Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Available online at www.sciencedirect.com
ScienceDirect
LWT 41 (2008) 669–677
LWT
www.elsevier.com/locate/lwt

In vitro evaluation of antioxidant activities of aqueous extracts from natural and cultured mycelia of *Cordyceps sinensis*

C.-H. Dong[a,b], Y.-J. Yao[a,*]

[a]*Key Laboratory of Systematic Mycology and Lichenology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China*
[b]*Graduate School of the Chinese Academy of Sciences, Beijing 10049, China*

Received 20 October 2006; received in revised form 28 April 2007; accepted 3 May 2007

Abstract

Cordyceps sinensis, one of the best known traditional Chinese medicines and health foods, has been highly valued for the treatment of a wide range of diseases and reported to have antioxidant properties. In the present study, the antioxidant activities of hot-water extracts from natural and cultured mycelia of *C. sinensis* were investigated and evaluated using six in vitro assays, including inhibition of linoleic acid peroxidation; scavenging abilities on DPPH•, hydroxyl and superoxide anion radicals; the reducing power and the chelating ability on ferrous ions. Among these assays, the extracts showed the best effect on the inhibition of linoleic peroxidation with the lowest IC50 values and with an inhibition rate over 90% at concentration of 0.8–1.6 mg/ml, more stable than that of α-tocopherol, a recognised natural antioxidant. The scavenging activities on superoxide anion and hydroxyl radicals of the two extracts were slightly lower than that of butylated hydroxytoluene. DPPH• scavenging activities of both extracts reached over 80% inhibition at 4–8 mg/ml. Both extracts showed moderate reducing power and ferrous ion chelating activity. The IC50 value of the extract from cultured mycelia in all the tests, except for linoleic acid peroxidation, was significantly lower than that of natural mycelia. There was no evident correlation between the antioxidant activity and the content of protein, polysaccharides and mannitol of extracts from *C. sinensis*; the antioxidant activity may be due to a combined effect of these or some other compounds. These results suggested that both the extracts from cultured and natural mycelia have direct and potent antioxidant activities and that the cultured mycelia of the fungus could be used for the antioxidant activity to reduce the human demands on the natural resources of the fungus, an endangered species.

Keywords: *Cordyceps sinensis*; Reactive oxygen species; Aqueous extracts; Tonic

1. Introduction

Cordyceps sinensis (Berk.) Sacc., an entomogenous fungus, is one of the best known traditional Chinese medicines and health foods. The fungus parasitises larvae of moths (*Lepidoptera*), especially *Hepialus armoricanus* Oberthür, and converts each larva into a sclerotium, from which the stroma and fruit-body grows (Pegler, Yao, & Li, 1994). The complex including the fungal stroma and the sclerotium, which appears as larva body owing to the intact exoskeleton of the insect, has been used as a health food and traditional medicine to invigorate the lung and nourish the kidney in China for hundreds of years, at least from the 17th century (Wang, 1694; Zhao, 1765).

C. sinensis is believed to have several effects on the human body and is mainly used as a tonic to strengthen the body, especially after a serious illness. However, recent studies have demonstrated that *C. sinensis* can be used to treat a wide range of conditions, including respiratory, renal, liver, nervous system and cardiovascular diseases; tumour; aging; and also hyposexuality and hyperlipidemia (reviewed in Zhu, Halpern, & Jones, 1998). It has been officially classified as a drug in the Chinese Pharmacopoeia since 1964 (Committee of Pharmacopoeia, Chinese Ministry of Health, 1964, 2005). The use of the fungal products in medicinal treatment and in health foods has become very popular since 1990s and more so since the outbreak of the Severe Acute Respiratory Syndrome (SARS) in China in

*Corresponding author. Tel.: +86 10 64807496; fax: +86 10 64807518.
E-mail address: yaoyj@sun.im.ac.cn (Y.-J. Yao).

0023-6438/$30.00
doi:10.1016/j.lwt.2007.05.002

2003. The market demand for *C. sinensis* is growing sharply in many countries, especially in Asia, in recent years (Sharma, 2004; Vinning & Tobgay, 2004).

C. sinensis is endemic to the Tibetan Plateau and may be found only from above 3000 m in altitude (Jiang & Yao, 2002; Wang, 1995; Yao, 2004) and the natural resources of the fungus are very limited due to its confined geographic distribution and over exploitation in recent years (Yao, 2004). The price of natural products of *C. sinensis* is now over US$12,000/kg (for average quality in 2006) in the market and is still increasing. As substitution to the natural products, living strains have been isolated from natural *C. sinensis* and cultivated in large quantity by fermentation technology (Wang, 1995; Zhu et al., 1998). The fermentation of mycelia has been proved as a promising method to meet the needs of human consumption and to reduce the pressure on natural resources of the species which is in danger (It has been officially classified as an endanger species by CITES Management Authority of China and China Customers, 2000).

