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Evaluation of Antioxidant Activity of *Armillaria tabescens*, *Leucopaxillus gentianeus* and *Suillus granulatus*: The mushroom Species from Anatolia

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Abstract

The antioxidant activity of extracts of *Armillaria tabescens*, *Leucopaxillus gentianeus* and *Suillus granulatus* were determined for the first time by using five complementary tests; namely, β-carotene-linoleic acid, DPPH$^{\bullet}$ scavenging, ABTS$^{\bullet+}$ scavenging, metal chelating and CUPRAC assays. In DPPH$^{\bullet}$ scavenging, ABTS$^{\bullet+}$ scavenging and CUPRAC assays, the ethyl acetate extract of *S. granulatus* showed the best activity 91.52±0.97%, 89.67±0.15% and 3.90±0.09 at 400 µg/mL concentration, respectively while in β-carotene-linoleic acid assay, the methanol extract of this mushroom exhibited higher activity. In addition, among the extracts of mushroom species, particularly the hexane extracts showed better activity in metal chelating activity. The hexane extract of *L. gentianeus* exhibited the highest metal chelating activity (70.68±0.34%) at 400 µg/mL concentration. In conclusion, the results showed the antioxidant importance of the studied mushroom species, growing naturally in Anatolia. Thus, particularly *S. granulatus* may have been helped people to protect against lipid peroxidation and free radical damage, and its extracts will probably use for the development of safe food products and additives.

Keywords:

Mushroom species, *Armillaria tabescens*, *Leucopaxillus gentianeus*, *Suillus granulatus*, Antioxidant activity

1. Introduction

Oxidation is essential to many living organisms for the production of energy to fuel biological processes. However, oxygen-centered free radicals and other reactive oxygen species that are continuously produced *in vivo*, result in cell death and tissue damage. Oxidative damage caused by free radicals may be related to aging and diseases, such as atherosclerosis, diabetes, cancer and cirrhosis [1]. Thus it is essential to develop natural antioxidants so that they can protect the human body from free radicals and many chronic diseases [2]. However synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *tert*-butylhydroquinone (TBHQ) have been restricted use in the food industry as they are suspected to be carcinogenic and liver damage [3]. Therefore, compounds from natural sources possessing antioxidant potential are being sought. Thus, the development and utilization of more effective antioxidants of natural origin are desired.

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Mushrooms have been the focus of researchers' interest since they are rich sources of antioxidant compounds such as phenolic acids, flavonoids and tocopherols [4]. Lectins, polysaccharides, polysaccharide-peptides, polysaccharide-protein complexes, lanostane-type triterpenoids, beside phenolic and flavonoid structured compounds were obtained from mushroom species. Most of them showed biological activities such as anticancer, antioxidant, antitumor, antiviral, antibacterial, antifungal, anti-inflammatory, immunomodulator activities, antibiotic effects and cholesterol-lowering properties [5-9].

Up to now, the antioxidant activities of mushrooms such as *Agaricus bisporus* [10], *Lactarius deliciosus*, *Lactarius sanguifluus*, *Lactarius semisanguifluus*, *Russula delica* and *Suillus bellini* [11], *Polyporus squamosus*, *Agaricus bisporus*, *Lepista nuda*, *Pleurotus ostreotus*, *Russula delica*, *Boletus badius* and *Verpa conica* [12], *Laetiporus sulpherus* [8], *Ganoderma lucidum*, *Ganoderma tsugae* and *Coriolus versicolor* [13] have been studied and significant antioxidant activity results were found.

In previous studies several biological activities such as antimicrobial [14] and ligninolytic enzyme activities [15] of *Armillaria tabescens* have been investigated. The phytochemical studies on the mushroom afforded emestrin-F, emestrin-G, 6-*O*-(4-*O*-methyl-*β*-*D*-glucopyranosyl)-8-hydroxy-2,7-dimethyl-4H-benzopyran-4-one, cephalosporolide-J [14], 4-dehydro-14-hydroxydihydromelleolide, 4-dehydro-dihydromelleolide, 14-hydroxydihydro-melleolide, 13-hydroxy-4-methoxymelleolide and 5*β*,10*α*-dihydroxy-1-orsellinatedihydro-melleolide [16] have been isolated. Moreover, nutritional value [17] trace element [18], fatty acid [19], organic acid [20] and organic elements and protein contend [21] of the *A. tabescens* were also studied.

