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Antioxidant properties of polysaccharides from *Ganoderma tsugae*

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Abstract

Ganoderma tsugae Murrill (Ganodermataceae) were available in the form of mature and baby Ling chih, mycelia and fermentation filtrate. From these four forms, hot water extracted and hot alkali extracted polysaccharides were prepared and their antioxidant properties were studied. Polysaccharides showed good antioxidant activity as evidenced by their particularly low EC_{50} values (<0.1 mg/ml). At 20 mg/ml, both extracted polysaccharides from mycelia showed reducing powers of 0.41–0.52 whereas reducing powers of other polysaccharides were in the range of 0.87 to 1.14. At 20 mg/ml, scavenging abilities on 1,1-diphenyl-2-picrylhydrazyl radicals increased to 93.7–100%, except for that of the hot water extracted polysaccharide, from filtrate, being 74.9%. At 20 mg/ml, scavenging abilities of both extracted polysaccharides from mycelia on hydroxyl radicals were 13.9 and 24.4%, respectively whereas scavenging abilities of the other polysaccharides were in the range of 39.0–55.2%. At 10 mg/ml, the chelating abilities of polysaccharides from mature and baby Ling chih, mycelia and filtrate were 93.9–100%, 97.6–100%, 85.1–88.0% and 51.2%, respectively. Overall, both extracts of polysaccharides possessed good antioxidant properties except for scavenging ability on hydroxyl radicals and can be developed as a new dietary supplement and functional food.

Keywords: *Ganoderma tsugae*; Polysaccharide; Antioxidant activity; Reducing power; Scavenging ability; Chelating ability

1. Introduction

Ganoderma tsugae Murrill (Ling chih, Sung-shan-ling-chih or reishi), one of the most famous traditional Chinese medicines, has attracted much attention on account of its biological activities (Wasser & Weis, 1999). 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. In addition, baby Ling chih does not cause ecological damage to trees due to no spores being discharged (Tseng, Lee, Li, & Mau, 2005). In polypropylene bag cultivation, *G. tsugae* requires a long time to produce fruit bodies. However, the submerged culture only requires a short time to obtain mycelia and a fermented filtrate. Both the fruit bodies and mycelia of *G. tsugae* are mainly prepared for use in the formulation of nutraceuticals and functional foods.

Plant polysaccharides have exhibited strong antioxidant properties and can be explored as novel potential antioxidants (Hu, Xu, & Hu, 2003; Ramarahnam, Osawa, Ochi, & Kawaishi, 1995; Wang & Luo, 2007). In addition, polysaccharides extracted from mushrooms, such as *Grifola frondosa* and *Auricularia auricular*, have also shown antioxidant properties as shown by their free radical scavenging ability (Fan, Zhang, Yu, & Ma, 2007; Lee et al., 2003; Liu, Ooi, & Chang, 1997). The methanolic extract from Ling chih, including *G. lucidum* and *G. tsugae*, was found to be high in antioxidant abilities (Mau, Lin, & Chen, 2002; Yen & Wu, 1999). Furthermore, methanolic, hot water and cold water extracts from *G. tsugae* possessed good antioxidant properties (Mau, Tsai, Tseng, & Huang, 2005a, 2005b; Tseng & Mau, 2007). However, the antioxidant properties of polysaccharides from *G. tsugae* were not available.

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Accordingly, our objective was to evaluate and compare the antioxidant properties of hot water extracted and hot alkali extracted polysaccharides from *G. tsugae* in the form of mature and baby fruit bodies, mycelia and fermentation 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.

2. Materials and methods

2.1. Mushroom fruit bodies, mycelia and fermentation filtrate

The pure culture of *Ganoderma tsugae* GT01 was originally obtained from the Department of Plant Pathology, Taiwan Agricultural Research Institute, Wufeng, Taichung County, Taiwan. Fresh mature (6 weeks old) and baby (2 weeks old) Ling chih was harvested from the mushroom room of the Department of Food Science and Biotechnology, 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.