There are increasing evidences that degenerative or pathological events, such as senescence, asthma and cancer, are associated with accumulation of an excess of reactive oxygen species (ROS), which results in oxidative damage to DNA, proteins and other macromolecules (Balaban, Nemoto, & Finkel, 2005; Klaunig & Kamendulis, 2004; Stevenson, Koch, & Britton, 2006). Antioxidants, which scavenge free radicals, are known to play important roles in preventing the diseases induced by ROS (Park et al., 2004; Willcox, Ash, & Catignani, 2004).

As a valued traditional Chinese medicine for the treatment of a wide range of diseases, *C. sinensis* has attracted many research interests in recent years for the antioxidant activity. Li, Su, Dong, and Tsim (2002) compared the antioxidant activities of extracts using phosphate buffered saline (PBS) at 37 °C from stroma and sclerotium of natural *C. sinensis*, and the results showed that mycelia from both the stroma and the sclerotium had similar potency in their antioxidant activities in xanthine oxidase, induction of hemolysis and lipid peroxidation assays. It was reported that aqueous extracts from natural *C. sinensis* could scavenge hydroxyl radicals (Cai, Chen, Yin, & Zhang, 2004) and that methanol extracts could do the same on hydroxyl and superoxide anion radicals (Zhang, Pu, Yin, & Zhong, 2003).

There are also some studies on the antioxidant activity of *C. sinensis* using unauthenticated material. A polysaccharide which can protect PC12 cells against hydrogen peroxide-induced neuronal cell toxicity was isolated by Li et al. (2003), but the *Cordyceps* mycelia used from Wan Fong Pharmaceutical Factory (Zhejiang, China) were derived from a fungal strain named *Cephalosporium sinensis* Chen sp. nov. [sic! A nomenclaturally illegitimate fungal name], which raised doubts whether it is truly a strain of *C. sinensis* (Jiang & Yao, 2003) and, indeed, was later proved to be a different species (Ke, 2005). Yamaguchi, Kagota, Nakamura, Shinozuka, and Kunitomo (2000a, 2000b) reported that both water and ethanol extracts from artificial cultivated fruit-bodies of *C. sinensis* from the Xinhui Xinhan Artificial *Cordyceps* Factory (Guangdong, China) could scavenge ROS by inhibiting malondialdehyde formation by the peroxynitrite generator SIN-1. Although the results have been referred to in many subsequent investigations (e.g. Buenz, Bauer, Osmundson, & Motley, 2004, 2005; Li et al., 2003), there is, however, a doubt that the fungal material used was authentic *C. sinensis*, due to the fact that reports exist of cultivation of fruit-bodies of this fungus not being repeatable and that the manufacturer is, in fact, selling products of *C. militaris*. Li, Li, Dong, and Tsim (2001) compared the antioxidant activities of natural *C. sinensis* and cultured *Cordyceps* mycelia from different sources by three tests (xanthine oxidase, induction of hemolysis and lipid peroxidation assays) using PBS at 37 °C for 12 h to extract the compounds from samples, and were able to show the similar effects of the cultured mycelia to the natural products. However, the cultured material used by Li et al. (2001) was derived from a wide range of strains, of which some are apparently not from a true *C. sinensis*, e.g. products from Chinese Medicine Factory of Jiangxi and from Hebei Boding Pharmaceutical Factory (see Jiang & Yao, 2003). Further, the traditional Chinese medicines are boiled in water for medications or as health foods and soaked in alcohol as drink. The extraction using different solvents and temperatures may have resulted in different compounds.

A direct correlation between antioxidant activity and metal chelating and also reducing power has been reported for extracts from some traditional Chinese medicines (Jung, Seog, Choi, Park, & Cho, 2006; Mau, Tsai, Tseng, & Huang, 2005), but there has been no report on metal chelating and reducing power of extracts from *C. sinensis* so far. Due to the increasing interest in the relationship between antioxidants and diseases, there is a need to get an overall measure of the antioxidant activity of extracts from *C. sinensis* using reliable fungal material.

In the present study, the antioxidant activities of the hot water extracts from natural and cultured mycelia of *C. sinensis* were investigated in vitro and evaluated by inhibition of linoleic acid peroxidation; scavenging abilities on DPPH•, hydroxyl and superoxide anion radicals; and the reducing power and the chelating ability on ferrous ions. The contents of protein, polysaccharides and mannitol of extracts from both natural and cultivated *C. sinensis* were also determined to reveal their correlation with antioxidant activity.

