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Antioxidant properties of various extracts from *Hypsizigus marmoreus*

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Abstract

Hypsizigus marmoreus (Peck) Bigelow (Tricholomataceae) is an edible mushroom currently available in Taiwan. The ethanolic, cold water and hot water extracts were prepared and their antioxidant properties studied. At 5 mg/ml, various extracts exhibited moderate antioxidant activities of 38.6–65.2% and their EC_{50} values were 3.74–6.59 mg/ml. At 5 mg/ml, the cold water extract showed a high reducing power of 0.99. With regard to the scavenging ability on 1,1-diphenyl-2-picrylhydrazyl radicals, extracts were effective in the order: ethanolic > hot water ≫ cold water extracts. With regard to the effect on hydroxyl radicals, the cold water extract was more effective than the hot water extract. EC_{50} values for chelating ability on ferrous ions for ethanolic, cold water and hot water extracts were 3.19, 0.37 and 0.40 mg/ml, respectively whereas values for chelating ability on cupric ions were 4.42–6.63 mg/ml. Total phenols were the major naturally occurring antioxidant components found and were in the order: cold water > hot water > ethanolic extracts. Based on EC_{50} values, the various extracts from *H. marmoreus* were effective antioxidants.

Keywords: *Hypsizigus marmoreus*; Antioxidant activity; Reducing power; Scavenging ability; Chelating ability; Antioxidant components

1. Introduction

Hypsizigus marmoreus (Peck) Bigelow (Tricholomataceae), also called bunashimeji and hon-shimeji, is one of the most popular edible mushrooms in Japan. Recently, this mushroom is successfully cultivated and commercially available in Taiwan. The cultivation methods for this mushroom are parallel to that for *Agrocybe aegerita* (Leu, 1992). Fruit bodies of *H. marmoreus* are usually produced in polypropylene bottles containing sterilised sawdust. After the completion of vegetative mycelial growth, bottle lids are removed and the colonised substrate subjected to environmental conditions known to stimulate fruiting (Stamets, 1993). The mushroom is packaged by placing an entire cluster or several clusters into each overwrapped package.

Fruit bodies of this mushroom comprise long stipes and closed caps, which are spotted or marbled. The cap of the fruit bodies is dark tan and its colour turns to gray-brown then creamy brown when mature (Stamets, 1993). The mushroom has become increasingly popular in Taiwan, due to its mildly sweet nutty flavour and crunchy texture, and probably the existence of some physiologically beneficial components.

This mushroom possesses antitumour (Ikekawa et al., 1992; Ikekawa, 1995; Tsuchida, Aoyagi, Odani, Mita, & Isemura, 1995), antifungal and antiproliferative activities (Lam & Ng, 2001), and free-radical scavenging properties (Fu, Shieh, & Ho, 2002; Liu, Ooi, & Chang, 1997). The nutritional value and taste components of *H. marmoreus* have been thoroughly studied (Lee, 2003). Although studies have been focused mainly on the therapeutic effects of this mushroom, little information is available about its antioxidant properties. Our objective was to evaluate the antioxidant properties of ethanolic, cold water and hot water extracts from *H. marmoreus*, including antioxidant activity, reducing power, scavenging abilities on radicals and chelating abilities on metal ions. The contents of potential antioxidant components in these extracts were also determined.

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2. Materials and methods

2.1. Mushrooms

Fresh fruit bodies of *H. marmoreus* were obtained from the Hon-shimeji Farm, Kuohsing, Nantou County, Taiwan and then freeze-dried. For each of ethanolic, cold water and hot water extractions, three mushroom samples (~50 g) were randomly selected and prepared for analyses. A coarse powder (20 mesh) was obtained using a mill (Retsch ultracentrifugal mill and sieving machine, Haan, Germany). For ethanolic extraction, a subsample (10 g) was extracted by stirring with 100 ml of 95% ethanol at 25 °C at 20*g* for 24 h and filtering through Whatman No. 1 filter paper. The residue was then extracted with two additional 100 ml portions of ethanol, as described above. The combined ethanolic extracts were then rotary evaporated at 40 °C to dryness.

For cold water extraction, a subsample (10 g) was extracted by stirring with 100 ml of cold water at 25 °C at 20*g* for 24 h, centrifuging at 5000*g* for 15 min and then filtering through Whatman No. 1 filter paper. The residue was then extracted with two additional 100 ml portions of cold water, as described above. For hot water extractions, a subsample (10 g) was extracted by stirring with 100 ml of boiling water at 100 °C at 20*g* for 10 min, centrifuging at 5000*g* for 15 min and filtering through Whatman No. 1 filter paper. The residue was then extracted with two additional 100 ml portions of boiling water, as described above. The combined cold water or hot water extracts were freeze dried. The dried extract was used directly for analyses of antioxidant components or redissolved in water or ethanol to a concentration of 50 mg/ml and stored at 4 °C for further use.

