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Antioxidant properties of methanolic extracts from *Ganoderma tsugae*

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Abstract

Ganoderma tsugae Murrill was available in the form of mature and baby Ling chih, mycelia and fermentation filtrate. From these four forms, methanolic extracts were prepared and their antioxidant properties were studied. Methanolic extracts from mature and baby Ling chih showed high antioxidant activities (96.8% and 93.6%) at 20 mg ml^{-1}, and had EC_{50} values of 0.53 and 1.11 mg ml^{-1}, respectively. EC_{50} values in reducing power were 5.00, 2.28, 0.93 and 2.15 mg ml^{-1} for Ling chih, baby Ling chih, mycelia and filtrate, respectively. Methanolic extracts from mature and baby Ling chih scavenged 1,1-diphenyl-2-picrylhydrazyl radicals by 88.4% and 93.8% at 5 mg ml^{-1}, whereas those from mycelia and filtrate scavenged by 85.7% and 79.3% at 10 mg ml^{-1}, respectively. EC_{50} values in chelating ability on ferrous ions were 4.82, 3.05, 1.10 and 3.41 mg ml^{-1} for Ling chih, baby Ling chih, mycelia and filtrate, respectively. Total phenols were the major naturally occurring antioxidant components found in all methanolic extracts and in the range of 24.0–35.6 mg g^{-1}. Based on EC_{50} values, *G. tsugae* was good in antioxidant properties except for the scavenging ability on hydroxyl radicals.
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Keywords: *Ganoderma tsugae*; Antioxidant activity; Reducing power; Scavenging ability; Chelating ability; Antioxidant components

1. Introduction

Ganoderma tsugae Murrill and *G. lucidum* (Curtis: Fries) Karsten, both called Ling chih or reishi, have been revered for centuries as a symbol of success and well being, meaning "marvelous herbs" or "mushroom of immortality" (Stamets, 1993). The varnished, dark red fruit body of Ling chih has a circular or kidney-shaped cap and sometimes an eccentric-lateral stipe (Arora, 1986). *G. tsugae* and *G. lucidum* are extremely similar in appearance (Arora, 1986). However, *G. tsugae* is much thicker than *G. lucidum*.

Ganoderma is highly valued as folk medicine and functional food for its antitumor and other physiological benefits. Recently, *Ganoderma* was found to be medically active in several therapeutic effects, including antiinflammatory, antitumor, antiviral (e.g., anti-HIV), antibacterial and antiparasitic, blood pressure regulation, cardiovascular disorders, immunomodulating, kidney tonic, hepatoprotective, nerve tonic, sexual potentiator and chronic bronchitis (Wasser & Weis, 1999).

Most recent studies have placed major emphases on the identification of pharmaceutical components in Ling chih and their mechanism of action in human bodies, and the means for its mass production to eliminate the ecological damage as a result of the infection of phytopathogenic Ling chih. Generally, the production of Ling chih includes a long-time cultivation in plastic bag for fruit bodies, and a short-time submerged fermentation for mycelia and fermentation filtrate. Currently, baby Ling chih, an immature fruit body, is becoming popular in Taiwan.

Normally, mature Ling chih is harvested from plastic bags at 1–2 months after fruiting, whereas baby Ling chih is harvested at 2–3 weeks after fruiting. The total

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yield of baby Ling chih is higher than that of mature Ling chih for the entire crop (Tseng, Tsai, Lee, & Mau, 2003). In addition, baby Ling chih will not cause ecological damage to trees because no spores are discharged. Recently, people in Mid-Taiwan have presented anecdotal evidence suggesting that baby Ling chih is more effective in therapeutic effects than mature Ling chih. It is thought that baby Ling chih is soft and contains more soluble substances than mature Ling chih that has become woody after 1 month of growth. Also, the nutritional values and taste components of these different forms of *G. tsugae* have been well studied (Tseng, Lee, Li, & Mau, 2005).

In addition to its therapeutic effects, Ling chih, including *G. lucidum* and *G. tsugae*, was found to be high in antioxidant abilities (Mau, Lin, & Chen, 2002a, 2002b; Yang, Lin, & Mau, 2002; Yen & Wu, 1999). However, the antioxidant properties of baby Ling chih, mycelia and fermentation filtrate are not reported. Our objective was to study and compare the antioxidant properties of methanolic extracts from *G. tsugae* in the form of mature and baby fruit bodies, mycelia and filtrate from the submerged culture. Antioxidant properties were assayed in terms of antioxidant activity by the conjugated diene method, reducing power, scavenging abilities on 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl radicals and chelating ability on ferrous ions. The contents of potential antioxidant components of methanolic extracts were also determined.

