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In vitro antioxidant, anticholinesterase and antimicrobial activity studies on three *Agaricus* species with fatty acid compositions and iron contents: A comparative study on the three most edible mushrooms

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ABSTRACT

The fatty acids of *Agaricus essettei*, *Agaricus bitorquis* and *Agaricus bisporus* were investigated by using GC and GC–MS. The dominant fatty acids were found to be linoleic (61.82–67.29%) and palmitic (12.67–14.71%) acids among the 13 fatty acids detected in the oils. Total unsaturation for the oils was calculated as 77.50%, 77.44%, and 79.72%, respectively. *In vitro* antioxidant, anticholinesterase and antimicrobial activities were also studied. The ethyl acetate extract of *Agaricus bitorquis* showed the highest activity in β-carotene-linoleic acid, DPPH· and $ABTS^{\cdot+}$ assays, while the hexane extract of *Agaricus bisporus* exhibited the best metal chelating activity. The ethyl acetate and hexane extract of *Agaricus bitorquis* and the hexane extract of *Agaricus essettei* showed meaningful butyrylcholinesterase activity being close to that of galantamine. The extracts were found to be effective on Gram (+) bacteria, especially against *Micrococcus luteus*, *Micrococcus flavus*, *Bacillus subtilis* and *Bacillus cereus*. In conclusion, *Agaricus bitorquis* and *Agaricus essettei* demonstrated higher iron content, and better antioxidant, anticholinesterase and antimicrobial activities than those of *Agaricus bisporus* commonly consumed mushroom. Hence, *Agaricus* species, particularly *Agaricus bitorquis* might be useful as antioxidant agents and moderate anticholinesterase agents, and their extracts will probably be used for development of dietary foods, food products and additives.

1. Introduction

Edible mushrooms are valuable healthy foods, having rich source of vitamins, proteins and minerals, especially in potassium and phosphorus. They are also low in calories and fats (Leon-Guzman et al., 1997). Moreover, lectins, polysaccharides, polysaccharide-peptides, polysaccharide-protein complexes, lanostane-type triterpenoids, phenolic and flavonoid have been isolated from the some edible mushroom species (Tong et al., 2009; Zhang et al., 2007). Furthermore, in previous studies various biologic activities such as antioxidant, antibacterial, antifungal (Türkoğlu et al., 2007), immunomodulatory, antiviral (Moradali et al., 2007), antitumor (Tong et al., 2009; Zhang et al., 2007), anti-inflammatory (Komura et al., 2010; Regina et al., 2008), cytotoxic (Zhang et al., 2007), antiaromatase (Chen et al., 2006) and anticholesterole (Jeong et al., 2010) activities of these compounds and/or complexes were investigated.

Agaricus bisporus, the most cultivated mushroom in the world, exhibits a high proportion of fatty acids. Literature survey shows that palmitic, stearic, oleic and linoleic acids are the most abundant fatty acids in *Agaricus* species (Barros et al., 2007; Pedneault et al., 2008; Yilmaz et al., 2006). Polyunsaturated fatty acids such as linoleic acid and linolenic acid called essential fatty acids are essential for human's basal metabolism and have many beneficial effects on human health (Parikh et al., 2005). Lack of dietary essential fatty acids or their inefficient metabolism has been implicated in etiology of disease including cardiovascular disease and progression of it (Browni, 2005). Therefore, investigation of the fatty acid content in edible mushrooms has become a topic of great interest.

Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *tert*-butylhydroquinone (TBHQ) are widely used in the food industry. However, the uses of these synthetic antioxidants are suspected that they are responsible for liver damage and carcinogenesis (Grice, 1988). Therefore, the investigation of the

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antioxidants without any side effect from the food consuming safely by people have been become important. On the other hand, excess amount of free radical species, which causes oxidative stress, is associated with pathology of many diseases including Alzheimer's disease (AD). AD is a progressive neurologic disorder characterized by cognitive deficit and behavioral abnormalities in the patient (Soholm, 1998). Up to date, pathogenesis of Alzheimer's disease has not been fully clarified. The only known valid hypothesis being accepted is the lack of amount of acetylcholine, a neuromediator. Thus, the acetylcholinesterase inhibitory drugs were used for the treatment of Alzheimer's disease. However, most of these drugs have side effects. Thus, the development and utilization of more effective antioxidants of natural origin as well as anticholinesterase compounds are desired. In a report it is suggested that the usage of antioxidants may reduce the progression of Alzheimer's disease and minimize neuronal degeneration (Atta-ur-Rahman and Choudhary, 2001) by inhibiting acetylcholinesterase and butyrylcholinesterase which are chief enzymes in pathogenesis of Alzheimer's disease. It is an advantageous, particularly for a food, to have both antioxidant activity accompanied with acetylcholinesterase and butyrylcholinesterase inhibitory activity.

Nowadays, the development of resistance by a pathogen to many of the commonly used antibiotics provides an impetus for further attempts to search for new antimicrobial agents to combat infections. The treatment of infectious diseases with antimicrobial agents continues to present problems in modern-day-medicine with many studies showing a significant increase in the incidence of bacterial resistance to several antibiotics (Finch, 1998; Kunin, 1993). Multiple drug resistance in human pathogenic microorganisms has developed due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. This situation forced scientists for searching new antimicrobial substances from various sources which have the potential of being sources of novel antimicrobial chemotherapeutic agents. There has been no much study on antimicrobial activity of *Agaricus* species to date. Thus, one of the aims of the study is to evaluate antimicrobial potential of *Agaricus* species against several Gram-positive and Gram-negative bacteria as well as against two yeast-like fungus, *Candida albicans* and *C. tropicalis*.