2.2. Antioxidant activity

The antioxidant activity was determined by the conjugated diene method (Lingnert, Vallentin, & Eriksson, 1979). Each extract (0.1–20 mg/ml) in water or ethanol (100 μl) was mixed with 2 ml of 10 mM linoleic acid emulsion (pH 6.6) in test tubes and placed in darkness at 37 °C to accelerate oxidation. After incubation for 0 h or 15 h, 0.1 ml of each tube was mixed with 7 ml of 80% methanol in deionised water, and the absorbance of the mixture was measured at 234 nm against a blank in a Hitachi U-2001 spectrophotometer. The antioxidant activity was calculated as follows:

$$\text{Antioxidant activity } (\%) = [(\Delta A_{234} \text{ of control} - \Delta A_{234} \text{ of sample})/\Delta A_{234} \text{ of control}] \times 100.$$

A value of 100% indicates the strongest antioxidant activity. EC_{50} value (mg extract/ml) is the effective concentration at which the antioxidant activity was 50% and was obtained by interpolation from linear regression analysis. Ascorbic acid (Sigma Chemical Co., St. Louis, MO), butylated hydroxyanisole (BHA, Sigma) and α-tocopherol (Sigma) were used for comparison.

2.3. Reducing power

The reducing power was determined according to the method of Oyaizu (1986). Each extract (0.1–20 mg/ml) in water or ethanol (2.5 ml) was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6, Wako Pure Chemical Co., Osaka, Japan) and 2.5 ml of 1% potassium ferricyanide (Sigma), and the mixture was incubated at 50 °C for 20 min. After 2.5 ml of 10% trichloroacetic acid (w/v, Wako) were added, the mixture was centrifuged at 200*g* for 10 min. The upper layer (5 ml) was mixed with 5 ml of deionised water and 1 ml of 0.1% ferric chloride (Wako), and the absorbance was measured at 700 nm against a blank. A higher absorbance indicated a higher reducing power. EC_{50} value (mg extract/ml) is the effective concentration at which the absorbance was 0.5 for reducing power and was obtained by interpolation from linear regression analysis. Ascorbic acid, BHA and α-tocopherol were used for comparison.

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

Each extract (0.1–20 mg/ml) in water or ethanol (4 ml) was mixed with 1 ml of methanolic solution containing 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma) radicals, resulting in a final concentration of 0.2 mM DPPH. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank (Shimada, Fujikawa, Yahara, & Nakamura, 1992). EC_{50} value (mg extract/ml) is the effective concentration at which DPPH radicals were scavenged by 50% and was obtained by interpolation from linear regression analysis. Ascorbic acid, BHA and α-tocopherol were used for comparison.

2.5. Scavenging ability on hydroxyl radicals

The hydroxyl radical reacted with the nitrone spin trap 5,5-dimethyl-1-pyrroline-N oxide (DMPO, Sigma) and the resultant DMPO-OH adducts were detected with an electron paramagnetic resonance (EPR) spectrometer. The EPR spectrum was recorded 8 min after mixing 200 μl of each extract (0.1–20 mg/ml) in water or ethanol with 200 μl of 10 mM H_2O_2 (Merck, Darmstadt, Germany), 200 μl of 10 mM Fe^{2+} (Sigma) and 200 μl of 10 mM DMPO using a Bruker EMX-10 EPR spectrometer at the following settings: 3480 G magnetic field, 1.0 G modulation amplitude, 0.5 s time constant, and 200 s scan period (Shi, Dalal, & Jain, 1991). BHA was used as for comparison.

2.6. Chelating ability on ferrous ions

Chelating ability was determined according to the method of Dinis, Madeira, and Almeida (1994). Each extract (0.1–20 mg/ml) in water or ethanol (1 ml) was mixed with 3.7 ml of methanol and 0.1 ml of 2 mM ferrous chloride (Merck). The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine (Sigma). After 10 min at room temperature, the absorbance of the mixture was determined at 562 nm against a blank. EC_{50} value (mg extract/ml) is the effective concentration at which ferrous ions were chelated by 50% and was obtained by interpolation from linear regression analysis. Citric acid (Sigma) and ethylenediaminetetraacetic acid (EDTA, Sigma) were used for comparison.

2.7. Chelating ability on cupric ions

Chelating ability was determined according to the method of Shimada et al. (1992). To 2 ml of the mixture consisting of 5 mM hexamine (Wako), 5 mM potassium chloride and 1.5 mM cupric sulphate was added 2 ml of each extract (0.1–20 mg/ml) in water or ethanol and 200 μl of 1 mM tetramethyl murexide (TMM, Sigma). After 3 min at room temperature, the absorbance of the mixture was determined at 485 nm against a blank. A lower absorbance indicates a higher chelating power. EC_{50} value (mg extract/ml) is the effective concentration at which cupric ions were chelated by 50% and was obtained by interpolation from linear regression analysis. Citric acid and EDTA were used for comparison.

