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Antioxidant and radical scavenging properties of extracts from *Ganoderma tsugae*

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Abstract

The antioxidant activities and scavenging effects on free radicals of extracts from *Ganoderma* were investigated. The methanolic extracts of *Ganoderma tsugae* (MEGT) showed the strongest antioxidant activity of five species of *Ganoderma* tested. MEGT exhibited substantial antioxidant activity in the linoleic acid and rat liver microsome peroxidation systems. The antioxidant activity of MEGT was stronger than α-tocopherol. MEGT had a strong chelating effect on Fe^{2+}. MEGT showed a marked and concentration-dependent scavenging activity on the 1,1-diphenyl-2-picrylhydrazyl radical. MEGT also exhibited a strong scavenging effect on the hydroxyl radical as measured by electron paramagnetic resonance spectrometry. The formation of 8-hydroxy-2′-deoxyguanosine, from 2′-deoxyguanosine induced by various prooxidants, was reduced by MEGT. In view of these results, the antioxidant and radical scavenging activities of *Ganoderma* may have an important role on inhibition of lipid peroxidation in biological systems.

1. Introduction

Lipid peroxidation is an important deteriorative reaction in food during storage and processing. It not only causes a loss of food quality but is also strongly associated with carcinogenesis, mutagenesis, aging, and atherosclerosis (Yagi, 1987). The role of active oxygen and free radicals in tissue damage in various human diseases, cancer and aging are becoming increasingly recognized (Halliwell, Gutteridge, & Cross, 1992). Active oxygen, in the form of superoxide, hydrogen peroxide, hydroxyl radical, and singlet oxygen, is a byproduct of normal metabolism and attacks biological molecules, leading to cell or tissue injury. Natural antioxidants from dietary plants are reported to prevent oxidative damage by free radical and active oxygen, and prevent the ocurrence of disease, cancer, and aging (Hirose, Imaida, Tamano, & Ito, 1994).

Recently, research on phytochemicals in food materials and their effects on health, especially the suppression of active oxygen species by natural antioxidants from teas, spices and herbs, has been intensively studied (Ho, Osawa, Huang, & Rosen, 1994). Chinese herbs have been commonly used for diet therapy for thousands of years. Some Chinese herbs are reported to exhibit strong antioxidant activity (Kim, Kim, Kim, Oh, & Jung, 1994; Su, 1992). *Ganoderma* is a traditional Chinese medicine and has been prescribed for treatment of chronic hepatopathy, hypertension, bronchitis, arthritis, neurasthenia and neoplasin in China and other countries of the Orient (Arisawa, Fujica, Saga, Fukumura, Hayashi, Shimizu & Morita, 1986). Ng, Kong, Ko, So, and Yick (1983) reported that *Ganoderma lucidum* had protective effects on carbon-tetrachloride-induced hepatotoxicity. However, little information is available about the antioxidant activity of *Ganoderma*. Therefore, the objectives of this study were to investigate the antioxidant activity and free radical scavenging activity of extracts from *Ganoderma*, especially *G. tsugae*.

2. Materials and methods

2.1. Samples

Five species of *Ganoderma* (dried fruiting bodies), including *G. formosanum* (Chang & Chen), *G. gibbasum* (Blumii: Nees) Patouillard, *G. lucidum* (Curtis: Fries) Karsten, *G. tsugae* Murrill, *Trametes versicolor* (Linnaeus: Fries) Pilat, were obtained from Ta-sun *Ganoderma*

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cultivation farm (Puli, Nanto, Taiwan). All samples were sliced and ground into a fine powder in a mill before extraction of antioxidants.

2.2. Extraction of antioxidants from Ganoderma

For comparison of antioxidant activity of different species of *Ganoderma*, 5 g of powder of each *Ganoderma* species was extracted with 100 ml methanol for 24 h, in a shaking incubator at room temperature. All extracts were filtered using Whatman no. 1 filter paper. The extraction was repeated twice, and the combined filtrates were evaporated to dryness in vacuo and weighed to determine the yield of soluble constituents.