2. Materials and methods

2.1. Mushroom fruit bodies, mycelia and fermentation filtrate

The pure culture of *G. tsugae* GT01 was originally obtained from the Department of Plant Pathology, Taiwan Agricultural Research Institute, Wufeng, Taichung County, Taiwan. Fresh mature (6-week-old) and baby (2-week-old) Ling chih were harvested from the mushroom room of the Department of Food Science, National Chung-Hsing University, Taichung, Taiwan, and air-dried in an oven at 40 °C for 2–3 days before sample preparation. Mycelia and fermentation filtrate, both in a freeze-dried form, were obtained from the Biotechnology Center, Grape King Inc., Chungli, Taiwan. For each of the mature and baby Ling chih, mycelia and filtrate, three dried samples (~50 g each) were randomly selected and prepared for analyses.

After a fine powder (20 mesh) was obtained using a mill (Retsch ultra centrifugal mill and sieving machine, Haan, Germany), a subsample (10 g) was extracted by stirring with 100 ml of methanol at 25 °C at 20*g* for 24 h and filtering through Whatman No. 4 filter paper. The residue was then extracted with two additional 100 ml portions of methanol as described above. The combined methanolic extracts were then rotary-evaporated at 40 °C to dryness. The dried extract was used directly for analyses of antioxidant components or redissolved in methanol to a concentration of 50 mg ml^{-1} 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 (1–20 mg ml^{-1}) in methanol (100 μl) was mixed with 2 ml of 10 mM linoleic acid emulsion (pH 6.5) in test tubes and placed in darkness at 37 °C to accelerate oxidation. After incubation for 15 h, 6 ml of 60% methanol (Mallinckrodt Baker, Paris, KY) in deionised water were added, and the absorbance of the mixture was measured at 234 nm against a blank in a Hitachi U-2001 spectrophotometer. The antioxidant activity (AOA) was calculated as follows: AOA (%) = [(ΔA_{234} of control − ΔA_{234} of sample)/ΔA_{234} of control] × 100. A control consisted of methanol and the reagent solution without methanolic extracts added and the procedure was carried out as described above. An AOA value of 100% indicates the strongest antioxidant activity. EC_{50} value (mg extract ml^{-1}) 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 (1–20 mg ml^{-1}) in methanol (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 in a Hitachi U-2001 spectrophotometer. A higher absorbance indicates a higher reducing power. EC_{50} value (mg extract ml^{-1}) 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 radicals

Each extract (1–20 mg ml^{-1}) in methanol (4 ml) was mixed with 1 ml of methanolic solution containing 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). The scavenging ability was calculated as follows: scavenging ability (%) = [(ΔA_{517} of control − ΔA_{517} of sample)/ΔA_{517} of control] × 100. EC_{50} value (mg extract ml^{-1}) 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 radicals reacted with the nitrone spin trap 5,5-dimethyl 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 2.5 min after mixing each extract (1–10 mg ml^{-1}) in methanol (200 μl) 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). The scavenging ability was calculated by subtracting the relative EPR signal intensity from 100. The relative EPR signal intensity was calculated as follows: relative EPR signal intensity (%) = [$h\Delta H^2$ (sample)/$h\Delta H^2$ (control)] × 100; h is the width of the peak, ΔH is the length of the peak. BHA was used for comparison.

2.6. Chelating ability on ferrous ions

Chelating ability was determined according to the method of Shimada et al. (1992). To 2 ml of the mixture, consisting of 30 mM hexamine (Wako), 30 mM potassium chloride (Sigma) and 9 mM ferrous sulphate (Union Chemical Works, Hsinchu, Taiwan), were added each extract (1–20 mg ml^{-1}) in methanol (2 ml) 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^{-1}) 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. Determination of antioxidant components

Ascorbic acid was determined according to the method of Klein and Perry (1982). Each methanolic extract (20 mg) was extracted with 10 ml of 1% metaphosphoric acid (Union) for 45 min at room temperature and filtered through Whatman No. 4 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 analysed as described by Rundhaug, Pung, Read, and Bertram (1988). Each methanolic extract (20 mg) was extracted with a solution of 1% pyrogallol (Wako) in 10 ml of methanol/dichloromethane (1:1, v/v) for 45 min at room temperature, filtered through Whatman No. 4 filter paper and the volume adjusted to 10 ml using the same solution. The filtrate was then passed through a filter unit (13 mm, Lida Corp., Kenosha, WI) and filtered using a 0.45 μm CA filter paper prior to injection onto a high-performance liquid chromatograph (HPLC).