To the best of our knowledge a few studies on *Leucopaxillus gentianeus* have been published. From fruiting bodies of the mushroom two cucurbitane triterpenes, namely, cucurbitacin D and 16-deoxycucurbitacin B were isolated [22]. In addition, the metal content of the mushroom [23] were also reported.

Antitumor [24], antioxidant, [25-27], antimicrobial [28], cytotoxic activity [29], anti-HIV-1 [30] and ligninolytic enzyme activities [31] of *Suillus granulatus* have been investigated. From the mushroom, flazine, 5*α*,8*α*-epidioxyergosta-6,22-dien-3*β*-ol, ergosta-5,7,22-trien-3*β*-ol, ergosta-5,7,22-trien-3*β*-*O*-*β*-*D*-glucopyranoside, uracil, thioacetic anhydride, stearic acid, 3-pyridinecarboxylic acid, D-allitol [30], 4-hydroxyphenylacetic acid, 4-hydroxy-benzaldehyde, 2,5-dihydroxybenzoic acid methyl ester, 5'-deoxy-5'-methylthioadenosine, indole-3-carboxylic acid metil ester, indole-3-carboxaldehyde, 1,3,5-trihydroxy-7-methylanthraquinone, nicotinamide and 3-geranylgeranyl-4-hydroxybenzoic acid [32] and 2-acetoxy-3-geranylgeranyl-1,4-dihydroxybenzene, 3-geranulgeranyl-1,2-dihydroxy-4-methoxybenzene, 1-acetoxy-6-geranylgeranyl-2,4-dihydroxybenzene, 6-geranylgeranyl-2,4-dihydroxy-1-methoxybenzene and 5,6-dihydroxy-2-methyl-2-[3',7'E]- 4',8',12'-trimethyltrideca-3',7',11'-trien-2(H)-chromene [28] have been isolated. Moreover, the nutritional composition [33-35], fatty acid composition [36-38], free amino acid composition [39-41], volatile composition [42], aroma compounds [43], metal content [44-48] and carboxylic and phenolic acids [27] were also studied.

Considering the phenolic profile of the mushroom species, we aimed to investigate the antioxidant activity by using five complementary methods. So far, the antioxidant activity of the mushrooms species have not been studied except *Suillus granulatus* extracts. In fact, it was only investigated against DPPH radical scavenging activity. The objective of this study is to compare antioxidant activity of the various extracts of mushroom species, with those of commercial and synthetic antioxidants which are commonly used in the food and pharmaceutical industries.

2. Materials and methods

2.1. Mushroom materials

Armillaria tabescens (Scop.) Emel was collected from Uşak-Sivaslı at 6th of October 2008. In addition, *Leucopaxillus gentianeus* (Quél.) Kotl. and *Suillus granulatus* (L.) Roussel were collected from Uşak-Banaz at 10th of December 2008 and 5th of May 2008, respectively. All mushroom species were identified by Professor Aziz Türkoğlu. Voucher specimens were deposited in the Herbarium of Department of Biology, Mugla Sıtkı Kocman University and coded as Türkoğlu 3921, Türkoğlu 4976 and Türkoğlu 3651 herbarium numbers, respectively.

2.2. Spectral measurements and chemicals used

Bioactivity measurements were carried out on a 96-well microplate reader, SpectraMax 340PC384, Molecular Devices (USA), at Department of Chemistry, Muğla Sıtkı Koçman University. The measurements and calculations of the activity results were evaluated by using Softmax PRO v5.2 software.

Ethanol, *n*-hexane, methanol, ammonium acetate, copper (II) chloride, potassium persulfate, ferrous chloride, and ethylenediaminetetraacetic acid (EDTA) were obtained from E. Merck (Darmstadt, Germany). β-carotene, linoleic acid, polyoxyethylene sorbitan monopalmitate (Tween-40), neocuproine, α-tocopherol, buthylatedhydroxylanisole (BHA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 3-(2-pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5′,5″-disulfonic acid disodium salt (Ferene), were obtained from Sigma Chemical Co. (Sigma-Aldrich GmbH, Sternheim, Germany). All other chemicals and solvents were in analytical grade.

