

IN VITRO ANTIOXIDANT ACTIVITY OF SOME EXTRACTS OBTAINED FROM *AGARICUS BISPORUS* BROWN, *PLEUROTUS OSTREATUS* AND *FOMES FOMENTARIUS*

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Abstract

The polyphenols and flavonoids from two edible mushrooms (*Agaricus bisporus* brown - 3 samples, *Pleurotus ostreatus* - 2 samples) and one un-edible mushroom (*Fomes fomentarius* - one sample) have been extracted with methanol. From each extract the quantity of these compounds has been determined by spectrophotometric methods. The antioxidant properties of each extract have been evaluated by *in vitro* tests: 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay, 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay, iron chelating activity test and the capacity to block 15-lipoxygenase. *Fomes fomentarius* sample contained the highest quantity of polyphenols (8.58 mg/g methanolic extract) and the sample 2 of *Pleurotus ostreatus* contained the highest quantity of flavonoids (2.62 mg/g methanolic extract). Excepting the test for inhibition of 15-lipoxygenase the antioxidant properties depend on the content of polyphenols.

Rezumat

Polifenolii și flavonoidele prezente în două specii de ciuperci comestibile (*Agaricus bisporus* brown - trei probe, *Pleurotus ostreatus* - două probe) și una necomestibilă (*Fomes fomentarius* - o probă) au fost separate prin extracție cu metanol. Determinarea cantitativă s-a efectuat prin metode spectrofotometrice. Acțiunea antioxidantă *in vitro* a fost evaluată prin: testul DPPH 2,2-difenil-1-picril-hidrazil-hidrat, testul ABTS (acid 2,2'-azino-bis-3-etilbenziazolin-6-sulfonic), capacitatea de chelatare a fierului și capacitatea de a bloca 15-lipoxigenaza. Proba de *Fomes fomentarius* a conținut cea mai mare cantitate de polifenoli (8,58 mg/g extract metanolic), iar proba 2 de *Pleurotus ostreatus* a conținut cea mai mare cantitate de flavonoide (2,62 mg/g extract metanolic). Cu excepția testului de inhibiție a 15-lipoxigenazei, capacitatea antioxidantă depinde de conținutul în polifenoli.

Keywords: mushrooms, polyphenols, flavonoids, antioxidant

Introduction

Nowadays mushrooms are considered nutraceuticals or functional foods due to the bioactive nutrients content. According to the International Life Sciences Institute of Europe mushrooms they are considered functional foods since it has been proven that they have both beneficial and nutritional effects on one or more functions of the body, thus improving health, well-being and lowering the risk of illness.

Mushrooms usually contain various amounts of proteins, lipids [17], carbohydrates [11], vitamins, and minerals and due to their low fat content they have a reduced energetic value, thus being recommended in many restricted diets. It is known that mushrooms accumulate substrate related minerals that are either extremely important for human body (Na, K, Ca,

Mg, P) or that are used for their own antioxidant protection (Cu, Zn, Mn, Se) [7].

Bioactive compounds content is dependent upon species, nutritive substrate type, pedo-climatic conditions, age of mycelium, processing and preservation conditions. All these factors lead to a wide variability in the biological potential [7].

Extracts or powders from mushrooms are often used for their effects of reducing cholesterol [25], lowering glucose level, of inhibiting inflammation [10, 12], and also for different approaches of improving antitumor activity [23] or neuro-degenerative diseases. Also, it is known that some mushrooms have antiaging properties [10].

Mushrooms also contain antioxidant compounds [21, 24, 28] such as polyphenols and flavones that are involved in antioxidant defence for humans. Such compounds have the ability to block reactive

oxygen species involved in lipid peroxidation, oxidative stress that leads to DNA, cell membrane proteins and cellular organelles damage. Oxidative stress arises from an imbalance between the production of reactive oxygen species and antioxidant systems in the human body, when the ability to inactivate these compounds is low [22].