2. Materials and methods

2.1. Chemicals

Linoleic acid, ferrozine, 2-deoxy-*o*-ribose and 1,1-diphenyl-2-picrylhydrazyl (DPPH•) were purchased from Sigma-Aldrich

(Steinheim, Germany); 2-thiobarbituric acid (TBA) from Acros Organics (Geel, Belgium); ethylenediaminetetraacetic acid (EDTA) from Amresco (Ohio, USA); sodium acetate trihydrate, acetic acid, hydrogen peroxide (H_2O_2), trichloroacetic acid (TCA) and ascorbic acid from Beijing Chemical Reagents Company (Beijing, China); butylated hydroxytoluene (BHT) from China National Pharmaceutical Group Shanghai Chemical Reagents Company (Shanghai, China); mannitol from Shanghai Zhengxiang Science and Technology Company (Shanghai, China); sodium periodate from Guangdong Shantou Xilong Chemical Factory (Shantou, China); detection Kit of superoxide anion radical scavenging activity from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All reagents were of analytical grade.

2.2. Natural and cultured mycelia of C. sinensis

Natural *C. sinensis* was collected from Xiaojin County, Sichuan Province, China, at an altitude of 3800–4500 m. After soil and plant debris on the fresh specimens were removed using a toothbrush, specimens were dried with silica gel in the field. The average weight per specimen is 0.273 g. The identity of these natural *C. sinensis* specimens were determined by the second author, YJY, who often acts as an authenticator on behalf of the Institute of Microbiology, Chinese Academy of Sciences, an authorised organisation for fungal identification by the Ministry of Health, People's republic of China, and the voucher specimens have been deposited at Herbarium Mycologicum, Academia Sinica (HMAS 132152), Beijing, China.

Cultured mycelia of *C. sinensis* were obtained by submerged culture using a strain (No. 762) originally isolated from fresh specimen collected from Sichuan, China, in laboratory (voucher specimen of cultured mycelium: HMAS 132153). The identity of the strain was confirmed by means of both morphological and molecular methods. The Internal Transcribed Spacer (ITS1-5.8S-ITS2) of nuclear ribosomal DNA (nrDNA) was amplified and sequenced from the culture. The sequence of the DNA fragment was compared with a data set generated in this laboratory containing ITS sequences from dried specimens and living strains of *C. sinensis* obtained from various regions of the Tibetan Plateau (Dong & Yao, 2005). The strain was maintained on potato dextrose agar (PDA) supplemented with 5 g/l wheat bran and 0.5 g/l peptone at 4 °C.

The strain was first incubated on the same medium as for the stock at 18 °C for 60 d in Petri dish. Seed cultures were grown in 500-ml Erlenmeyer flasks containing 100 ml of liquid medium, inoculated with a 5-mm agar disc from the 60-d culture. The flasks were rotated at 100 rpm, at 18 °C for 15 d and then 100 ml medium in 500-ml Erlenmeyer flask was inoculated with 10 ml of seed culture mycelium and incubated on a rotary shaker at 100 rpm and 18 °C for 25 d.

The mycelium was harvested by centrifugation for 15 min at 8000*g* to separate it from the liquid medium. After repeated washing with distilled water, the mycelial pellets were lyophilised using a VirTis freeze dryer (VirTis Co., Gardiner, New York) for later experiments.

2.3. Preparation of freeze-dried extracts from C. sinensis

All samples were dried at 40 °C for 24 h before grinding into a fine powder, which was than extracted with 10 volumes of distilled water at 95–100 °C for 2 h. After vacuum filtration, the filtrate was collected and the residue was re-extracted under the same conditions twice again. The filtrates were combined and condensed to one-third of their total volume with a rotary evaporator under reduced pressure at 50 °C, and the resultant extract was lyophilised to dryness in vacuum. The lyophilised power was stored in dark at 4 °C before use.

2.4. Determination of polysaccharide, protein and mannitol contents

The contents of polysaccharide, protein and mannitol in extracts were determined using the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), Lowry assay (Winters & Minchin, 2005) and colorimetric method (Li, Bao, & Wang, 1999), successively. In brief, for the contents of mannitol, one millilitre of the solution containing 0.2 mg of extract and 1 ml of 0.015 mol/l sodium periodate were mixed. After 10 min, 2 ml of 5.5 mmol/l rhamnose and 4 ml of fresh Nash reagent (1000 ml of 2 mol/l ammonium acetate mixed with 2 ml of acetic acid and 2 ml of acetyl acetone) were added to the mixture which was placed in a water bath at 53 °C for 15 min. The absorbance was measured at a wavelength of 412 nm on a Unico-2100 spectrophotometer (Shanghai, China). Blank test was prepared by substituting distilled water for the extract solution. A standard curve was prepared using a mannitol standard. One millilitre of solution containing up to 50 μg/ml of mannitol was determined by the above method and the mannitol content of samples was calculated by the linear regression equation from the standard curve.

