Food and Chemical Toxicology 48 (2010) 1230–1233

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Evaluation of metal concentration and antioxidant activity of three edible mushrooms from Mugla, Turkey

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ARTICLE INFO

Article history:
Received 30 September 2009
Accepted 16 December 2009

Keywords:
Amanita caesarea
Clitocybe geotropa
Leucoagaricus pudicus
Metal concentration
Antioxidant activity

ABSTRACT

This study is designed for the determination of metal concentrations, antioxidant activity potentials and total phenolics of *Amanita caesarea*, *Clitocybe geotropa* and *Leucoagaricus pudicus*. Concentrations of four heavy metals (Pb, Cd, Cr, Ni) and five minor elements (Zn, Fe, Mn, Cu, Co) are determined. In the case of *A. caesarea*, Cr and Ni concentrations are found in a high level. Concentrations of the metals are found to be within safe limits for *C. geotropa*. In β-carotene/linoleic acid test, *L. pudicus* showed the highest activity potential. In DPPH system, *A. caesarea* showed 79.4% scavenging ability. Additionally, reducing power and chelating capacity of the mushrooms increased with concentration. The strongest super-oxide anion scavenger was *A. caesarea*. In the case of total phenolics, *L. pudicus* found to have the highest content.

1. Introduction

Mushrooms have been long known to accumulate high levels of heavy metals (Cocchi and Vescovi, 1997–2005; Cocchi et al., 2002). For instance, radioactive heavy metals in fruit bodies of edible mushrooms were already reported in the 1960s (Grüter, 1964). Several actors may affect the accumulation and concentration of trace elements and heavy metals in mushrooms. Concentrations of the elements are generally assumed to be species-dependent, but substrate composition is also considered to be an important factor (Stijve et al., 2004).

Several studies have been carried out to detect and explain the presence and distribution of several heavy metals in edible mushrooms, in particular arsenic, cadmium, caesium, copper, iron, lead, manganese, mercury, selenium, rubidium, and zinc (Blanusa et al., 2001; Falandysz et al., 2004; Stijve, 2001; Svoboda and Kalac, 2003).

Oxygen-centered free radicals and other reactive oxygen species are continuously produced *in vivo*. Although almost all organisms are well-protected against free-radical damage by enzymes such as super-oxide dismutase and catalase or by compounds such as ascorbic acid, tocopherols, and glutathione, these systems are insufficient to prevent damage entirely. Therefore, an antioxidant supplement in the human diet is important to prevent or reduce oxidative damage (Yang et al., 2002). Mushrooms are widely recognized as a functional food and as a source of various physiologically active compounds. Recently, certain mushrooms have been found to possess antioxidant activity (Cheung et al., 2003; Ferreira et al., 2007; Mau et al., 2002; Yang et al., 2002).

The aim of present work is to evaluate the antioxidant potentials and metal contents of the methanol extracts of *Amanita caesarea* (Scop.: Fr.) Pers., *Clitocybe geotropa* (Bull.: Fr.) Quél., and *Leucoagaricus pudicus* (Bull.) Bon by five different antioxidant test systems namely; β-carotene/linoleic acid, DPPH, reducing power, chelating effect and super-oxide anion radical scavenging, in addition to their total phenolic contents.

2. Materials and methods

2.1. Chemicals

Potassium ferricyanide, ferrous chloride, ferric chloride, Folin–Ciocalteu's reagent (FCR), methanol, and trichloroacetic acid (TCA) were obtained from E. Merck (Darmstadt. Germany). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT) and α-tocopherol were obtained from Sigma Chemical Co. (Sigma–Aldrich GmbH, Sternheim, Germany). All other chemicals and solvents were of analytical grade.

2.2. Mushrooms

Fruiting bodies of edible mushrooms were collected in 2004 in Mugla, Turkey.

For the extraction procedure, the air-dried fruiting bodies of the mushroom samples (10 g) were extracted by using a Soxhlet extractor for 5 h with methanol and then filtered. After that, methanolic extracts were evaporated at 40 °C to dryness and kept in the dark at +4 °C until tested. Extract yields of the mushrooms were 32.7%, 42.4% and 40.2% (w/w), respectively.