Mycelia were grown in a 500 l fermentor with a 350 l working volume. The medium consisted of 2% glucose, 1% corn starch, 0.5% yeast extract, 0.5% peptone, 0.3% ammonium sulfate, 0.3% magnesium sulfate heptahydrate, 0.3% potassium dihydrogen phosphate and pH 4.5. The working conditions were: temperature, 28 °C; aeration rate, 0.5 vvm; agitation speed, 90 rpm and the inoculum rate, 10 ml/l. After 5 days of incubation, the mycelia were harvested at the reducing sugar concentration of 0.1 g/l. The mycelia were separated from the filtrate using centrifugation (4 °C, 8000*g* for 15 min) and then washed with deionised water. Finally, the mycelia and fermentation filtrate were freeze-dried to a powder form. For each of mature and baby Ling chih, mycelia and filtrate, three dried samples (*ca.* 50 g each) were randomly selected and prepared for analyses.

2.2. Preparation of polysaccharides

After a coarse powder (20 mesh) was obtained using a mill (Retsch ultracentrifugal mill and sieving machine, Haan, Germany), a subsample (100 g) was heated with 5000 ml deionised water or aqueous 0.1 N sodium hydroxide solution (Wako Pure Chemical Co., Osaka, Japan), at reflux, for 3 h. The mixture was cooled to room temperature and filtered through Whatman No. 4 filter paper. The residue was then refluxed with two additional 100 ml portions of deionised water or aqueous alkaline solution as described above. The filtrate was dialysed using a Cellu Sep T2 tubular membrane (MWCO: 6,000–8,000, Membrane Filtration Products, Inc., Seguin, TX) for 24 h. The retentate was concentrated to a small volume and then mixed with 3 volumes of 95% ethanol to yield a 70% ethanolic solution. The precipitate thus obtained was lyophilised and ground to obtain a coarse powder of hot water extracted or hot alkali extracted polysaccharides (60 mesh) from mature and baby Ling chih, mycelia and filtrate. Each polysaccharide powder was redissolved in deionised water to a concentration of 20 mg/ml and stored at 4 °C for further uses.

2.3. Antioxidant activity

The antioxidant activity was determined by the conjugated diene method (Lingnert, Vallentin, & Eriksson, 1979). Each polysaccharide powder (0.1–20 mg/ml, 100 μl), in deionised water, was mixed with 2 ml of 10 mM linoleic acid (Sigma Chemical Co., St. Louis, MO) emulsion in 0.2 M sodium phosphate buffer (Sigma, 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 was added into each tube, 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 (%) = $[(\Delta A_{234}\text{of control} - \Delta A_{234}\text{ of sample})/\Delta A_{234}\text{ of control}] \times 100$. A blank is the deionised water only and a control consisted of water and the reagent solution without the extract. An AOA value of 100% indicates the strongest antioxidant activity. The 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, butylated hydroxyanisole (BHA) and α-tocopherol (all from Sigma) were used for comparison.

2.4. Reducing power

The reducing power was determined according to the method of Oyaizu (1986). Each polysaccharide powder (0.1–20 mg/ml, 2.5 ml), in deionised water, was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6, Wako) 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 indicates a higher reducing power. The EC_{50} value (mg extract/ml) is the effective concentration at which the absorbance was 0.5 for the reducing power. Ascorbic acid, BHA and α-tocopherol were used for comparison.

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

Each polysaccharide powder (0.1–20 mg/ml, 4 ml) in deionised water was mixed with 1 ml of methanolic solution

containing DPPH l (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, and Nakamura (1992). The scavenging ability was calculated as follows: scavenging ability (%) = [(ΔA_{517} of control − ΔA_{517} of sample)/ΔA_{517} of control] × 100. The EC_{50} value (mg extract/ml) is the effective concentration at which the DPPH radicals were scavenged by 50%. Ascorbic acid, BHA and α-tocopherol were used for comparison.

