Antioxidant and free-radical scavenging activity of submerged mycelium extracts from aphyllophoroid mushrooms

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Abstract. Antioxidant properties were studied from ten submerged cultivated mycelium Basidiomycetes strains of aphyllophoroid mushrooms using the ß-carotene bleaching method and ten strains using DPPH free-radical scavenging assay. Three different solvents: ethanol, water (culture liquid), and ethyl acetate were used for extraction. The yield of extracts from biomasses depended on the mushroom species and solvent used. Water extracts from *Stereum hirsutum* 524 and *Ganoderma lucidum* 545 showed high (74 % and 81 %) antioxidant activities (AA) at 2 mg/ml using the ß-carotene bleaching method. When the ethanol extracts were tested, the highest AA were found in *Ganoderma lucidum* 545, *Stereum hirsutum* 524, and *Trametes versicolor* 1013 extracts (77 %, 68 %, and 72 %, respectively) at a concentration of 2 mg/ml. Water and ethanol extracts from *Ganoderma lucidum* showed the highest scavenging ability (70 % and 56 %, respectively) on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals at a minimal sample concentration of 0.5 mg/ml. The scavenging capacity of extracts varied from 1 to 85 % depending on the mushroom species, solvent used, and concentration. *Inonotus tamaricis* and *Trametes gibbosa,* exerted high scavenging abilities at low-effective concentrations.

Key words: antioxidant activity, aphyllophoroid mushrooms, free-radical scavenging, submerged mycelium, water and ethanol extracts

Introduction

Reactive oxygen species (superoxide, hydrogen peroxide, and hydroxyl radicals), are known mutagens produced by radiation air pollutions and are also formed during normal cellular metabolism. When present in high concentrations they become toxic. In order to protect the cells against excessive levels of free radicals, mammalian cells possess intracellular defence systems including the enzymes superoxide dismutase (SOD), catalase or glutathione peroxidases, and antioxidant compounds such as ascorbic acid, tocopherols, and glutathione (Niki *et al.* 1994; Mau *et al.* 2002). Therefore, an antioxidant supplement in the human diet is important to prevent or to reduce oxidative damage (Yang *et al.* 2002). Exogenous addition of compounds such as vitamins (A, E, and β-carotene), minerals (selenium, zinc), or proteins (transferrin,

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ceruloplasmin, and albumin) can provide additional protection (Ostrovidov *et al.* 2000). These natural antioxidants or other compounds that can neutralize free radicals may be very important in the prevention of vascular diseases, some forms of cancers and oxidative stress responsible for DNA, and protein and membrane damage (Nakayama *et al.* 1993; Halliwell 1997). The Higher Basidiomycetes mushrooms, as natural sources of many fungal active metabolites, are potential objects for biotechnological and mycopharmacological research (Badalyan 2001). Many of them are also of interest commercially as myconutriceutical and mycocosmeceutical products (Wasser *et al.* 2000, 2001; Wasser 2002).

Synthetic antioxidants have been used in the stabilization of foods. The most commonly used synthetic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ) that are applied in fat and oily foods to prevent oxidative deterioration (Loliger 1991). Originally, BHA appeared to have tumor-initiating as well as tumor-promoting action. However, recently it has been established that tumour formation appears to involve only tumor promotion caused by BHA and BHT (Botterweck *et al.* 2000). For this reason, to prevent pathological conditions, it is important to include a certain amount of antioxidants in the human diet. Mushrooms have long been appreciated for their flavour and texture. Now they are recognized as a nutritious food as well as an important source of biologically active compounds of medicinal value (Breene 1990). A number of studies have focused on the search for effective sources of antioxidants from edible mushroom fruiting bodies. However, mushroom cultivation takes several months to complete fruiting body development in solid-state fermentation. Therefore, an alternative and promising approach is a search for the new and safe natural antioxidants from mushroom submerged cultures since this approach permits controlled production of these compounds in a compact space and shorter time with higher yield, as well as with easier recovery and purification. Surprisingly, very scarce information is available on fungi capability to produce antioxidants in submerged cultures (Song *et al.* 2002; Badalyan 2003; Mau *et al.* 2005).

Therefore, in this study the antioxidant and free-radical scavenging activity of eleven aphyllophoroid mushrooms has been investigated in their submerged cultivation.