2.3. Extraction

Each mushroom species were extracted by using soxhlet apparatus with *n*-hexane, ethyl acetate and methanol, successively. For each species, the filtered extracts were evaporated to dryness *in vacuum*. The *n*-hexane, the ethyl acetate and the methanol extracts were used for antioxidant activities.

2.4. Determination of Antioxidant activity

2.4.1. β-carotene/linoleic acid bleaching assay

The total antioxidant activity was evaluated using β-carotene-linoleic acid test system [49] with slight modifications. β-Carotene (0.5 mg) in 1 mL of chloroform was added to 25 μL of linoleic acid and 200 mg of Tween 40 emulsifier mixture. After evaporation of chloroform under vacuum, 100 mL of distilled water saturated with oxygen, was added by vigorous shaking. One-sixty microliters of this mixture was transferred into 40 μL of the samples at different concentrations. As soon as the emulsion was added into each tube, the zero time absorbance was measured at 470 nm using a 96-well microplate reader. Absorbance of the emulsion was read again at the same wavelength after incubation of the plate for 2 h at 50 ºC. Ethanol was used as a control. BHA and *α*-tocopherol were used as antioxidant standards for comparison of the activity.

The bleaching rate (R) of β-carotene was calculated according to the following equation:

$$R = \frac{\ln \frac{a}{b}}{t}$$

Where: ln=natural log, a=absorbance at time zero, b=absorbance at time t (120 min). Antioxidant activity was calculated in terms of percent inhibition relative to the control, using following equation:

$$\text{Antioxidant activity (\%)} = \frac{R_{control} - R_{sample}}{R_{control}} \text{x100}$$

where $R_{Control}$ is the bleaching rate of the β-carotene without test material and R_{Sample} is the absorbance of the β-carotene in presence of the sample.

2.4.2. DPPH free radical scavenging assay

The free radical scavenging activity was determined spectrophotometrically by the DPPH assay described by Blois [50] with slight modification. In its radical form, DPPH absorbs at 517 nm, but upon reduction by an antioxidant or a radical species, its absorption decreases. Briefly, 120 µL of ethanol and 40 µL of sample solutions, dissolved in ethanol, at different concentrations were mixed. The reaction was then initiated by the addition of 0.4 mM 40 µL DPPH prepared in ethanol. Thirty minutes later, absorbance was measured at 517 nm by using a 96-well microplate reader. Ethanol was used as a control. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability of scavenging the DPPH radical was calculated by using the following equation:

$$\text{DPPH}^{\bullet} \text{ scavenging effect (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \text{x100}$$

where $A_{Control}$ is the initial concentration of the $\text{DPPH}^{\bullet}$ and A_{Sample} is the absorbance of the remaining concentration of $\text{DPPH}^{\bullet}$ in presence of the sample. BHA and α-tocopherol were used as antioxidant standards for comparison of the activity.

2.4.3. ABTS cation radical decolorization assay

The spectrophotometric analysis of $\text{ABTS}^{\bullet+}$ scavenging activity was determined according to the method of [51] with slight modifications. The $\text{ABTS}^{\bullet+}$ was produced by the reaction between 7 mM ABTS in H_2O and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. The radical cation was stable in this form for more than 2 days when stored in the dark at room temperature. Before usage, the $\text{ABTS}^{\bullet+}$ solution was diluted to get an absorbance of 0.708±0.025 at 734 nm with ethanol. Then, 160 µL of $\text{ABTS}^{\bullet+}$ solution was added to 40 µL of sample solution in ethanol at different concentrations. After 10 min, by using a 96-well microplate reader, the percentage inhibition at 734 nm was calculated for each concentration relative to a blank absorbance (ethanol). The scavenging capability of $\text{ABTS}^{\bullet+}$ was calculated using the following equation:

$$\text{ABTS}^{\bullet+} \text{ scavenging effect (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{Control}$ is the initial concentration of the $\text{ABTS}^{\bullet+}$ and A_{Sample} is the absorbance of the remaining concentration of $\text{ABTS}^{\bullet+}$ in presence of the sample. BHA and α-tocopherol were used as antioxidant standards for comparison of the activity.