Agaricus bisporus was well investigated in many studies by various researchers. Especially, its fatty acid profile and its antioxidant activity was studied (Leon-Guzman et al., 1997; Barros et al., 2007; Yilmaz et al., 2006; Saiqa et al., 2008). However, *A. bitorquis* and *A. essettei* have not been investigated in detail. There is only a recent study on *A. bitorquis* (Saiqa et al., 2008). Furthermore, antioxidant and antimicrobial activities of the *A. bitorquis* and *A. essettei* and anticholinesterase activity of all *Agaricus* species tested were studied for the first time in this study.

Agaricus bisporus together with other *Agaricus* species are the most edible mushroom in the world, due to their high proportion of fatty acids and their nutritional value. Regarding to the consumption of *Agaricus* species in Turkey as well as in some other countries we aimed to investigate the fatty acid compositions, and iron contents of *A. bitorquis*, *A. essettei* and *A. bisporus* with antioxidant, anticholinesterase and antimicrobial activities by comparing with those of commercial antioxidants and that of galantamine. The objective of this study is also to make comparison of the fatty acid compositions, iron contents and the tested biologic activities of *A. bitorquis* and *A. essettei* with those of *A. bisporus*.

2. Materials and methods

2.1. Chemicals and spectral measurements

Quercetin, potassium persulfate, ferrous chloride, ferric chloride, pyrocatechol, quercetin, copper (II) chloride, ethylenediaminetetraacetic acid (EDTA) and boron trifluoride-methanol complex (BF_3:MeOH) were obtained from E. Merck (Darmstadt, Germany). β-Carotene, linoleic acid, polyoxyethylene sorbitan monopalmitate (Tween-40), Folin–Ciocalteu's reagent (FCR), 3-(2-pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5',5''-disulfonic acid disodium salt (Ferene), neocuproine and ammonium acetate butylated hydroxytoluene (BHT), 1,1-diphenyl-2-picrylhydrazyl (DPPH·), Electric eel acetylcholinesterase (AChE, Type-VI-S, EC 3.1.1.7, 425.84 U/mg), horse serum butyrylcholinesterase (BChE, EC 3.1.1.8, 11.4 U/mg), 5,5′-dithiobis (2-nitrobenzoic) acid (DTNB), acetylthiocholine iodide and butyrylthiocholine chloride, galantamine were obtained from Sigma Chemical Co. (Sigma–Aldrich GmbH, Sternheim, Germany). 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was obtained from Fluka Chemie (Fluka Chemie GmbH, Sternheim, Germany). All other chemicals and solvents were in analytical grade.

GC analyses were performed on a Shimadzu GC-17 AAF, V3, 230 V series gas chromatography (Japan), GC–MS analyses were carried out on Varian Saturn 2100 (USA), and Bioactivity measurements were carried out on a 96-well microplate reader, SpectraMax 340PC^{384}, Molecular Devices (USA), at Department of Chemistry, Muğla University. The measurements and calculations of the activity results were evaluated by using Softmax PRO v5.2 software.

2.2. Mushroom materials and preparation of the extracts

Agaricus bisporus (J.E. Lange) Pilát, *Agaricus bitorquis* (Quél.) Sacc. and *Agaricus essettei* Bon. were identified by Dr. Aziz Türkoğlu and collected from Banaz-Uşak, Turkey in December 2007 Voucher specimens were deposited in the Herbarium of Department of Biology, University of Nevşehir and coded as Türkoğlu 4003, Türkoğlu 4004 and Türkoğlu 4005 Herbarium numbers, respectively.

Each *Agaricus* species were extracted separately with 2.5 L hexane for four times (24 h × 4) at room temperature (25 °C), filtered and evaporated to dryness *in vacuum*. The residue mushroom materials were similarly extracted, filtered and evaporated by using ethyl acetate and aqueous methanol solvents, successively. The yields of the extracts were given in Table 1.

Table 1
Yield percentages, total iron content, total phenolic and total flavonoid contents of the extracts of the three *Agaricus* species.[a]

Mushrooms extracts	Yields (%)	Fe content mg Fe^{3+}/kg mushroom	Phenolic contents μg PEs/mg extract [b]	Flavonoid contents μg QEs/mg extract [c]	Phenolic contents mg PEs/100 g mushroom [b]	Flavonoid contents mg QEs/100 g mushroom [b]
A. bisporus						
Hexane	0.68	206.20 ± 1.14	9.76 ± 1.00	5.12 ± 0.55	383.83 ± 2.36	544.27 ± 2.69
Ethyl acetate	0.65	42.38 ± 0.56	62.71 ± 0.23			
Methanol	5.84	59.87 ± 0.55	85.45 ± 0.36			
A. bitorquis						
Hexane	0.36	2964.54 ± 4.40	13.06 ± 0.46	4.34 ± 0.36	315.69 ± 3.56	378.90 ± 3.45
Ethyl acetate	0.92	56.21 ± 0.22	37.94 ± 0.12			
Methanol	10.33	25.10 ± 0.11	33.15 ± 0.10			
A. essettei						
Hexane	0.67	2618.46 ± 4.49	10.93 ± 0.59	7.09 ± 0.46	325.32 ± 3.00	668.68 ± 3.66
Ethyl acetate	0.72	23.49 ± 0.42	31.29 ± 0.31			
Methanol	12.00	27.11 ± 0.30	53.45 ± 0.20			

[a] Values expressed are means ± standard deviation of three parallel measurements ($p < 0.05$).
[b] PEs, pyrocatechol equivalents.
[c] QEs, quercetin equivalents.