2.3. Determination of antioxidant activity

The antioxidant activities of extracts from *Ganoderma* were determined by the thiocyanate method (Yen & Chen, 1995). 0.5 ml aliquots of each sample were mixed with linoleic acid emulsion (2.5 ml, 0.02 M, pH 7.0) and phosphate buffer (2 ml, 0.2 M, pH 7.0) and incubated at 37°C. The antioxidant activity was evaluated from the peroxide value determined by measuring the absorbance at 500 nm after coloring with $FeCl_2$ and thiocyanate at various intervals during incubation.

The antioxidant activity of methanolic extracts of *G. tsugae* (MEGT) on the inhibition of oxidation of microsome induced by nicotinamide adenine dinucleotide phosphate (NADPH)/adenosine diphosphate (ADP)/$FeCl_3$ was also evaluated. Rat liver microsomes were prepared from a male Sprague–Dawley rat (200 g) according to the method described by Quinlan, Halliwell, Moorhouse, and Gutteridge (1988) with some modification. Briefly, the rat liver was homogenized by a Polytron homogenizer and the homogenate was suspended in phosphate buffer. The microsomes were purified by three cycles of high speed centrifugation and stored at −80°C until used. Antioxidant activity assay was carried out by incubating MEGT (0–1000 ppm) with liver microsomal fractions (0.5 mg protein/ml) suspended in 1 ml 10 mM phosphate buffer pH 7.4, in the presence of NADPH and ADP (0.6 mM) and $FeCl_3$ (25 μM) for 1 h at 37°C. The inhibition of peroxidation was determined by measuring malondialdehyde formation using the thiobarbituric acid (TBA) methods (Aruoma, Murcia, Butler, & Halliwell, 1993). All test data are presented as an average of triplicate analyses.

2.4. Scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical

The effect of MEGT on DPPH radical was estimated according Hatano, Kagawa, Yasuhara, and Okuda (1988). MEGT (0.01–4 mg) in 4 ml methanol was added to a 1 ml solution of DPPH radical in methanol (final concentration of DPPH was 0.2 mM). The mixture was shaken vigorously and allow to stand for 30 min; the absorbance of the resulting solution was measured at 517 nm with a spectrophotometer (Hitachi U-2000). All tests and analyses were run in three replicates and averaged.

2.5. Hydroxyl radical scavenging activity

The generated hydroxyl radical rapidly reacted with nitrone spin trap 5,5-dimethylpyrrolidine *N*-oxide (DMPO). The resultant DMPO-OH adduct was detected by electron paramagnetic resonance (EPR) spectrometry using an EPR spectrometer (Bruker ER 200D 10/12, Germany) set under the following condition: 3480-G magnetic field, 1.0-G modulation anplitude, 0.5 s time constant, and 200 s scan period (Shi, Dalal, & Jain, 1991). The EPR spectrum was recorded 2.5 min after a 0.2 ml MEGT (1–20 mg/0.2 ml) was mixed with H_2O_2 (10 mM, 0.2 ml), Fe^{2+} (10 mM, 0.2 ml) and DMPO (0.3 M, 0.2 ml) in a phosphate buffer solution (pH 7.2).

2.6. Chelating activity on Fe^{2+}

The chelating activity of MEGT on Fe^{2+} was measured as reported by Decker and Welch (1990). One milliliter of MEGT (0.5–6 mg/ml) was mixed with 3.7 ml of deionized water, and then the mixture was reacted with $FeCl_2$ (2 mM, 0.1 ml) and ferrozine (5 mM, 0.2 ml) for 10 min, and the absorbance at 562 nm determined spectrophotometrically. Chelating activity of MEGT on Fe^{2+} was calculated as follows: Chelating activity (%) = [1−(absorbance of sample at 562 nm)/(absorbance of control at 562 nm)]×100.