2.6. Scavenging ability on hydroxyl radicals

The hydroxyl radical 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 200 μl of each polysaccharide powder (0.1–20 mg/ml, 200 μl), in deionised water, with 10 mM hydrogen peroxide (Merck, Darmstadt, Germany), 200 μl of 10 mM ferrous sulfate (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 as follows: Scavenging ability (%) = 100 − the relative EPR signal intensity. The relative EPR signal intensity (%) = [$h\Delta H^2$ (sample)/$h\Delta H^2$ (control)] × 100; wherein h is the width of the peak and ΔH is the length of the peak. The EC_{50} value (mg extract/ml) is the effective concentration at which the hydroxyl radicals were scavenged by 50%. BHA was used for a comparison.

2.7. Chelating ability on ferrous ions

Chelating ability was determined according to the method of Dinis, Madeira, and Almeida (1994). Each polysaccharide powder (0.1–20 mg/ml, 1 ml), in deionised water, 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 5 mM ferrozine (0.2 ml, Sigma). After 10 min at room temperature, the absorbance of the mixture was determined at 562 nm against a blank. A lower absorbance indicates a higher chelating power. The EC_{50} value (mg extract/ml) is the effective concentration at which ferrous ions were chelated by 50%. Citric acid (Sigma) and ethylenediaminetetraacetic acid (EDTA, Sigma) were used for comparison.

2.8. Statistical analysis

For each hot water extracted and hot alkali extracted polysaccharide from mature, baby Ling chih, mycelia and filtrate, three samples were prepared for assays of every antioxidant attribute. The experimental data were subjected to an analysis of variance (ANOVA) for a completely random design (CRD), to determine the least significant difference (LSD) at the level of 0.05.

3. Results and discussion

3.1. Extraction yields

Using hot water or hot alkali solution as the extractant and after dialysis, the yields of both polysaccharides, precipitated from the 70% ethanolic solution, were in the descending order of mycelia > filtrate > Ling chih > baby Ling chih (Table 1). However, the yields of hot alkali extracted polysaccharides were higher than the corresponding yields of hot water extracted polysaccharides. It seems that hot alkaline treatment was effective in degrading the cell wall and water insoluble materials into water soluble components. Tseng (2004) found that the molecular weight ranges of hot water extracted and hot alkali extracted polysaccharides were 2.8×10^4 to 1.8×10^5 Da.

The difference between the methods for the preparation of hot water extracts and hot water extracted polysaccharides was the dialysis process. The yields of hot water extracts from Ling chih, baby Ling chih, mycelia and filtrate were 6.22, 9.91, 32.31 and 83.57%, respectively (Mau et al., 2005a). However, after dialysis, small components in hot water extracts were removed and the yields of hot water extracted polysaccharides was remarkably reduced. Contents of soluble polysaccharides were 5.06%, 11.3%, 11.6% and 14.7% for Ling chih, baby Ling chih, mycelia and filtrate, respectively (Tseng et al., 2005). Contents of hot water extracted polysaccharides were far less than those contents. It seems that hot water treatment might degrade soluble polysaccharides into smaller components and those were then removed during the dialysis. However, hot alkali extracted polysaccharides were obtained from the residual fiber, which was high in chitin (Tseng et al., 2005).

3.2. Antioxidant activity

Using the conjugated diene method, polysaccharides showed high antioxidant activities of 78.3–87.8% at 0.1 mg/ml, except for the antioxidant activity of hot alkali

Table 1
Extraction yield of polysaccharides from *Ganoderma tsugae*

	Extraction %[a] (w/w)	
	Hot water extracted	Hot alkali extracted
Ling chih	1.73 ± 0.08 C[b]	7.28 ± 0.06 C
Baby Ling chih	1.51 ± 0.09 D	6.32 ± 0.08 D
Mycelia	8.32 ± 0.10 A	14.45 ± 0.13 A
Filtrate	6.22 ± 0.07 B	8.42 ± 0.09 B

[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 ($P < 0.05$).

extracted polysaccharides being 54.8% (Fig. 1). However, antioxidant activities of ascorbic acid, BHA and α-tocopherol were 32.0, 89.2 and 88.9% at 0.1 mg/ml, respectively. It is obvious that both polysaccharides prepared were as effective as BHA and α-tocopherol in inhibiting the peroxidation of linoleic acid.