2.3. Determination of Fe^{3+} content by spectrophometric method

Ferric ion content of the mushrooms was measured according to thiocyanate method. As known, the complexation of ferric ions with thiocyanate anions gives a red color. Briefly, 10 g mushroom sample was weighted and burned by using an oven at 800 °C for an hour. Each sample was dissolved with 9 mL of HNO_3 (65%) and 1 mL of H_2O_2 (30%) and finally diluted to 25 mL with deionized water (18.2 MΩ cm^{-1}). Five thousand microliter of this sample was added to a test tube containing 1 mL 2 M SCN and 3.5 mL deionized water. After five minutes the absorbance was red at 470 nm. When the absorbance of the sample was found outside the range, the sample was diluted and tried again until finding the absorbance between 0.200 and 0.800 values. Fe^{3+} ion content of the sample was calculated from the following graph:

$$\text{Absorbance} = 0.08237\ Fe^{3+}\text{ions}\ (\mu M) + 0.00058\ (R^2 : 0.9987)$$

2.4. Derivatization of fatty acids

The hexane extract (100 mg) was dissolved in 0.5 M NaOH (2 mL) in a 25 mL flask. After the flask was heated by using a water bath (50 °C), 2 mL BF_3:MeOH was added. The mixture was boiled for 2 min, and then left until it cooled down, and then the volume was completed to 25 mL with saturated NaCl solution. Esters were extracted with *n*-hexane; thus, the organic layer was separated. The hexane layer was washed with a potassium bicarbonate solution (4 mL, 2%) and dried with anhydrous Na_2SO_4 and filtered. The organic solvent was removed under reduced pressure by a rotary evaporator to give methyl esters (Yilmaz et al., 2006).

2.5. Gas chromatography (GC)

A Flame Ionization Detector (FID) and a DB-1 fused silica capillary non-polar column (30 m × 0.25 id., film thickness 0.25 μm) were used for GC analyses of the methyl derivatives of fatty acid. Injector and detector temperatures were 250 and 270 °C, respectively, carrier gas was He at a flow rate of 1.4 mL/min; sample size, 1.0 μL; split ratio, 50:1. The initial oven temperature was held at 100 °C for 5 min, then increased up to 238 °C with 3 °C/min increments and held at this temperature for 9 min. The percentage compositions of methyl derivatives of the fatty acid methyl derivatives were determined with GC Solution computer program.

2.6. Gas chromatography–Mass spectrometry (GC–MS)

An Ion trap mass spectrometer (MS) and a DB-1 MS fused silica non-polar capillary column (30 m × 0.25 mm ID, film thickness 0.25 μm) were used for the GC–MS analyses of the methyl derivatives of fatty acids. For GC–MS detection, an electron ionization system with ionization energy of 70 eV was used. Carrier gas was helium (15 psi) at a flow rate of 1.3 mL/min. Injector and MS transfer line temperatures were set at 220 and 290 °C, respectively. The oven temperature was held at 100 °C for 5 min, then increased up to 238 °C with 3 °C/min increments and held at this temperature for 9 min. Diluted samples (1/25, w/v, in hexane) of 0.5 μL were injected manually in the split mode. Split ratio was 50:1. EI-MS were taken at 70 eV ionization energy. Mass range was from *m/z* 50 to 650 amu. Scan time 0.5 s with 0.1 inters scan delays. The library search was carried out using NIST and Wiley 2005 (gas chromatography–mass spectrometry) GC–MS libraries. Supelco™ 37 components of (fatty acid methyl ester) FAME mixture (Catalog no: 47885-U) was used for the comparison of the GC chromatograms. The relative percentages of separated compounds were calculated from total ion chromatography by the computerized integrator.

2.7. Determination of total phenolic concentration

The concentrations of phenolic content in all extracts were expressed as microgram of pyrocatechol equivalents (PEs), determined by using FCR (Slinkard and Singleton, 1977). One milliliter of the solution (contains 1 mg) of the extracts in methanol was added to 46 mL of distilled water and 1 mL FCR, and mixed thoroughly. After 3 min, 3 mL of sodium carbonate (2%) were added to the mixture and shaken intermittently for 2 h at room temperature. The absorbance was read at 760 nm. The concentration of phenolic compounds was calculated according to the following equation that was obtained from standard pyrocatechol graph:

$$\text{Absorbance} = 0.08237\ \text{pyrocatechol}(\mu g) + 0.00058\ (R^2 : 0.9985)$$

2.8. Determination of total flavonoid concentration

Measurement of flavonoid concentration of the extracts was based on the complexation with Al^{3+} and the results were expressed as quercetin equivalents (Türkoğlu et al., 2007). An aliquot of 1 mL of the solution (contains 1 mg) extracts in methanol was added to test tubes containing 0.1 mL of 10% aluminum nitrate, 0.1 mL of 1 M potassium acetate and 3.8 mL of 80% methanol. After 40 min at room temperature, the absorbance was determined at 415 nm. Quercetin was used as a standard. The concentrations of flavonoid compounds were calculated according to following equation that was obtained from the standard quercetin graph:

$$\text{Absorbance} = 0.06648\ \text{quercetin}(\mu g) - 0.01586\ (R^2 : 0.9972)$$

2.9. Bioassays

2.9.1. Determination of the antioxidant activity with the β-carotene bleaching method

The total antioxidant activity was evaluated using β-carotene-linoleic acid test system (Marco, 1968) 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, 50 mL of distilled water saturated with oxygen was added by vigorous shaking. One-sixty microliters of this mixture were transferred into 40 μL of the samples at different concentrations. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a 96-well microplate reader. The absorbance of the emulsion was read again at the same wavelength after the incubation of the plate for 2 h at 50 °C. Ethanol was used as a control. The extract concentration providing 50% antioxidant activity (EC_{50}) was calculated from the graph of antioxidant activity percentage against extract concentration. BHT, α-tocopherol and quercetin 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). The antioxidant activity was calculated in terms of percent inhibition relative to the control, using following equation:

$$\text{Antioxidant activity}\ (\%) = \frac{R_{\text{Control}} - R_{\text{Sample}}}{R_{\text{Control}}} \times 100$$