The HPLC system consisted of a Hitachi D-6200 pump, a Hitachi L-5000 LC controller, a Rheodyne 7161 injector, a 20 μl sample loop, a Hitachi D-2500 chromato-integrator, a Hitachi L-4000 UV detector, and a Prodigy 5 ODS-2 column (4.6 × 250 mm, 5 μm, Phenomenex Inc., Torrance, CA). The mobile phase was acetone/methanol/acetonitrile, 1:2:2 (v/v/v), at a flow rate of 0.7 ml min^{-1} and UV detection was at 470 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 methanolic extract (50 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 sulphate, 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 1.0 ml min^{-1} 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 methanolic extract (20 mg) was dissolved in a solution of 5 ml of

1.3% 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 3 min, 100 μl of 50% Folin–Ciocalteau's phenol reagent (Sigma) were added to the mixture. After 30 min standing, the 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).

2.8. Statistical analysis

For each methanolic extract from mature and baby Ling chih, mycelia and filtrate, 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 as described by Steel, Torrie, and Dickey (1997), to determine the least significant difference at the level of 0.05.

3. Results and discussion

3.1. Antioxidant activity

Following the extraction with methanol, the yields were in a descending order: filtrate ≫ mycelia ≫ Ling chih ∼ baby Ling chih (Table 1). The highest yield of filtrate was mainly due to the fact that most components contained in the filtrate were small and water-soluble. The methanolic yields from mycelia of *Antrodia camphorata* and *Agaricus blazei* were 31.1% and 35.1–41.2%, respectively (Huang, 2000). The methanolic yields from mycelia of *Grifola frondosa*, *Morchella esculenta* and *Termitomyces albuminosus* were 24.5%, 13.5% and 24.2%, respectively (Mau, Chang, Huang, & Chen, 2004). Apparently, the methanolic yield from mycelia of *G. tsugae* seemed to be in the range of higher yields. With regard to methanolic extracts from fruit bodies, the yields from Ling chih and baby Ling chih were similar to that from *Coriolus versicolor* (9.16%), and higher than those from *G. lucidum* (5.61–6.15%) and *G. tsugae* (3.97%) (Mau et al., 2002a). The discrepancy in the yields from fruit bodies of *G. tsugae* might be due to the difference in strains used and harvest times. However, the yields from mycelia were higher than those from some fruit bodies that contained high contents of crude fibre such as *G. lucidum*, *G. tsugae*, *Lentinula edodes* and *Pleurotus* spp. (Mau, Lin, & Chen, 2001a, 2002a; Yang, Lin, & Mau, 2001, 2002).

Table 1
Extraction yield of methanolic extracts from *Ganoderma tsugae*

	Amount[a] (g)	Extraction % (w/w)
Ling chih	0.85 ± 0.08	8.46 C[b]
Baby Ling chih	0.98 ± 0.06	9.79 C
Mycelia	4.06 ± 0.01	40.64 B
Filtrate	7.93 ± 0.20	79.30 A

[a] Extracted from dried materials (10.00 g). Each value is expressed as mean ± standard deviation ($n = 3$).

[b] Means with different letters within a column are significantly different ($p < 0.05$).

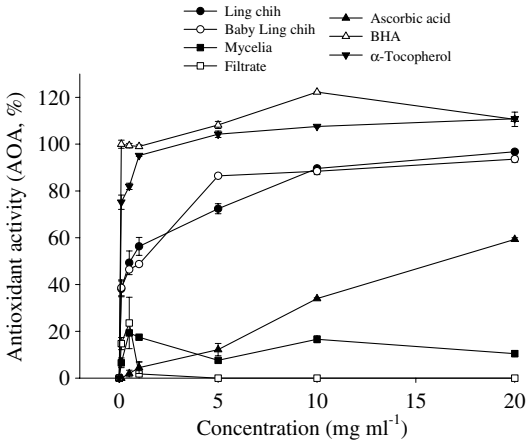

Fig. 1. Antioxidant activity of methanolic extracts from *Ganoderma tsugae* (conjugated diene method). Each value is expressed as mean ± standard deviation ($n = 3$).

Using the conjugated diene method, the methanolic extracts from mature and baby Ling chih showed moderate antioxidant activities (49.3% and 46.4%) at 0.5 mg ml^{-1} and high antioxidant activities (96.8% and 93.6%, respectively) at 20 mg ml^{-1} (Fig. 1). On the other hand, the methanolic extract from mycelia showed a low antioxidant activity of 10.4–19.3% at 0.5–20 mg ml^{-1}, whereas no antioxidant activity was found in the methanolic extract from filtrate. However, the antioxidant activities were 99.9% at 0.1 mg ml^{-1} for BHA, 95.1% at 1 mg ml^{-1} for α-tocopherol and 59.3% at 20 mg ml^{-1} for ascorbic acid.