Materials and Methods

Organisms and cultivation conditions

Nine aphyllophoroid species isolated from various ecological niches were tested in this study: *Cerrena maxima* 1004, *Daedalea quercina* 943, *Ganoderma lucidum* 545, *Hericium erinaceus* 819, *Inonotus tamaricis* 905, *Phellinus robustus* 531, *Stereum hirsutum* 524, *Trametes gibbosa* 514, and *T. versicolor* 1013 (Table 1).

All strains are preserved in the culture collection (HAI) of the Laboratory of Biotechnology and Biodiversity of Fungi, Institute of Evolution, University of Haifa, Israel (Wasser *et al.* 2002)

The inocula were prepared by growing fungi on a rotary shaker at 150 rpm in 250 ml flasks containing 100 ml of medium (g/l): 15 glucose, 3 peptone, 0.8 KH_2PO_4, 0.2 K_2HPO_4, 0.5 $MgSO_4{\cdot}7H_2O$, and 5 yeast extract. The medium was adjusted to pH 5.8-6.0 before sterilization. After 5-7 days of cultivation, mycelial pellets were harvested, homogenized with a Waring laboratory blender and the homogenates were used to inoculate 1 litre of the same medium containing 20 g glucose in 2-l Erlenmeyer flasks. Aphyllophoroid mushrooms submerged cultivation was carried out at 27 °C on a rotary shaker at 150 rpm. After 8-11 days of fungi submerged cultivation, mycelial biomasses were harvested with filtration and dried at 50 °C to a constant weight. The dried mycelia were milled to a powder form and freeze-dried along with the culture liquids.

Table 1. Investigated species of aphyllophoroid mushrooms

Family	Species	Origin	Habitat	HAI number of srain
Polyporaceae	*Cerrena maxima*	Cuba; Piuar del Rio	unknown	1004
	Daedalea quercina	Slovenia	*Fagus*	943
	Trametes gibbosa	Ukraine, Kiev	unknown	514
	Trametes versicolor	Russia, Samara	*Betula pendula*	1013
Ganodermataceae	*Ganoderma lucidum*	Israel, Massada	*Quercus* sp.	545
Hymenochaetaceae	*Inonotus tamaricis*	Israel, Haifa	unknown	906
	Phellinus robustus	Ukraine, Kiev	*Quercus* sp.	531
Hericiaceae	*Hericium erinaceus*	Norway, Oslo	unknown	819
Stereaceae	*Stereum hirsutum*	Ukraine, Kiev	*Quercus* sp.	524

Extraction

Culture liquid (instead of water) and ethanol were used to extract the antioxidant compounds from mushroom mycelia. We supposed that the secretion and accumulation of antioxidants take place in submerged cultivation of tested fungi. Therefore, to evaluate correctly an overall antioxidant potential of mushrooms the extraction of water-soluble antioxidant compounds from mycelia was accomplished with culture liquids of corresponding strains. At the first stage, each mushroom mycelia was extracted 3 h on a water bath at 80 °C with a corresponding culture liquid (1g/10ml). After extraction, insoluble compounds were separated by centrifugation at 6,000 x g for 15 min and filtrated through the Wathman filter paper N 4. Filtrates were evaporated. The residues after centrifugation were extracted during 3 h on a rotary shaker at 150 rpm at 27 °C with ethanol (80 %). After extraction the solutions were centrifuged, filtrated, and then the organic solvent was evaporated.

Antioxidant activity assay

The antioxidant activity of mushroom extracts was determined according to the ß-carotene bleaching method described by Velioglu *et al.* (1998). A reagent mixture, containing 1 ml of β-carotene (Sigma, USA) solution (0.2 mg/ml in chloroform), 0.02 ml of linoleic acid (Sigma) and 0.2 ml of Tween 80 (Sigma), was evaporated to dryness under a nitrogen stream and 50 ml of oxygenated distilled water was added to the mixture. To determine antioxidant activity, 4.8 ml of reagent mixture and 0.2 ml of mushroom crude extracts with different concentrations (2-8 mg/ml) were added. Pure methanol or water (0.2 ml) was used as a control, whereas the blank contained all mentioned chemicals except β-carotene. All these mixtures were then incubated at 50 °C for 2 h to form liposome solutions. The absorbance of an aliquot (1 ml) of these solutions at 470 nm was monitored by a spectrophotometer at 20 min time intervals. Butylated Hydroxianisole (BHA) (Sigma) (2 mg/ml in methanol) was used as the standard. The bleaching rate (R) of β-carotene was used as the standard and calculated according to Equation (1)

$$R = \ln (a/b)/t \qquad \text{Equation (1)}$$

where: ln - natural log, a - absorbance at time 0, b - absorbance at time t, and t – incubation interval 20, 40, 60, 80, 100, or 120 min.

The antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control, using Equation (2).

$$AA = [(R_{control} - R_{sample})/R_{control}] \times 100 \qquad \text{Equation (2)}$$

Scavenging activity on 1, 1-diphenyl-2-picrylhydrazyl radicals

The scavenging activity of ethanol and water extracts from mushrooms was measured on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals (Chu *et al.* 2000). An aliquot of 0.5 ml of 0.1 mM DPPH radical (Sigma) in methanol was added to a test tube with 1 ml of mushroom ethanol or water extract of different concentrations (0.5 to 9 mg/ml). Methanol or water was used instead of the mushroom sample as a control. The reaction mixture was vortex-mixed at room temperature and the absorbance was determined immediately after mixing by measuring at 520 nm with a spectrophotometer. Inhibition of free radicals by DPPH was calculated as follows:

$$I (\%) = (A_{blank} - A_{sample}/ A_{blank}) \times 100 \qquad \text{Equation (3)}$$

where I is inhibition (%), A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound.

The extract concentration providing 50 % inhibition (EC_{50}) was calculated from the graph of scavenging activity of radical percentage against extract concentration. Butylated hydroxyanisole (BHA) in concentration of 1 mg/ml methanol was used as the standard. Each value is expressed as mean±standard deviation (n=3).

Results and Discussion

Extraction yield

Nutrition medium selected for the submerged cultivation of aphyllophoroid fungi during the screening program ensured growth of all selected mushrooms. However, the yield of mycelial biomass after 8-11 days of fungi cultivation in identical culture conditions varied from 4.4 to 13.6 g/l (Table 2). The highest biomass accumulation was reached by *Phellinus robustus* 13.6 (15.1 g/l). Biomass yield was two times lower in cultivation of *Cerrena maxima* 1004 in the same medium.

The extraction of soluble compounds from dried and milled submerged mushroom mycelia was performed with water (culture liquid), and ethanol. Significant differences in yield among the extracts received from different mushroom species were revealed. Moreover, the yield of extracts received from mushroom biomasses significantly depended on the solvent used. Culture liquid appeared to be the most appropriate solvent for the extraction. It could solubilize up to 52.3 % of the material from submerged mycelium of *Ganoderma lucidum* 545 (Table 2). High levels of water extracts were also received with *Cerrena maxima* 1004 and *Inonotus tamaricis* 906. Subsequent extraction of mushroom biomass with ethanol increased the yield of extract by 3.6-20.9 %. Extraction with ethanol was most effective with *Stereum hirsutum* 524 biomasses but ethanol appeared to be a very weak solubilizer with *Hericium erinaceus* 819, *Cerrena maxima* 1004, *Daedalea quercina* 943 mycelia.

Table 2. Yield of extracts (%) received from dried submerged cultivated mushroom mycelia

Species	Biomass (g)	Water (CL) (%)	Ethanol (%)
Cerrena maxima 1004	5.0	32.4	8.4
Daedalea quercina 943	10.4	18.2	9.1
Ganoderma lucidum 545	6.5	52.3	12.0
Hericium erinaceus 819	7.0	34.2	3.6
Inonotus tamaricis 906	9.1	39.6	16.3
Phellinus robustus 531	13.6	23.9	12.1
Stereum hirsutum 524	7.4	29.0	20.9
Trametes gibbosa 514	7.2	31.1	13.2
Trametes versicolor 1013	5.5	26.5	10.2

Antioxidant activity

Data presented in Table 3 show the antioxidant activity of water (culture liquid) extracts received from submerged mycelia of 10 aphyllophoroid strains using the ß-carotene bleaching method. The antioxidant capability of these extracts significantly depended on mushroom species. Very high AA (more than 70%) was revealed when *Ganoderma lucidum* 545, *Inonotus tamaricis* 906, and *Stereum hirsutum* 524 water extracts in concentration of 2 mg/ml were used. Slightly lower AA was observed in water extracts from *Trametes dibbosa* 514, and *Trametes versicolor* 1013 mycelial biomasses. The inhibition values of all these extracts practically did not change with an increase of their concentration in the reagent mixture. Meanwhile, the water extracts of *Trametes gibbosa* 514, and *Trametes versicolor* 1013 at the same concentration showed very low AA. However, the AA of water extracts from them and several other mushrooms increased with extract concentration from 2 mg/ml to 8 mg/ml (Table 3).