2.8. Determination of antioxidant components

Ascorbic acid was determined according to the method of Klein and Perry (1982). Each dried water or ethanol extract (100 mg) was extracted with 10 ml of 1% metaphosphoric acid (Union) for 45 min at room temperature and filtered through Whatman No. 1 filter paper. The filtrate (1 ml) was mixed with 9 ml of 2,6-dichloroindophenol (Sigma) and the absorbance was measured within 15 s at 515 nm against a blank. Content of ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid.

β-Carotene was extracted and analyzed as described by Rundhaug, Pung, Read, and Bertram (1988). Each dried extract (100 mg) was extracted with 1 ml of ethanol, 2 ml of *n*-hexane containing BHA (25 μg/ml) and 1 ml of deionised water at 20*g* for 45 min at room temperature, and then centrifuged at 400*g* for 10 min. After the removal of the *n*-hexane layer by N_2 gas, the volume was adjusted to 1 ml using *n*-hexane and filtered through a syringe-driven filter unit (13 mm, Millipore, Billerica, MA) using a 0.45-μm PVDF non-sterile filter paper. Immediately after filtration, the filtrate was injected onto a high performance liquid chromatograph (HPLC). The HPLC system consisted of a Shimadzu LC-10AT VP pump, a Shimadzu FCV-10AL VP controller, a Rheodyne 7725i injector, a 20-μl-sample loop, a Hitachi D-2500 chromato-integrator, a Shimadzu SPD-10A VP UV–vis detector, and a LiChrospher 100 RP-18 column (4.6 × 250 mm, 5 μm, Merck). The mobile phase was methanol/toluene, 3:1 (v/v), at a flow rate of 1.5 ml/min and UV detection was at 450 nm. Content of β-carotene was calculated on the basis of the calibration curve of authentic β-carotene (Sigma).

Tocopherols were extracted and analysed according to the method of Carpenter (1979). Each extract (100 mg) was suspended in 6 ml of pyrogallol (6% in 95% ethanol) and 4 ml of 60% aqueous potassium hydroxide solution, and the resulting mixture was saponified at 70 °C for 20 min. Deionised water (15 ml) was added and the mixture was extracted with 15 ml of *n*-hexane. The organic layer was washed with deionised water to neutral, dried over anhydrous sodium sulfate, and rotary evaporated to dryness. The residue was redissolved in 5 ml of n-hexane and filtered prior to HPLC injection, in the same manner as in the β-carotene assay.

The HPLC system was the same as for the β-carotene assay. The mobile phase was acetonitrile/methanol, 85:15 (v/v), at a flow rate of 1.5 ml/min and UV detection was at 295 nm. Content of each tocopherol was calculated on the basis of the calibration curve of each authentic tocopherol (Sigma).

Total phenols were determined according to the method of Taga, Miller, and Pratt (1984). Each extract (100 mg) was dissolved in a solution of 5 ml of 0.3% HCl in methanol/deionised water (60:40, v/v) and the resulting mixture (100 μl) was added to 2 ml of 2% aqueous sodium carbonate solution. After 2 min, 100 μl of 50% Folin–Ciocalteau reagent (Sigma) were added to the mixture. After 30 min of standing, absorbance was measured at 750 nm against a blank. The content of total phenols was calculated on the basis of the calibration curve of gallic acid (Sigma).

2.9. Statistical analysis

For each of ethanolic, cold water and hot water extractions, three samples were prepared for assays of every antioxidant attribute and component. The experimental data were subjected to an analysis of variance for a completely random design to determine the least significant difference at the level of 0.05.

3. Results and discussion

3.1. Extraction yield

Using various extractants, the yields were: hot water (48.2%) > cold water (41.3%) > ethanolic extracts (17.1%) (Table 1). The higher yields of hot water and cold water extracts might be due to the fact that *H. marmoreus* contained more water-soluble substances.

Table 1
Extraction yields of various extracts from *Hypsizigus marmoreus*

Solvent	Extraction yield[A] (%, w/w)
Ethanol	17.1 ± 0.32c[B]
Cold water	41.3 ± 0.22b
Hot water	48.2 ± 0.13a

[A] Extracted from freeze-dried materials. Each value is expressed as mean ± standard error ($n = 3$).
[B] Means with different letters within a column are significantly different ($P < 0.05$).

3.2. Antioxidant activity

Using the conjugated diene method, antioxidant activities of various extracts from *H. marmoreus* increased at increased concentrations (Fig. 1). At 5 mg/ml, ethanolic, cold water and hot water extracts exhibited moderate antioxidant activities of 56.4%, 65.2% and 38.6%, respectively. From 10 to 20 mg/ml, antioxidant activities of various extracts increased from 72.8–85.0% to 87.2–95.6%. With regard to antioxidant activities, the cold water extract was more effective than the hot water extract and comparable to the ethanolic extract. It seems that water could extract more antioxidant components than ethanol from *H. marmoreus*, whereas hot water might cause the thermal destruction of certain antioxidant components. In comparison, antioxidant activities were 99.2% and 87.7% at 0.1 mg/ml for BHA and α-tocopherol, respectively and 82.4% at 10 mg/ml for ascorbic acid.