Similarly, using the same method, the methanolic extract from fruit bodies of *Agrocybe cylindracea* showed moderate (37.0–50.6% at 0.1–1.0 mg ml^{-1}) and high antioxidant activity (90.0–97.3% at 5–20 mg ml^{-1}) (Huang, Tsai, & Mau, 2002). However, the methanolic extract from its mycelia and filtrate show a rapid and concentration-dependent increase from 21.5% and 24.0% at 0.5 mg ml^{-1} to 67.4% and 90.9% at 20 mg ml^{-1}, respectively (Huang et al., 2002). With regard to antioxidant activity, it seems that the methanolic extracts from the mycelia and filtrate of *G. tsugae* were much lower than those from *A. cylindracea*.

Yen and Wu (1999) found that, using the thiocyanate method, the antioxidant activity of the methanolic extract from *G. tsugae* fruit bodies was better than four other *Ganoderma* spp. and α-tocopherol, and slightly lower than BHA. Further, in the linoleic acid and rat liver microsome peroxidation systems, at 200 ppm (0.2 mg ml^{-1}), the methanolic extract from *G. tsugae* showed a good antioxidant activity of 92.1%, much

higher than that of α-tocopherol (57.1%) (Yen & Wu, 1999). Using the 1,3-diethyl-2-thiobarbituric acid method, the methanolic extracts from *G. lucidum* and *G. tsugae* both showed good antioxidant activities as evidenced by relatively low percentages of lipid peroxidation (2.30–6.41%) at 0.6 mg ml^{-1} (Mau et al., 2002a). Obviously, Ling chih consistently showed a good antioxidant activity in several methods assayed.

Huang (2000) mentioned that the methanolic extract from *Antrodia camphorata* (Chang-chih) exhibited an outstanding antioxidant activity of 91.2–93.0% (fruit bodies) and 87.7% (mycelia) at as little as 0.5 mg ml^{-1}, whereas that from *Agaricus blazei* (Brazilian mushrooms) also showed an excellent activity of 91.8% (fruit bodies) and 93.6% (mycelia) at 1.0 mg ml^{-1}. The antioxidant activities of methanolic extracts from red and silver ears increased as the concentration increased from 1.0 to 2.0 mg ml^{-1} and reached a plateau of 89.9–94.6% and 50.9–54.2% at 2.0–5.0 mg ml^{-1}, respectively (Mau, Chao, & Wu, 2001b). Similarly, that of methanolic extract from black ears reached a plateau of 75.1–76.6% at 3.0–5.0 mg ml^{-1} (Mau et al., 2001b). Compared to *A. camphorata* and Brazilian mushrooms, the tested Ling chih and baby Ling chih contained less of the components effective in inhibiting the oxidation of linoleic acid.

3.2. Reducing power

Reducing powers of the methanolic extracts increased in two patterns with increased concentrations, i.e., a fast increase for baby Ling chih, mycelia and filtrate and a slow increase for Ling chih (Fig. 2). At 5 mg ml^{-1}, the reducing powers of methanolic extracts were 0.50, 0.93, 1.05 and 1.00 for Ling chih, baby Ling chih, mycelia and filtrate, respectively. However, the reducing power of the methanolic extract from Ling chih steadily increased to 1.05 at 20 mg ml^{-1}. BHA showed an excellent reducing power of 1.00 at 0.1 mg ml^{-1}, and remained at the level of 1.11–1.21 to 20 mg ml^{-1}. At 0.5 to 20 mg ml^{-1}, ascorbic acid and α-tocopherol showed a slight increase in reducing power, from 0.88 to 1.05 and from 0.67 to 0.87, respectively.

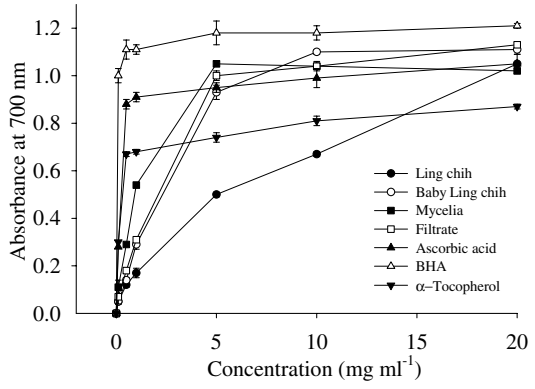

Fig. 2. Reducing power of methanolic extracts from *Ganoderma tsugae*. Each value is expressed as mean ± standard deviation (n = 3).

Huang et al. (2002) found that the reducing power of the methanolic extract from *A. cylindracea* fruit bodies increased to 0.99 at 5 mg ml^{-1}, whereas reducing powers of those from its mycelia and filtrate eventually increase to more than 1.0 at 20 mg ml^{-1}. Huang (2000) reported that the methanolic extract from *A. camphorata* showed an excellent reducing power of 0.92–0.94 at 5 mg ml^{-1}, whereas that from Brazilian mushrooms showed a reducing power of 0.79 at 5 mg ml^{-1}.