Mau et al. (2005a) found that at 20 mg/ml, the hot water extracts from mature and baby Ling chih and filtrate showed high antioxidant activities of 78.5, 78.2 and 45.8%, respectively, whereas no activity was found in the hot water extract from mycelia. It seems that the dialysis process could remove small components without antioxidant activity from the hot water extracts and keep polysaccharides with high antioxidant activity.

3.3. Reducing power

Reducing powers of both polysaccharides increased in two patterns with increased concentrations, i.e., a fast increase for Ling chih, baby Ling chih and filtrate and a slow increase for mycelia (Fig. 2). At 20 mg/ml, hot water extracted and hot alkali extracted polysaccharides from mycelia showed reducing powers of 0.41–0.52, whereas reducing powers of other polysaccharides were in the range of 0.87 to 1.14. However, BHA showed a reducing power of 0.96 at 0.1 mg/ml and reducing powers of ascorbic acid and α-tocopherol were 0.90 and 1.02 at 1 mg/ml, respectively.

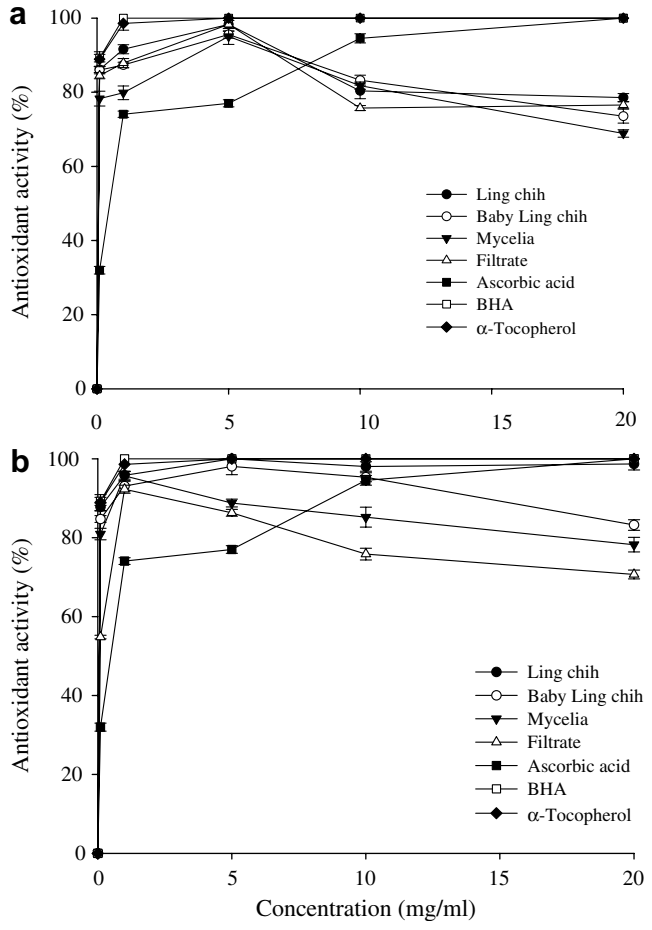

Fig. 1. Antioxidant activity of hot water extracted (a) and hot alkali extracted polysaccharides (b) from *Ganoderma tsugae*. Each value is expressed as mean ± standard deviation ($n = 3$).