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doi:10.1016/j.fct.2009.12.033

2.3. Determination of metal concentration

For trace metal analysis, samples were cleaned, cut and dried at 105 °C for 24 h. Dried samples were homogenized using an agate homogenizer and stored in pre-cleaned polyethylene bottles. Deionized water (18.2 MΩ cm^{-1}) from a Milli-Q system (Human Power I Plus, Korea) was used to prepare all of the aqueous solutions. Mineral acids and oxidants (HNO_3 and H_2O_2) were of the highest quality (Merck, Darmstadt, Germany). All of the plastics and glasswares were cleaned by soaking in a 10% nitric acid solution overnight and then rinsed with deionized water. For the elemental analysis, a Perkin–Elmer Optima 2000 ICP-OES was used.

For digestion, CEM Mars 5 microwave closed system was used. Samples (0.25 g) were digested with 9 ml of HNO_3 (65%) and 1 ml of H_2O_2 (30%) in microwave digestion system for 7 min and finally diluted to 50 ml with deionized water. A blank digestion was carried out in a similar way. For the digestion, temperature of the microwave system was increased up to 180 °C in 5 min and kept at this level for 2 min. This procedure was carried out twice (Yamac et al., 2007).

2.4. Total antioxidant activity by the β-carotene–linoleic acid method

In this assay antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Dapkevicius et al., 1998). A stock solution of β-carotene–linoleic acid mixture was prepared as following: 0.5 mg β-carotene was dissolved in 1 ml of chloroform (HPLC grade). Twenty-five microliters of linoleic acid and 200 mg Tween 40 was added. Chloroform was completely evaporated using a vacuum evaporator. Then 100 ml of oxygenated distilled water was added with vigorous shaking; 4.6 ml of this reaction mixture was dispersed to test tubes and 0.4 ml of various concentrations (2.5–10.0 mg ml^{-1}) of the extracts in methanol were added and the emulsion system was incubated for up to 2 h at 50 °C. The same procedure was repeated with the positive control BHT, α-tocopherol, quercetin and a blank. After this incubation period, absorbance of the mixtures was measured at 490 nm. Measurement of absorbance was continued until the color of β-carotene disappeared. The bleaching rate (R) of β-carotene was calculated according to Eq. (1).

$$R = \ln(a/b)/t \quad (1)$$

where, ln = natural log, a = absorbance at time 0, b = absorbance at time t (30, 60, 90, 120 min) (Cheung et al., 2003). The antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control using Eq. (2).

$$AA = \left[(R_{Control} - R_{Sample})/R_{Control}\right] \times 100 \quad (2)$$

Antioxidative activities of the extracts were compared with those of BHT, α-tocopherol and quercetin at 0.5 mg ml^{-1} and blank consisting of only 0.4 ml methanol.

2.5. Scavenging effect on 1,1-diphenyl-2-picrylhydrazyl

The hydrogen atoms or electrons donation ability of the corresponding extracts and some pure compounds was measured from the bleaching of purple coloured methanol solution of DPPH. This spectrophotometric assay uses the stable radical diphenylpicrylhydrazyl (DPPH) as a reagent (Burits and Bucar, 2000; Cuendet et al., 1997). One milliliter of various concentrations (2–20 mg ml^{-1}) of the extracts in methanol was added to 4 ml of a 0.004% (w/v) methanol solution of DPPH. After a 30 min incubation period at room temperature the absorbance was read against a blank at 517 nm. Inhibition of free-radical DPPH in percent (I %) was calculated in following way:

$$I\,\% = 100 \times (A_{Control} - A_{Sample})/A_{Control} \quad (3)$$

where, $A_{Control}$ is the absorbance of the control reaction (containing all reagents except the test compound), and A_{Sample} is the absorbance of the test compound. BHT, quercetin and α-tocopherol were used as a control.

2.6. Reducing power

The reducing power was determined according to the method of Oyaizu (1986). Each of the extracts (2–20 mg ml^{-1}) in methanol (1 ml) were mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. Reaction mixture was incubated at 50 °C for 20 min and then 2.5 ml of 10% trichloroacetic acid was added. The mixture was centrifuged at 200g (MSE Mistral 2000, London, UK) for 10 min. The upper layer (2.5 ml) was mixed with 2.5 ml of deionized water and 0.5 ml of 0.1% ferric chloride, and the absorbance was measured at 700 nm against a blank. BHT, ascorbic acid and α-tocopherol were used as a control.

2.7. Chelating effects on ferrous ions

The chelating effect was determined according to the method of Dinis et al. (1994). Briefly, 1 ml of the various concentrations (1–4 mg ml^{-1}) of extracts in methanol were added in 2 mM $FeCl_2$ solution (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml) and total volume was adjusted to 5 ml with methanol. Then, the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was measured spectrophotometrically at 562 nm. The inhibition percentage of ferrozine–Fe^{2+} complex formation was calculated by using the formula given below:

$$\text{Metal chelating effect}\ (\%) = \left[(A_{Control} - A_{Sample})/A_{Control}\right] \times 100 \quad (4)$$

where $A_{Control}$ is the absorbance of control and A_{Sample} is the absorbance of the compounds tested. EDTA was used as the control agent.