Methanolic extracts from other medicinal mushrooms, including *G. lucidum*, *G. lucidum* antler and *G. tsugae*, exhibited strong reducing powers of 0.81, 1.03, and 1.05 at 1.5 mg ml^{-1}, respectively (Mau et al., 2002a). The discrepancy in reducing power between the methanolic extract from *G. tsugae* in Mau et al. (2002a) and that in this research might be due to the difference in the strains used. However, a good reducing power of 0.79 was observed with the methanolic extract from another medicinal mushroom, *Coriolus versicolor* (Yun chih), at 4.0 mg ml^{-1} (Mau et al., 2002a). Among methanolic extracts from four speciality mushrooms, basket stinkhorn showed an excellent reducing power of 1.09 at 3 mg ml^{-1} (Mau, Lin, & Song, 2002b). Reducing powers of methanolic extracts from maitake, lion's mane, and white matsutake were 0.88, 0.79, and 0.50 at 6 mg ml^{-1}, respectively (Mau et al., 2002b).

Among methanolic extracts from commercial mushrooms, abalone and tree oyster mushrooms exhibited excellent reducing powers of 0.65 and 0.81 at 5 mg ml^{-1}, respectively (Yang et al., 2002). Reducing powers of methanolic extracts from two strains of winter mushrooms were 0.35 and 0.43 at 5 mg ml^{-1}, whereas reducing powers of 0.42 and 0.57 were observed with those from two strains of shiitake at 5 mg ml^{-1} (Yang et al., 2002). Mau et al. (2001b) reported that methanolic extracts from ear mushrooms, excluding silver ears, showed reducing powers of 0.67–0.82 at 5 mg ml^{-1}. The reducing power of that from silver ears was 0.32 at 5 mg ml^{-1} (Mau et al., 2001b). Methanolic extracts from black, red, and jin ears showed good reducing powers of 0.67–0.74 at 5 mg ml^{-1} (Mau et al., 2001b). With regard to reducing power, it can be concluded that most mushrooms showed higher absorbances at low concentrations of methanolic extracts.

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

The methanolic extracts from Ling chih and baby Ling chih showed high DPPH radical scavenging abilities of 88.4% and 93.8% at 5 mg ml^{-1}, respectively (Fig. 3). The methanolic extracts from mycelia and filtrate scavenged DPPH radicals by 85.7% and 79.3% at

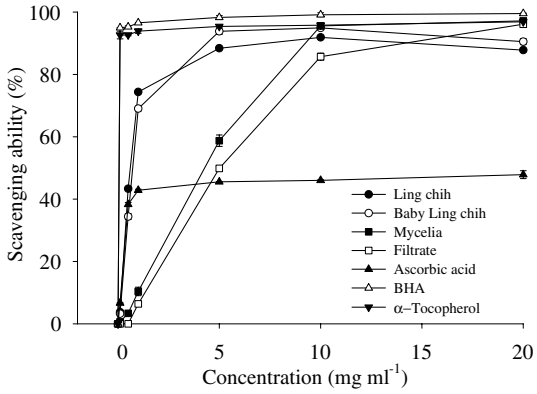

Fig. 3. Scavenging ability of methanolic extracts from *Ganoderma tsugae* on 1,1-diphenyl-2-picrylhydrazyl radicals. Each value is expressed as mean ± standard deviation ($n = 3$).

10 mg ml^{-1}, respectively. It seems that the scavenging abilities of the methanolic extracts from Ling chih and baby Ling chih were relatively comparable, and more effective than those of the methanolic extracts from mycelia and filtrate that were similar. At 0.1 mg ml^{-1}, BHA and α-tocopherol showed excellent scavenging abilities of 95.0% and 92.6%, respectively. However, at 0.5–20 mg ml^{-1}, ascorbic acid showed a plateau of scavenging ability of 38.3–47.8%.

Huang et al. (2002) found that the scavenging ability of the methanolic extract from *A. cylindracea* fruit bodies was 89.0% at 1 mg ml^{-1}, better than those from Ling chih and baby Ling chih. Similarly, the methanolic extracts from mycelia and filtrate from *A. cylindracea* scavenged 91.4% and 94.9% of DPPH radicals at 20 mg ml^{-1}, respectively. Excellent scavenging abilities (96.3–99.1% and 97.1%) were observed with methanolic extracts from *A. camphorata* and Brazilian mushrooms at 2.5 mg ml^{-1} (Huang, 2000), respectively. Scavenging abilities of methanolic extracts from other medicinal mushrooms were measured at up to 0.64 mg ml^{-1} and were 24.6%, 67.6%, 74.4%, and 73.5% for *C. versicolor*, *G. lucidum*, *G. lucidum* antler and *G. tsugae*, respectively (Mau et al., 2002a). However, at 0.5 mg ml^{-1}, the scavenging ability of the methanolic extract from *G. tsugae* was 43.4%, similar to that from Ling chih (47.3%) in Fig. 3.

