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A comparative study on the methods of antioxidant activity in wild edible mushrooms from the Batak Mountain, Bulgaria

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A comparative study on the methods of antioxidant activity in methanolic extracts from seven species of wild edible mushrooms (*Boletus pinophilus*, *Cantharellus aurora*, *Cantharellus tubaeformis*, *Cantharellus cibarius*, *Craterellus cornucopioides*, *Morchella esculenta* and *Tricholoma equestre*) from the Batak Mountain (Bulgaria) was performed. The total antioxidant capacity was estimated by ABTS•+ (2,2′azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)), DPPH• (1,1-diphenyl-2-picrylhydrazyl radical), FRAP (ferric reducing/antioxidant power) and CUPRAC (cupric ion reducing antioxidant capacity) methods. The total phenolic content (TPC) of the mushrooms was evaluated by Folin-Ciocalteau's phenol reagent and gallic acid was used as standard. Trolox equivalent antioxidant capacity (TEAC) values of the mushrooms ranged from 4.10 to 69.74 mmol TE/ g dw, from 1.55 to 20.23 mmol TE/ g dw, from 7.72 to 35.31 mmol TE/ g dw, and from 7.97 to 64.25 mmol TE/ g dw for ABTS•+, DPPH•, FRAP and CUPRAC, respectively. TPC ranged from 0.68 to 11.92 mg of gallic acid equivalents (GAE)/g dw. In conclusion, the mushroom with the highest TEAC values was *Boletus pinophilus* and with the lowest was *Cantharellus cibarius*.

Keywords*: mushrooms, antioxidant activity, TEAC, TPC*

INTRODUCTION

Reactive oxygen species are involved in oxidizing the biomolecules in human body and that can cause damage of the cells and tissues. They are also the major factor of lipid peroxidation which leads not only to deterioration of the food quality (flavor, colour, nutritional value), but also can produce some toxic compounds (aldehydes, ketones, etc.) [1, 2, 3]. Therefore, it is of importance to be used antioxidants that can cease the processes of oxidation. Recently, new products which possess antioxidative properties are becoming of a great interest in many studies and similar constituents are observed in the mushrooms.

Mushrooms have been used both in food industry and medicine for many years. Not only do they have an unique taste and flavor but also are a rich source of different nutrients such as lipids, proteins, carbohydrates, minerals, as well as various antioxidants (phenolic acids, flavonoids, tocopherols, ascorbic acid and carotenoids) [4]. Besides that, mushrooms are known to accumulate some secondary metabolites such as phenolic compounds, mainly phenolic acids and flavonoids [5, 6, 7]. They also can produce some novel constituents with helpful biological properties. Mushrooms are established to treat various diseases and to display antitumor, antibacterial and antiviral effects [2, 8]. Because of that, they can be considered as a functional food, which contain myriad of antioxidants that can help to reduce the production of oxygen-derived free radicals in the human body [3]. More and more studies are focused on antioxidant capacity of mushrooms and it is established that they show strong antioxidant effect that can be compared to some well-known natural antioxidants such as ascorbic acid and tocopherols [9]. Therefore, it is really important to be examined the antioxidant activity of some globally widespread mushroom species.

There is a high diversity of mushrooms in Bulgaria and the most common ones are *Boletus pinophilus*, *Cantharellus aurora*, *Cantharellus tubaeformis*, *Cantharellus cibarius*, *Craterellus cornucopioides*, *Morchella esculenta* and *Tricholoma equestre*. However, there is no information about the antioxidant activity of these Bulgarian species of wild edible mushrooms, which can contribute to the whole evaluation of their functional properties. The aim of the present research is to be performed a comparative study on the methods of antioxidant activity of methanol extracts of seven species wild edible mushrooms from The Batak Mountain, Bulgaria using ABTS•+ (2,2′azinobis-(3-ethylbenzthiazoline-6-sulfonic

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acid)), DPPH• (1,1-diphenyl-2-picrylhydrazyl radical), FRAP (ferric reducing/antioxidant power) and CUPRAC (cupric ion reducing antioxidant capacity) method as well as determination of their total phenolic contents by Folin-Ciocalteau method.

EXPERIMENTAL

Samples

Mushroom samples were collected in the period of 2014 - 2017 year from the Batak Mountain, Bulgaria. Three samples from every mushroom species were collected and used for the analysis. They were air-dried in an oven at 60ºC and stored at room temperature before analysis.