2.7. Effect of MEGT on oxidation of 2′-deoxyguanosine

The effect of MEGT on oxidation of 2′-deoxyguanosine (2′-dG) to 8-hydroxy-2′-deoxyguanosine (8-OH-2′-dG) was assayed, using a modified method of Kasai and Nishimura (1984). The reaction mixture (0.7 ml) containing MEGT (0–8 mg), 2′-dG (0.5 mM), KH_2PO_4–K_2HPO_4 buffer (0.1 M, pH 7.4), was initiated by a Fenton reaction model system [H_2O_2 (25 mM), $FeCl_3$ (0.65 mM) and EDTA (3.25 mM)] with additional ascorbic acid (7.5 mM). The entire mixture was incubated at 37°C for 30 min. Control reactions lacking MEGT and ascorbic acid were also carried out. Placing the samples in an ice-bath terminated the reactions; the samples were filtered through a 0.45 μm filter before use. The filtrate (5 μl) was analysed by HPLC (Hitachi, Japan), using a LiChrosphere RP-18 column (150 × 4mm, 5 μm) and UV detector set at 254 nm. The column was equilibrated and eluted with 50 mM KH_2PO_4 (pH 4.6)/methanol (93.5:6.5, v/v) at a flow rate of 0.5 ml/min. 2′-dG and 8-OH-2′-dG were identified by

comparison of their retention times with those of known standards, and their ratio determined by peak areas from the chromatograms. All analyses were run in three replicates and averaged.

2.8. Statistical analysis

Statistical analysis involved use of the Statistical analysis systems (SAS, 1985) software package. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's multiple range tests.

3. Results and discussion

3.1. Antioxidant activity of different species of Ganoderma

The yield of methanolic extracts from five species of *Ganoderma* is shown in Table 1. *G. lucidum* had the highest yield of 10.3% while the other four species had yields around 3.3–4.8%. Comparison of antioxidant activity of methanolic extracts from five species of *Ganoderma* in the peroxidation of linoleic acid with commercial antioxidants BHA and α-tocopherol is shown in Fig. 1. The antioxidant activity of the five species of *Ganoderma* under study was in the order of *G. tsugae* > *G. formosanum* > *G. gibbasum* > *G. lucidum* > *T. versicolor*. The antioxidant activity of a methanolic extract of *G. tsugae* (MEGT) was slightly lower than that of BHA but was stronger than that of α-tocopherol. We therefore, selected *G. tsugae* for the following study.

As shown in Fig. 1, MEGT exhibited a stronger antioxidant activity in the linoleic acid peroxidation system. In order to extend this observation to biological system, the antioxidant activity of MEGT was evaluated in NADPH, ADP and $FeCl_3$-induced peroxidation in rat liver microsomes. Fig. 2 shows a profile of the antioxidant activity of MEGT in the rat liver microsome peroxidation system as compared to that of α-tocopherol. There was a rapid and concentration-dependent increase in the antioxidant activity of MEGT until a concentration of 200 ppm, beyond which there was no significant increase ($p > 0.05$) even up to 400 ppm. α-Tocopherol, on the other hand, exhibited a concentration-dependent increase in activity up to 400 ppm. The antioxidant activity of MEGT (92.1%) at a concentration of 200 ppm was stronger than that of α-tocopherol (57.1%) at a similar concentration range. The inhibition of lipid peroxidation can be explained on the basis of competition between MEGT, the antioxidant, and ADP, the oxidant, for binding Fe^{2+} (Kanner, German,

Table 1
Yield and ratio of methanolic extracts from different species of *Ganoderma*

Samples	Yield (mg)[a]	Ratio (%)
G. tsugae	210.2	4.20
G. formosanum	237.5	4.75
T. versicolor	162.4	3.25
G. gibbasum	202.4	4.05
G. lucidum	517.1	10.3

[a] Extracted from different species of *Ganoderma* (5.0 g). Values are means of duplicate analyses.