In vitro and *in vivo* studies of methanolic extracts obtained from fungi revealed scavenger action against free radicals due to polyphenols [6]. Most of these studies used mushrooms collected from forests, thus the data indicated a high variability of the active components with implications on both *in vitro* and *in vivo* therapeutic actions.

Lee proved that the extracts of *Fomes fomentarius* have protective action on a diabetic rat model. This mushroom is used in traditional medicine in Asia as a diuretic, febrifuge, anti-tumour and anti-inflammatory [13].

Based on these observations our study aimed to evaluate the composition of polyphenols and flavonoids, as well as *in vitro* antioxidant activity on samples of mushrooms from different areas. Six mushroom samples belonging to three species of which two are cultivated and edible (*Pleurotus ostreatus*, *Agaricus bisporus* brown) and one parasite on trees (*Fomes fomentarius*) were analysed. Among the active principles found in mushrooms polyphenols and flavonoids were determined as they confer antioxidant activity to extracts and once entered the living organisms they contribute to their defence against oxidants and free radicals.

In vitro antioxidant potential was evaluated by: free radical scavenger activity assessment (DPPH test, ABTS assay), reducing capacity test and 15-lipoxygenase inhibition capacity assay.

Materials and Methods

All used reagents were of analytical grade. An UV-VIS spectrophotometer Jasco V550 ABL&E has been used for spectrophotometric determinations.

Agaricus bisporus brown samples were from different manufacturers and were selected in different stages of development. Therefore, the Abb 1 sample were large mushrooms, whereas the mushrooms from Abb 2 were small and sample Abb 3 is represented by medium-small mushrooms with intense brown coloured cap.

Pleurotus ostreatus samples were as follows: Po 1 sample - cultivated mushrooms with light-grey cap and Po 2 sample was represented by mushrooms collected from the forest and had a dark-brown cap. These differences in cap colour are caused by micelle composition variations and especially by environmental conditions. This phenomenon was observed also for fungi that grow naturally in

nature, when the soil composition, humidity and exposure to light can affect cap colour and often cause confusion with toxic mushrooms.

Mushroom samples processing: Mushroom samples included in research were: 3 samples of *Agaricus bisporus* brown (Abb 1, Abb 2, Abb 3), 2 samples of *Pleurotus ostreatus* (Po 1, Po3) and 1 sample of *Fomes fomentarius* (Ff). Mushroom species were identified using Moser criteria [18]. 5 g of dried mushrooms were extracted with 100 mL of methanol. The resulting methanol extract was evaporated under pressure and the solutions obtained by dissolving the residue in methanol or dimethylsulphoxide (DMSO) have been used for chemical analysis and for *in vitro* evaluation of the antioxidant activity.

Determination of total polyphenols was performed by Singleton and Rossi method, using Folin-Ciocalteu reagent. For the calibration curve, gallic acid (1-5 mg/mL) was used in similar conditions as the samples. The results were expressed in mg gallic acid equivalent/1 g dry methanol extract [26].

Determination of flavonoids was performed by Hatanao method using NaNO₂ solution and AlCl₃ solution. For the calibration curve, catechine (2.5 to 17.5 mg/mL) was used in similar conditions as the samples. The results were expressed as mg of catechine equivalent/1 g dry methanol extract [8].

Determination of free radicals scavenger activity - DPPH test was performed by Hatanao method. The scavenger activity was calculated according to the following formula:

$$\% \text{ activity} = (A_{\text{DPPH}} - A_p) \cdot 100 / A_{\text{DPPH}}$$

Where: A_{DPPH} - the absorbance of alcoholic DPPH solution, A_p - the absorbance after 10 minutes of DPPH solution treated with methanol extract. For each sample EC₅₀ value (expressed in µg extract/mL) was calculated by plotting DPPH radical scavenger activity variation and the concentration of the extract. Gallic acid was used as positive control [8].

Determination of free radicals scavenger activity - ABTS assay was performed by Re modified method. The scavenger activity was calculated according to the formula:

$$\% \text{ activity} = (A_{\text{ABTS}} - A_p) \cdot 100 / A_{\text{ABTS}}$$

Where: A_{ABTS} - absorbance of alcoholic solution of ABTS, A_p - absorbance of ABTS solution treated with methanol extract, after 6 minutes. For each sample EC₅₀ value was calculated as µg extract/mL. Gallic acid was used as positive control [20].