Chemicals and reagents

Chromatographic grade methanol was used for HPLC analyses (VWR, Austria). Water for HPLC was prepared with Millipore purifier (Millipore, USA). Ammonium acetate (NH_4Ac), cupper(II) chloride, gallic acid, glacial acetic acid, sodium acetate trihydrate, ferric chloride hexahydrate, hydrochloric acid, 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox,) and reagents 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), neocuproine (2,9-dimethyl-1,10-phenanthroline), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), Folin–Ciocalteu reagent, phosphate buffered saline, pH 7.4, (PBS), were purchased from Sigma-Aldrich.

Preparation of extracts

The milled mushroom material was weighed with 0.0001 g precision and 2 g were used for analysis. The initial milled material was extracted via ultrasonic for 30 minutes with 40 mL methanol at room temperature. All samples were filtered under vacuum. The extraction was repeated three times. The supernatants were combined and evaporated under vacuum with temperature of the water bath 40°C. The volume of all samples was adjusted to 60 mL and passed through a membrane filter with pore size of 0.45 μm prior for analysis.

Total phenolic contents (TPC)

The total phenolic content in crude extracts was determined with colorimetric method using Folin-Ciocalteau's reagent [10] with slight modifications. Calibration curve was achieved using as standard ethanolic solution of gallic acid at concentrations between 25 and 1000 μg/mL. Briefly, 100 μL of extract or gallic acid standard was mixed with 2.4 mL distilled water, 500 μL of 0.2 M Folin-Ciocalteu's reagent and 2 mL of 7.5 % sodium carbonate (Na_2CO_3) solution. The tested samples were incubated for 2 h in dark at room temperature. The absorbance of the samples was measured at 765 nm with a spectrophotometer (Camspec M508, England), using a blank sample. The total phenolic contents was expressed as mg gallic acid equivalent per grams of dry weight (mg GAE/g dw) based on the calibration curve.

Trolox Equivalent Antioxidant Capacity (TEAC) ABTS method

The Trolox Equivalent Antioxidant Capacity (TEAC) was determined by using the colorimetric method reported from Re et al. (1999) [11]. For this assay, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) cation radical (ABTS•+) solution was prepared by dissolving 7 mM of ABTS in 2.45 mM $K_2S_2O_8$. This mixture was shaken for 12 – 16 h at ambient temperature in the dark until obtaining a stable oxidative state. For the study of the extracts, the ABTS•+ stock solution was diluted with PBS until absorbance became 0.70 ± 0.02 at 734 nm. Sample analysis was performed as follows: 2 mL of ABTS solution and 20μL of sample or standard were mixed. Absorbance of sample was measured at 734 nm with a spectrophotometer Camspec M508, England after samples incubation at 25°C for 5 min. The calibration curve was plotted by using 6-hydroxy-2,5,7,8- -tetramethylchromane-2-carboxylic acid (Trolox) as a standard. The results were expressed as mmol Trolox equivalents per g of dry weight (mmol TE/g dw).

Cupric ion reducing antioxidant capacity (CUPRAC) method

The mushroom extracts were investigated by cupric ion reducing antioxidant capacity (CUPRAC) method described by Apak et al. (2006) [12]. In test tubes were mixed 1 mL $CuCl_2$ solution (10 mM), 1 mL neocuproine alcoholic solution (7.5 mM) and 1M NH_4Ac buffer solution (pH = 7), 0.2 mL tested extract or Trolox and 0.9 mL water (final volume, 4.1 mL). Absorbance against a blank sample was measured at 450 nm with a spectrophotometer Camspec M508, England after 30 min in dark at room temperature. Calibration curve was achieved using trolox as a standard ethanolic solution at concentration ranges between 0.045 and 1.5 mM. Trolox equivalent antioxidant capacity was plotted as mmol Trolox equivalents per g of dry weight (mmol TE/g dw).

Ferric reducing antioxidant power (FRAP) method

All samples were investigated by ferric reducing antioxidant power (FRAP) method described by Benzie and Strain (1996) [13]. The FRAP reagent was freshly prepared before analyzes by mixing 0.3

M acetate buffer (pH 3.6), 10 mM 2,4,6- tripyridyl-*s*-triazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in distilled water in a ratio 10:1:1. In test tubes were mixed 0.15 mL tested extract or standard Trolox and 2.85 mL FRAP reagent. Absorbance against a blank sample was measured at 593 nm with a spectrophotometer Camspec M508, England after 15 min in dark at room temperature. Calibration curve was achieved using Trolox as a standard ethanolic solution at concentration ranges between 0.045 and 1.5 mM. Trolox equivalent antioxidant capacity was plotted as mmol Trolox equivalents per g of dry weight (mmol TE/g dw).