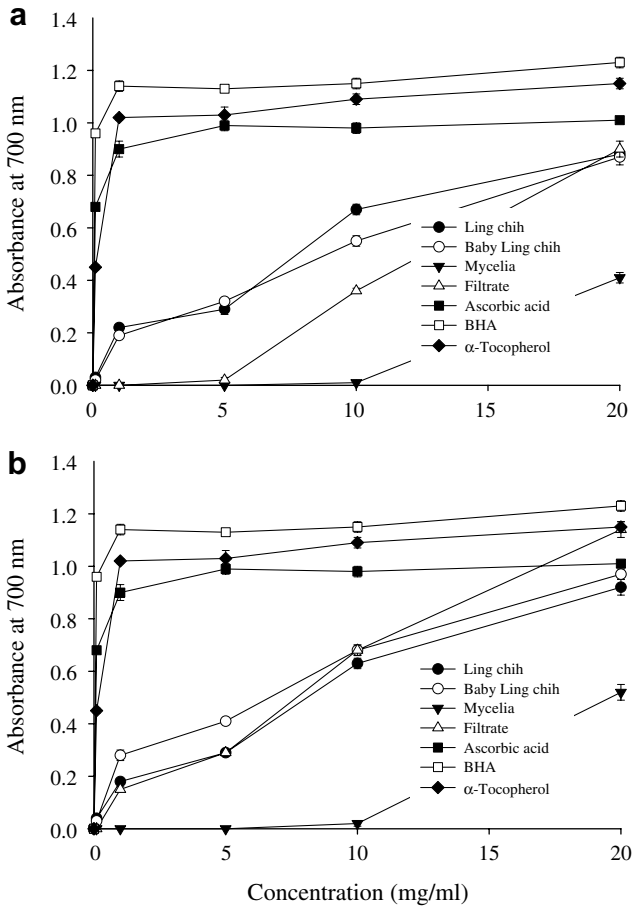

Fig. 2. Reducing power of hot water extracted (a) and hot alkali extracted polysaccharides (b) from *Ganoderma tsugae*. Each value is expressed as mean ± standard deviation ($n = 3$).

Mau et al. (2005a) found that at 5 mg/ml, the hot water extracts from mature and baby Ling chih, mycelia and filtrate showed reducing powers of 1.08, 1.04, 0.95 and 1.12, respectively. With regard to reducing power, hot water extracted polysaccharides were less effective than hot water extracts. Apparently, high reducing power might be due to small components in the hot water extracts.

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

At 5 mg/ml, polysaccharides showed scavenging abilities of 36.4–58.4% on DPPH radicals (Fig. 3). At 20 mg/ml, scavenging abilities increased to 93.7–100%, except for that of the hot water extracted polysaccharide from filtrate, which was 74.9%. However, at 0.1–20 mg/ml, scavenging abilities of ascorbic acid, BHA and