2.4.4. Cupric reducing antioxidant capacity (CUPRAC)

The cupric reducing antioxidant capacity was determined according to the method of [52], with sligh modifications. To each well, in a 96 well plate, 50 µL 10 mM Cu (II), 50 µL 7.5 mM neocuprine, and 60 µL NH_4Ac buffer (1 mol L^{-1}, pH 7.0) solutions were added. Forty microliter

extract at different concentrations were added to the initial mixture so as to make the final volume 200 µL. After 1 h, the absorbance at 450 nm was recorded against a reagent blank by using a 96-well microplate reader. Results were given as absorbance comparing that those of BHA and α-tocopherol used as antioxidant standards.

2.4.5. Metal chelating activity

The chelating activity of the extracts on Fe^{2+} was measured as reported by Decker and Welch [53] with slight modifications. The extracts solution (80 µL dissolved in ethanol in different concentrations) were added to 40 µL 0.2 mM $FeCl_2$. The reaction was initiated by the addition of 80 µL 0.5 mM ferene. The mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture reached equilibrium, the absorbance was measured at 593 nm. The metal chelating activity was calculated using the following equation:

$$\text{Metal chelating activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{Control}$ is the absorbance of control devoid of sample and A_{Sample} is the absorbance of sample in the presence of the chelator. EDTA was used as standard for comparison of the activity.

2.5. Statistical analysis

All data on antioxidant activity tests were the average of triplicate analyses. Data were recorded as mean ± standard deviation. Significant differences between means were determined by student's-*t* test, p values <0.05 were regarded as significant.

3. Results and Discussion

There are several methods for determination of antioxidant activities. The chemical complexity of extracts, often a mixture of dozens of compounds with different functional groups, polarity and chemical behaviour, could lead to scattered results, depending on the test employed. Therefore, an approach with multiple assays for evaluating the antioxidant potential of extracts would be more informative and even necessary. In this study, mainly five methods, β-carotene bleaching method, DPPH radical scavenging activity, ABTS cation radical scavenging activity, metal chelating activity, and cupric reducing power were used. The activity results were compared with those of BHA and α-tocopherol. According to results in all tests the antioxidant activity increased with increasing amounts of the extracts. Results were found to be statistically significant (p<0.05) when compared with that of controls in each test.

Figure 1 shows the total antioxidant activity of the extracts of mushrooms, compared with α-tocopherol and BHA, which were determined by the β-carotene bleaching method. Total antioxidant activity increased with increasing amount of the extracts. Antioxidants and lipid peroxidation inhibitors can be tested in β-carotene-linoleic acid assay by transferring $H^{\bullet}$ to the media. In β-carotene-linoleic acid assay, the methanol and ethyl acetate extract of *S. granulatus* showed the highest lipid peroxidation inhibition activity indicating 87.26±1.85% and 87.18±2.22% inhibition at 50 µg mL^{-1} concentration. The methanol and ethyl acetate extract of *S. granulatus* exhibited close activity to those of antioxidant standards in all concentrations. As for *L. gentianeus*, the ethyl acetate extract also showed the highest activity among its extracts indicating 63.58±1.65% inhibition at 50 µg/mL concentration followed by methanol extract (42.78±1.65%). Among the *A. tabescens* extracts, however, the methanol extract was found to be the highest (53.81±1.44%) at same concentration.