DPPH method

Antioxidant activity was measured according to Brand-Williams et al. (1995) [14] procedure. In the test tubes were mixed 150 µL of extract or Trolox and 2.85 mL of 0.12 mM DPPH (2,2-diphenyl- 1-picrylhydrazyl) reagent, which was prepared with 4.8 mg DPPH dissolved in 100 mL CH_3OH). The mixtures were shaken and then incubated for 30 min at room temperature. The absorbance was recorded at 517 nm with a spectrophotometer Camspec M508, England. To quantify the antioxidant activity a standard Trolox curve is used with concentration from 0.045 to 1.5 mmol Trolox. The results were expressed as mmol TE/g dw.

Statistical analysis

R 3.5.2 program for Windows was used for statistical data processing. All the analyses were made in triplicate. Statistical differences between samples were tested using ANOVA. Dates were expressed as mean ± standard deviation (SD). The level of significance was set at $p<0.05$. Multiple pair-wise comparisons of means among species were obtained by Post-Hoc analysis, i.e. it was used TukeyHSD test with $p<0.05$ in conjunction with an ANOVA.

RESULTS AND DISCUSSION

Mushroom extracts were obtained by ultrasound-assisted solvent extraction with methanol and were subjected to estimation for their antioxidant activity. Four different tests were used for the analysis and the antioxidant capacity of methanol extracts of the examined wild edible mushrooms is shown in Figure 1.

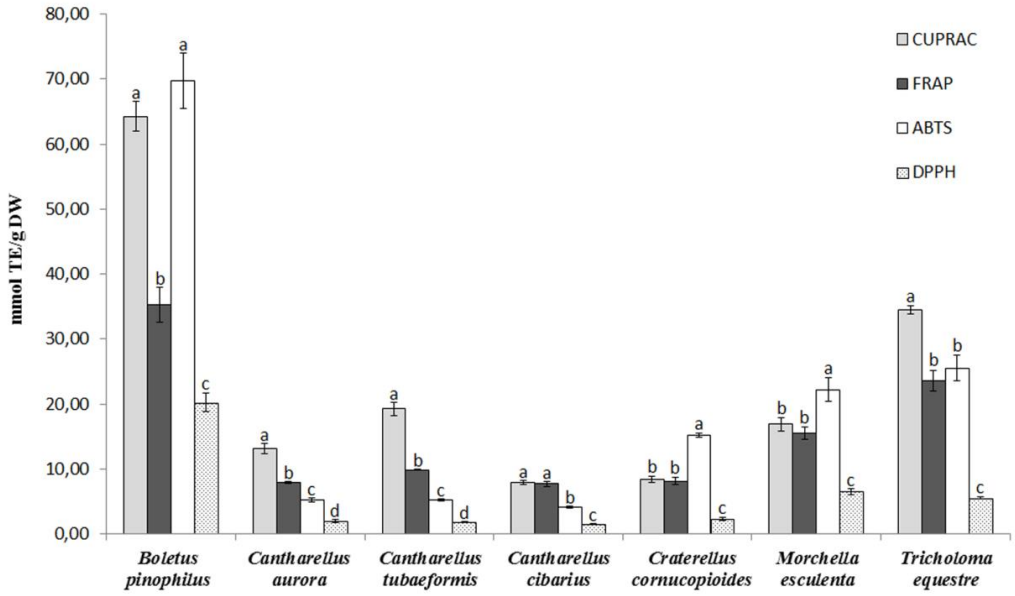

Fig. 1 Antioxidant capacity of methanol extracts of wild edible mushrooms.
Letters represent the results of Tukey's post hoc comparisons of mean values among the species ($p < 0.05$).

The scavenging activity of mushroom extracts towards DPPH, ABTS, FRAP and CUPRAC was expressed in mmol TE/g dw. Regarding the scavenging capacity of the used methods the highest activity belongs to the methanol extract from *Boletus pinophilus* – 20.23 mmol TE/g dw for DPPH, 69.74 mmol TE/g dw for ABTS, 35.31 mmol TE/g dw for FRAP and 64.25 mmol TE/g dw for CUPRAC.

The DPPH radical scavenging activity is used for estimation of the antioxidant capacity of extracts against oxidation which is caused by free radicals [9]. The DPPH values for the other mushroom extracts ranged from 1.55 (*Cantharellus cibarius*) to 6.51 mmol TE/g dw (*Morchella esculenta*) and follow the order: *Boletus pinophilus* > *Morchella esculenta* > *Tricholoma equestre* > *Craterellus cornucopioides* > *Cantharellus aurora*

> *Cantharellus tubaeformis* > *Cantharellus cibarius*.