Determination of the reducing capacity was performed by Oyazu method. EC₅₀ value was calculated (as mg extract/mL) by plotting the absorbance at 700 nm depending on the concentration. Gallic acid was used as positive control [19].

Determination of inhibition of 15-lipoxygenase activity: was performed by Maltreud modified method. The capacity of 15-lipoxygenase inhibition was calculated according to the formula:

$$\% \text{ activity} = (A_{\text{EFI}} - A_{\text{ECI}}) \cdot 100/A_{\text{EFI}}$$

Where: A_{EFI} - the difference between the absorbance of the enzyme solution without inhibitor at second 90 and absorbance at second 30; A_{ECI} - the difference between the absorbance of the enzyme solution treated with inhibitor at second 90 and absorbance at second 30. The value of EC_{50} expressed as mg extract/mL was calculated for each sample. Gallic acid was used as positive control [16]. For each sample three measurements were performed and the average value \pm standard deviations were calculated.

Results and Discussion

Chemical composition assessment

The samples of mushrooms contain relatively small amounts of polyphenols and flavonoids (see Table I) compared to medicinal herbs. However, these substances are not negligible with regard to the

source and the presence of other compounds with biological activity that are found in the composition of mushrooms. Our results indicate that polyphenols found in the samples included in our study are lower than in other mushrooms species compared to what Gursoy [7] found in samples of *Morchella conica* (gallic acid equivalents (EAG) 25.38 $\mu\text{g}/\text{mg}$ extract). Therewith, in 2007 Ferreira obtained much smaller amounts of flavonoids in *Lactarius deliciosus* (EAG 17.25 mg/g extract) [6]. The highest amount of polyphenols was determined in *F. fomentarius* sample. Although it is a non-edible mushroom, it can be used to obtain extracts with protective action but firstly the possible toxic effects need to be evaluated.

The amount of polyphenols varied within the same genus, so *A. bisporus* brown contains a higher amount of polyphenols than *A. arvensis* [2], but tests show a slightly lower antioxidant action than that of sample *A. arvensis* grown in natural conditions. For our study the quantity of polyphenols was higher when the cap of mushrooms was dark coloured and presented small-medium size.

Table I

Polyphenols and flavonoids found in mushroom samples included in the study

Sample	Polyphenols (mg EAG/g ^a)	Flavonoids (mg EC/g ^b)
<i>Agaricus bisporus</i> brown - Abb 1	6.33 \pm 0.10	1.09 \pm 0.02
<i>Agaricus bisporus</i> brown - Abb 2	7.47 \pm 0.06	0.97 \pm 0.01
<i>Agaricus bisporus</i> brown - Abb 3	7.90 \pm 0.09	1.52 \pm 0.01
<i>Pleurotus ostreatus</i> - Po 1	5.47 \pm 0.05	2.45 \pm 0.04
<i>Pleurotus ostreatus</i> - Po 2	6.17 \pm 0.10	2.62 \pm 0.05
<i>Fomes fomentarius</i> - Ff	8.58 \pm 0.08	1.20 \pm 0.01

^a mg EAG% - mg polyphenols expressed as gallic acid equivalents/g dried methanolic extract

^b mg EC% - mg flavonoids expressed as catechine equivalents/g dried methanolic extract

Po 2 sample contains high amounts of polyphenols and flavonoids as compared to Po 1 sample, due the fact that naturally grown mushrooms are able to synthesize large amounts of active compounds as a mean of adapting to various environmental conditions. The same phenomenon was highlighted by Barros [2] who revealed a greater amount of polyphenols in mushrooms harvested from the forest compared to those from controlled cultivation. These variations are the result of environmental influences as polyphenols and flavonoids are often synthesized as protective substances to resist to extreme environmental conditions.

