

EXPERIMENTAL PAPER

Antioxidant activities and polyphenolics content of *Flammulina velutipes* mushroom extracts

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S u m m a r y

A study was conducted in order to determine the content of phenolic compounds and antioxidative activity of extracts from *Flammulina velutipes*. Water and ethanolic, methanolic, acetone (70% v/v) extracts were prepared from lyophilized fruiting bodies of mushrooms. Different extraction techniques were used: ultrasonication and stirring by a rotary shaker at ambient temperature and at 50°C, and at boiling point for each solvent. Next, total phenolics by Folin-Ciocalteu method, antioxidant capacity (EC) by ferric reducing antioxidant power assay (FRAP) and scavenging ability on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals were analysed in these extracts. It was observed that the extraction method and the kind of solvent influenced the antioxidant activity and concentration of total phenolics in the extracts. The highest concentration of phenolic compounds (7.58 ± 0.17 mg GAE/g extract) were observed for water extract prepared with use of ultrasonification method (at 50°C for 1h). Results showed that water extracts possessed high equivalent capacity (EC) for all applied extraction methods. The highest EC value (9.2 ± 0.18 mM Fe^{2+} /g extract) was observed in water extract prepared by stirring at ambient temperature for 1 h. This extract was characterized by high level of total phenolics. The acetone extracts, prepared by ultrasonic extraction at 50°C for 1 h, at 10 mg/ml showed the highest scavenging activity ($57.53 \pm 1.18\%$), although, the value was lower than that of ascorbic acid at 10 mg/ml ($60.50 \pm 1.32\%$).

Key words: *Flammulina velutipes*, mushroom extracts, total phenols content, DPPH, FRAP

INTRODUCTION

Nowadays, mushrooms as important natural resources of immunomodulating, anticancer agents and bioactive compounds. They have been cultured at a large scale in Asia. *Flammulina velutipes*, one of the most popular edible mushrooms, has attracted considerable attention of biochemistry and pharmacology due to its biological activities [1]. A lot of bioactive compounds such as polysaccharides, protein-glucan complex, sterols and lectins of medicinal and pharmaceutical properties (immunomodulating, antitumor, antioxidant, thrombolytic, fibrinolytic, antibacterial, antifungal, antiviral, haploidic, mitogenic) were isolated from *F. velutipes* [2]. Several medicinal properties of mycelial and fruiting body samples of *F. velutipes* have been established in *in-vitro* experiment such as immunomodulatory effect via induction of cytokines and antifungal, antibacterial, antiviral, antioxidant, antiprotozoal, mitogenic activities [3]. Polysaccharides and a low-weight protein-bound polysaccharide with high antitumor activity were also isolated from this mushroom [4]. Flammulin, a basic simple protein from *F. velutipes* markedly inhibits tumor cells [5]. An epidemiological study in Nagano Prefecture (Japan) showed that cancer death rate among farmers producing *F. velutipes* was remarkably lower than that of other people in the Prefecture and other places in Japan [4]. Anti-inflammatory, antitumor and immunomodulating drugs as well as dietary supplements were produced from this mushrooms [6]. Bioactive substances produced by mushrooms show many medicinal effects [7, 8] including antimicrobial [9-11] and antioxidative properties [12-15]. Antioxidants are substances that delay, prevent or reverse oxidative damage to a target molecule and can be synthesized *in vivo* or taken from the diet. They are known to prevent many chronic diseases including cancer, diabetes and neurodegenerative disorders [16]. In the last decades, there has been a great interest in use of natural antioxidants as food supplements and scientific interest in fungal biomolecules as antioxidants has grown recently [13,17-22], although, the chemical nature of such compounds is still unclear. Phenolic compounds are the major naturally occurring antioxidant components found in medicinal mushrooms [17, 19, 23] and the majority of those identified in commercial mushrooms are phenolic acids [13, 24]. Other, non-phenolic compounds, including terpenoids [25] and polysaccharides [26] have also been designated as mushroom antioxidants. Kim et al. [24] studied phenolic compounds of *F. velutipes*. The polyphenols found in this mushrooms were: gallic acid, pyrogallol, homogentisic acid, 5-sulfosalicylic acid, protocatechuic acid, chlorogenic acid, caffeic acid, ferulic acid and quercetin. Most previous studies on fungal antioxidants have been oriented towards commercial mushrooms used as food [13], but recent scientific literature shows even higher antioxidative potential of wild-growing edible mushrooms [11, 22, 27].