With regard to ethanolic extracts, *P. citrinopileatus* exhibited moderate (69.9%) at 5 mg/ml and moderate to high antioxidant activities (71.7–87.9%) at 10–20 mg/ml (Huang, 2003). In addition, *Agaricus bisporus, Pleurotus eryngii, Pleurotus ferulae* and *Pleurotus ostreatus* showed moderate (60.0%, 64.5%, 54.0% and 58.6%) at 5 mg/ml and moderate to high antioxidant activities (74.2–84.0%, 73.4–84.0%, 64.5–79.2% and 68.5–84.6%, respectively) at 10–20 mg/ml (Lo, 2005). It is obvious that these mushrooms showed similar effect on the inhibition of linoleic acid oxidation. However, with regard to cold water extracts, antioxidant activities of *P. citrinopileatus* were 53.4% at 5 mg/ml and 58.6–63.6% at 10–20 mg/ml (Huang, 2003), lower than those of *H. marmoreus* shown in Fig. 1.

With regard to hot water extracts, mature and baby Ling chih (*Ganoderma tsugae*) showed low (32.3% and 16.7%) at 5 mg/ml and moderate to high antioxidant activities (64.4–78.5% and 57.1–78.2%, respectively) at 10–20 mg/ml (Mau, Tsai, Tseng, & Huang, 2005). Antioxidant activities of *Pleurotus citrinopileatus* were 63.7% at 5 mg/ml and maintained the level of 68.1–75.6% at 10–20 mg/ml (Huang, 2003). In addition, antioxidant activities of *Agrocybe cylindracea* were 44.3% at 5 mg/ml and 44.5–63.6% at 10–20 mg/ml (Tsai, Huang, & Mau, 2006). It seems that the antioxidant activity of *H. marmoreus* was comparable to that of *P. citrinopileatus* and higher than those of *G. tsugae* and *A. cylindracea*.

Taylor and Richardson (1980) found that cysteine exhibited better protection of a linoleate emulsion than BHA, butylated hydroxytoluene (BHT) and α-tocopherol against the oxidation by haemoglobin. Lee (2003) reported that *H. marmoreus* contained 10.5 mg/g of free cystine, which is readily soluble in water and thereby, probably contributing to the better antioxidant activities of cold and hot water extracts.

3.3. Reducing power

Reducing powers of various extracts increased in two patterns with increased concentrations, i.e., a fast increase for cold water extract and a slow increase for ethanolic and hot water extracts (Fig. 2). At 5 mg/ml, the reducing power of the cold water extract was 0.99 whereas those of ethanolic and hot water extracts were 0.27 and 0.36, respectively. The reducing power of the hot water extract was 1.01 at 10 mg/ml, whereas that of the ethanolic extract was 0.74 at 20 mg/ml. In comparison, reducing powers were 0.92 and 0.93 for ascorbic acid and BHA at 0.1 mg/ml, respectively, and 1.01 for α-tocopherol at 0.5 mg/ml.

With regard to ethanolic extracts, the reducing power of *P. citrinopileatus* were 1.03 at 5 mg/ml (Huang, 2003)

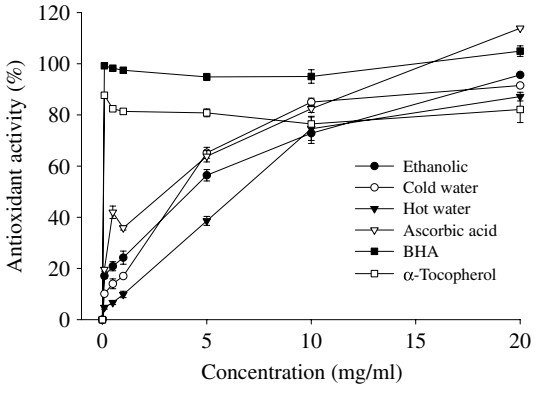

Fig. 1. Antioxidant activity of various extracts from *Hypsizigus marmoreus*. Each value is expressed as mean ± SE ($n = 3$).

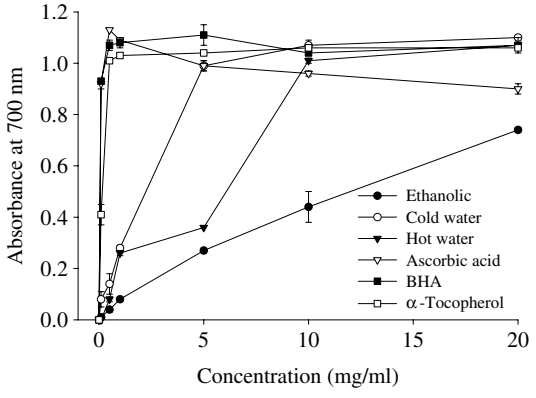

Fig. 2. Reducing power of various extracts from *Hypsizigus marmoreus*. Each value is expressed as mean ± SE ($n = 3$).

whereas *A. bisporus, P. eryngii, P. ferulae* and *P. ostreatus* showed reducing powers of 0.76, 0.75, 0.70 and 0.61 at 20 mg/ml, respectively (Lo, 2005). It can be seen that the reducing power of *H. marmoreus* was lower than that of *P. citrinopileatus* and higher than those of*A. bisporus, P. eryngii, P. ferulae* and *P. ostreatus*.