2.9.2. DPPH free radical scavenging activity

The free radical scavenging activity was determined spectrophotometrically by the DPPH· assay (Blois, 1958) 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 40 μL of DPPH· (0.4 mM) prepared in ethanol. After thirty minutes, the 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:

$$\text{DPPH radical scavenging effect}\ (\%) = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100$$

where A_{Control} is the initial concentration of the DPPH· and A_{Sample} is the absorbance of the remaining concentration of DPPH· in the presence of the extract and positive controls. The extract concentration providing 50% radical scavenging activity (EC_{50}) was calculated from the graph of DPPH radical scavenging effect percentage against extract concentration. BHT, α-tocopherol and quercetin were used as antioxidant standards for comparison of the activity.

2.9.3. ABTS cation radical decolorization assay

The spectrophotometric analysis of $ABTS^{\cdot+}$ scavenging activity was determined according to the method of Re et al. (1999), with slight modifications. The $ABTS^{\cdot+}$ was produced by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. Oxidation of ABTS commenced immediately, but the absorbance was not maximal and stable until more than 6 h had elapsed. The radical cation was stable in this form for more than 2 days in storage in the dark at room temperature. Before usage, the $ABTS^{\cdot+}$ solution was diluted to get an absorbance of 0.708 ± 0.025 at 734 nm with ethanol. Then, 160 μL of $ABTS^{\cdot+}$ solution was added to 40 μL of sample solution in ethanol at different concentrations. After 10 min the absorbance was measured at 734 nm by using a 96-well microplate reader. The percentage inhibitions were calculated for each concentration relative to a blank absorbance (ethanol). The scavenging capability of $ABTS^{\cdot+}$ was calculated using the following equation:

$$\text{ABTS}^{\cdot+}\text{scavenging effect}\ (\%) = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100$$

where A_{Control} is the initial concentration of the $ABTS^{\cdot+}$ and A_{Sample} is the absorbance of the remaining concentration of $ABTS^{\cdot+}$ in the presence of sample. The extract concentration providing 50% radical scavenging activity (EC_{50}) was calculated from the graph of $ABTS^{\cdot+}$ scavenging effect percentage against extract concentration. BHT, α-tocopherol and quercetin were used as antioxidant standards for comparison of the activity.

2.9.4. Cupric reducing antioxidant capacity (CUPRAC)

The cupric reducing antioxidant capacity of the extracts was determined according to the CUPRAC method (Apak et al., 2004) with slight modifications. To each well, in a 96 well plate, 50 μL 10 mM Cu (II), 50 μL 7.5 mM neocuproine, and 60 μL NH_4Ac buffer (1 M, pH 7.0) solutions were added. Forty microliter extract at different concentrations was 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 absorbances and compared with BHT, α-tocopherol and quercetin used as antioxidant standards.

2.9.5. Ferrous ions chelating activity

The chelating activity of the extracts on Fe^{2+} was measured by using Ferrin (Decker and Welch, 1990) 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 at room temperature for 10 min. After the mixture reached equilibrium, the absorbance was measured at 593 nm.The metal chelation activity was calculated using the following equation:

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

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. The extract concentration providing 50% metal chelating activity (EC_{50}) was calculated from the graph of Fe^{2+} chelating effects percentage against extract concentration. EDTA and quercetin were used as antioxidant standards for comparison of the activity.

2.9.6. Determination of anticholinesterase activity

Acetylcholinesterase and butyrylcholinesterase inhibitory activities were measured by slightly modifying the spectrophotometric method of Ellman et al. (1961) AChE from electric eel and BChE from horse serum were used, while acetylthiocholine iodide and butyrylthiocholine chloride were employed as substrates of the reaction. DTNB (5,5′-Dithio-bis(2-nitrobenzoic)acid was used for the measurement of the cholinesterase activity. Briefly, 150 μL of 100 mM sodium phosphate buffer (pH 8.0), 10 μL of sample solution dissolved in ethanol at different concentrations and 20 μL AChE (5.32 × 10-3 U) or BChE (6.85 × 10-3 U) solution were mixed and incubated for 15 min at 25 °C, and then 10 μL of DTNB (0.5 mM) was added. The reaction was then initiated by the addition of 10 μL of acetylthiocholine iodide (0.71 mM) or 10 μL of butyrylthiocholine chloride (0.2 mM). The hydrolysis of these substrates was monitored spectrophotometrically by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine iodide or butyrylthiocholine chloride, respectively, at a wavelength of 412 nm utilizing a 96-well microplate reader. Percentage of inhibition of AChE or BChE enzymes was determined by comparison of reaction rates of samples relative to blank sample (ethanol in phosphate buffer pH 8) using the formula $(E - S)/E \times 100$, where E is the activity of enzyme without test sample, and S is the activity of enzyme with test sample. The experiments were carried out in triplicate. Galantamine was used as a reference compound.