Some studies analyzing the total phenols and antioxidant activity of wild and commercial mushrooms have been published [28-35]. Our objective was to study and compare total phenolics and antioxidant properties of mushroom extracts prepared from *Flammulina velutipes*. We intend to find the most appropriate solvent and extraction method for extracting phenols and obtaining extracts with high level of antioxidant activities.

MATERIALS AND METHODS

Mushroom material

Fruit bodies of *Flammulina velutipes* (Curtis: Fries) Singer were collected from alder stump in the Janowskie Forests (East Poland) in November 2011. Mushrooms were authenticated by professor Janusz Kalbarczyk, Department of Fruits, Vegetables and Mushrooms Technology, University of Life Sciences in Lublin.

Sample preparation

The fruit bodies of *F. velutipes* were cleaned, cut into small pieces and next freeze-dried. Then, all dried fruit bodies of mushrooms were then ground and stored in air-tight plastic bag in a desiccator at room temperature for further analysis.

Extraction

Four different solvents: water, ethanol, methanol and acetone (70% v/v) were used for extraction. For the extraction optimization, sample of mushroom powders (3 g) was extracted with 100 ml of solvent using five different methods:

- A: stirring with solvent at ambient temperature at 160 rpm for 1 h
- B: stirring with solvent at 50°C at 160 rpm for 1 h
- C: extraction using the sonic bath at ambient temperature for 1 h
- D: extraction using the sonic bath at 50°C for 1 h
- E: extraction with boiling solvent for 1 h

After extraction samples were centrifuged at 5000 x g for 30 min and filtered through Whatman No. 1 filter paper. Supernatants were evaporated at 40°C to dryness and redissolved in the corresponding solvent at the concentration of 50 mg/ml. Water extract were thickened and next freeze-dried. The obtained extracts were kept in the dark at 4°C until total phenolic content and antioxidant activity were analysed.

Determination of total phenolics content

The concentration of phenolic compounds in extracts of mushrooms, expressed as gallic acid equivalents (GAE), were measured according to the method of Singleton and Rosi (1965) [36] with modification. The extract solution (1 ml) was mixed with 1 ml of Folin and Ciocalteu's phenol reagent, next with 1 ml of saturated Na_2CO_3 solution and with 7 ml of distilled water. The reaction was kept in the dark for 60 min and a absorbance was read at 725 nm. The calibration curve was constructed with different concentration of gallic acid as a standard. Total phenolic content of the sample was expressed as gallic acid equivalent (GAE) to 1 g per extract.

Scavenging ability on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals

The scavenging ability on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals was determined according to the method of Shimada et al. [37]. Each extract (2-10 mg/ml) was mixed with 1 ml of methanolic solution containing DPPH radicals (0.2 mM). The mixture was shaken vigorously and left to stand for 25 min in the dark. Next, the absorbance was measured at 517 nm against blank sample. The scavenging ability was calculated as follows: Scavenging ability (%) = $[(\Delta A_{517} \text{ of blank} - \Delta A_{517} \text{ of sample}) / \Delta A_{517} \text{ of blank}] \times 100$. Ascorbic acid was used as a control.