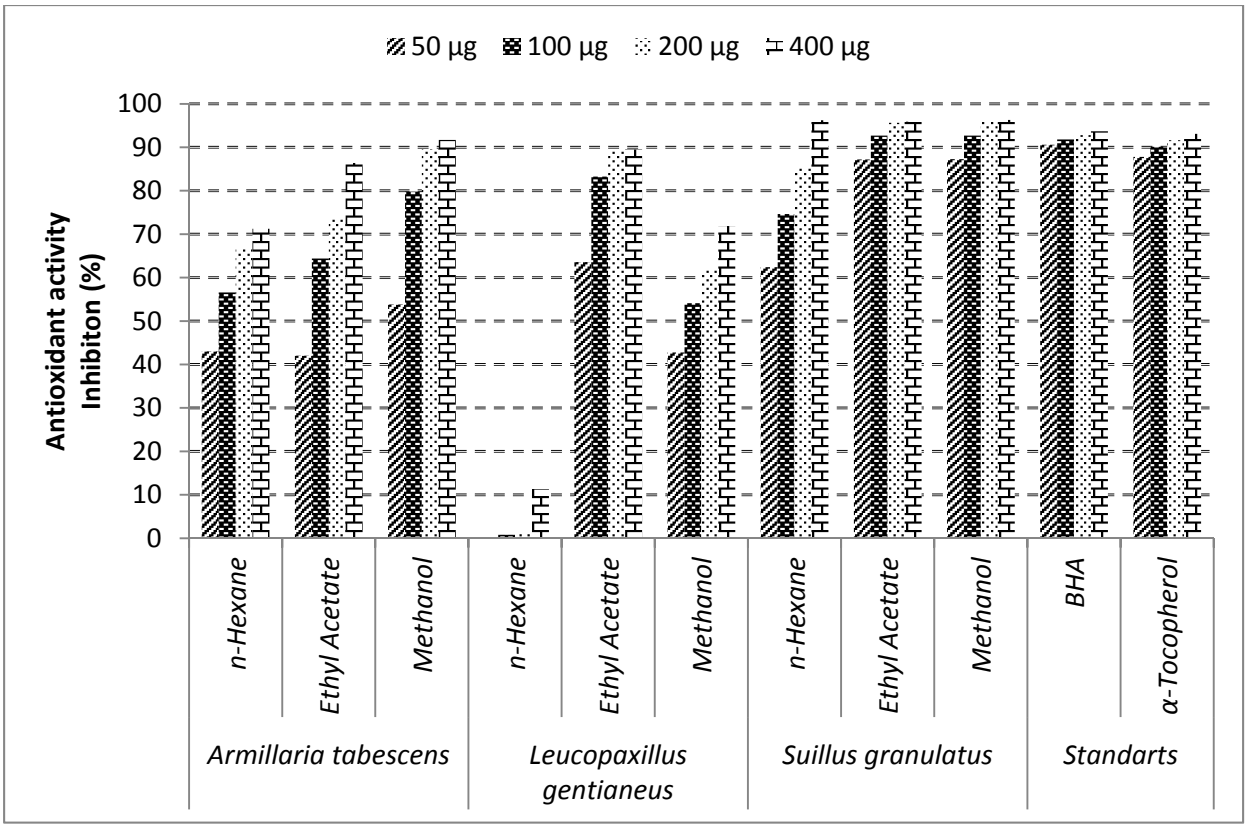

Fig. 1. The inhibition (%) of linoleic acid oxidation in β-carotene/linoleic acid assay of the extracts of mushroom species

The antioxidants and phenolics are easily tested with both radical scavenging activities by transferring electron to the media. DPPH free radical and ABTS cation radical scavenging activities of the extracts of mushrooms were given in **Table 1**. In $DPPH^{\bullet}$ assay, the ethyl acetate extract of *S. granulatus* showed the highest activity (91.52±0.97%) followed by the *n*-hexane extract of this mushroom (70.30±0.33%) and the methanol extract of *L. gentianeus* (65.74±1.02%) at 400 µg mL^{-1} concentration. In $ABTS^{\bullet+}$ assay, however, the ethyl acetate extract of *S. granulatus* and the methanol extract of *L. gentianeus*, the methanol extract of *A. tabescens* and the *n*-hexane extract of *S. granulatus* exhibited better radical scavenging activity, indicating 89.67±0.15, 89.44±0.17, 88.48±1.12 and 87.78±0.36% inhibitions at 400 µg/mL concentration, respectively (**Table 1**).

Figure 2 shows the cupric reducing antioxidant capacity (CUPRAC). This method is based on the measurement of absorbance at 450 nm by the formation of a stable complex between neocuproine and copper (I), the latter is formed by the reduction of copper (II) in the presence of neocuproine. In other words highest absorbance shows highest activity. The ethyl acetate extract of *S. granulatus* was found to be the best reductant indicating 3.90±0.09 absorbance at 400 µg/mL concentration followed by its hexane extract (2.27±0.09) and the hexane extract of *A. tabescens* (1.07±0.01).