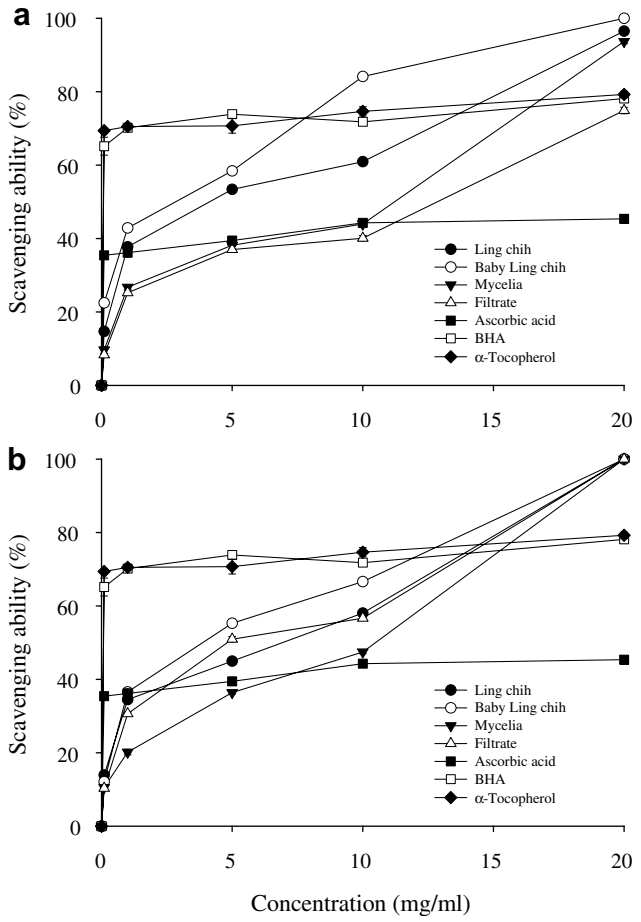

Fig. 3. Scavenging ability of hot water extracted (a) and hot alkali extracted polysaccharides (b) from *Ganoderma tsugae* on 1,1-diphenyl-2-picrylhydrazyl radicals. Each value is expressed as mean ± standard deviation ($n = 3$).

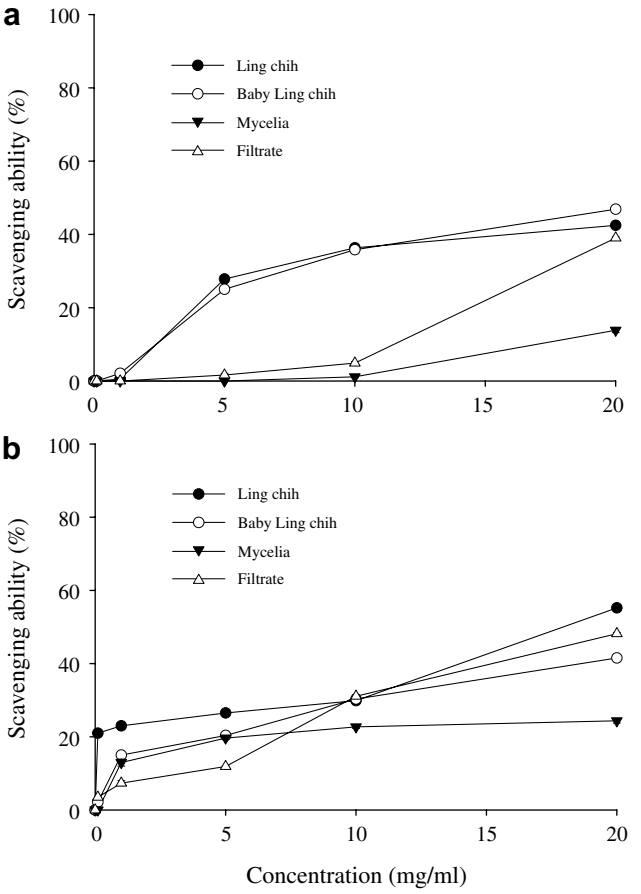

Fig. 4. Scavenging ability of hot water extracted (a) and hot alkali extracted polysaccharides (b) from *Ganoderma tsugae* on hydroxyl free radicals. Each value is expressed as mean ± standard deviation ($n = 3$).

α-tocopherol were 35.4–45.4%, 65.1–78.1% and 69.4–79.2%, respectively.

Mau et al. (2005a) found that at 5 mg/ml, scavenging abilities of the hot water extracts from mature and baby Ling chih, mycelia and filtrate were 71.9, 67.1, 59.6 and 50.0%, respectively. It seems that scavenging abilities of hot water extracts and hot water extracted polysaccharides were comparable.

3.5. Scavenging ability on hydroxyl radicals

At 20 mg/ml, the scavenging abilities of hot water extracted and hot alkali extracted polysaccharides from mycelia on hydroxyl radicals were 13.9 and 24.4%, respectively, whereas scavenging abilities of other polysaccharides were in the range of 39.0 to 55.2% (Fig. 4). However, the scavenging ability of BHA was 22.8% at 20 mg/ml.