2.9.7. Antimicrobial activity

The following strains of bacteria were used: *Pseudomonas aeruginosa* NRRL B-23, *Salmonella enteritidis* RSKK 171, *Escherichia coli* ATCC 25922, *Morganella morganii* (clinical isolate), *Yersinia enterecolitica* RSKK 1501, *Klebsiella pneumoniae* ATCC 27736, *Proteus vulgaris* RSKK 96026, *Staphylococcus aureus* ATCC 25923, *Micrococcus luteus* NRRL B-4375, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* RSKK 863, *Candida albicans* ATCC 10231 and *Candida tropicalis* (clinical isolate). The bacteria were obtained from the culture collection of the Microbiology Department of Pamukkale University. The antimicrobial activity of the extracts was assayed by the standard disk diffusion method (Murray et al., 1995). For the investigation of the antibacterial and anticandidal activity, the dried mushroom extracts were dissolved in dimethylsulfoxide (DMSO) and sterilized by filtration through a 0.22 μm membrane filter. Empty sterilized disks of 6 mm (Schleicher and Schuell, No. 2668, Germany) were each impregnated with 25 μL of extracts. All the microorganisms mentioned above were incubated at 37 ± 0.1 °C (30 ± 0.1 °C for *M. luteus* NRRL B-4375 and *M. flavus*) for 24 h by inoculation into Muller Hinton Broth and the yeast cultures were incubated Sabouraund Dextrose Broth at 28 ± 0.1 °C for 48 h. The culture suspensions were prepared and adjusted by comparing against 0.5 Mac-Farland turbidity standard tubes. Muller Hinton Agar (MHA) and Sabouraund Dextrose Agar (SDA) (15 mL) were poured into each sterile petri dish after injecting cultures (0.1 mL) of bacteria and yeast and distributing medium in petri dishes homogeneously. The disks injected with samples were placed on the inoculated agar by pressing slightly. Petri dishes were kept at 4 °C for 2 h, plates injected with the yeast cultures were incubated at 28 °C for 48 h, and the bacteria were incubated at 37 °C (30 °C for *M. luteus* NRRL B-4375 and *M. flavus*) for 24 h. At the end of the period, inhibition zones formed on the medium were evaluated in mm. Disks of DMSO was used as control. Studies performed in duplicate and the inhibition zones were compared with those of reference disks. Reference disks used are as follows: Nystatin (100 U), Ampicillin (10 μg), Penicillin (10 U), Gentamicin (10 μg), Oxacilin (1 μg), Tetracycline (30 μg).

2.10. Statistical analysis

All data on both antioxidant and anticholinesterase activity tests were the average of triplicate analyses. The 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

3.1. Iron concentration

Iron concentration of the mushroom species presented herein was determined via the thiocyanate method by using spectrophotometer. Edible mushrooms are known as a good source of iron. The sources of iron are important for the vegetarian people in order to meet their iron requirement. The results were given as mg Fe^{3+} per kg mushroom. *A. bitorquis* has the richest iron content (2964.54 ± 4.40 mg/kg mushroom) among the three *Agaricus* species. *A. essettei* has close iron content (2618.46 ± 4.49 mg/kg mushroom) to that of *A. bitorquis*. In contrast, iron content of *A. bisporus* (206.20 ± 1.14 mg/kg mushroom) was approximately 14-fold poorer than that of *A. bitorquis* as shown on Table 1.

3.2. Fatty acid composition

The fatty acid compositions of the three *Agaricus* species were given in Table 2. Thirteen fatty acids were detected by using GC and GC–MS in *Agaricus* species tested herein. The dominants were found to be linoleic acid (61.82–67.29%) and palmitic acid (12.67–14.71%). The total unsaturated fatty acid percentages were found to be between 77.44% and 79.72%. Oleic (6.07–8.11%), palmitoleic (4.16–5.12%) and stearic acids (3.72–3.97%) were also found in the hexane extracts of mushrooms tested. Our results were found to be compatible with previous studies on *Agaricus* species (Barros et al., 2007; Pedneault et al., 2008; Yilmaz et al., 2006; Saiqa et al., 2008). Other fatty acids such as $C_{8:0}$, $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, $C_{15:0}$, $C_{17:0}$, $C_{18:3}$ and $C_{20:0}$ were also found in the *Agaricus* species, but all of them were in small quantity (less than 3.0% in concentration).

All *Agaricus* species studied were characterized by a high concentration of unsaturated fatty acids more than 75% of total fatty acid content. The linoleic:oleic acid ratio could provide an important criterion from a chemotaxonomic viewpoint and could be

Table 2
The fatty acid compositions (%) of the three *Agaricus* species.

Fatty acids	*A. bisporus* (%)	*A. bitorquis* (%)	*A. essettei* (%)
Caprylic acid ($C_{8:0}$)	1.08	0.86	*tr*
Capric acid ($C_{10:0}$)	0.85	0.93	0.74
Lauric acid ($C_{12:0}$)	0.11	1.03	0.25
Myristic acid ($C_{14:0}$)	0.94	0.79	1.02
Pentadecanoic acid ($C_{15:0}$)	0.23	0.39	0.19
Palmitic acid ($C_{16:0}$)	13.35	12.67	14.71
Palmitoleic acid ($C_{16:1}$)	4.84	4.16	5.12
Heptadecanoic acid ($C_{17:0}$)	*tr*	–	–
Stearic acid ($C_{18:0}$)	3.72	3.94	3.97
Oleic acid ($C_{18:1}$)	6.07	6.87	8.11
Linoleic acid ($C_{18:2}$)	67.29	64.38	61.82
Linolenic acid ($C_{18:3}$)	1.52	2.03	2.45
Arachidic acid ($C_{20:0}$)	0.92	1.95	1.62
Total saturation	20.28	22.56	22.5
Total unsaturation	79.72	77.44	77.50
Saturation/unsaturation	0.25	0.29	0.29
L/O[a]	11.09	9.37	7.62

[a] L/O: linoleic acid-oleic acid ratio; *tr* = trace (*tr* < 0.01%).

useful for the taxonomical differentiation between species of the same genus.

Unsaturated fatty acids increase nutritional values of mushrooms. The mushrooms which contain high concentration of polyunsaturated fatty acids are recommended to people who are on a diet due to their high blood cholesterol. Polyunsaturated fatty acid concentrations of *A. bitorquis* and *A. essettei* were close to that of *A. bisporus* (Table 2). These results show that *A. bitorquis* and *A. essettei* are as valuable as *A. bisporus*.