Therefore, mushrooms naturally grown are not a good source for the production of food supplements or drugs because their growth and development

depend on the environmental conditions and the chemical composition range more than in culture.

In order to obtain mushrooms with a high content of polyphenols and flavonoids mushrooms producers should grow them in conditions close to natural ones. For example, *P. ostreatus* grows rapidly if it is cultivated at higher temperatures, but the content of polyphenols is very low. Reduction of ambient temperature by a few degrees causes a slowdown of growth with accumulation of larger amounts of polyphenols [28].

In vitro antioxidant capacity tests

Polyphenols and flavonoids from the tested extracts have the capacity to block the DPPH and ABTS radicals, depending on the concentration (Figure 1, Figure 2), although the scavenger effect is more intense for ABTS test. The lower value of EC_{50} indicates a higher activity.

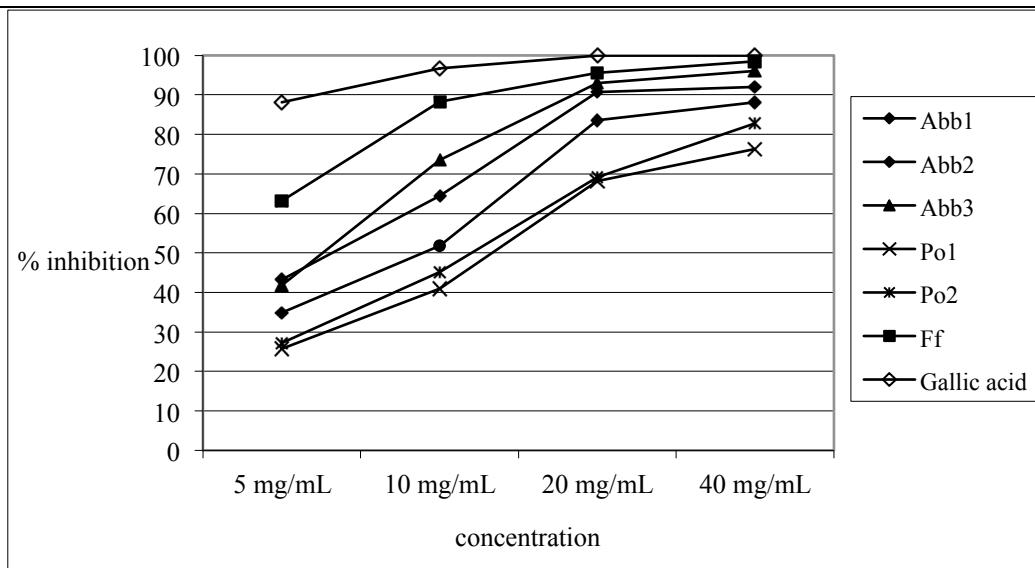


Figure 1.

Scavenger capacity of extracts from mushrooms samples in DPPH assay, depending on the concentration of methanolic solutions (mg/mL)

Our extracts have a lower scavenger capacity than gallic acid, given the EC_{50} values calculated for each case (Table II). Obtaining possible lower EC_{50} values depends on the extent of purification of the extracts, which will ultimately increase the price of the product made with such an extract.

DPPH radical scavenger action of *Agaricus bisporus* samples was close to that determined by other

researchers [4, 12, 14, 27] who have been analysed several species of the *Agaricus* genus. Studies by Barros [2] that analysed other species of *Agaricus*, harvested from the forest, showed a radical scavenger action and reducing capacity very close to our own results.