With regard to cold water extracts, the reducing power of *P. citrinopileatus* was 1.19 at 5 mg/ml (Huang, 2003), higher than that of *H. marmoreus* (Fig. 2). With regard to hot water extracts, mature and baby Ling chih exhibited reducing powers of 1.08 and 1.04 at 5 mg/ml, respectively (Mau et al., 2005). Reducing powers of *A. cylindracea* were 0.87 at 5 mg/ml and 0.99 at 10 mg/ml (Tsai et al., 2006). Moreover, *P. citrinopileatus* showed a reducing power of 1.10 at 5 mg/ml (Huang, 2003). Apparently, the reducing power of *H. marmoreus* was slightly lower than those of *A. cylindracea*, *G. tsugae* and *P. citrinopileatus*.

3.4. Scavenging ability on 1,1-diphenyl-2-picrylhydrazyl radicals

At 5 mg/ml, scavenging abilities on DPPH radicals were 59.7%, 34.0% and 44.2% for ethanolic, cold water and hot water extracts from *H. marmoreus*, respectively (Fig. 3a). At 10–20 mg/ml, three different patterns were found on scavenging abilities, which were 92.4–93.2%, 44.8–55.3% and 64.1–77.2% for ethanolic, cold water and hot water extracts, respectively. In comparison, at 0.1–20 mg/ml, scavenging abilities of BHA, α-tocopherol and ascorbic acid were 75.6–77.9%, 67.6–81.0% and 38.2–45.3%, respectively.

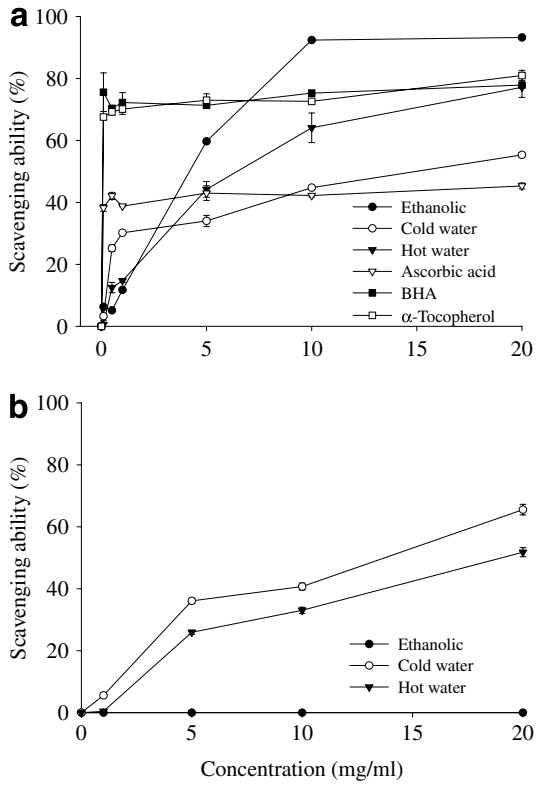

Fig. 3. Scavenging ability of various extracts from *Hypsizigus marmoreus* on 1,1-diphenyl-2-picrylhydrazyl (a) and hydroxyl radicals (b). Each value is expressed as mean ± SE ($n = 3$).

With regard to ethanolic extracts, *P. citrinopileatus* scavenged DPPH radicals by 94.9% at 5 mg/ml (Huang, 2003) whereas scavenging abilities of *A. bisporus, P. eryngii, P. ferulae* and *P. ostreatus* were in the range of 46.6–68.4% at 5 mg/ml (Lo, 2005). At 10–20 mg/ml, scavenging abilities of *A. bisporus, P. eryngii, P. ferulae* and *P. ostreatus* were 69.2–74.4%, 78.7–92.2%, 69.5–81.9% and 75.5–77.9%, respectively (Lo, 2005). It seems that the scavenging ability of *H. marmoreus* was less effective than that of *P. citrinopileatus* but comparable to those of *A. bisporus, P. eryngii, P. ferulae* and *P. ostreatus* at 5 mg/ml and more effective at 10–20 mg/ml.

With regard to cold water extracts, at 20 mg/ml, the scavenging ability of *H. marmoreus* was similar to that of *P. citrinopileatus* (50.7%, Huang, 2003). With regard to hot water extracts, at 0.5 mg/ml, scavenging abilities of *G. tsugae* and *A. cylindracea* were 53.8–61.2% (Mau et al., 2005) and 47.3% (Tsai et al., 2006), whereas at 5 mg/ml, that of *P. citrinopileatus* was 41.8% (Huang, 2003). Apparently, the scavenging ability of *H. marmoreus* was less effective than those of *G. tsugae* and *A. cylindracea* but more effective than that of *P. citrinopileatus*.