2.8. Super-oxide anion radical scavenging activity

Measurement of super-oxide anion scavenging abilities of the extracts was based on a method described by Liu et al. (1991). Super-oxide radicals were generated in 3 ml of Tris–HCl buffer (16 mM, pH 8.0) containing 1 ml of NBT (50 μM) solution, 1 ml of NADH (78 μM) solution and 1 ml of extract solution (4 and 10 mg ml^{-1}) in water. The reaction was started by adding 1 ml of PMS solution (10 μM) to the mixture. The reaction mixture was incubated at 25 °C for 5 min and the absorbance at 560 nm was measured against blank samples. The inhibition percentage of super-oxide anion generation was calculated by using the following formula:

$$\%\ \text{Inhibition} = \left[(A_{Control} - A_{Sample})/A_{Control}\right] \times 100 \quad (5)$$

where, $A_{Control}$ is the absorbance of the control reaction (containing all reagents except the test compound), and A_{Sample} is the absorbance of the test compound. Quercetin was used as the control agent.

2.9. Determination of total phenolics

Phenolic contents of the methanol extracts were determined by employing the methods given in the literature (Chandler and Dodds, 1983; Slinkard and Singleton, 1977). One milliliter of extract solution containing 2 g extract was added to a volumetric flask. Then, 45 ml distilled water and 1 ml Folin–Ciocalteu reagent was added and flask was shaken vigorously. After 3 min, a 3 ml solution of Na_2CO_3 (2%) was added and the mixture was allowed to stand for 2 h with intermittent shaking. Absorbance was measured at 760 nm. The concentrations of phenolic compounds were calculated according to the following equation obtained from the standard pyrocatechol graph:

$$\text{Absorbance} = 0.00246\ \text{pyrocatechol}(\mu g) + 0.00325 \quad (R^2 :\ 0.9996) \quad (6)$$

3. Results and discussion

3.1. Metal concentration

Metal concentrations of the mushroom species presented here were determined via a microwave digestion system. By this experimental process, concentrations of four heavy metals (Pb, Cd, Cr, and Ni) and five elements (Zn, Fe, Mn, Cu, and Co) have been determined. Data obtained from the analysis have been shown in Table 1.

In the case of *A. caesarea*, iron was the most abundant element with a concentration value of 4660 mg kg^{-1} dry weight. This is followed by Mn and Zn, respectively. Among the elements tested, Co has the lowest concentration value. In the case of heavy metals, amounts of Cr and Ni were too close to each other and showed the highest concentrations for this mushroom. Additionally, amount of Cd was determined as 1.9 mg kg^{-1}.

As far as our literature survey could as certain, cadmium, arsenite and arsenate levels of *A. caesarea* has previously been evaluated (Cocchi et al., 2006; Slejkovec et al., 1997). According to Cocchi et al., Cd levels of *A. caesarea* exceeded the maximum amount recommended by WHO and the average amount of lead present in this species was, in general, below the maximum allowed concentration. As can be seen from Table 1, Cd levels of *A. caesarea* collected from Mugla-Turkey found to be within the safe limits. But the amounts of Cr and Ni force the critical limits arranged by WHO.

The levels of iron were also found to be the highest in *C. geotropa* and *L. pudicus*. As can be seen from Table 1, these species

Table 1
Metal concentrations of the mushroom species.[a]

Mushroom	Pb	Cd	Zn	Fe	Mn	Cu	Cr	Ni	Co
Amanita caesarea	5.0 ± 0.0[b]	1.9 ± 0.0	123.8 ± 0.4	4660.0 ± 14.0	166.8 ± 0.7	38.6 ± 1.4	16.4 ± 0.0	14.2 ± 0.1	2.8 ± 0.0
Clitocybe geotropa	3.2 ± 0.3	0.7 ± 0.0	130.4 ± 1.3	662.0 ± 7.0	35.2 ± 0.0	65.6 ± 3.5	7.2 ± 0.2	4.5 ± 0.0	0.5 ± 0.0
Leucoagaricus pudicus	4.0 ± 0.3	3.7 ± 0.0	139.4 ± 0.7	794.0 ± 16.0	34.4 ± 0.2	31.4 ± 1.2	3.4 ± 0.0	11.0 ± 0.1	1.2 ± 0.0

[a] mg kg^{-1}, Dry weight basis.
[b] Means ± S.D., $n = 5$.

showed a similar minor element concentration profile except Cu for *C. geotropa*.