Ferric-reducing antioxidant power (FRAP) assay

Antioxidant capacity was determined by ferric-reducing antioxidant assay (FRAP) according to Benzie and Strain [38] and modified by Alvarez-Parrilla et al. [39] with some modifications. FRAP reagent was prepared daily by mixing 0.3 mM acetate buffer (pH 3.6) with 10 mM 2,4,6-tripyridyl-s triazine (TPTZ) solution in 40 mM HCl and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (10:1:1 ratio). Assay solutions were prepared by mixing 1 ml of FRAP reagent with 4 ml of mixture of water and: extract, standard or methanol as a blank (3:1 ratio). Methanolic solutions of Fe^{2+} of different concentrations were prepared from $\text{Fe}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$ stock solution in order to obtain the calibration curve. The mixed solution was incubated at 37°C in the dark for 30 min. The ferrous tripyridyltriazine complex was measured by reading the absorbance at 593 nm. The reducing power was expressed as an equivalent capacity (EC) which was the ability to reduce ferric ions expressed as mM Fe^{2+} /g of extract.

Statistical analysis

All analyses were performed in triplicate. The data were recorded as means \pm SD (standard deviation).

RESULT AND DISCUSSION

Previous studies reported that extracts prepared from mushrooms had free radical scavenging and antioxidative activities *in vitro* and *in vivo* [40–42]. Among the active compounds, phenolic compounds have been known as potent antioxidants which evidently exist in the fruiting body of certain mushroom species [13, 43]. The concentration of total phenolics in the extracts from *F. velutipes*, expressed as mg GAE/g extract, is shown in figure 1. Results indicated that concentration of polyphenols was dependent on the solvent and method of extraction. The range of amount of phenolic compounds was the highest (4.56 ± 0.21 – 7.58 ± 0.16 mg GAE/g of extract) in water extracts from *F. velutipes*, next for ethanolic extracts (4.73 ± 0.13 – 6.31 ± 0.34 mg GAE/g of extract), and for acetone extracts (2.96 ± 0.20 – 5.47 ± 0.21 mg GAE/1 g of extract). Methanol extracts (1.62 ± 0.14 – 2.89 ± 0.14 mg GAE/g of extract) had the smallest amount of total phenolic contents. Water extracts of *F. velutipes* prepared by different methods with exception for method E (extraction with boiling solvent for 1 h) have higher amount of total phenols compared with extracts prepared with use of other solvents. The highest concentration of phenolic compounds (7.58 ± 0.16 mg GAE/1 g extract) were observed for water extract using ultrasonification method (at 50°C for 1 h). Similar results obtained Lee et al. [44]. In their study, ethanolic extract from mushroom *Pleurotus citrinopileatus* contained lower amount of total phenols than water extracts prepared at room temperature. This results were in agreement with Yim et al. [45] where water extracts from mushrooms *Pleurotus ostreatus*, *Hygrocybe conica*, *Schizophyllum commune* and *Lentinus ciliatus* had the highest amount of total phenolics.

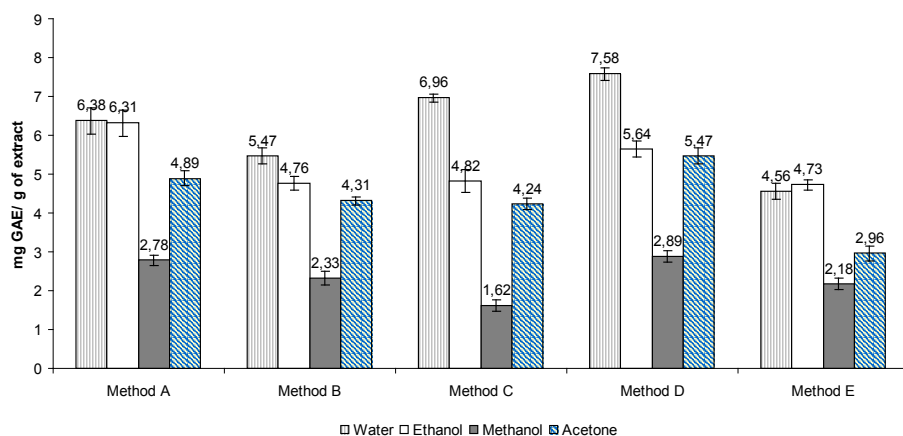


Figure 1.