At 6.4 mg ml^{-1}, the methanolic extract from basket stinkhorn scavenged DPPH radicals by 92.1%, whereas scavenging abilities of methanolic extracts from other speciality mushrooms were 63.3–67.8% (Mau et al., 2002b). In addition, at 6.4 mg ml^{-1}, the methanolic extract from tree oyster mushrooms scavenged DPPH radicals by 81.8% (Yang et al., 2002). However, scavenging abilities of methanolic extracts from other commercial mushrooms were 42.9–69.9% (Yang et al., 2002). At 1 mg ml^{-1}, methanolic extracts from black and red ears scavenged DPPH radicals completely (100%) whereas those from snow and jin ears scavenged DPPH radicals by 94.5% at 0.4 mg ml^{-1} and 95.4% at 3 mg ml^{-1}, respectively (Mau et al., 2001b). However, silver ears were less effective in scavenging DPPH radicals (71.5% at 5 mg ml^{-1}) (Mau et al., 2001b).

3.4. Scavenging ability on hydroxyl radicals

All methanolic extracts from *G. tsugae* showed a slight scavenging ability on hydroxyl radicals and this ability was not concentration-dependent (Table 2). However, the scavenging ability of BHA was 38.2% at 20 mg ml^{-1}. Similar results were also found in the methanolic extract from *A. cylindracea* fruit bodies, mycelia and filtrate (Huang et al., 2002). Mau et al. (2001b) also indicated that ear mushrooms were not good scavengers for hydroxyl radicals. Similarly, methanolic extracts from *A. camphorata* and Brazilian mushrooms did not scavenge hydroxyl radicals (Huang, 2000). At 40 mg ml^{-1}, methanolic extracts from speciality mushrooms scavenged hydroxyl radicals by 39.6–75.0% (Mau et al., 2002b), whereas those from commercial mushrooms showed scavenging abilities of 29.2–36.6% (Yang et al., 2002). However, at 16 mg ml^{-1}, methanolic extracts from medicinal mushrooms, such as *C. versicolor*, *G. lucidum*, and *G. tsugae*, scavenged hydroxyl radicals by 38.0–52.6% (Mau et al., 2002a). These results indicated that *G. tsugae* might scavenge hydroxyl radicals. However, a study focussed on the scavenging ability of different strains of *G. tsugae* on hydroxyl radicals is needed to screen the strain with the highest scavenging ability.

Table 2
Scavenging ability of methanolic extracts from *Ganoderma tsugae* on hydroxyl radicals

Amount (mg ml^{-1})	Scavenging ability[a] (%)			
	Ling chih	Baby Ling chih	Mycelia	Filtrate
0.1	7.96 ± 0.78 A[b]	0.10 ± 0.70 C	2.42 ± 0.13 E	1.69 ± 0.21 D
0.5	9.66 ± 0.66 A	6.90 ± 0.27 A	7.81 ± 0.18 B	6.48 ± 0.49 B
1.0	4.54 ± 0.07 B	2.69 ± 0.16 B	20.1 ± 0.38 A	23.3 ± 0.44 A
5.0	1.49 ± 0.22 C	0.00 ± 0.00 C	6.36 ± 0.28 C	4.32 ± 0.47 C
10.0	0.00 ± 0.00 C	0.00 ± 0.00 C	3.65 ± 0.34 D	4.22 ± 0.18 C

[a] Scavenging ability (%) = 100 − the relative EPR signal intensity. The relative EPR signal intensity (%) = $[h\Delta H^2$ (sample)/$h\Delta H^2$ (control)] × 100; h = the width of the peak, ΔH = the length of the peak.

[b] Each value is expressed as mean ± standard deviation ($n = 3$). Means with different letters within a column are significantly different ($p < 0.05$).

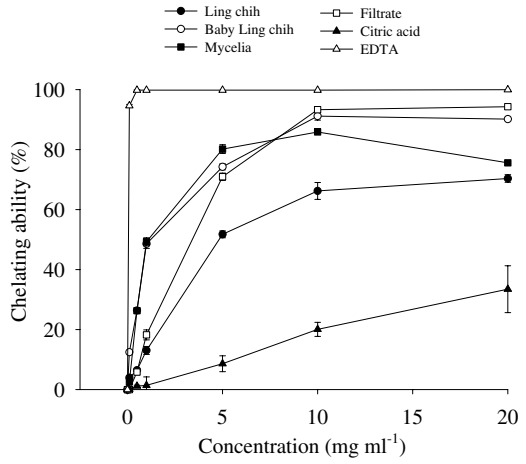

Fig. 4. Chelating ability of methanolic extracts from *Ganoderma tsugae* on ferrous ions. Each value is expressed as mean ± standard deviation ($n = 3$).