2.5. Antioxidant activity in a linoleic acid system

The antioxidant capacity of the extracts on inhibition of linoleic acid peroxidation was assayed using the thiocyanate method (Yen, Chang, & Su, 2003). Linoleic acid emulsion was prepared with linoleic acid (0.2804 g) and Tween 20 (0.2804 g) in PBS (50 ml, 0.2 mol/l, pH 7.0). A reaction solution, containing different concentrations of extracts (0.5 ml), linoleic acid emulsion (2.5 ml) and PBS (2 ml 0.2 mol/l, pH 7.0) were mixed with a homogeniser. The reaction mixture was incubated at 37 °C in dark to accelerate the oxidation process and samples were withdrawn every 24 h, up to 5 d for evaluation of their oxidative

rancidity by the thiocyanate method. To 9.7 ml of ethanol (75%), 0.1 ml of ammonium thiocyanate solution (300 g/l), 0.1 ml of sample solution and 0.1 ml of ferrous chloride solution (20 mmol/l in hydrochloric acid) were added in sequence. The solution was stirred for 3 min and its absorption value at 500 nm was taken as the peroxide value. The inhibition of α-tocopherol on linoleic acid peroxidation was also assayed at the same concentration for comparison. The solution without adding extracts or α-tocopherol was used as blank. All the tests were performed in triplicate.

The inhibition percentage was calculated as following:

$$\text{Inhibition (\%)} = \frac{Ab - As}{Ab} \times 100,$$

where *Ab* is the absorbance of the blank and *As* is the absorbance of the aqueous extract or α-tocopherol at 500 nm.

2.6. Scavenging effect on superoxide anion radicals

Superoxide anion radical scavenging activity was determined using a commercial kit (Jiancheng Bioengineering Institute, Nanjing, China). The superoxide anion radicals were generated by the xanthine/xanthine oxidase system and reacted with 2,4-iodiphenyl-3,4-nitrophenyl-5-phenyltetrazolium chloride to form formazan, a coloured compound which can be spectrophotometrically quantified at 550 nm. The production of formazan is inversely related to the superoxide anion radical scavenging activity of the samples tested. The final results were expressed as the inhibition degree of formazan production. The scavenging activity of mannitol solution at the same concentration as the samples was also determined. BHT was used as positive control and distilled water in place of extracts or BHT as blank.

The percentage inhibition of superoxide anion radicals was calculated using the same formula as the above, but with the absorbance of the aqueous extract or BHT at 550 nm.

2.7. Scavenging effect on hydroxyl radicals

The determination of scavenging effect on hydroxyl radicals was carried out as described by Halliwell, Gutteridge, & Arugma (1987). The reaction mixture, in a final volume of 1.0 ml, containing 0.4 ml of 20 mmol/l sodium phosphate buffer (pH 7.4), 0.1 ml of 0.25–2 mg/ml extracts, 0.1 ml of 60 mmol/l deoxyribose, 0.1 ml of 10 mmol/l hydrogen peroxide, 0.1 ml of 1 mmol/l ferric chloride, 0.1 ml of 1.04 mmol/l EDTA and 0.1 ml of 2 mmol/l ascorbic acid, was incubated at 37 °C for 1 h. Solutions of ferric chloride and ascorbic acid were made up immediately before use in de-aerated water. The reaction was stopped by adding 1 ml of 17 mmol/l TBA and 1 ml of 17 mmol/l TCA. The mixture was then boiled for 15 min, cooled in ice, and then measured for the absorbance at 532 nm. The same was done to determine the scavenging activity of mannitol solution. BHT was used as positive control. Distilled water in place of extracts or BHT was used as blank and the sample solution without adding deoxyribose as sample blank.

$$\text{Scavenging activity (\%)} = \frac{Ab - (As - Asb)}{Ab} \times 100,$$

where *Ab*, *As* and *Asb* are the absorbances at 532 nm of the blank, extract or BHT, and sample blank respectively.

2.8. Scavenging effect on DPPH• radicals

The effect of extract from *C. sinensis* on DPPH• radicals was studied following the method of Blois (1958) with some modifications. A 0.5 mmol/l solution of DPPH• in ethanol and in 0.05 mol/l acetate buffer (pH = 5.5) were prepared. Extracting solution of 0.1 ml at different concentrations was mixed with 2 ml of acetate buffer, 1.9 ml of absolute ethanol and 1 ml of DPPH• solution. The mixture was shaken immediately after adding DPPH• and allowed to stand at room temperature in dark for 30 min. The decrease in absorbance at 517 nm was then measured. BHA and α-tocopherol were used as positive controls and the sample solution without DPPH• was used as sample blank. The radical scavenging activity was measured as a decrease in the absorbance of DPPH• and was calculated using the same equation in Section 2.7 above, with *Ab*, *As* and *Asb* as the absorbance at 517 nm of DPPH• of the blank, extract or control, and sample blank respectively.