The antioxidant capacity by ABTS radical cation of the other examined methanol extracts of mushrooms ranged from 4.10 (*Cantharellus cibarius*) to 25.52 mmol TE/g dw (*Tricholoma equestre*). The ABTS values are in the following order: *Boletus pinophilus* > *Tricholoma equestre* > *Morchella esculenta* > *Craterellus cornucopioides* > *Cantharellus tubaeformis* > *Cantharellus aurora* > *Cantharellus cibarius*.

The FRAP values for the mushrooms apart from *Boletus pinophilus* ranged from 7.72 (*Cantharellus cibarius*) to 23.54 mmol TE/g dw (*Tricholoma equestre*). The values of the examined mushrooms are as following: *Boletus pinophilus* > *Tricholoma equestre* > *Morchella esculenta* > *Cantharellus tubaeformis* > *Craterellus cornucopioides* > *Cantharellus aurora* > *Cantharellus cibarius*.

The CUPRAC values for the mushrooms apart from *Boletus pinophilus* ranged from 7.97 (*Cantharellus cibarius*) to 34.52 mmol TE/g dw (*Tricholoma equestre*). The antioxidant activity of the mushrooms tested by CUPRAC is as following: *Boletus pinophilus* > *Tricholoma equestre* > *Cantharellus tubaeformis* > *Morchella esculenta* > *Cantharellus aurora* > *Craterellus cornucopioides* > *Cantharellus cibarius.*

As can be seen in the Figure 1 the highest value for antioxidant capacity of the mushroom from species *Boletus pinophilus*, *Craterellus cornucopioides* and *Morchella esculenta* were observed when using ABTS method, while in the other species the highest values were obtained by using CUPRAC. Therefore, those methods for assaying antioxidant capacity of the methanol extract from mushrooms are more suitable.

The results for assessing *Boletus pinophilus* by FRAP were lower than those by Keleş et al. (2011) [15] who observed that the FRAP value of genus *Boletus* ranged from 47528.57 (*Boletus pseudosulphureus*) to 62771.43 μmol/g (*Boletus erythropus* var. *erythropus*). *Cantharellus cibarius* was observed to have the worst antioxidant capacity for all used methods. This was confirmed by previous studies where this species possessed the highest EC_{50} value [4].

Total phenolic content of the examined seven species of mushrooms is shown in Figure 2.

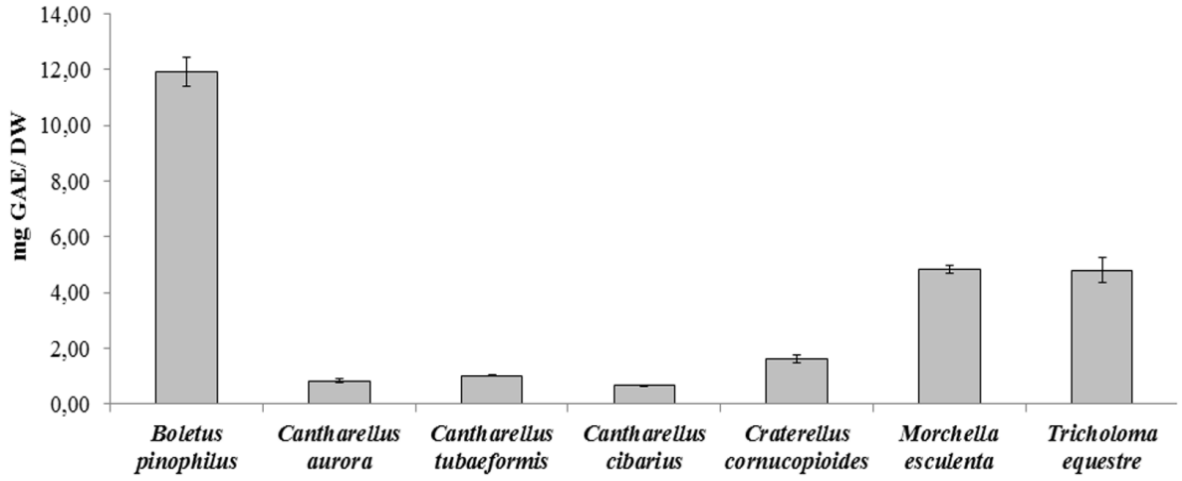

Fig. 2 Total phenolic contents of extracts from wild edible mushrooms

TPC of the seven mushroom species ranged from 0.68 to 11.92 mg GAE/g dw. The highest TPC was found in the methanol extract from *Boletus pinophilus*, followed by *Morchella esculenta* (4.83 mg GAE/g dw), *Tricholoma equestre* (4.80 mg GAE/g dw), *Craterellus cornucopioides* (1.62 mg GAE/g dw), *Cantharellus tubaeformis* (1.03 mg GAE/g dw), *Cantharellus aurora* (0.83 mg GAE/g dw) and *Cantharellus cibarius* was observed to have the lowest value.

