to other extracts obtained with different solvents, the raw methanolic extracts were similar to samples from *Polysporus squamosus*, *Lepista nuda*, or *Boletus badius* (at concentration of 200 µg mL⁻¹) (Elmastas *et al.*, 2007).

Cupric reducing capacity: It is important to eliminate cellular copper ions because of their negative action on nervous system, a possible major cause for neurodegenerative problems (Sharma and Vig, 2014). The extract obtained from mushrooms cultivated on substrate P4 led to results comparable with control sample (gallic acid, 1 mg mL⁻¹) of approximately 1.9 mg mL⁻¹. Substrate P3 determined results are significantly higher (p<0.5), with almost 45% compared to control sample (Fig. 1b). P3 extract (substrate based on *V. pannonica* straws) has an Optical Density (OD) with 70% lower than a similar mixture of wheat straws and beech shavings (substrate P3). These values obtained on extracts corresponding to substrate P2 are compared with previous results of *Auricularia auricular-judae* species at concentration of 1 mg mL⁻¹ (hot water extract). Mushrooms cultivated on substrate P3 gave higher results than hot water extracts of species like *Ganoderma lucidum*, *Lentinula edodes*, or *Volvariella volvaceae*. Also, the results are approximately 40% higher than quercetin (1 mg mL⁻¹) (Abdullah *et al.*, 2012).

Inhibition of lipid peroxidation: Inhibition of lipid peroxidation represents a standard parameter of antioxidant capacity of a product, it is a directly dependent process to free-radicals

action taking place at the membrane polyunsaturated acids affecting the integrity and permeability of cell membrane. At this point, metals like iron or copper react and favor the apparition of oxygen reactive species with a direct effect on the lipid layer (Abdullah *et al.*, 2012). The obtained results contradict the other determined biochemical parameters. Our extracts presented an inhibitory effect of lipid peroxidation lower than standard gallic acid (1 mg mL^{-1}) in ratios between 29 and 85% (Fig. 1c). The lowest value was determined for the extract obtained from those mushrooms cultivated on a substrate with almost equal quantities of wheat straws and beech shavings. This result is contradictory to that one corresponding to substrate with *V. pannonica* straws and beech shavings (1:1); the average difference between these two limits is about 65%. The ascending order between substrates was $P3 < P2 < P4 < P1$.

Our results are not promising compared with methanolic extracts from marketed *P. ostreatus*. These values are comparable with concentrations of freeze-dried extract of $2\text{-}3 \text{ mg mL}^{-1}$ (Vamanu, 2013a). On the contrary, our results (P1 and/or P4) can be compared with those determined for *Boletus edulis* at concentrations of $0.2\text{-}0.4 \text{ mg mL}^{-1}$ also, they are comparable with ascorbic acid at maximum concentration of 1 mg mL^{-1} (Vamanu and Nita, 2013).

Free radical scavenging activity: The DPPH radical scavenging activity. If we take a mushroom extract, the presence of some secondary compounds like different carbohydrates and/or proteins seem to alter the accuracy of the results expressed by DPPH radical use (Marinova and Batchvarov, 2011). Thus, the results presented in Fig. 1d show a low activity on DPPH radical. Extract P1 had a maximum scavenging activity of 47.83% with approximately 45% lower than standard and the lowest activity was determined in *V. pannonica* straws (substrate P2) inconsistent with the amount of antioxidants determined. These results were influenced by other determinations by straws/shavings ratio and also by the type of straws.

Compared with marketed species extracts, extract P1 had similar results with up to 50% scavenging activity causing an average value of EC_{50} index of 5 mg mL^{-1} . Likewise, similar results were obtained for methanolic extracts from wild edible mushroom, *Sparassis crispa* (Wulf.) Fr., found in North Western Himalayas, India (Joshi and Sagar, 2014).

ABTS radical scavenging activity: The ABTS radical usage to determine radical scavenging activity of a product is a frequently used method. In comparison to DPPH scavenging activity, this method is more specific and the obtained results are considered much closer to the real potential of a hydroalcoholic extract (Vamanu, 2014). The obtained results (Fig. 1d) are much lower compared to first free radical. Substrates P1 and P4 determined similar results between 3 and 3.5%. A minimum ratio of 50% wood residues in substrate determined a maximum activity of 20%, about 75% lower than the standard gallic acid (1 mg mL^{-1}).

Scavenging effect of extract P3 was similar to the ones determined on methanolic and warm water extracts from dried fruiting bodies of paddy straw mushroom (*Volvariella volvacea*), values available for $20 \text{ } \mu\text{g mL}^{-1}$ concentration (Punitha and Rajasekaran, 2014). Also, this result was confirmed for methanolic extracts from *Termitomyces microcarpus* and *T. heimii*, too, for same concentrations. Noteworthy was the close value of BHT used as control sample, being one of the known antioxidants used for this type of determinations (Johnsy and Kaviyarasan, 2014).