In the case of heavy metals, Cr found to be the highest one for *C. geotropa* with a value of 7.2 mg kg^{-1}. This is followed by Ni and Pb. Levels of Co and Cd were found lower than 1.0 mg kg^{-1} for this mushroom.

Heavy metal concentrations of *C. geotropa*, have been investigated by Cocchi et al. (2006) and Yakiz et al. (2008). Based on the study reported by Yakiz et al. (2008); Ca, Cu, Fe, K, Mg, Mn, Na, P, and Zn levels had been found to be within the safe limits. Data given in this study is highly in agreement with those presented in this report.

It is extremely important to point out that, Ni level of *L. pudicus* found as 11.0 mg kg^{-1}. This is also the highest heavy metal concentration obtained from this mushroom. We could not reach any record for this species in the literature. Therefore, data given in this study could be assumed as the first report on *L. pudicus*.

3.2. Antioxidant activity

Among the methanolic extracts of the mushroom species evaluated here, *L. pudicus* showed the highest linoleic acid preventing capacity against the oxidative stress available in the media (Table 2). Antioxidant activity of this mushroom was found as 90.1% in the concentration value of 10.0 mg ml^{-1}. This is closely followed by *C. geotropa*. Linoleic acid preventing capacity of *A. caesarea* was determined as 79.6%.

The radical scavenging of mushrooms extracts was tested using a methanolic solution of the "stable" free-radical, DPPH. Unlike laboratory-generated free radicals such as the hydroxyl radical and super-oxide anion, DPPH has the advantage of being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition (Amarowicz et al., 2004). In this system, *A. caesarea* was able to scavenge the free-radical DPPH in the percentage of 79.4% at 20.0 mg ml^{-1} concentration (Table 3). Radical scavenging capacities of *C. geotropa* and *L. pudicus* found almost equal and 64% at the same concentration value.

In the present study, assay of reducing activity was based on the reduction of Fe^{3+}/ferricyanide complex to the ferrous form in presence of reductants (antioxidants) in the tested samples. The Fe^{2+} was then monitored by measuring the formation of Perl's Prussian blue at 700 nm (Oyaizu, 1986). Table 4 shows the reducing power of mushroom methanolic extracts as a function of their concentration. The reducing power of the mushroom methanolic extracts increased with concentration. At 20.0 mg ml^{-1} concentration, the absorbance values were higher than 1.0 for the all extracts. According to the results, the most active mushroom was *A. caesarea* with an absorbance value of 1.5. At this concentration value, this mushroom was followed by *L. pudicus* and *C. geotropa*, respectively.

Metal ions can initiate lipid peroxidation and start a chain reaction that leads to the deterioration of food (Gordon, 1990). The catalysis of metal ions also correlates with incidents of cancer and arthritis (Halliwell et al., 1995). Ferrous ions, the most effective pro-oxidants, are commonly found in food systems (Yamaguchi et al., 1998). In the present study, the chelating ability of the mushroom extracts toward ferrous ions was investigated. Table 5 shows the chelating effects of the mushroom species compared with EDTA as standard on ferrous ions. As can be seen from the table, chelating capacity of the extracts was increased with the increasing concentration. Except *C. geotropa*, chelating effect of the methanol extracts was higher than 90% at 4.0 mg ml^{-1} concentration. The most active mushroom was *L. pudicus* with a value of 99.0%.

Super-oxide anion radical is normally formed first in cellular oxidation reactions. Although it is not highly reactive, it can produce hydrogen peroxide and hydroxyl radical through dismutation and other types of reaction and is the source of free radicals formed *in vivo*. Not only super-oxide anion radical but also its derivatives are cell-damaging, which can cause damage to DNA and membrane of cell. Therefore, it is of great important to scavenge super-oxide anion radical (Macdonald et al., 2003).

Table 6 shows the percentage inhibition of super-oxide anion radicals by the mushroom species at different concentrations (0.2–10.0 mg ml^{-1}). According to the results, the strongest super-oxide anion scavenger was *A. caesarea* at 10.0 mg ml^{-1} concentra-

Table 2
Antioxidant activity (%) of the methanolic extracts of mushrooms in β-carotene–linoleic acid test system.[a]

Mushroom	Sample concentration (mg ml^{-1})			
	0.5	2.5	5.0	10.0
Amanita caesarea	–	70.1 ± 1.2	73.9 ± 1.5	79.6 ± 1.0
Clitocybe geotropa	–	61.3 ± 1.9	83.2 ± 0.9	86.1 ± 0.9
Leucoagaricus pudicus	–	81.8 ± 4.8	88.6 ± 1.0	90.1 ± 2.2
BHT	96.0 ± 0.6	–	–	–
α-Tocopherol	96.4 ± 0.3	–	–	–
Quercetin	98.4 ± 0.6	–	–	–

[a] Values expressed are means ± S.D. of three parallel measurements.