3.3. Bioassays

3.3.1. Antioxidant activity

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 behavior, 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 antioxidant capacity were used. Since, the phenolic compounds such as flavonoids, phenolic acids, and tannins are known as powerful chain breaking antioxidants and may contribute directly to antioxidative action (Shahidi and Wanasundara, 1992), total phenolic content and total flavonoid contents of the mushrooms were also evaluated as pyrocatechol and quercetin equivalents, respectively (Table1).

As expected, methanol and ethyl acetate extracts of the mushrooms were found to be richer in the content of phenolics and flavonoids than their hexane extracts. Among the three species, methanol extract of *A. bisporus* had a higher phenolic content (59.87 ± 0.55 μg PEs/mg extract) than others, while the least phenolics containing one was the hexane extract of the same species (9.76 ± 1.00 μg PEs/mg extract). In other words, when 100 g mushroom consumed 383.80 μg pyracotechol equivalent phenolics have been taken from *A. bisporus* as well as 315.69 μg from *A. bitorquis* and 349.55 μg from *A. essettei*. Thus, it seems that *A. bisporus* has the richest phenolic content. Nevertheless, the phenolic content of the other two mushrooms are close to that of *A. bisporus*.

The most flavonoid rich extract was found to be methanol extract of *A. bisporus* (85.45 ± 0.36 μg QEs/mg extract), while hexane extract of *A. bitorquis* (4.34 ± 0.36 μg QEs/mg extract) was the poorest. On the other hand, when the flavonoid content of the mushroom species at 100 g consumption is considered, the flavonoid content of *A. essettei* (668.68 μg quercetin equivalents/100 g mushroom) was found to be the richest, followed by *A. bisporus* (544.27 μg PEs) and *A bitorquis* (378.90 μg).

Table 3 shows the antioxidant activity of the extracts of three mushrooms tested, which were determined by the β-carotene-linoleic acid assay for lipid peroxidation activity, and DPPH and ABTS assays for radical scavenging activity by comparing with α-tocopherol, BHT and quercetin. The results were given as half maximum effective concentration (EC_{50}). All the species proved to have antioxidant activity, but none of them demonstrated better activity than the antioxidant standards. In β-carotene-linoleic acid, methanol extracts of *A. bisporus* (EC_{50}: 293.78 ± 0.76 μg/mL) showed the highest lipid peroxidation inhibition activity among all the tested extracts, followed by the methanol extract of *A. essettei* (EC_{50}: 296.92 ± 0.50 μg/mL) and ethyl acetate extract of *A. bitorquis* (EC_{50}: 378.48 ± 0.59 μg/mL). β-carotene-linoleic acid method reveals the level of inhibition of lipid peroxidation, and it is important to understand the type of antioxidant giving H**.**radicals to the medium to terminate the radical degradation (Huang et al., 2005). This method is also important to understand the antioxidants which scavenge singlet oxygen causing radicals in lipids.

The ethyl acetate extract of *A. bitorquis* was found to be the most active extract in DPPH and in ABTS assays, demonstrating 0.395 ± 0.17, and 0.087 ± 0.17 mg/mL EC_{50} values, respectively. As results of lipid peroxidation inhibitory activity and antiradical activities, the ethyl acetate extract of *A. bitorquis* was found to be the most active mushroom among the others. However, the hexane extract of *A. bisporus* indicated the best metal chelating activity among the others tested, even it was better than quercetin (Table 3). In fact, hexane extracts of all species showed better activity than quercetin. As it is known transition ions, such as ferrous and cupric, accelerate lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals via the Fenton reaction

Table 3
Antioxidant activity of various extracts of the three *Agaricus* species by the β-carotene-linoleic acid, $DPPH^{\cdot}$, $ABTS^{\cdot+}$, and metal chelating assays.[a]

Samples	β-carotene-linoleic acid assay EC_{50} (μg/mL)	$DPPH^{\cdot}$ assay EC_{50} (mg/mL)	$ABTS^{\cdot+}$ assay EC_{50} (mg/mL)	Fe^{2+}-Ferrin assay EC_{50} (μg/mL)
A. bisporus				
Hexane extract	928.99 ± 2.00	2.685 ± 0.47	1.135 ± 0.15	**34.62 ± 0.65**
Ethyl acetate extract	312.83 ± 0.90	1.167 ± 0.13	0.516 ± 0.07	207.13 ± 0.87
Methanol extract	**293.78 ± 0.76**	**0.988 ± 0.13**	**0.241 ± 0.07**	310.00 ± 0.87
A. bitorquis				
Hexane extract	997.93 ± 1.90	3.931 ± 0.46	1.631 ± 0.33	**62.61 ± 0.71**
Ethyl acetate extract	**178.48 ± 0.09**	**0.395 ± 0.17**	**0.087 ± 0.00**	290.00 ± 0.87
Methanol extract	510.79 ± 1.56	0.590 ± 0.31	0.158 ± 0.03	261.74 ± 1.13
A. essettei				
Hexane extract	719.87 ± 0.95	7.719 ± 0.81	1.386 ± 0.11	**67.93 ± 1.18**
Ethyl acetate extract	498.35 ± 0.70	1.211 ± 0.05	**0.287 ± 0.03**	276.85 ± 1.05
Methanol extract	**296.92 ± 0.50**	**0.921 ± 0.07**	0.347 ± 0.02	264.04 ± 1.11
Standards				
α-Tocopherol [b]	2.10 ± 0.09	7.31 ± 0.17[c]	4.31 ± 0.10[c]	nt
BHT [b]	1.34 ± 0.09	45.37 ± 0.47[c]	4.10 ± 0.06[c]	nt
Quercetin [b]	1.81 ± 0.11	2.07 ± 0.10[c]	1.18 ± 0.03[c]	250.09 ± 0.87
EDTA [b]	nt	nt	nt	6.50 ± 0.07

nt = not tested.