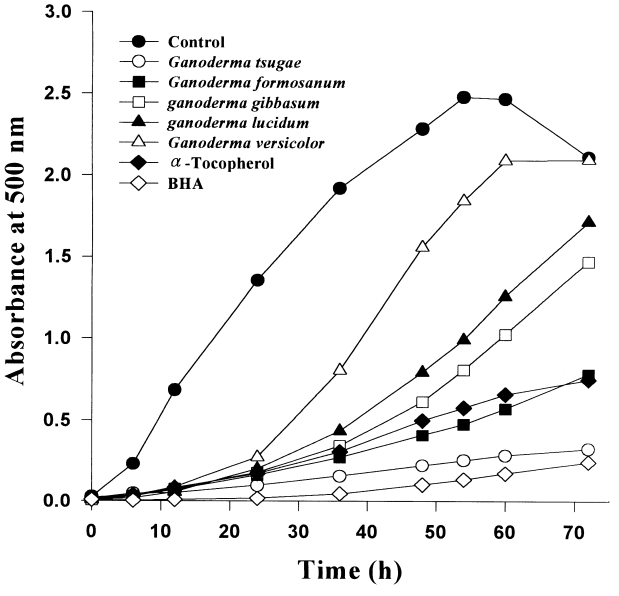

Fig. 1. Antioxidant activities of methanolic extracts from various species of *Ganoderma* in the linoleic acid peroxidation system as measured by the thiocyanate method.

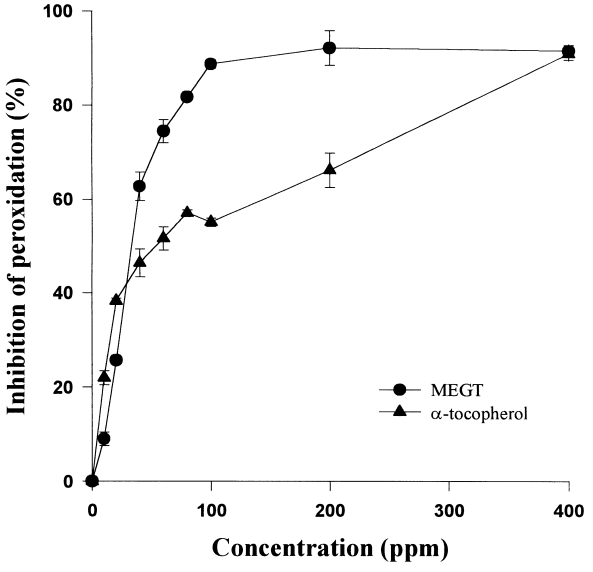

Fig. 2. Antioxidant activities of methanolic extracts from *G. tsugae* (MEGT) and α-tocopherol in the rat liver microsome peroxidation system induced by NADH, ADP, and $FeCl_3$.

& Kinsella, 1987) with MEGT having a higher affinity for the same.

3.2. Radical scavenging activity of MEGT

The profile of scavenging activity of MEGT on DPPH is shown in Fig. 3. The radical scavenging activity of MEGT on DPPH increased with an increasing concentration of MEGT. MEGT exhibited 42 and 75% radical scavenging activity at a concentration of 200 and 500 ppm, respectively. However, BHA and α-tocopherol showed 96% radical scavenging activity at a concentration of 200 ppm. This result shows that MEGT is also a free radical inhibitor, particularly of the peroxy radical, which is the major propagator of the autoxidation chain of fat, thus terminating the chain reaction.