Table 3
Scavenging effect (%) of mushroom species on 1,1-diphenyl-2-picrylhydrazyl.[a]

Mushroom	Sample concentration (mg ml^{-1})				
	0.1	2	4	8	20
Amanita caesarea	–	9.1 ± 1.4	23.3 ± 1.1	43.3 ± 3.8	79.4 ± 1.4
Clitocybe geotropa	–	8.4 ± 0.1	19.4 ± 1.4	33.2 ± 0.8	64.8 ± 1.9
Leucoagaricus pudicus	–	6.7 ± 0.6	16.7 ± 1.1	30.3 ± 1.4	64.6 ± 0.8
BHT	30.8 ± 0.3	–	–	–	–
α-Tocopherol	52.9 ± 2.2	–	–	–	–
Quercetin	95.9 ± 0.5	–	–	–	–

[a] Values expressed are means ± S.D. of three parallel measurements.

Table 4
Reducing power (absorbance of 700 nm) of mushroom species.[a]

Mushroom	Sample concentration (mg ml^{-1})				
	0.2	2	4	8	20
Amanita caesarea	–	0.3 ± 0.0	0.5 ± 0.0	0.7 ± 0.1	1.5 ± 0.1
Clitocybe geotropa	–	0.3 ± 0.0	0.4 ± 0.0	0.7 ± 0.0	1.2 ± 0.2
Leucoagaricus pudicus	–	0.3 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	1.3 ± 0.0
BHT	0.8 ± 0.0	–	–	–	–
α-Tocopherol	0.5 ± 0.0	–	–	–	–
Ascorbic acid	1.2 ± 0.1	–	–	–	–

[a] Values expressed are means ± S.D. of three parallel measurements.

Table 5
Chelating effect (%) of mushroom species.[a]

Mushroom	Sample concentration (mg ml^{-1})			
	0.25	1	2	4
Amanita caesarea	–	60.1 ± 2.7	74.1 ± 3.3	94.1 ± 0.9
Clitocybe geotropa	–	28.0 ± 0.2	37.2 ± 3.8	43.8 ± 0.6
Leucoagaricus pudicus	–	88.0 ± 1.1	97.6 ± 0.4	99.0 ± 0.1
EDTA	99.4 ± 0.1	–	–	–

[a] Values expressed are means ± S.D. of three parallel measurements.

Table 6
Superoxide anion radical scavenging effect (%) of mushroom species.[a]

Mushroom	Sample concentration (mg ml^{-1})		
	0.2	4	10
Amanita caesarea	–	45.4 ± 1.7	61.1 ± 2.4
Clitocybe geotropa	–	22.8 ± 1.0	44.3 ± 1.0
Leucoagaricus pudicus	–	32.0 ± 0.9	45.0 ± 2.2
Quercetin	76.7 ± 0.9	–	–

[a] Values expressed are means ± S.D. of three parallel measurements.

tion. Scavenging capacities of *C. geotropa* and *L. pudicus* found almost equal.

As far as our literature survey could as certain, there is no report on these mushroom species in the literature. Therefore, data given for the mushrooms here could be assumed as the first report on this topic.

3.3. Assay for total phenolics

Phenolic compounds such as flavonoids, phenolic acids, and tannins are considered to be major contributors to the antioxidant capacity of plants. These antioxidants also possess diverse biological activities, such as anti-inflammatory, anti-atherosclerotic and anti-carcinogenic activities. These activities may be related to their antioxidant activity (Chung et al., 1998). Thus, the total phenolic and flavonoid contents of the mushrooms was also evaluated.

In this assay, *L. pudicus* (2.2 μg pyrocatechol equivalents/mg extract) found to have the highest phenolic content among the mushroom species evaluated. This is closely followed by *C. geotropa* (2.1 μg pyrocatechol equivalents/mg extract) and *A. caesarea* (1.9 μg pyrocatechol equivalents/mg extract). It is extremely important to point out that, data obtained from this part is especially shows a correlation with those obtained from the β-carotene/linoleic acid test system.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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