[a] EC_{50} values represent the means ± standard deviation of three parallel measurements ($p < 0.05$), the values written in bold show the highest activity in its own group for each assay.

[b] Reference compounds.

[c] Positive controls were given in μg/mL concentration.

Table 4
Cupric reducing antioxidant capacity (CUPRAC) of various extracts of the three *Agaricus* species[a], BHT and -tocopherol.[a]

Sample	0.00 μg	100 μg	200 μg	400 μg	800 μg
A. bisporus					
Hexane extract	0.07 ± 0.01	0.42 ± 0.01	0.55 ± 0.02	0.77 ± 0.01	1.19 ± 0.03
Ethyl acetate extract	0.07 ± 0.01	0.22 ± 0.01	0.34 ± 0.01	0.55 ± 0.02	1.02 ± 0.05
Methanol extract	0.07 ± 0.01	0.19 ± 0.00	0.26 ± 0.01	0.37 ± 0.01	0.73 ± 0.01
A. bitorquis					
Hexane extract	0.07 ± 0.01	0.27 ± 0.01	0.37 ± 0.01	0.64 ± 0.01	1.05 ± 0.01
Ethyl acetate extract	0.07 ± 0.01	0.53 ± 0.02	0.66 ± 0.01	1.12 ± 0.00	1.89 ± 0.02
Methanol extract	0.07 ± 0.01	0.18 ± 0.03	0.26 ± 0.01	0.48 ± 0.04	0.81 ± 0.08
A. essettei					
Hexane extract	0.07 ± 0.01	0.20 ± 0.01	0.38 ± 0.05	0.66 ± 0.07	0.93 ± 0.06
Ethyl acetate extract	0.07 ± 0.01	0.15 ± 0.00	0.26 ± 0.03	0.43 ± 0.05	0.84 ± 0.10
Methanol extract	0.07 ± 0.01	0.13 ± 0.01	0.20 ± 0.01	0.31 ± 0.02	0.58 ± 0.03
Standards					
BHT[b]	0.07 ± 0.01	3.51 ± 0.01	3.73 ± 0.01	3.81 ± 0.01	3.99 ± 0.01
α-Tocopherol[b]	0.07 ± 0.01	1.85 ± 0.01	2.22 ± 0.01	2.85 ± 0.01	3.21 ± 0.01

[a] Values expressed as absorbance at 450 nm are means ± standard deviation of three parallel measurements. ($p < 0.05$).
[b] Reference compounds.

Table 5
Acetylcholinesterase and butyrylcholinesterase inhibitory activities of various extracts of the three *Agaricus* species.[a]

Sample	AChE assay EC_{50} (mg/mL)	BChE assay EC_{50} (mg/mL)
A. bisporus		
Hexane extract	–	**0.188 ± 0.001**
Ethyl acetate extract	**2.277 ± 0.01**	0.393 ± 0.001
Methanol extract	–	1.289 ± 0.01
A. bitorquis		
Hexane extract	3.352 ± 0.01	0.066 ± 0.00
Ethyl acetate extract	**0.745 ± 0.002**	**0.046 ± 0.00**
Methanol extract	1.791 ± 0.01	0.411 ± 0.03
A. essettei		
Hexane extract	1.093 ± 0.01	**0.045 ± 0.00**
Ethyl acetate extract	**0.918 ± 0.002**	0.756 ± 0.001
Methanol extract	3.032 ± 0.01	0.884 ± 0.002
Standard		
Galantamine[b]	0.005 ± 0.00	0.050 ± 0.00

– = not active.
[a] EC_{50} values represent the means ± standard deviation of three parallel measurements ($p < 0.05$), the values written in bold show the highest activity in its own group for both assays.
[b] Standard drug.

(Halliwell and Gutteridge, 1984). Therefore, chelating agents known as secondary antioxidants are important to retard the radicalic degradation.

Table 4 shows the cupric reducing antioxidant capacity which was 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. The difference between the extracts and control was statistically significant ($p < 0.05$). Activity increases with increasing the amount of the extracts. The ethyl acetate extract of *A. bitorquis* was also found to be the best active extract in this assay.

Accordingly one says from the results that *A. bitorquis* showed the best antioxidant activity among the others.

3.3.2. Acetylcholinesterase and butyrylcholinesterase inhibitory activity

Table 5 shows the acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities of the extracts, compared with those of galantamine used as a standard drug for the treatment of mild Alzheimer's disease. Against AChE enzyme, ethyl acetate extracts of the three species were found to be active. The most

Table 6
Antimicrobial activity of the methanol extracts of the three *Agaricus* species (200 μg/disk) against the bacterial strains tested by disk-diffusion method.

Bacteria	Inhibition zone diameter (mm)								
	A. bisporus	*A. bitorquis*	*A. essettei*	N	A	P	G	O	T
Pseudomonas aeruginosa NRRL B-23	–	–	–	nt	nt	nt	16	nt	8
Salmonella enteritidis RSKK 171	–	–	–	nt	–	nt	nt	nt	12
Escherichia coli ATCC 35218	–	–	–	nt	10	11	nt	nt	8
Morganella morganii	–	–	–	nt	nt	nt	–	nt	–
Yersinia enterecolitica RSKK 1501	–	16 ± 0	–	nt	20	18	nt	nt	7
Klebsiella pneumoniae ATCC 27736	–	14 ± 0	–	nt	–	nt	nt	nt	5
Proteus vulgaris RSKK 96026	–	16 ± 0	–	nt	–	nt	nt	nt	16
Staphylococcus aureus ATCC 25923	–	12 ± 0	11 ± 0	nt	nt	31	nt	21	20
Staphylococcus aureus ATCC 12598	–	7 ± 1	9 ± 1	nt	nt	28	nt	18	21
Micrococcus luteus NRRL B-4375	20 ± 1	21 ± 1	20 ± 1	nt	30	31	nt	22	19
Micrococcus flavus	22 ± 0	20 ± 0	20 ± 0	nt	29	31	nt	24	20
Bacillus subtilis ATCC 6633	19 ± 1	18 ± 0	19 ± 0	nt	nt	12	nt	8	17
Bacillus cereus RSKK 863	21 ± 0	19 ± 0	19 ± 0	nt	nt	22	nt	14	19
Candida albicans	16 ± 0	18 ± 1	10 ± 1	19	nt	nt	nt	nt	nt
Candida tropicalis	11 ± 0	14 ± 0	11 ± 1	19	nt	nt	nt	nt	nt