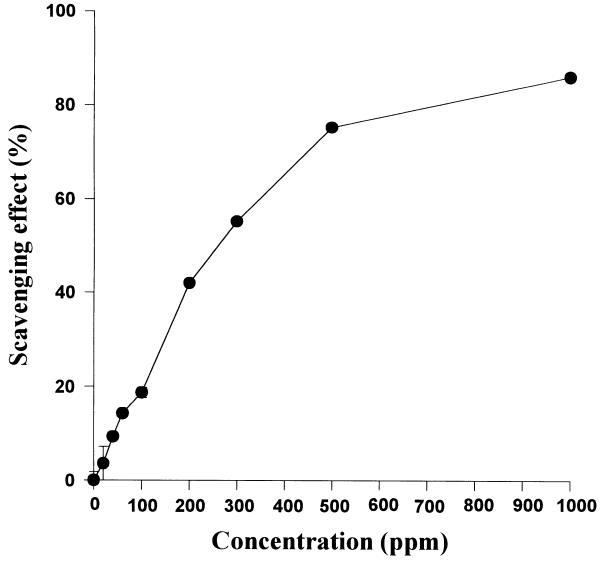

Fig. 3. Scavenging effect of methanolic extracts from *G. tsugae* on DPPH radicals with different concentrations.

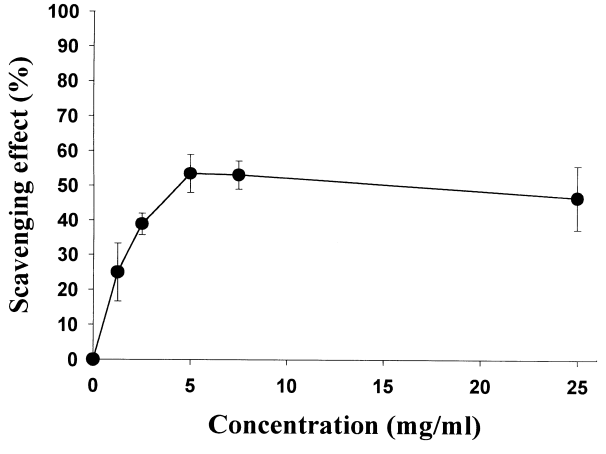

Fig. 4. Effect of methanolic extracts from *G. tsugae* on EPR signal intensity of DMPO-OH spin adducts with different concentrations.

3.3. Scavenging of hydroxyl radical by MEGT

The hydroxyl radical is an extremely reactive free radical formed in biological systems and reacts rapidly with almost every type of molecule found in living cells, such as sugars, amino acids, phospholipids, DNA bases, and organic acids. Fig. 4 shows the effect of MEGT on the EPR signal intensity of DMPO-OH adducts. MEGT exhibited a concentration-dependent increase in hydroxyl radical scavenging activity up to a concentration of 5 mg/ml. The scavenging activity was 53.4% at a concentration of 5 mg/ml. However, no significant differences ($p > 0.05$) were found in scavenging effect at higher concentrations from 5 to 25 mg/ml. This result is higher than that of methanolic extracts of peanut hulls which scavenge 30% of hydroxyl radical at a concentration of 5 mg/ml (Yen & Duh, 1994). Namiki (1990) indicated that the hydroxyl radical is the major active oxygen species causing lipid oxidation. Therefore, the antioxidant activity of MEGT may also be due to its scavenging activity on the hydroxyl radical.

3.4. Chelating activity on Fe^{2+} ion

As shown in Fig. 5, MEGT showed a strong chelating activity on Fe^{2+} ion. It displayed 95.3% chelating effect on Fe^{2+} ion at a concentration of 600 ppm. EDTA and citric acid are known metal ion chelators; we therefore compared the chelating effect of MEGT with both. The results indicated that the chelating activity of MEGT (89.9%) at 400 ppm was found to be weaker than EDTA (99.9%) but was dramatically higher than citric acid (2.0%) at the same concentration. This indicates that the chelating activity of MEGT on a metal ion may play an important role in its antioxidant activity.