2.9. Measurement of reducing power

The reducing power of the extracts was determined by the method of Yen and Chen (1995) with some modification. Extracts (2–10 mg/ml) in PBS (2.5 ml, 0.2 mol/l, pH 6.6) were added to potassium ferricyanide (2.5 ml, 10 mg/ml), and the mixture was incubated at 50 °C for 20 min. After 2.5 ml of 10 mg/ml TCA was added, the mixture was centrifuged at 1160*g* for 10 min and then 2.5 ml of the supernatant was mixed with 2.5 ml of deionized water and 0.5 ml of 1.0 mg/ml ferric chloride. The absorbance was then measured at 700 nm against a blank in a spectrophotometer. A higher absorbance of the reaction mixture indicates a higher reducing power. BHT and α-tocopherol were used as controls.

2.10. Ferrous ion chelating activity assay

The chelating activity of extracts on ferrous ion was measured as reported by Decker and Welch (1990). One millilitre of extracts (0.125–8 mg/ml) was mixed with 3.7 ml of deionised water and then the mixture was reacted with ferrous chloride (2 mmol/l, 0.1 ml) and ferrozine (5 mmol/l, 0.2 ml) for 20 min. The absorbance at 562 nm was determined spectrophotometrically. EDTA was used as positive control and chelating activity on ferrous ion was calculated using the same equation given in Section 2.5

above, with *Ab* as the absorbance of the blank without extract or EDTA and *As* as the absorbance in the presence of the extract or EDTA.

2.11. Statistical analysis

Experimental results recorded were means ± standard deviation (SD) of triple determinations. The data were analysed by one-way analysis of variance (ANOVA). Tests of significant differences were determined by Duncan's multiple range tests at $p = 0.05$ or independent sample *T*-test ($p = 0.05$). The IC50 values were calculated by using median-effect analysis and CalcuSyn software (Biosoft). Results were processed by SPSS 11.0 (SPSS Inc.) and Origin 7.0 (OriginLab Corporation).

3. Results

3.1. Extraction yield, and contents of polysaccharides, protein and mannitol of hot water extracts from C. sinensis

In the hot water extraction, the cultured mycelia produced a significantly higher total yield than that of natural mycelium ($p < 0.05$, Table 1). The contents of polysaccharides of extracts from cultural mycelia were significantly higher, according to the results of independent sample T-test, than that of natural mycelia and an inverse result was obtained for the content of protein and mannitol (Table 1).

3.2. Antioxidant activity on linoleic acid peroxidation

The time–course plots for the antioxidant activity of various concentrations of extracts from *C. sinensis* (from 0.1 to 1.6 mg/ml) determined using the thiocyanate method were compared with that of α-tocopherol (Table 2). The antioxidant activity of both extracts and of α-tocopherol showed a similar trend during the entire incubation period. The percentage of inhibition increased with the increasing concentration and reached the maximum at 48 h, and then declined to the termination. There was no significant difference between the inhibition given by both the extracts at each concentration, except that at 0.1 mg/ml, where the inhibition of the extract from natural *C. sinensis* was significantly higher than that of the extract from cultured mycelia ($p < 0.05$). The percentage of inhibition of

Table 1
Total yield and contents of polysaccharides, protein and mannitol of hot water extracts from *Cordyceps sinensis*

Extracts	Total yield (g/100 g of mycelium)	Content of protein (g/100 g of extract)	Content of polysaccharides (g/100 g of extract)	Content of mannitol (g/100 g of extract)
Natural mycelia	30.46 ± 0.74	18.37 ± 0.57	18.37 ± 0.51	32.22 ± 1.50
Cultured mycelia	39.11 ± 1.69	15.14 ± 0.42	28.43 ± 0.05	21.77 ± 0.73

Table 2
Inhibition of linoleic peroxidation by α-tocopherol and water extracts from *Cordyceps sinensis*

Time (h)	Concentration of samples (mg/ml)				
	0.1	0.2	0.4	0.8	1.6
Inhibition rate of extract from cultural mycelia					
24	22.32 ± 1.68	61.99 ± 1.34	81.47 ± 0.00	100 ± 0.00	100 ± 0.00
48	24.31 ± 4.65	64.90 ± 0.52	92.50 ± 0.26	100 ± 0.00	100 ± 0.00
72	11.81 ± 0.12	51.75 ± 4.15	91.36 ± 0.35	100 ± 0.00	100 ± 0.00
96	7.74 ± 0.06	45.54 ± 3.92	80.90 ± 0.44	99.10 ± 1.31	100 ± 0.00
120	14.86 ± 2.82	37.47 ± 0.94	73.39 ± 0.94	90.52 ± 0.71	97.84 ± 0.71
Inhibition rate of extract from natural mycelia					
24	23.75 ± 1.68	63.42 ± 6.72	81.71 ± 3.02	97.15 ± 1.34	100 ± 0.00
48	43.14 ± 0.26	62.34 ± 1.03	89.58 ± 1.29	100 ± 0.00	100 ± 0.00
72	37.08 ± 1.38	58.11 ± 1.27	88.75 ± 0.81	100 ± 0.00	100 ± 0.00
96	31.10 ± 2.61	45.50 ± 3.92	89.20 ± 0.00	100 ± 0.00	100 ± 0.00
120	22.84 ± 0.94	33.48 ± 1.41	82.87 ± 0.71	99.33 ± 0.00	98.34 ± 0.00
Inhibition rate of VE					
24	35.23 ± 0.90	38.79 ± 0.57	68.21 ± 0.62	79.83 ± 1.71	86.48 ± 0.98
48	57.81 ± 0.64	60.96 ± 0.00	74.77 ± 1.70	97.30 ± 0.42	91.74 ± 1.06
72	54.41 ± 1.66	55.59 ± 0.42	57.65 ± 0.42	85.74 ± 0.62	82.94 ± 0.83
96	55.50 ± 0.80	55.80 ± 0.40	59.70 ± 0.80	63.10 ± 0.80	68.50 ± 0.40
120	22.22 ± 1.52	34.23 ± 0.76	31.18 ± 3.55	37.63 ± 0.51	40.32 ± 0.76