3.5. Chelating ability on ferrous ions

The chelating abilities of the methanolic extracts from baby Ling chih and filtrate were 74.2% and 71.0% at 5 mg ml^{-1} and 90.2% and 94.3% at 20 mg ml^{-1}, respectively (Fig. 4). The methanolic extract from mycelia showed high chelating abilities of 80.2–85.9% at 5–10 mg ml^{-1}, but its chelating ability was down to 75.6% at 20 mg ml^{-1}. The methanolic extract from Ling chih chelated ferrous ions by 70.4% at 20 mg ml^{-1}. However, EDTA showed excellent chelating ability of 94.6% at a concentration as low as 0.10 mg ml^{-1}. Citric acid was not a good chelating agent for ferrous ions and its chelating ability was 33.5% at 20 mg ml^{-1}. Since ferrous ions are the most effective pro-oxidants in the food system (Yamaguchi, Tatsumi, Kato, & Yoshimitsu, 1988), the high chelating abilities of methanolic extracts from *G. tsugae* would be beneficial.

Huang et al. (2002) found that all the methanolic extracts from *A. cylindracea* fruit bodies, mycelia and filtrate were good chelators for ferrous ions and their chelating abilities were 90.6%, 84.6% and 96.3% at 5 mg ml^{-1}, respectively. Yen and Wu (1999) reported that the methanolic extract of *G. tsugae* chelated 95.3% of ferrous ions at 600 ppm (0.6 mg ml^{-1}). However, Yen and Wu (1999) used the method of Decker and Welch (1990) to determine the chelating ability instead of the method of Shimada et al. (1992).

Methanolic extracts from *A. camphorata* chelated ferrous ions by 64.4–74.5% at 5 mg ml^{-1}, whereas that from Brazilian mushrooms showed an excellent chelating ability of 98.6% at 2.5 mg ml^{-1} (Huang, 2000). The methanolic extract from *C. versicolor* was not a good ferrous chelator (13.2% at 2.4 mg ml^{-1}), whereas other medicinal mushrooms, including *G. lucidum*, *G. lucidum* antler, and *G. tsugae*, chelated 44.8–67.7% of ferrous ions at 2.4 mg ml^{-1} (Mau et al., 2002a). The methanolic extract from maitake chelated 70.3% of ferrous ions at 6 mg ml^{-1} whereas, at 24 mg ml^{-1}, methanolic extracts from black stinkhorn, lion's mane, and white matsutake chelated ferrous ions by 46.4–52.0% (Mau et al., 2002b). For commercial mushrooms, including winter, abalone, and tree oyster mushrooms and shiitake, their methanolic extracts chelated 45.6–81.6% of ferrous ions at 1.6 mg ml^{-1}(Yang et al., 2002). Methanolic extracts from ear mushrooms were good chelators for ferrous ions (85.1–96.5% at 5 mg ml^{-1}) (Mau et al., 2001b).

3.6. EC_{50} values in antioxidant properties

The antioxidant properties assayed herein are summarized in Table 3 except for scavenging ability on hydroxyl radicals, and the results were normalized and expressed as EC_{50} values (mg methanolic extract per ml) for comparison. With regard to EC_{50} values in antioxidant activity by the conjugated diene method, the methanolic extract from Ling chih was better than baby Ling chih. However, EC_{50} values were not available for those from mycelia and filtrate. Effectiveness in reducing powers inversely correlated with EC_{50} value and was in a descending order: mycelia > filtrate ~ baby Ling chih > Ling chih. Scavenging abilities on DPPH radicals were excellent for methanolic extracts from Ling chih and baby Ling chih while EC_{50} values for those from

Table 3
EC_{50} values of methanolic extracts from *G. tsugae* in antioxidant properties

	EC_{50} value[a] (mg extract ml^{-1})			
	Ling chih	Baby Ling chih	Mycelia	Filtrate
Antioxidant activity (conjugated diene method)	0.53 ± 0.18 B[b]	1.11 ± 0.02 A	>20	>20
Reducing power	5.00 ± 0.06 A	2.28 ± 0.09 B	0.93 ± 0.01 C	2.15 ± 0.01 B
Scavenging ability on DPPH radicals	0.61 ± <0.01 C	0.73 ± 0.01 C	4.28 ± 0.14 B	5.00 ± <0.01 A
Chelating ability on ferrous ions	4.82 ± 0.08 A	3.05 ± 1.20 B	1.10 ± 0.12 C	3.41 ± 0.07 B

[a] EC_{50} value: the effective concentration at which the antioxidant activity was 50%; the absorbance was 0.5 for reducing power; 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals were scavenged by 50%; and ferrous ions were chelated by 50%, respectively. EC_{50} value was obtained by interpolation from linear regression analysis.