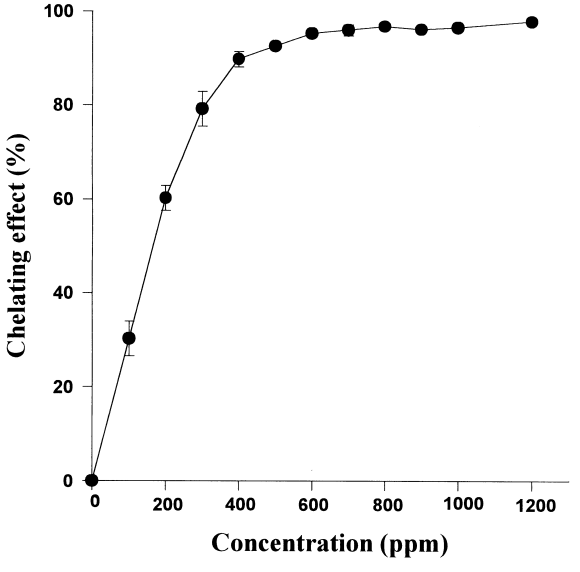

Fig. 5. Chelating effect of methanolic extracts from *G. tsugae* on Fe^{2+} ion with different concentrations.

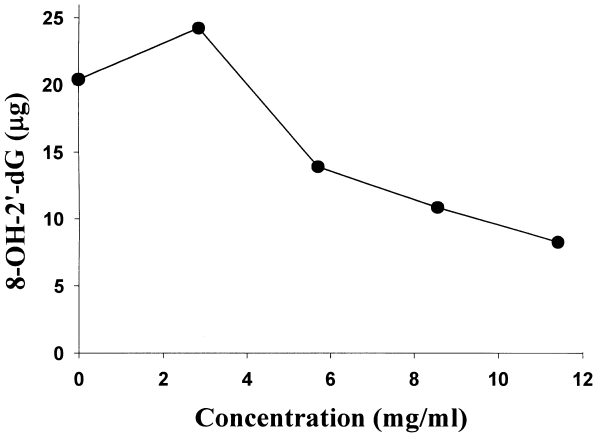

Fig. 6. Effect of methanolic extracts from *G. tsugae* on the hydroxylation of 2′-deoxyguanosine to 8-hydroxy-2′-deoxyguanosine (8-OH-2′-dG) induced by Fe^{3+}/H_2O_2/ascorbic acid.

3.5. Effect of MEGT on the oxidation of 2′-dG to 8-OH-2′-dG

The influence of MEGT on the formation of 8-OH-2′-dG from 2′-dG induced by Fe^{3+}/H_2O_2/ascorbic acid, was tested and the results are shown in Fig. 6. 8-OH-2′-dG is an useful index for detection oxidative damage of DNA in vivo and in vitro (Kasai & Nishimura, 1984). Therefore, the content of 8-OH-2′-dG can be used to investigate the extent of oxidative damage to DNA bases.

The results shown in Fig. 6 indicate that a low concentration of MEGT (<2.9 mg/ml) is enough to enhance the oxidation of 2′-dG to 8-OH-2′-dG in the test system induced by Fe^{3+}/H_2O_2/ascorbic acid. The formation of 8-OH-2′-dG, however, was inhibited with increasing concentration of MEGT, and 59.6% 8-OH-2′-dG can be reduced with MEGT at a concentration of 11.4 mg/ml. The inhibition of MEGT was lower than trolox, an antioxidant, which reduced formation of 8-OH-2′-dG by 39% at a concentration of 2.9 mg/ml.

Stadler, Turesky, Muller, Markovic, and Leung-Morgenthaler (1994) showed that incubation of 2′-dG with caffeic acid led to more formation of 8-OH-2′-dG, resulting from the reducing ability of caffeic acid. Coffee also accelerated the oxidation of 2′-dG to 8-OH-2'-dG at lower dosages. However, the formation of 8-OH-2'-dG was decreased by increasing the dosage of coffee, which may be due to interaction of the highly electrophilic hydroxy radical with coffee constituents. In the present study, MEGT exhibited an inhibitory effect on the oxidation of 2′-dG at higher concentration, which might be due to its scavenging effect on the hydroxyl radical and chelating effect on the metal ion.

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