Hot water extracts from mature and baby Ling chih, mycelia and filtrate scavenged hydroxyl radicals by 72.4, 73.7, 55.9 and 46.0% at 20 mg/ml (Mau et al., 2005a). It is obvious that hot water extracts contained other small components responsible for this hydroxyl radical scavenging ability in addition to polysaccharides.

3.6. Chelating ability on ferrous ions

At 5 mg/ml, both polysaccharides from mature and baby Ling chih chelated ferrous ions by 73.0–75.6% whereas chelating abilities of polysaccharides from mycelia and filtrate were 50.8–68.1% and 41.1–47.5%, respectively (Fig. 5). At 10 mg/ml, chelating abilities of polysaccharides from mature and baby Ling chih, mycelia and filtrate were 93.9–100%, 97.6–100%, 85.1–88.0% and 51.2%, respectively. However, EDTA showed an excellent chelating ability of 90.1% at 0.1 mg/ml. Citric acid was not a good chelating agent for ferrous ions in this assay and its chelating ability was 17.2% at 20 mg/ml.

Hot water extracts from mature and baby Ling chih chelated 42.6 and 39.5% of ferrous ions at 20 mg/ml, respectively, whereas those from mycelia and filtrate chelated 4.9 and 17.2% of ferrous ions at 20 mg/ml, respectively (Mau et al., 2005a). Obviously, hot water extracted and hot alkali extracted polysaccharides were good chelating agents for ferrous ions. Since ferrous ions are the most effective pro-oxidants in the food system (Yamaguchi,

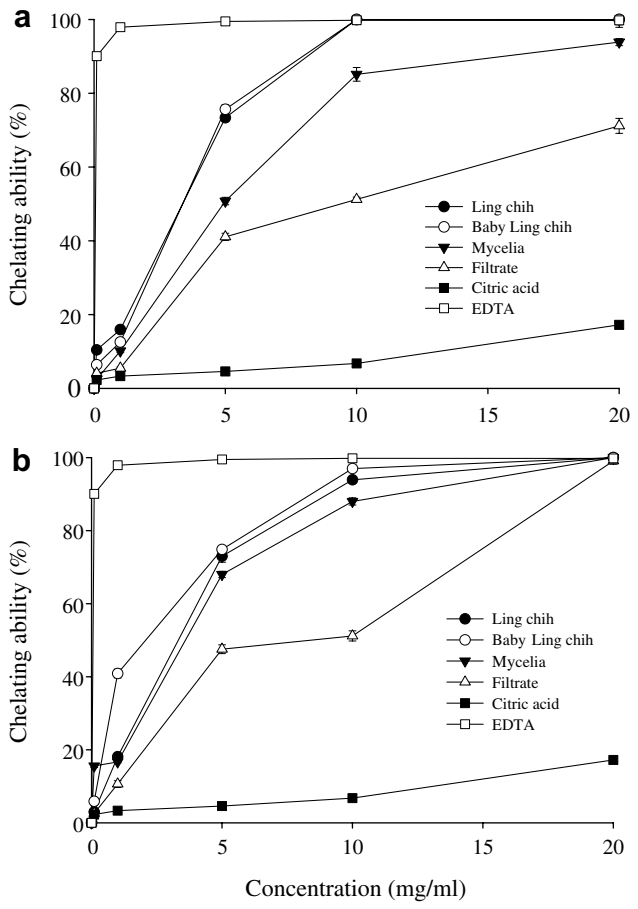

Fig. 5. Chelating ability of hot water extracted (a) and hot alkali extracted polysaccharides (b) from *Ganoderma tsugae* on ferrous ions. Each value is expressed as mean ± standard deviation ($n = 3$).

Tatsumi, Karo, & Yoshimitsu, 1988), the high ferrous-ion chelating abilities of polysaccharides from *G. tsugae* would be somewhat beneficial.