The results are in agreement with those reported by Barros et al. (2008) [4] who established the phenolic contents of five edible mushrooms and observed that *Boletus edulis* had the highest phenolic contents (5.03 mg/g) and *Cantharellus cibarius* had the lowest (0.88 mg/g). On the other hand, the results for the seven Bulgarian edible mushrooms are lower than some culinary-medicinal mushrooms studied by Abdullah et al. (2012) [16] who established that the total phenolic contents ranged from 6.19 (*Auricularia auricular-judae*) to 63.51 mg GAE/g extract (*Ganoderma lucidum*).

Some studies revealed that the antioxidant activity is related to the content of polyphenols [17, 18]. The obtained results from the present study confirmed this statement – the highest total phenolic content was observed in the specie *Boletus pinophilus* and the highest values for antioxidant

capacity were noticed in the same mushroom. The correlation between total phenolic contents and antioxidant capacity of the examined mushrooms is shown in Table 1.

Table 1 Correlation between total phenolics and antioxidant capacity of the examined mushrooms.

	mg GAE/g dw	CUPRAC	FRAP	ABTS	DPPH
mg GAE/g dw	1	0.9381	0.9671	0.9889	0.9846
CUPRAC		1	0.9729	0.9350	0.9349
FRAP			1	0.9495	0.9319
ABTS				1	0.9861
DPPH					1

Table 2 Antioxidant Potency Composite Index (APC index)*

Mushroom species	CUPRAC	FRAP	ABTS	DPPH	TPC	Average
Boletus pinophilus	100	100	100	100	100	100
Cantharellus aurora	20.34	22.46	7.44	8.52	7.00	13.15
Cantharellus tubaeformis	29.96	27.87	7.49	3.29	8.61	15.44
Cantharellus cibarius	12.41	21.86	5.89	2.04	5.70	9.58
Craterellus cornucopioides	13.02	22.88	21.81	7.14	13.62	15.69
Morchella esculenta	26.27	43.87	31.83	61.81	40.58	40.87
Tricholoma equestre	53.77	66.65	36.59	46.22	40.27	48.70

* APC index = (sample score/best score)×100

From the Table 1 can be concluded that the correlation between the values for total phenolic contents and the antioxidant activity of the mushrooms is very positive as well as between the different antioxidant capacity assays. It can be suggested that total phenolic content is closely related to the antioxidant activity of the examined mushroom species, determined by the four used methods. All correlation coefficients shown in Table 1 are high, therefore all four methods are suitable for determination of TEAC in the mushrooms regardless of their different mechanism of action.

The different methods for assaying the antioxidant activity are not easy to compare because of the different mechanisms of action [19]. The antioxidant capacity of the investigated mushrooms showed different rank orders, for that reason antioxidant potency composite index (APC index) was calculated. APC indices of the examined mushrooms are presented in Table 2.

Regarding the average values for APC index the antioxidant capacity of the examined mushrooms can be expressed in the following order: *Boletus pinophilus* > *Morchella esculenta* > *Tricholoma equestre* > *Craterellus cornucopioides* > *Cantharellus tubaeformis* > *Cantharellus aurora* > *Cantharellus cibarius.* Therefore, *Boletus pinophilus* was observed to have the highest antioxidant capacity and *Cantharellus cibarius* had the lowest. Despite, can be concluded that the examined seven mushrooms from the Batak

mountain are a good source of bioactives and have rather positive antioxidant activity.

CONCLUSION

A comparative study on the methods of antioxidant capacity of seven species of Bulgarian wild edible mushrooms from the Batak Mountain was performed. Different determination methods were used and *Boletus pinophilus* was proved to have the highest mean values for all of the used methods. The latter specie also had the highest value of total phenolic contents.

Total phenolic content in the examined wild edible mushrooms was very positive correlated with CUPRAC, FRAP, ABTS and DPPH values. Antioxidant potency composite index was calculated and based on the overall antioxidant index can be concluded that the extract from *Boletus pinophilus* had the highest and *Cantharellus cibarius* had the lowest antioxidant activity.

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