When the ethanol extracts from the same strains biomasses were tested for the AA the highest inhibition values were received with *Ganoderma lucidum* 545 ethanol extracts followed by *Stereum hirsutum* 68 and *Inonotus tamaricis* 906, 77%, 68%, and 65%, respectively, at an extract concentration of 2 mg/ml (Table 3). In contrast to these fungi, no antioxidant activity was exhibited *Phellinus robustus* 531 extract at the same concentration, it showed only 17% of inhibition. The AA of ethanol extracts from mushroom biomasses highly depended not only on mushroom species, but it varied in wide ranges with a variation of the extract concentration in the reagent mixture too. When the concentration of ethanol extract increased from 2 mg/ml to 4-8 mg/ml, the AA of extracts from *Phellinus robustus* 531 and *Trametes gibbosa* 514 increased from 17 to 71% and from 36 to 79%, respectively (Table 3). The comparison of the antioxidant potential of extracts received from mushroom biomasses with two different solvents show that the water extracts of *Phellinus robustus* 531 had higher activity than their ethanol extracts. Analogically, the water extracts of *Lentinus edodes* and *Volvariella volvacea* fruiting bodies had significantly higher antioxidant activities than their methanol extracts (Cheung *et al.* 2003). The fact that the mushroom water extracts possessed higher antioxidant activity than the ethanol ones was explained by their significantly higher total phenolic contents. In our experiments the ethanol extracts received from the mycelial biomasses of *Trametes gibbosa* 514, and *Trametes versicolor* 1013 showed higher antioxidant activity compared with the water extracts.

Table 3. Antioxidant activity (%) of water and ethanol extracts from dried submerged mushroom mycelia

Species	Extract concentration (mg/ml)					
	Water extract			Ethanol extract		
	2.0	4.0	8.0	2.0	4.0	8.0
Cerrena maxima 1004	52	69	77	55	64	84
Daedalea quercina 943	68	72	71	61	63	67
Ganoderma lucidum 545	81	81	83	77	92	91
Hericium erinaceus 819	63	70	91	59	74	89
Inonotus tamaricis 906	74	82	89	65	78	91
Phellinus robustus 531	39	63	68	17	45	71
Stereum hirsutum 524	74	81	86	68	78	83
Trametes gibbosa 514	16	43	65	36	65	79
Trametes versicolor 1013	29	63	69	73	79	91
BHA	97.8	-	-	-	-	-

Free-radical scavenging activity

Free-radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. DPPH, a stable free radical with a characteristic absorption at 520 nm, was used to study the radical scavenging effects of extracts. When water extracts from mushroom mycelia were evaluated, the highest activity at a minimal sample concentration of 0.5 mg/ml was shown with extracts from *Ganoderma lucidum* 545 (69 %) and *Daedalea quercina* 943 (49 %) (Table 4). Under the same culture conditions, grown mycelial biomasses of *Phellinus robustus* 531 showed very weak scavenging ability toward DPPH, only 11 %. The data obtained show that, for the majority of screened mushrooms, the sample concentration of 1.5 mg/ml is sufficient to reveal maximal scavenging activity. This concentration appeared to be much lower than that in experiments of Ferreira *et al.* (2007) with wild edible mushrooms and quite comparable to those shown for mycelium of *Ganoderma tsugae* (Mau *et al.* 2005).

The data presented in Table 4 show that the scavenging effect of ethanolic extracts obtained from mushroom submerged mycelia ranged from 1 % (*Stereum hirsutum* 524) to 56 % (*Ganoderma lucidum* 545) at a sample concentration of 0.5 mg/ml. In accordance with data of other authors (Mau *et al.* 2002; Cheung *et al.* 2003; Ferreira *et al.* 2007), the scavenging effects of ethanolic extracts obtained from mushroom submerged mycelia increased when concentration increase. The scavenging ability of extract from *Ganoderma lucidum* 545 and *Hericium erinaceus* 819 increased by 21 and 27 %, respectively, when the sample concentration increased to 1.5 mg/ml. Such a sample concentration was sufficient to reveal maximal scavenging ability of the majority of the ethanolic extracts tested. The scavenging effect of ethanol extracts from *Trametes gibbosa* 514, and *Trametes versicolor* 1013 increased until a concentration of 3 mg/ml and afterwards decreased with further elevation of sample concentrations. This finding differs from the data of several authors (Mau *et al.* 2004; Ferreira *et al.* 2007), which showed no decrease in scavenging effect of methanolic extract in concentrations up to 20-50 mg/ml.