3.7. EC_{50} values in antioxidant properties

The antioxidant properties assayed herein were summarised in Table 2 and the results were normalised and expressed as EC_{50} values for comparison. Effectiveness in antioxidant properties inversely correlated with EC_{50} value. Hot water extracted and hot alkali extracted polysaccharides showed good antioxidant activity as evidenced by their particularly low EC_{50} values (<0.1 mg/ml). With regard to effectiveness in reducing powers of both polysaccharides, mature and baby Ling chih were comparable and more effective than filtrate, which was in turn more effective than mycelia.

With regard to scavenging ability on DPPH radicals, EC_{50} values of polysaccharides from mature and baby Ling chih were less than 7 mg/ml whereas those of polysaccharides from mycelia were 10.49–11.22 mg/ml. However, for filtrate, EC_{50} value of hot water extracted and hot alkali extracted polysaccharides were 12.85 and 4.81 mg/ml. Both extracted polysaccharides showed scavenging ability on hydroxyl radicals but EC_{50} values were higher than 20 mg/ml, except for that of hot alkali extracted polysaccharide from mature Ling chih being 17.95 mg/ml. Chelating abilities of both polysaccharides from four forms of *G. tsugae* on ferrous ions were good as shown by their low EC_{50} values (<10 mg/ml). Effectiveness in chelating abilities was in the descending order of mature Ling chih ~ baby Ling chih > mycelia > filtrate. Overall, both extracted polysaccharides possessed good antioxidant properties, except for scavenging ability on hydroxyl radicals and can be developed as a new dietary supplement and functional food.

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

Table 2
EC_{50} values of polysaccharides from *Ganoderma tsugae* in antioxidant properties

	EC_{50}[a] (mg extract/ml)			
	Ling chih	Baby Ling chih	Mycelia	Filtrate
Hot water extracted polysaccharides				
Antioxidant activity	<0.1[b]	<0.1	<0.1	<0.1
Reducing power	7.76 ± 0.12 C	8.91 ± 0.04 B	>20	12.59 ± 0.11 A
Scavenging ability on DPPH radicals	4.13 ± 0.07 C	2.84 ± 0.02 D	11.22 ± 0.11 B	12.85 ± 0.06 A
Scavenging ability on OH radicals	>20	>20	>20	>20
Chelating ability on ferrous ions	3.37 ± 0.01 C	3.37 ± 0.02 C	4.92 ± 0.02 B	9.40 ± 0.05 A
Hot alkali extracted polysaccharides				
Antioxidant activity	<0.1	<0.1	<0.1	<0.1
Reducing power	8.09 ± 0.14 B	6.67 ± 0.11 D	19.60 ± 0.05 A	7.69 ± 0.03 C
Scavenging ability on DPPH radicals	6.93 ± 0.04 B	3.87 ± 0.02 D	10.49 ± 0.14 A	4.81 ± 0.08 C
Scavenging ability on OH radicals	17.95 ± 0.16 A	>20	>20	>20
Chelating ability on ferrous ions	3.32 ± 0.02 C	2.08 ± 0.04 D	3.60 ± 0.01 B	8.39 ± 0.03 A

[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) or hydroxyl (OH) 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 are significantly different ($P < 0.05$).

levels in foods. However, *G. tsugae* in the form of Ling chih, baby Ling chih, mycelia and filtrate and their hot water extracted and hot alkali extracted polysaccharides could be used in g levels as food or a food ingredient. Therefore, in addition to their therapeutic effects, *G. tsugae* in human diets might serve as possible protective agents to help humans reduce oxidative damage. The hot water extracted and hot alkali extracted polysaccharides prepared could be developed as a new dietary supplement and functional food. In addition, both extracted polysaccharides could be added in emulsion for antioxidation prevention (Kishk & Al-Sayed, 2007) or formulated into bread as a health-promoting functional food (Fan et al., 2007). To study the antioxidant mechanisms by polysaccharides, the composition and structure confirmation of hot water extracted and hot alkali polysaccharides are in progress.

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