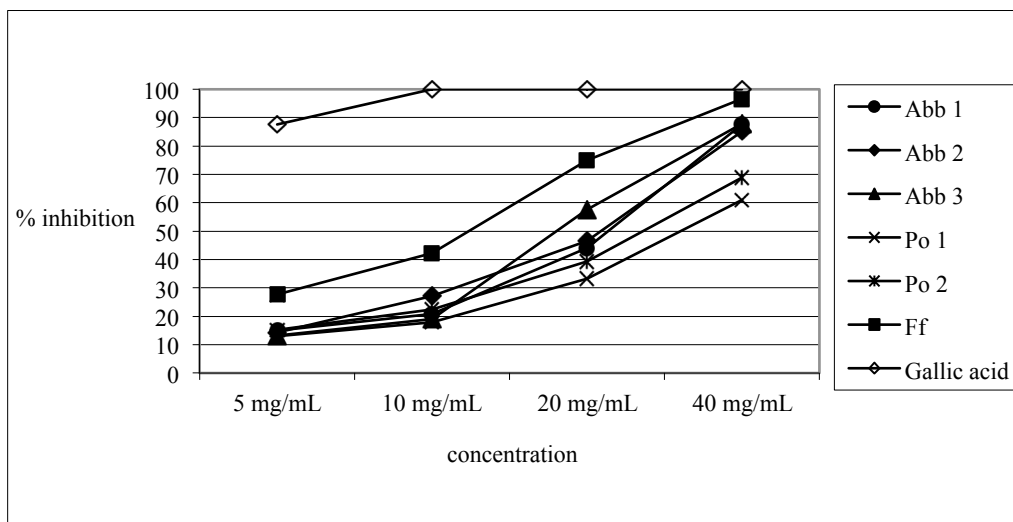


Figure 2.

Scavenger capacity of extracts from mushrooms samples in ABTS assay, depending on the concentration of methanolic solutions (mg/mL)

Unlike the previous test in ABTS assay, EC_{50} value for gallic acid final solution was $0.79 \pm 0.01 \mu\text{g/mL}$, whereas for Ff sample (the most active) was $115.30 \pm 1.33 \mu\text{g/mL}$ (Table II).

Radical scavenging potential depends on the polyphenol content of the samples, rather than the content of flavonoids, thus Ff and Abb 3 samples

with a high content in polyphenols showed the most intense action, while sample Po 1 containing less polyphenols, but more flavonoids, showed a lower inhibitory capacity. Gursoy obtained the same results for several species of *Morchella* that showed very good DPPH scavenger and reducing capacity, but dependent on the content of polyphenols [7].

On the other hand, Chen showed that ergothioneine present in samples of *P. ostreatus* and *A. bisporus* contributes to antioxidant action of the extracts

made from these fungi thereby enhancing polyphenol and flavonoid activity [3].

Table II

The values of EC₅₀ (µg/mL) for each extract and for gallic acid

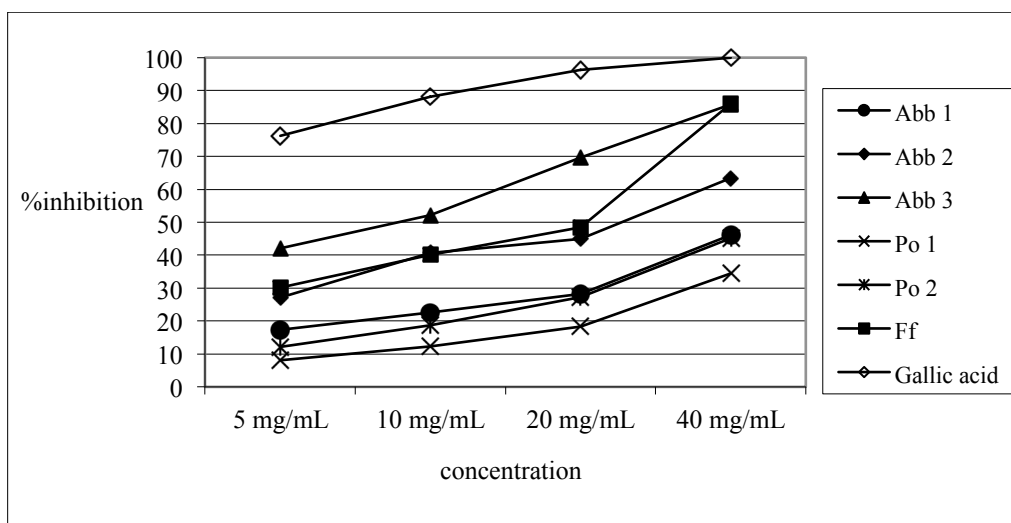
Sample	EC ₅₀ (µg/mL)			
	DPPH	ABTS	Reducing capacity	15-lipoxygenase inhibition
Abb 1	929.98 ± 20.32	226.60 ± 2.97	2.78 ± 0.01	699.88 ± 4.66
Abb 2	623.94 ± 18.25	211.49 ± 2.69	2.91 ± 0.02	414.89 ± 24.95
Abb 3	598.05 ± 7.70	174.25 ± 1.78	1.44 ± 0.01	154.47 ± 13.87
Po 1	1257.71 ± 17.56	276.19 ± 2.61	3.28 ± 0.02	977.82 ± 27.42
Po 2	1461.06 ± 26.50	286.49 ± 2.31	3.30 ± 0.01	893.91 ± 21.22
Ff	199.93 ± 4.54	115.30 ± 1.33	1.61 ± 0.06	376.71 ± 24.22
Gallic acid	1.47 ± 0.04	0.79 ± 0.01	0.05 ± 0.001	36.22 ± 2.64