N: Nystatin (100 U), A: Ampicillin (10 μg), P: Penicillin (10 U), G: Gentamicin (10 μg), O: Oxacillin (1 μg), T: Tetracycline (30 μg), nt: Not tested, –: No inhibition.

active one was ethyl acetate extract of *A. bitorquis* demonstrating a 0.745 ± 0.002 mg/mL EC_{50} value. Against BChE enzyme, the most active extract was found to be ethyl acetate extract of *A. bitorquis* (EC_{50}: 0.046 ± 0.000 mg/mL), as well. Hexane extract of *A. bisporus* demonstrated the best activity among its studied extracts with an EC_{50} of 0.188 ± 0.001 mg/mL. Generally, the extracts exhibited better activity against BChE enzyme. Moreover, three extracts namely; hexane extracts of *A. bisporus* and *A. bitorquis* and ethyl acetate extract of *A. bitorquis* indicated a competitive butyrylcholinesterase inhibitory activity with that of galantamine.

3.3.3. Antimicrobial activity

The antimicrobial effect of methanol extract of *Agaricus* species was tested against six species of Gram-positive bacteria, seven species of Gram-negative bacteria and two species of yeast. As summarized in Table 6, the inhibition zones of *Agaricus* species which were obtained against all test microorganisms were in the range of 7–22 mm. As it is seen in Table 6, while methanol extracts from both *A. bisporus* and *A. essettei* did not show any antibacterial activity against Gram-negative bacteria at test concentration, Gram-positive bacteria were inhibited by these extracts. But, only *A. bitorquis* extract has some effects against three of Gram-negative bacteria namely *Y. enterecolitica* RSKK 1501, *K. pneumoniae* ATCC 27736 and *P. vulgaris* RSKK 96026. The results in Table 6 revealed that Gram-positive bacteria were more sensitive to the mushroom extracts than Gram-negative bacteria and the diameters of inhibition zones of the extracts against gram-positive bacteria were found to be very similar in the three *Agaricus* extracts. In general, the methanol extract of *A. bitorquis* demonstrated the growth of both the Gram-positive and the Gram-negative bacteria with the exception of four gram-negative bacteria namely *P. aeruginosa, S. enteritidis, E. coli* and *M. morganii*. The highest inhibitory activity was determined against *Micrococcus* species, especially against *M. flavus* (22 ± 0 mm, inhibition zone diameter) by *A. bisporus* extract. On the other hand, the weakest inhibitory activity was determined against *S. aureus* ATCC 12598 (7 ± 1 mm, inhibition zone diameter). Yeast species *Candida albicans* and *C. tropicalis* were also sensitive to the methanol extracts of the three mushrooms.

4. Conclusion

The results presented in this study are the first information on the antioxidant, anticholinesterase and antimicrobial activities of *Agaricus bitorquis* and *A. essettei*. The fatty acid composition and iron content of these species were also studied for the first time. In addition, the results of these two species were also compared to that of *A. bisporus* which is mostly consumed mushroom in the world.

Among the tested three species, the iron content of the *A. bitorquis* and *A. essettei* found to be 12–14 folds higher than *A. bisporus*. Since, the iron content of the mushroom gives its one of the nutritional value, *A. bitorquis* and *A. essettei* should be considered as valuable mushrooms.

The phenolic and flavonoid contents and antioxidant potential increases the nutritional value of the food, as well. According to these results, when 100 g of *A. bisporus* mushroom is consumed, 383.83 mg PEs phenolic compounds will be taken by the person. Similarly, the results for the other two *Agaricus* species were also found to be close to that of *A. bisporus*. As for the flavonoid content, when 100 g of *A. bisporus* is consumed, 544.27 mg QEs flavonoid compound will be taken by the person. *A. essettei* is found to be richer flavonoid content than *A. bisporus*.

Among the tested *Agaricus* species particularly the ethyl acetate extract of *A. bitorquis* demonstrated the highest antioxidant activity in five assays. The same mushroom also showed the best acetylcholinesterase and butyrylcholinesterase inhibitory activity, as well. Moreover, it exhibited antimicrobial activity against more bacteria particularly against gram positive bacteria. Briefly, the fact is that *A. bitorquis* demonstrates the best antioxidant, the best anticholinesterase and the best antimicrobial activities among the other two mushrooms tested in this study. Demonstrating these activities enhances its nutritional value.

In conclusion, the results showed the antioxidant, anticholinesterase and antimicrobial importance of *Agaricus* species tested, and they are commonly consumed as edible mushrooms in Anatolia as well as in the whole world with their delicious taste. Thus, *Agaricus* species particularly *A. bitorquis* may protect people against lipid peroxidation and free radical damage, as well as against amnesia. Its extracts will probably be used for the development of safe food products and additives. However, further studies, especially *in vivo* activity tests on extracts and isolated constituents are needed.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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