The EC_{50} values, representing the extract concentrations at which the β-carotene oxidation was 50 %, are given in Table 5. However, most of the tested extracts (water and ethanol) showed very high antioxidant activity at the lowest concentration used and, therefore, these values could not be precisely defined. For instance, the EC_{50}s of the water extracts of *Phellinus robustus* as well as the ethanol extract of *Trametes versicolor* appeared to be lower than the lowest concentration used (2 mg/ml). EC_{50}s were possible to be calculated only for few ethanol and water extracts.

Table 4. Scavenging ability (% of inhibition) of hot water and ethanol extracts from the submerged mushroom mycelia

Species	**Extract concentration** (mg/ml)							
	Water (culture liquide) extract				Ethanol extract			
	0.5	1.5	3.0	9.0	0.5	1.5	3.0	9.0
Daedalea quercina 943	49±2.3	62±2.8	65±2.6	39±1.8	55±2.5	66±1.9	59±1.8	32±1.9
Ganoderma lucidum 545	69±1.6	70±1.8	60±1.5	32±2.5	56±2.2	77±1.9	68±2.4	49±2.2
Hericium erinaceus 819	41±2.7	46±1.5	41±1.5	21±2.2	30±4.3	57±3.7	54±2.4	47±2.2
Inonotus tamaricis 906	46±1.9	50±1.6	43±1.8	33±2.9	54±1.4	56±2.6	63±1.9	67±1.0
Phellinus robustus 531	23±1.4	41±3.0	60±3.2	58±3.7	12±2.5	36±2.2	53±1.5	58±1.7
Stereum hirsutum 524	11±3.3	23±3.6	45±5.7	61±3.0	1±0.8	21±2.3	32±3.3	68±1.6
Trametes gibbosa 514	32±1.8	63±4.4	62±5.1	54±4.8	34±1.9	53±2.9	55±4.4	34±3.6
*Trametes versicolor*1013	28±3.1	53±5.1	61±6.1	53±3.7	17±2.2	28±1.9	50±3.4	41±3.3
BHA (1 mg/ml)	95±2.6	-	-	-	-	-	-	-

The values of effective concentrations for DPPH scavenging effects showed evidence that the EC_{50} of both water and ethanol extracts from several mushrooms (*Daedalea quercina* 943, *Inonotus tamaricis* 906, *Trametes gibbosa* 514) appeared to be close to 1 mg/ml. Hence, the scavenging abilities of these mushrooms are remarkable since EC_{50} values established for these fungi extracts are lower than those from mushroom mycelia in other studies (Mau *et al.* 2004). These results suggest that the tested fungi differ with chemical nature of bioactive compounds, their spectrum and/or ratio in submerged mycelium.

Table 5. EC50 values of water and ethanol extracts in DPPH scavenging assays

Species	**EC50 value** (mg extract/ml)			
	β-carotene bleaching method		DPPH scavenging assay	
	Water extract	Ethanol extract	Water extract	Ethanol extract
Cerrena maxima 1004	<2*	<2*	-	-
Daedalea quercina 943	<2*	<2*	0.7±0.1	5.0±0.3
Ganoderma lucidum 545	<2*	<2*	2.4±0.3	2.2±0.3
Hericium erinaceus 819	<2*	<2*	2.1±0.3	1.2±0.1
Inonotus tamaricis 906	<2*	<2*	0.9±0.1	0.5±0.1
Phellinus robustus 531	<2*	4.6	2.0±0.2	2.4±0.3
Stereum hirsutum 524	<2*	<2*	3.3±0.9	6.0±0.4
Trametes gibbosa 514	5.0	2.2	1.1±0.1	1.3±0.2
Trametes versicolor 1013	3.0	<2*	>9	6.8±0.9

*EC_{50} less than the lowest concentration used (2 mg/ml)

This study shows that the submerged mycelium of most investigated species possess high antioxidant and free-radical scavenging potentials and may serve as good source of safe natural antioxidants. Further studies are needed to determine the physiological mechanisms regulating antioxidant accumulation, to isolate active component(s), and to establish the pharmacological efficacy of promising mushroom extracts.

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