Concentration of total phenolics (mg GAE/g of extract) of mushrooms extracts obtained with use of different extraction methods

GAE – gallic acid equivalents; the concentration of total phenols in the extracts expressed as mg GAE/g of extract

In this study, antioxidant activity was measured by the FRAP method, which measures the capacity of the antioxidant to reduce a Fe^{3+} -TPTZ complex to a Fe^{2+} -TPTZ. In this way, a higher Fe^{3+} -TPTZ reduction means a higher antioxidant activity. The original FRAP methodology proposed by Benzie and Strain [38] established 4 min interval before their determination of the FRAP value. However, Alvarez-Parrilla et al. [39] reported that the reaction of reduction of Fe^{3+} to Fe^{2+} follows a slow kinetic mechanism. For this reasons, FRAP values were determined at 30 min, as suggested in the publications [39, 46]. The reducing power estimated by FRAP method was expressed as an equivalent capacity (EC) which was the ability to reduce ferric ions expressed as mM Fe^{2+} /g extract. Data are shown in figure 2. Results showed that water extracts possessed high equivalent capacity (EC) for all applied extraction methods. The highest EC value (9.2 ± 0.18 mM Fe^{2+} /g extract) was observed in water extract prepared by stirring at ambient temperature for 1 h. This extracts were characterized by high level of total phenolics. Except water extracts, acetone extracts presented higher FRAP values compared with ethanolic and methanolic extracts. Only in case of stirring at ambient temperature data showed that ethanolic extract possessed higher equivalent capacity (4.07 ± 0.20 mM Fe^{2+} /g extract) than acetone and methanolic extracts (3.42 ± 0.23 mM Fe^{2+} /g extract and 2.62 ± 0.17 mM Fe^{2+} /g extract respectively).

In table 1, the scavenging activity of the DPPH radical due to its reduction by different extracts is presented. The mushroom extracts were shown to scavenge the stable DPPH radical after 30 min to different extents over a concentration range of 2–10 mg/ml. Water extracts prepared by stirring at ambient temperature at concentrations ranges 2-10 mg/ml showed higher scavenging activity toward DPPH radical (from $25.67 \pm 0.64\%$ to $56.28 \pm 0.49\%$) than extracts from other solvents. In general, extracts from *F. velutipes* obtained at 50°C by stirring and ultrasonication showed higher scavenging activity than extracts prepared at ambient temperature. Only water extract prepared by stirring at ambient temperature (method A) showed highest scavenging activity. It has been shown previously that it contains high amounts of polysaccharide and soluble protein compounds [47] which could skew results from other assays. The acetone extracts, prepared by ultrasonic extraction the at 50°C for 1 h, at 10 mg/ml showed the highest scavenging activity ($57.53 \pm 1.18\%$) but the value was lower than that of ascorbic acid at 10 mg/ml ($60.50 \pm 1.32\%$; tab. 1). All extracts obtained by extraction using the sonic bath at 50°C for 1 h (method D) and extraction with boiling solvent for 1 h (method E), at 10 mg/ml showed above 50% of reduction DPPH radicals.

Table 1.