Table 1. Antioxidant activity (%) of the extracts of mushroom species by the DPPH and ABTS assays [a]

		DPPH assay				ABTS assay			
Mushroom	**Extracts**	**50 µg**	**100 µg**	**200 µg**	**400 µg**	**50 µg**	**100 µg**	**200 µg**	**400 µg**
Armillaria tabescens	*n*-Hexane	1.72±0.17	2.76±0.83	4.40±0.46	5.07±1.93	12.73±0.81	15.95±0.48	24.36±0.57	37.65±1.87
	Ethyl Acetate	5.75±0.11	10.20±0.35	18.12±1.57	18.14±0.45	17.50±2.61	19.36±0.45	29.54±0.65	50.81±0.56
	Methanol	10.01±0.06	19.72±0.64	30.17±0.74	34.52±0.39	36.41±1.65	48.50±0.30	61.22±0.43	88.48±1.12
Leucopaxillus gentianeus	*n*-Hexane	-	-	-	-	8.84±0.36	10.36±0.52	17.05±1.67	18.95±0.92
	Ethyl Acetate	10.33±0.38	18.80±0.54	26.62±0.97	32.14±0.74	48.10±0.59	52.96±1.33	76.35±0.08	81,20±0.33
	Methanol	10.35±1.98	22.03±1.53	41.16±1.68	65.74±1.02	62.74±1.64	87.82±0.66	89.37±0.29	89.44±0.17
Suillus granulatus	*n*-Hexane	30.38±0.54	53.74±2.14	62.83±0.52	70.30±0.33	63.73±1.49	85.15±1.32	86.87±0.31	87.78±0.36
	Ethyl Acetate	68.87±1.01	72.64±0.13	76.79±0.07	91.52±0.97	79.86±2.03	81.65±0.06	85.36±0.09	89.67±0.15
	Methanol	20.60±0.88	36.93±0.80	62.44±0.70	64.66±0.31	48.93±1.51	82.14±0.95	86.97±0.09	87.64±0.35
Standarts	BHA [b]	59.01±0.01	79.30±0.50	90.82±0.22	94.13±0.10	94.10±0.01	97.90±0.01	98.11±0.90	99.73±0.10
	α-Tocopherol [b]	84.10±0.01	95.91±0.01	96.10±0.90	96.70±0.10	95.11±0.01	97.91±0.02	98.12±0.90	99.70±0.10

[a] Values expressed are means ± SD of three parallel measurements (p<0.05).
[b] Reference compounds.