peroxidation in linoleic acid system of both extracts at concentration of 0.8 and 1.6 mg/ml was found to be over 90%, which was significantly higher than that of α-tocopherol of the same concentration ($p<0.05$), and the inhibition was maintained over 90% toward the end of the test, whereas the inhibition of α-tocopherol decreased evidently after being incubated for 48 h.

3.3. Scavenging effect on superoxide anion radicals

Extracts from both cultured and natural mycelia of *C. sinensis* exhibited dose-dependence of superoxide anion radical scavenging activity, just little lower than that of BHT, a commercial synthetic antioxidant (Fig. 1). The scavenging effect of the extract from cultured mycelia was significantly better than that of the extract from natural *C. sinensis* at all the tested concentration, except 2 mg/ml, at which there was no significant difference. There was almost no scavenging effect for the mannitol solution at the same concentration as the samples.

3.4. Scavenging effect on hydroxyl radicals

As shown in Fig. 1, both the extracts from *C. sinensis* and the mannitol solution had scavenging activity toward hydroxyl radicals in a dose-dependent manner (0.25–2.0 mg/ml), relatively lower than that of BHT and the scavenging activity of the both extract from *C. sinensis* was significantly higher ($p<0.05$) than that of the mannitol solution at the same concentration. There was no significant difference at all tested concentrations between the extracts from both cultured and natural mycelia.

3.5. Scavenging effect on DPPH• radicals

DPPH• radical scavenging activity of the extracts was evident at all of the tested concentrations but lower than that of α-tocopherol and BHT (Fig. 2). The scavenging effect increased with the increasing concentration up to a certain extent (4 mg/ml) and then levelled off with further increases. The extract from cultural mycelia showed

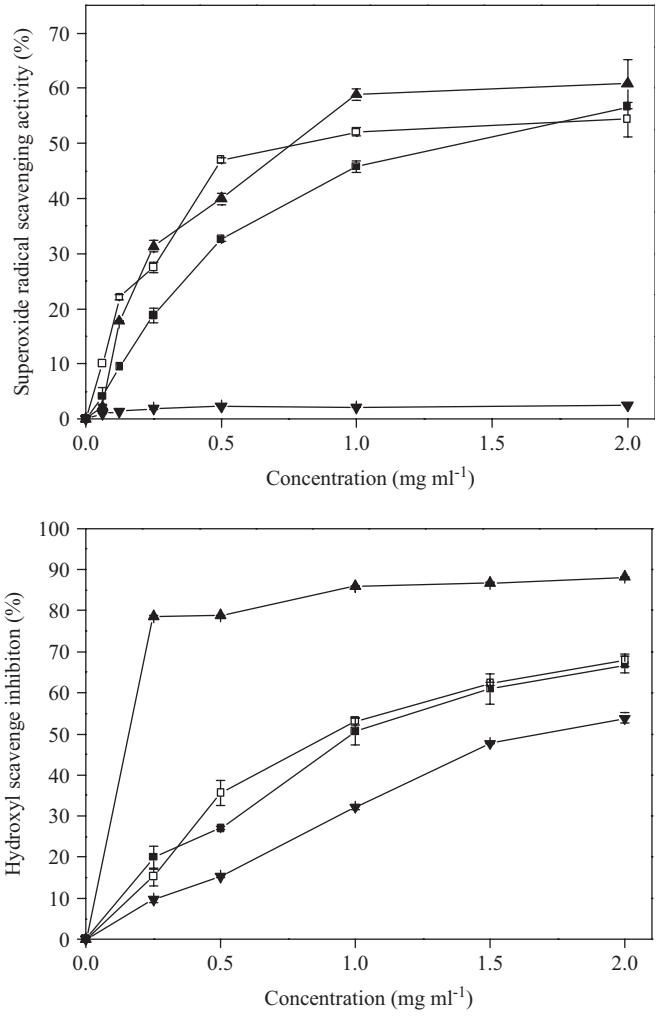

Fig. 1. Superoxide anion and hydroxyl radical scavenging activity of extracts from *Cordyceps sinensis*: (■) extract from natural mycelia, (□) extract from cultured mycelia, (▲) BHT, (▼) mannitol solution.