Effect of different solvent extracts on DPPH radical scavenging activity of the *Flammulina velutipes* fruiting bodies

Extraction method	Extract concentration mg/ml	% Inhibition of DPPH radical \pm SD			
		Solvent			
		Water	Ethanol 70%	Methanol 70%	Acetone 70%
Method A	2	25.67 \pm 0.64	2.68 \pm 0.79	7.53 \pm 0.60	9.05 \pm 0.91
	4	32.12 \pm 0.65	11.00 \pm 0.76	19.00 \pm 0.65	22.34 \pm 0.81
	8	54.76 \pm 0.91	16.49 \pm 0.94	32.34 \pm 0.60	41.08 \pm 0.33
	10	56.28 \pm 0.49	24.63 \pm 1.04	40.78 \pm 0.81	44.76 \pm 0.72
Method B	2	28.43 \pm 0.67	24.05 \pm 0.90	23.14 \pm 0.62	23.17 \pm 0.68
	4	30.87 \pm 0.48	27.07 \pm 0.58	29.01 \pm 0.59	29.22 \pm 0.90
	8	34.77 \pm 0.22	36.39 \pm 0.64	42.55 \pm 0.50	42.12 \pm 0.43
	10	39.66 \pm 0.59	43.21 \pm 0.79	46.66 \pm 0.60	49.19 \pm 0.60
Method C	2	9.05 \pm 0.42	12.96 \pm 0.56	11.74 \pm 0.49	10.51 \pm 0.53
	4	33.99 \pm 0.49	21.64 \pm 0.44	20.62 \pm 0.46	18.66 \pm 0.31
	8	51.79 \pm 0.25	32.15 \pm 0.44	34.27 \pm 0.88	32.40 \pm 0.7
	10	53.03 \pm 0.	33.99 \pm 0.	42.34 \pm 0.74	37.90 \pm 0.44
Method D	2	35.84 \pm 0.45	29.85 \pm 0.69	29.14 \pm 0.36	31.24 \pm 0.79
	4	45.58 \pm 0.26	38.13 \pm 0.58	38.28 \pm 0.84	38.01 \pm 0.51
	8	51.42 \pm 0.47	48.88 \pm 0.59	50.11 \pm 0.49	50.67 \pm 0.90
	10	55.05 \pm 0.37	55.62 \pm 0.41	55.02 \pm 0.68	57.53 \pm 1.18
Method E	2	40.22 \pm 0.59	29.07 \pm 0.63	25.72 \pm 0.67	29.87 \pm 0.80
	4	44.33 \pm 0.48	34.32 \pm 0.66	33.90 \pm 0.57	33.41 \pm 0.57
	8	53.31 \pm 0.82	46.19 \pm 0.54	47.91 \pm 1.20	47.91 \pm 0.51
	10	55.37 \pm 0.34	50.72 \pm 0.46	53.61 \pm 0.70	56.09 \pm 0.63
Ascorbic acid as a control	2		37.47 \pm 0.81		
	4		48.07 \pm 1.01		
	8		55.67 \pm 1.15		
	10		60.50 \pm 1.32		

Method of extraction is one of the main factors conditioning the obtainment of high quality natural antioxidants. Methods that are simple, rapid and environmentally friendly should be preferred. The ideal method shorten the extraction time, decreases the solvent consumption and increases the extracted yield. In this study ultrasonication, stirring and boiling at solvent were applied to mushroom powders. Data in figure 1 show that extraction by ultrasonication yielded higher amount of total phenolics in extracts than stirring at the same temperature or boiling in

solvent. Water extracts from *F. velutipes* obtained by ultrasonication were characterized by higher total phenolics content than received by stirring at the same temperature (6.96 ± 0.10 mg GAE/g and 6.38 ± 0.34 mg GAE/g, respectively, at ambient temperature (7.58 ± 0.17 mg GAE/g and 5.47 ± 0.20 mg GAE/g respectively at 50°C). For acetone and methanolic extracts prepared at 50°C , ultrasonication was a better methods than stirring. In this case, the amount of total phenols reached the level of 5.47 ± 0.20 mg GAE/g and 2.89 ± 0.14 mg GAE/g for acetone and methanolic extracts, respectively. These results were in agreement with other authors [48] who extracted antioxidants from *Rosmarinus officinalis*. It was considered that with ultrasonication, the high frequency disrupted the structure of the plant cell wall, resulting in the increased contact between the solvent and the plant material. Similar results were obtained by other authors [49]. In that study, the effect of extraction methods on amount of natural antioxidants in extracts was estimated. Authors reported that ultrasonication was a much better method than stirring.