Herraiz, Galisteo, and Chamorro (2003), found out that an essential amino acid L-tryptophan could react with phenolic aldehydes in food to form phenolic tetrahydro-β-carboline alkaloids that scavenged 2,2-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) effectively. Lee (2003) reported that *H. marmoreus* contained 5.03 mg/g dry weight of free L-tryptophan. Therefore, the presence of L-tryptophan in various extracts might most likely account for the scavenging ability on DPPH radicals. However, the better ability of ethanolic extract might be due to more hydrogen-donating components extracted by ethanol.

3.5. Scavenging ability on hydroxyl radicals

At 5–20 mg/ml, scavenging abilities of cold water and hot water extracts from *H. marmoreus* on hydroxyl radicals slowly increased from 36.1 to 65.5% and 25.9 to 51.8%, respectively whereas ethanolic extracts showed no abilities (Fig. 3b). At 20 mg/ml, the scavenging ability of BHA was only 17.8%.

With regard to ethanolic extracts, *P. citrinopileatus, P. ferulae* and *P. ostreatus* did not show any ability (Huang, 2003; Lo, 2005) whereas *A. bisporus* and *P. eryngii* exhibited low scavenging abilities of 20.3 and 16.1% at 20 mg/ml (Lo, 2005). With regard to cold water extracts, at 5–20 mg/ml, scavenging abilities of *P. citrinopileatus* were 39.9–70.3%, comparable to those of *H. marmoreus* (Fig. 3b).

With regard to hot water extracts, at 5–20 mg/ml, scavenging abilities of mature and baby Ling chih were 19.6–23.2% to 72.4–73.7% (Mau et al., 2005), that of *A. cylindracea* was 17.0 to 43.4% (Tsai et al., 2006), and that of *P. citrinopileatus* was 42.0 to 80.1% (Huang, 2003). Lo (2005) found that at 5–20 mg/ml, scavenging abilities of *A. bisporus*, *P. eryngii* and *P. ostreatus* were 11.3–22.6% to 38.2–48.0%, whereas that of *P. ferulae* was 33.9 to 67.9%. Therefore, scavenging abilities of hot water extracts were in the descending order: *P. citrinopileatus* > *G. tsugae* ~ *P. ferulae* > *H. marmoreus* ~ *A. cylindracea* ~ *A. bisporus* ~ *P. eryngii* ~ *P. ostreatus*.

Halliwell, Gutteridge, and Aruoma (1987), indicated that mannitol, histidine and adenosine monophosphate (AMP) showed scavenging abilities on hydroxyl radicals. Lee (2003) found that contents of mannitol, free histidine and AMP in *H. marmoreus* were 20.0, 2.60 and 0.61 mg/g dry weight, respectively. The presence of these components in water extracts might be responsible for their better hydroxyl ion scavenging abilities.

3.6. Chelating abilities on ferrous ions and cupric ions

Chelating abilities of ethanolic, cold water and hot water extracts from *H. marmoreus* on ferrous ions were 3.30%, 62.9% and 58.5% at 0.5 mg/ml, respectively (Fig. 4a). At 5 mg/ml, chelating abilities of cold water and hot water extracts were 94.1% and 92.6%, respectively and at 10 mg/ml, that of ethanolic extracts was 94.2%. However, EDTA was an excellent chelating agent for ferrous ions and its chelating ability was 99.4% at 0.1 mg/ml. Citric acid showed a low chelating ability of 15.5% at 20 mg/ml.

With regard to ethanolic extracts, *P. citrinopileatus* chelated ferrous ions by 46.4% at 5 mg/ml and 75.7% at 20 mg/ml (Huang, 2003). Lo (2005) found that at 5–20 mg/ml, chelating abilities of *A. bisporus, P. eryngii, P. ferulae* and *P. ostreatus* were 41.4–64.0% to 73.1–82.3%. It is obvious that *H. marmoreus* showed better chelating ability than other mushrooms mentioned above.

With regard to cold water extracts, *P. citrinopileatus* showed chelating abilities of 66.6% at 5 mg/ml and 90.3% at 20 mg/ml (Huang, 2003), less effective than *H. marmoreus* in Fig. 4a. With regard to hot water extracts, at 20 mg/ml, *G. tsugae* and *A. cylindracea* chelated ferrous ions by 39.5–42.6% and 45.8%, respectively (Mau et al., 2005; Tsai et al., 2006). At 1–5 mg/ml, chelating abilities of *P. citrinopileatus* were 25.8–82.1% (Huang, 2003). It seems that chelating ability of *H. marmoreus* on ferrous ions was more effective to that of *P. citrinopileatus* and much more effective than those of *G. tsugae* and *A. cylindracea*.