[b] Each value is expressed as mean ± standard deviation ($n = 3$). Means with different letters within a row at a specific EC_{50} are significantly different ($p < 0.05$).

Table 4
Contents of ascorbic acid, β-carotene, tocopherols and total phenols of methanolic extracts from *Ganoderma tsugae*

Compound	Content (mg g^{-1})			
	Ling chih	Baby Ling chih	Mycelia	Filtrate
Ascorbic acid	0.05 ± 0.04 B[a]	0.02 ± 0.01 B	nd[b]	0.20 ± 0.03 A
β-Carotene	0.24 ± 0.02 A	0.17 ± 0.03 B	0.09 ± 0.02 C	nd
α-Tocopherol	0.24 ± 0.01 A	0.12 ± 0.09 B	0.08 ± 0.03 B	nd
γ-Tocopherol	0.43 ± 0.20 A	0.14 ± 0.20 B	nd	nd
δ-Tocopherol	0.06 ± 0.03 A	0.01 ± 0.01 A	0.07 ± 0.05 A	nd
Total phenols	24.0 ± 0.15 C	30.5 ± 0.50 B	35.6 ± 0.34 A	30.7 ± 0.40 B

[a] Each value is expressed as mean ± standard deviation ($n = 3$). Means with different letters within a row are significantly different ($p < 0.05$).
[b] Not detected.

mycelia and filtrate were significantly higher. The chelating abilities on ferrous ions were in a descending order: mycelia > baby Ling chih ~ filtrate > Ling chih. All EC_{50} values were below 10 mg ml^{-1}, except for EC_{50} values of mycelia and filtrate in antioxidant activity, indicating that these forms of *G. tsugae* were good in antioxidant properties, except for the scavenging ability on hydroxyl radicals.

When the extraction yields were taken into consideration, EC_{50} values (mg dried mycelia per ml) in antioxidant activity were 6.26 and 11.3 mg ml^{-1} for Ling chih and baby Ling chih. EC_{50} values in reducing power were 59.1, 23.3, 2.29 and 2.71 mg ml^{-1} for Ling chih, baby Ling chih, mycelia and filtrate. EC_{50} values, in scavenging ability on DPPH radicals, were 7.21, 7.46, 10.5 and 6.31 mg ml^{-1} for Ling chih, baby Ling chih, mycelia and filtrate. EC_{50} values in chelating ability on ferrous ions were 57.0, 31.2, 2.71 and 4.30 mg ml^{-1} for Ling chih, baby Ling chih, mycelia and filtrate.

Although BHA and α-tocopherol were good in antioxidant activity, reducing power and scavenging ability on DPPH radicals and EDTA was excellent for chelating ferrous ions, they are additives and used or present in mg levels in foods. However, *G. tsugae*, in the form of Ling chih, baby Ling chih, mycelia and filtrate, could be used in g levels as food or a food ingredient. Therefore, *G. tsugae* in human diets might serve as possible protective agents to help humans to reduce oxidative damage.

3.7. Antioxidant components

Naturally occurring antioxidant components, including ascorbic acid, tocopherols, and total phenols, were found in methanolic extracts from Ling chih and baby Ling chih (Table 4). Total phenols were the major naturally occurring antioxidant components found in methanolic extracts from Ling chih, baby Ling chih, mycelia and filtrate and in the range of 24.0–35.6 mg g^{-1}. However, ascorbic acid and γ-tocopherol were not detected in the methanolic extract from mycelia, and β-carotene and tocopherols were not found in that from filtrate. Total antioxidant components varied among methanolic extracts and were 25.0, 31.0, 35.8 and 30.9 mg g^{-1} for Ling chih, baby Ling chih, mycelia and filtrate, respectively.

It seems that contents of total antioxidant components and total phenols did not correlate well with the discrepancy in antioxidant attributes. However, phenols such as BHT and gallate are known to be effective antioxidants (Madhavi, Singhal, & Kulkarni, 1996). Yen, Duh, and Tsai (1993) found that the antioxidant activity of the methanolic extract from peanut hulls correlated with its content of total phenols. Therefore, the high content of total phenols in all methanolic extracts might explain high antioxidant properties in *G. tsugae*. To study the antioxidant mechanisms by some specific phenolic components, the fractionation of the methanolic extract and further identification are in progress. Nevertheless, on the basis of the results obtained, upon the consumption of *G. tsugae*, the alleged antioxidant properties might be somewhat beneficial to the antioxidant protection system of the human body.

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