CONCLUSIONS

The results obtained in the present work denote that wild growing edible mushrooms may constitute a good source of healthy compounds or phenols intake in diet. Due to these characteristics, edible mushrooms could be considered as a complement in the diet for the benefits they present. Our results suggest that a kind of solvent and the extraction methods influence the total phenolics content and antioxidant activity of extracts. It was demonstrated that extracts from *F. velutipes* could exhibit antioxidant activity through both mechanisms of free radical scavenging and reducing power which contributed to total phenolic compounds existing in the extracts. Data show that water was the best solvent to obtain the most effective natural antioxidant from *F. velutipes* and ultrasonication was a better method as compared with stirring at the same temperature. Furthermore, the knowledge gained from this study is expected to be beneficial for producing the extraction of natural antioxidants from fruiting bodies of *F. velutipes*. Based on this results obtained, extracts from mushroom *F. velutipes* were effective in antioxidant properties.

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CZYNNIKI WPŁYWAJĄCE NA WŁAŚCIWOŚCI PRZECIWOXTLENIAJĄCE I OGÓLNĄ ZAWARTOŚĆ ZWIĄZKÓW POLIFENOLOWYCH W EKSTRAKTACH Z GRZYBA *FLAMMULINA VELUTIPES*

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Streszczenie

Praca miała na celu określenie ogólnej zawartości związków polifenolowych i właściwości przeciwutleniających ekstraktów z grzyba *Flammulina velutipes*. Z liofilizowanych owocników *F. velutipes* przygotowano ekstrakty wodne, etanolowe (70% v/v), metanolowe (70% v/v) i acetonowe (70% v/v). Zastosowano różne techniki ekstrakcji: wytrząsanie i ekstrakcję przy użyciu łaźni ultradźwiękowej w temperaturze pokojowej i w 50°C oraz ekstrakcję we wrzącym rozpuszczalniku. W otrzymanych ekstraktach zbadano ogólną zawartość związków polifenolowych metodą Folin-Ciocalteu i ich właściwości przeciwutleniające (EC – *equivalent capacity*) metodą FRAP (*ferric reducing antioxidant power assay*) oraz zdolność do neutralizacji wolnych rodników z użyciem DPPH (1,1-diphenyl-2-picrylhydrazyl). Zaobserwowano, że takie czynniki jak warunki ekstrakcji czy rodzaj użytego rozpuszczalnika mają wpływ na właściwości przeciwutleniające i ogólną zawartość związków polifenolowych w otrzymanych ekstraktach. Najwyższą ilość związków polifenolowych uzyskano w wodnych ekstraktach otrzymanych z użyciem łaźni ultradźwiękowej w 50°C ($7,58 \pm 0,17$ mg GAE/g ekstraktu). Wyniki pokazują, że wodne ekstrakty, otrzymane wszystkimi zastosowanymi metodami ekstrakcji, posiadają wysokie właściwości przeciwutleniające. Najwyższą wartość EC ($9,2 \pm 0,18$ mM Fe²⁺/g ekstraktu) zaobserwowano dla wodnych ekstraktów przygotowanych w pokojowej temperaturze z użyciem wytrząsarki. Te ekstrakty charakteryzowały się również wysoką zawartością związków polifenolowych. Ekstrakty acetonowe otrzymane poprzez zastosowanie łaźni ultradźwiękowej w 50°C przy stężeniu 10 mg/ml wykazywały najwyższą zdolność do redukcji wolnego rodnika DPPH ($57,53 \pm 1,18\%$) ale niższą niż kwas askorbinowy przy takim samym stężeniu ($60,50 \pm 1,32\%$).

Słowa kluczowe: *Flammulina velutipes*, ekstrakty grzybowe, polifenole, DPPH, FRAP