Chelating abilities of cold water, ethanolic and hot water extracts on cupric ions were 57.5%, 52.3% and 40.6% at 5 mg/ml and 95.2%, 41.4% and 82.4% at 20 mg/ml, respectively (Fig. 4b). However, EDTA chelated cupric ions by 58.9–57.3% at 5–20 mg/ml whereas at 20 mg/ml, the chelating ability of citric acid was 7.41%. With regard

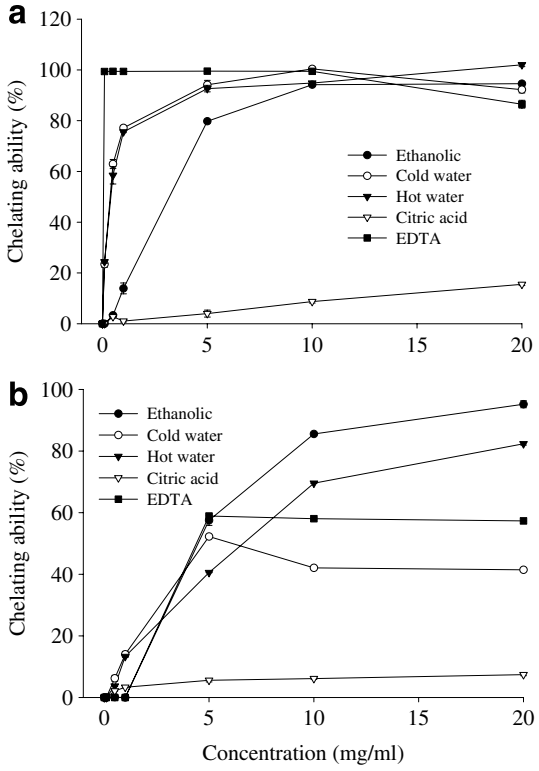

Fig. 4. Chelating ability of various extracts from *Hypsizigus marmoreus* on ferrous (a) and cupric ions (b). Each value is expressed as mean ± SE ($n = 3$).

to hot water extracts, at 20 mg/ml, *G. tsugae* and *A. cylindracea* chelated 34.2–40.0% and 34.8% of cupric ions, respectively (Tsai, 2002). It seems that *H. marmoreus* showed better chelating ability than *G. tsugae* and *A. cylindracea*.

Histidine was reported to be effective in decreasing the binding of cupric ions to low-density lipoprotein (LDL) and thereby, preventing the oxidation of LDL (Chen & Frei, 1997; Wagner & Heinecke, 1997). In addition, α-tocopherol could also reduce cupric ions to eliminate lipid oxidation (Yoshida, Tsuchiya, & Niki, 1994). Lee (2003) found that both histidine and α-tocopherol were present in *H. marmoreus* and their existence might account for the better chelating abilities on cupric ions for various extracts. Since ferrous and cupric ions are the most effective pro-oxidants in foods (Yamaguchi, Tatsumi, Asano, Kato, & Ueno, 1988), and ferrous ions are commonly found in foods, the high ferrous ion and moderate cupric ion chelating abilities of the various extracts from *H. marmoreus* would be beneficial.

3.7. EC_{50} values in antioxidant properties

The antioxidant properties assayed herein are summarised in Table 2 and the results are normalised and expressed as EC_{50} values (mg various extracts per ml) for comparison. Effectiveness of antioxidant properties are inversely correlated with their EC_{50} values. With regard to EC_{50} values of antioxidant activities by the conjugated

Table 2
EC_{50} values of various extracts from *Hypsizigus marmoreus* for antioxidant properties

	EC_{50} value[A] (mg extract/ml)		
	Ethanolic	Cold water	Hot water
Antioxidant activity	4.20 ± 0.15b[B]	3.74 ± 0.07c	6.59 ± 0.13a
Reducing power	12.0 ± 0.03a	2.24 ± 0.02c	6.08 ± 0.01b
Scavenging ability on DPPH radicals	4.19 ± 0.02c	15.0 ± 0.02a	6.48 ± 0.17b
Scavenging ability on OH radicals	–[C]	13.75 ± 0.06b	19.0 ± 0.05a
Chelating ability on ferrous ions	3.19 ± 0.06a	0.37 ± 0.06b	0.40 ± 0.08b
Chelating ability on cupric ions	4.42 ± 0.03c	4.76 ± 0.02b	6.63 ± 0.01a

[A] EC_{50} value, the effective concentration at which the antioxidant activity was 50%; the absorbance was 0.5 for reducing power; the 1,1-diphenyl-2-picrylhydrazyl (DPPH) or hydroxyl (OH) radicals were scavenged by 50%; and ferrous or cupric ions were chelated by 50%. EC_{50} value was obtained by interpolation from linear regression analysis.
[B] Each value is expressed as mean ± SE ($n = 3$). Means with different letters within a row are significantly different ($P < 0.05$).
[C] No effect.

diene method, effectiveness was in the order: cold water > ethanolic > hot water extracts. Effectiveness in reducing power were in a descending order: cold water > hot water > ethanolic extracts. With regard to the scavenging ability on DPPH radicals, various extracts were effective in the order: ethanolic > hot water ≫ cold water extracts, whereas with regard to the effect on hydroxyl radicals, the cold water extract was more effective than the hot water extract. With regard to chelating ability on ferrous ions, cold water and hot water extracts were similar and more effective than the ethanolic extract. Effectiveness in the chelating ability on cupric ions was in the order: ethanolic > cold water > hot water extracts.