Superoxide radical scavenging assay: This activity is presented in Fig. 1d and it can be considered high. Although it is not a very toxic radical, its inhibition is important because it can

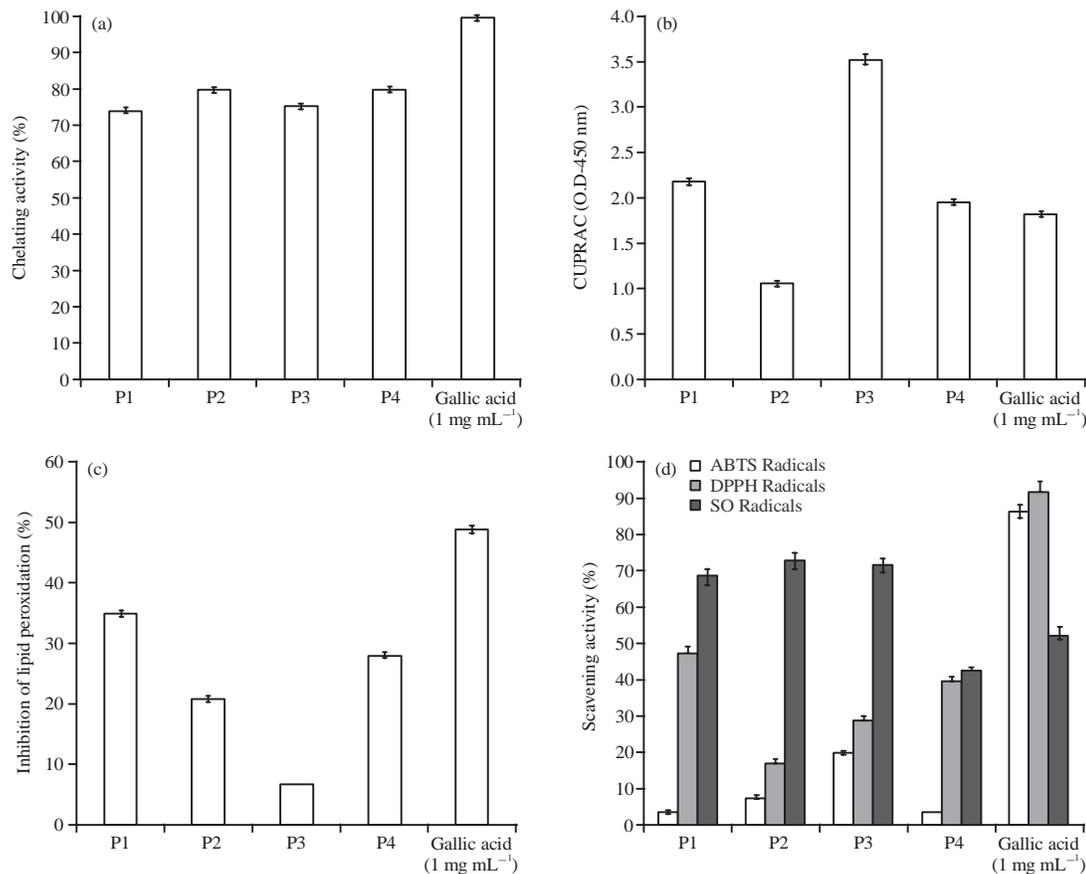


Fig. 1(a-c): (a) Chelating activity of *Pleurotus ostreatus*, (b) Reducing activity of *Pleurotus ostreatus*, (c) Inhibition of lipid peroxidation of *Pleurotus ostreatus* and (d) ABTS, DPPH and SO scavenging activities of *Pleurotus ostreatus* methanolic extracts

lead to other reactive species of oxygen. The most important is hydroxyl radical which has a high reactivity and is a main cause for cell oxidative stress (Menaga *et al.*, 2013). The presence of straws in substrate determined the apparition of biomolecules in tested mushrooms with similar values, an average of 70%; this value is almost 39% higher than the one obtained on extract P4. Also significant is the difference compared to standard (gallic acid), an average of 25% lower.

Results of extracts P1-P3 were similar to that of *T. microcarpus* species at a concentration of 200 $\mu\text{g mL}^{-1}$. For extract P4, superoxide radical scavenging activity was similar to methanolic extracts from *T. heimii* at the same concentration (Johnsy and Kaviyarasan, 2014). Maximum values were similar to hydroalcoholic extracts from medicinal fungus *Xylaria nigripes* at lower concentration of approximately 25 $\mu\text{g mL}^{-1}$ (Ko *et al.*, 2009).

CONCLUSION

Our results showed a high antioxidant capacity of raw methanolic extracts from *P. ostreatus*. These were more specific for hydroxyl radicals than for other free radicals. The antioxidant potential was similar to other marketed species, both of *P. ostreatus* and other edible species. Generally, we can conclude that the presence of a high quantity of lignin components reduces the

presence of biologically active molecules, directly correlated to a decreased biological activity. Higher reducing capacity than the control indicates the presence of important quantities of reductones (ascorbic acid) not directly related to the substrate formulas.

Our results also showed a high biological value of the fruiting bodies of *P. ostreatus* mushroom. This is a strong barrier against dysfunctions caused by an increased presence of free radicals at cell level. Future studies are intended to identify extracts that can be directly used and are toxic free.

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