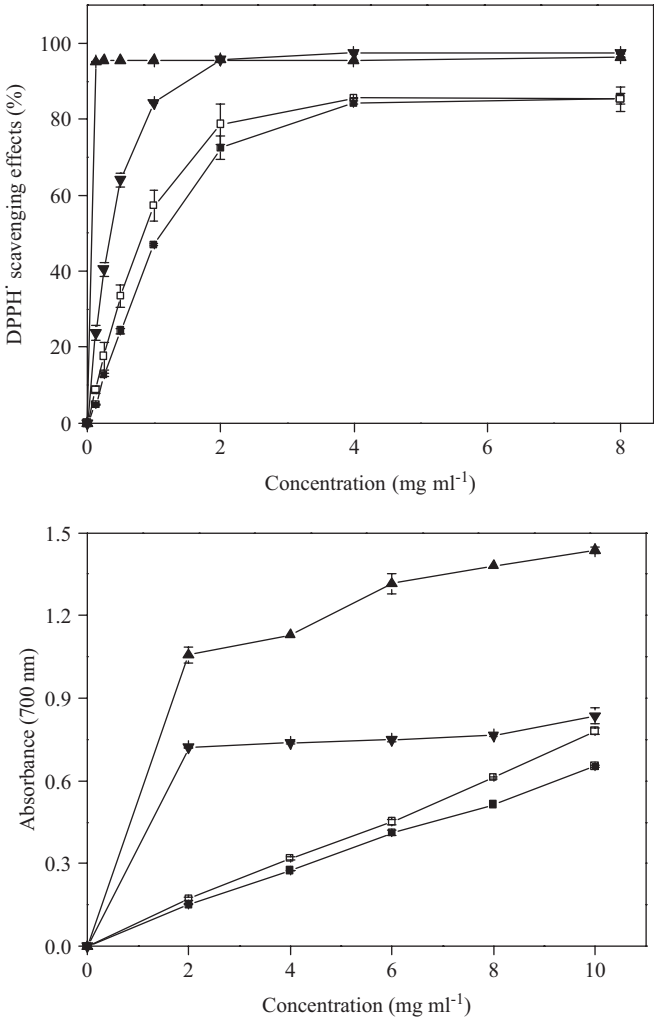

Fig. 2. Scavenging effect on DPPH• radicals and reducing power of extracts from *Cordyceps sinensis*: (■) extract from natural mycelia, (□) extract from cultured mycelia, (▲) BHT, (▼) α-tocopherol.

stronger DPPH• radical scavenging activity than that of natural mycelia but without significant difference.

3.6. Reducing power

The reducing power of the extracts from *C. sinensis* was shown in Fig. 2. Like the other antioxidant activity, the reductive potential of both extracts exhibited a dose-dependent activity within a concentration range of 0–10 mg/ml. The extract from cultured mycelia had a higher reducing power than that from natural mycelia, but there was no significant difference ($p<0.05$) between the two extracts.

3.7. Fe^{2+} chelating activity

Both of the extracts showed a moderate ferrous ion chelating ability and reached 41.86% and 53.86% at 8 mg/ml, respectively (Fig. 3). However, EDTA showed a better chelating ability at all the tested concentrations. The chelating ability of the extract from natural mycelia was less effective than that of cultured mycelia.

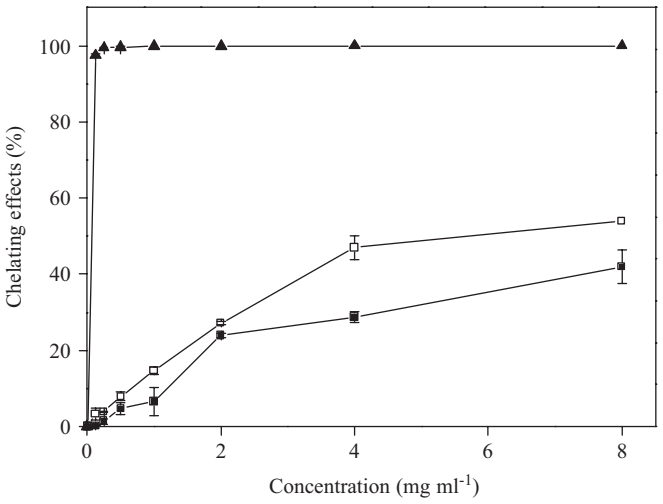

Fig. 3. Ferrous ion chelating activity of extracts of *Cordyceps sinensis*: (■) extract from natural mycelia, (□) extract from cultured mycelia, (▲) EDTA.