Generally, EC_{50} values of lower than 10 mg/ml indicated that the various extracts were effective in antioxidant properties. However, EC_{50} values of the ethanolic extract in reducing power, of the cold water extract in scavenging abilities of DPPH and hydroxyl radicals and of the hot water extract in scavenging ability on DPPH radicals were higher than 10 mg/ml. In addition, chelating abilities of cold water and hot water extracts on ferrous ions were below 1 mg/ml. Among the antioxidant properties assayed, the cold extract was most effective in antioxidant activity, reducing power, scavenging ability on hydroxyl radicals and chelating ability on ferrous ions. The hot water extract was most effective in chelating ability on ferrous ions whereas the ethanolic extract was most effective in scavenging ability on DPPH radicals and chelating ability on cupric ions.

When the extraction yields were taken into consideration, EC_{50} values in antioxidant activity (mg dried sample per ml) were 24.6, 9.05 and 13.7 mg/ml for ethanolic, cold water and hot water extracts, respectively. EC_{50} values in reducing power were 70.3, 5.42 and 12.6 mg/ml for ethanolic, cold water and hot water extracts, respectively. EC_{50} values in scavenging ability on DPPH radicals were 24.6, 36.2 and 13.4 mg/ml for ethanolic, cold water and hot water extracts, respectively. EC_{50} values in scavenging ability on hydroxyl radicals were 33.3 and 39.5 mg/ml for cold water and hot water extracts, respectively. EC_{50} in chelating ability on ferrous ions were 18.7, 0.83 and 0.90 mg/ml for ethanolic, cold water and hot water extracts, respectively. EC_{50} in chelating ability on cupric ions were 25.9, 11.5 and 13.7 mg/ml for ethanolic, cold water and hot water extracts, respectively. EC_{50} values of cold water and hot water extracts were below 40 mg/ml on the basis of dry sample, due to their high extraction yields (41.32 and 48.24%, respectively) in Table 1.

Although BHA, ascorbic acid and/or α-tocopherol were good in antioxidant activity, reducing power and scavenging ability on DPPH radicals and EDTA was a good chelator for ferrous and cupric ions, they were additives and used or present in mg levels in foods. Various extracts of *H. marmoreus* could be used in gram or hundreds of gram levels as food or a food ingredient. Therefore, mushrooms in human diets might supply as health protection to help human reduce oxidative damage.

3.8. Antioxidant components

Naturally occurring antioxidant components, including ascorbic acid, β-carotene, tocopherols, and total phenols, were found in the ethanolic extract from *H. marmoreus* (Table 3). However, β-carotene and tocopherols were not detected in the water extracts due to their fat-soluble nature. The contents of ascorbic acid were in the order: cold water > ethanolic > hot water extracts, due to the fact that ascorbic acid is easily degraded by heat. Total phenols were

Table 3
Contents of ascorbic acid, β-carotene, tocopherols and total phenols of various extracts from *Hypsizigus marmoreus*

Compound	Content[A] (mg/g)		
	Ethanolic	Cold water	Hot water
Ascorbic acid	0.13 ± 0.01a	0.29 ± 0.03a	0.07 ± 0.02c
β-Carotene	0.02 ± <0.01	nd[B]	nd
α-Tocopherol	0.15 ± 0.01a	0.04 ± <0.01b	nd
δ-Tocopherol	0.03 ± 0.01	nd	nd
Total phenols	12.9 ± 0.95c	30.8 ± 0.45a	19.2 ± 0.26b

[A] Each value is expressed as mean ± SE ($n = 3$). Means with different letters within a row are significantly different ($P < 0.05$).
[B] Not detected.

the major naturally-occurring antioxidant components found and were in the order: cold water > hot water > ethanolic extracts. The highest amount of total phenols in the cold water extract might explain its high antioxidant ability, reducing power and chelating abilities. The contents of total antioxidant components assayed were in the order: cold water (31.2 mg/g) > hot water (19.3 mg/g) > ethanolic extracts (13.3 mg/g).

Phenols such as BHT and gallate are known to be effective antioxidants (Madhavi, Singhal, & Kulkarni, 1996). Due to their scavenging abilities on free radicals and chelating abilities on ferrous and cupric ions, phenols possessed good antioxidant, antimutagenic and anticancer properties (Ahmad & Mukhtar, 1999; Lotito & Fraga, 1998). Therefore, high contents of total phenols in the extracts were responsible for their effective antioxidant properties. To study the antioxidant mechanisms of some specific phenolic components, the fractionation of the extracts and further identification are in progress. Nevertheless, on the basis of the results obtained, consumption of *H. marmoreus* might be somewhat beneficial to the antioxidant protection system of the human body against oxidative damage.

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