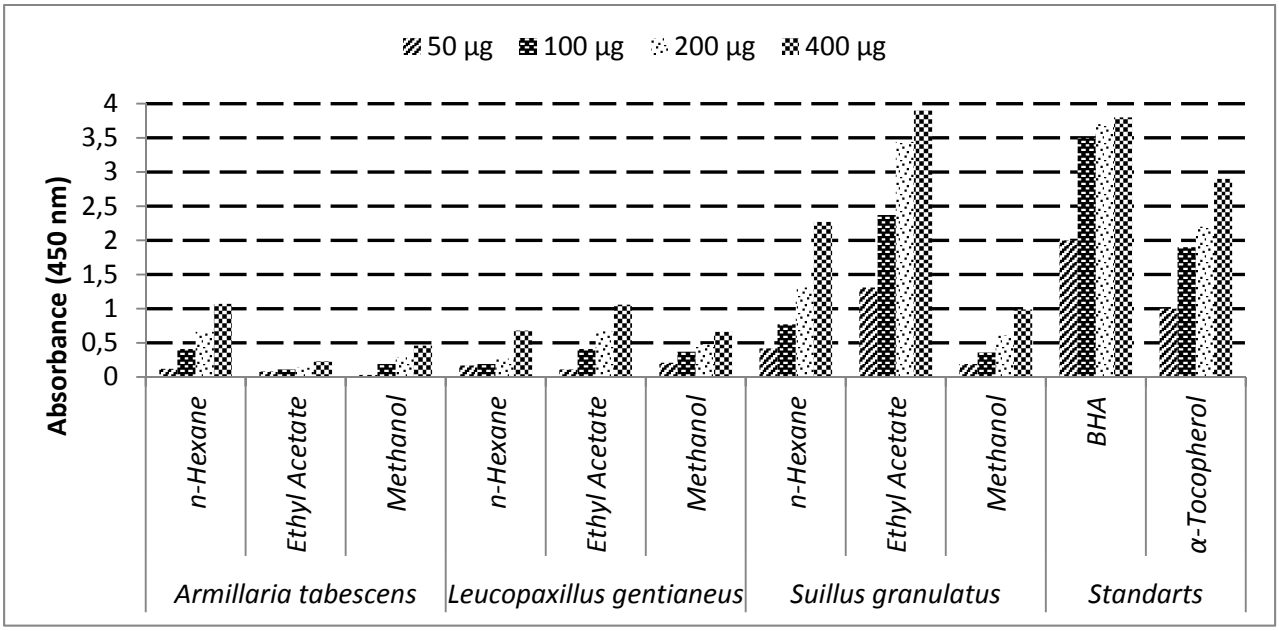

Fig. 2. The cupric reducing antioxidant capacity (CUPRAC) by Cu^{2+} Cu^{+} transformation of the extracts of mushroom species

Ferrous ions are also commonly found in food systems and considered to be the most effective pro-oxidants. For example, the ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals via the Fenton Reaction [54, 55]. In fact, the reaction is very slow, when catalyzed by ferrous state iron it accelerates. **Table 2** shows the chelating effects of the extracts of mushroom species compared with EDTA on ferrous ions. Among all extract of mushroom species, generally the *n*-hexane extracts showed better activities. The *n*-hexane extract of *L. gentianeus* exhibited the highest metal chelating activity (70.68±0.34%) at 400 µg mL^{-1} concentration, although its ethyl acetate extract showed no chelation activity. The *n*-hexane extract of *S. granulatus* indicated 58.42±0.63% inhibition followed by the *n*-hexane extract of *A. tabescens* (57.13±1.87%).

Table 2. Metal chelating activity (Inhibition %) by Ferrene–Fe^{2+} assays of the extracts of mushroom species [a].

Mushroom Species	Extracts	Inhibition % Metal Chelating Activity			
		50 µg	100 µg	200 µg	400 µg
Armillaria tabescens	*n*-Hexane	22.94±1.56	34.70±0.72	38.57±1.03	57.13±1.87
	Ethyl Acetate	-	-	-	4.75±0.62
	Methanol	-	17.46±0.24	20.62±0.47	35.31±0.45
Leucopaxillus gentianeus	*n*-Hexane	22.27±0.18	44.25±0.14	57.49±1.26	70.68±0.34
	Ethyl Acetate	-	-	-	-
	Methanol	14.54±0.49	21.05±2.10	32.68±0.42	43.31±2.14
Suillus granulatus	*n*-Hexane	20.39±0.70	26.95±1.08	40.12±0.16	58.42±0.63
	Ethyl Acetate	-	-	6.14±2.69	8.01±0.03
	Methanol	7.42±0.66	10.08±1.98	13.78±1.62	20.37±1.97
Standart	EDTA[b]	92.5±1.40	94.7±0.60	95.2±0.10	96.3±0.10

[a] Values expressed are means±S.E.M. of three parallel measurements. (p<0.05)
[b] Reference compounds.

4. Conclusions

The results presented in this study are the first information on the antioxidant activities of *Armillaria tabescens* and *Leucopaxillus gentianeus* except *Suillus granulatus*. Among the tested five methods, the highest activity was observed for inhibition of lipid peroxidation in β-carotene–linoleic acid system almost by all extracts of the mushrooms. Particularly, ethyl acetate extract of the *Suillus granulatus* was found to be the most active one, showing better activity than that of the standards, this finding should be related to the highest phenolic content of the extract. On the other hand, antioxidant activity by $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and CUPRAC activities, giving electrons to the media, was well correlated with β-carotene-linoleic acid assay, giving $H^{\bullet}$ to the media. On the contrary, the ethyl acetate extracts of the mushrooms showed stronger activity in these assays. It should be noted that the *n*-hexane extract of the *Leucopaxillus gentianeus* showed very high metal chelating ability on ferrous ion, but indicated less activity than that of EDTA.

In conclusion, the results showed the antioxidant importance of mushrooms, naturally growing in Anatolia. According to the results these species may have been helped people to protect against lipid peroxidation and free radical damage, and its extracts will probably use for the development of safe food products and additives. However, further studies, especially *in vivo* antioxidant activity tests on extracts and isolated constituents are needed.

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