In terms of ability to reduce ferric ion, all extracts were more active compared to the scavengers effect (Table II). This observation is of extreme importance since iron acts as an inducer of oxidative processes in biological environments. Sarikurkcu and Lee reported similar results during his research on mushroom extracts [15, 24].

15-lipoxygenase is a hemin enzyme involved in generating peroxy radicals, which in turn will initiate the oxidation thus affecting the structure and stability of lipids, proteins and nucleic acids, and finally determining the occurrence of

pathological phenomena of many living organisms. *In vivo* 15-lipoxygenase is involved in the oxidation of low density lipoproteins (LDL) lipid fraction components involved in the production of atherosclerosis.

In order to assess the ability to inhibit 15-lipoxygenase, the Maltreud modified method was used [16], by altering the time of contact between the enzyme and inhibitor before the addition of the substrate, thus increasing the action of the inhibitor (Figure 3).

**Figure 3.**

The capacity of extracts from mushrooms samples to block 15-lipoxygenase, depending on the concentration of methanolic solutions (mg/mL)

The compounds with reducing properties present in the extracts confer the capacity to inhibit 15-lipoxygenase. Compared to other tests, the effects of gallic acid on the 15-lipoxygenase was reduced, EC₅₀ value being 36.22 ± 2.64 µg/mL (Table II).

Originally, scavenger and reducing power assays indicated a direct correlation between polyphenol content and the intensity of antioxidant potential, whereas the results of 15-lipoxygenase inhibition test do not indicate this type of relationship, which

emphasizes that the interaction between the enzyme and the inhibitor is determined by complex effects of substances present in the analysed extracts.

Analysis of data on antioxidant activity for *A. bisporus* and *P. ostreatus* is lower for cultivated mushrooms than that of the same edible species grown in the wild [5, 7, 24].

On the basis of the existing results, polyphenols and flavonoids found in mushroom extracts are lower than in the case of plant extracts, but *in vitro*

antioxidant tests showed their ability to block radicals or oxidizing substances activity.

Conclusions

Our results do not overlap with those shown in other studies since the samples are not singular, but a mixture of substances, with a variable composition. Although, sometimes the species were similar, important factors such as the growth environment, the substrate composition, the age and time elapsed from harvest to analysis, induced variations between the results obtained in different studies. The effects might overlap only if a full analysis of the powder and mushroom extracts is carried out; thereby obtaining standardized extracts that will allow an easy comparison of their biological action.

The data suggest that similar medicinal plants or mushrooms could be a source of bioactive compounds with antioxidant properties, especially *A. bisporus* brown and *P. ostreatus* that are edible mushrooms. *F. fomentarius*, although is a parasitic fungus on trees, has a high content in antioxidants, therefore beneficial extracts such as those made from *Ganoderma sp.* could be obtained by using a proper procedure to isolate the toxic components. Further studies are needed to reveal possible toxicity and standardization requirements of mushroom extracts should be elaborated.

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