3.8. IC50 value in antioxidant properties

The antioxidant properties assayed herein were summarised in Table 3, except for chelating ability on ferrous ions, and the results were normalised and expressed as IC50 value (mg/ml) for comparison. For the extract from cultured mycelia of *C. sinensis*, IC50 value followed the ascending sequence: linoleic acid peroxidation < DPPH• < hydroxyl radical < superoxide anion < reducing power, but for that of natural mycelia, the sequence was linoleic acid peroxidation < hydroxyl radical < DPPH• < superoxide anion < reducing power. The IC50 value of extract from cultured mycelia of all tested antioxidant activities, except linoleic acid peroxidation, was lower than that of natural mycelia.

4. Discussion

Antioxidant activity has become one of the focuses of the study on mechanisms of the nutraceutical and therapeutical effects of traditional Chinese medicines (Mau et al., 2005) and there are numerous antioxidant methods and modifications for evaluation of antioxidant activity (Huang, Ou, & Prior, 2005). Due to the complexity of the oxidation–antioxidation processes, it is obvious that no single method is capable of providing a comprehensive picture of the antioxidant profile of a studied sample. In the present study the antioxidant properties of hot water extracts from authentic natural and cultured mycelia of *C. sinensis* were demonstrated by using a range of testing systems in vitro. The results suggest that both the extracts from cultured and natural mycelia have direct and potent antioxidant activities.

As shown in Table 2, hot water extracts from both natural and cultured mycelia of *C. sinensis* demonstrated excellent inhibition of the linoleic peroxidation and a much higher effect than the hot water extract from *Cordyceps militaris* (L.) Link (Zhan, Dong, & Yao, 2006), and also a much more stable effect than that of α-tocopherol, a well-known natural antioxidant, at concentration of 0.8–1.6 mg/ml. Their IC50 values were also the lowest among all the assays performed (Table 3). These results showed that the

Table 3
IC50 values of extracts from *Cordyceps sinensis* in antioxidant properties

	IC50 value[a] (mg/ml)				
	Linoleic acid peroxidation[b]	Superoxide anion radicals	Hydroxyl radicals	DPPH•	Reducing power
Extract from cultural mycelia	0.15±0.01 a	1.00±0.08 b	0.96±0.06 b	0.93±0.01 b	5.73±0.14 b
Extract from natural mycelia	0.14±0.01 b	1.24±0.05 a	1.03±0.03 a	1.23±0.01 a	7.27±0.08 a
BHT or α-tocopherol[c]	0.09±0.01 c	0.83±0.06 b	<0.01 c	<0.01 c	0.15±0.01 c

[a]IC50 value is the effective concentration at which DPPH•, hydroxyl or superoxide anion radicals were scavenged and linoleic acid peroxidation was inhibited by 50% or the absorbance was 0.5 at 700 nm for reducing power. Means with different letters within a column are significantly different ($p<0.05$).

[b]Calculated with the inhibition at 48 h.

[c]Linoleic acid peroxidation and reducing power were shown as the IC50 value of α-tocopherol and others were of BHT.

extracts from *C. sinensis* have great potential in inhibition of the linoleic peroxidation.

Superoxide anion radicals are known to be very harmful to cellular components as a precursor of more ROS. Although the scavenging activities of the hot water extracts from both natural and cultured mycelia of *C. sinensis* were just a little lower than that of BHT, but they were much higher than that of methanol extracts reported by Zhang et al. (2003), IC50 of 8.60–12.4 mg/ml compared with 1.00 ± 0.08 and 1.24 ± 0.05 of this study (Table 3). Further, the IC50 values of hydroxyl radical scavenging of the hot water extracts from *C. sinensis* (Table 3, as 0.96 ± 0.06 and 1.03 ± 0.03 mg/ml, respectively) were also much lower than that of methanol extract reported by Zhang et al. (2003, as 7.49–10.25 mg/ml). It seems that the water extracts had more positive antioxidant activities than methanol extracts from *C. sinensis* although only two assays of the latter were performed by Zhang et al. (2003).

The scavenging activity on DPPH•, a stable free radical, is a widely used index and a quick method to evaluate antioxidant activity (Mokbel & Hashinaga, 2006). The highest scavenging activities on DPPH• radical were 55.4% for the water extract of *Lentinus edodes* (Berk.) Singer and 37.9% for V*olvariella volvacea* (Bull.) Singer at the concentration of 1–9 mg/ml (Cheung, Cheung, & Ooi, 2003). At 1–20 mg/ml, the scavenging abilities of hot water extracts from *Agrocybe cylindracea* (DC) Gillet fruit bodies, mycelia and filtrate on DPPH• radical were in the range of 58.3–66.2%, 47.7–76.1% and 53.5–73.5%, respectively (Tsai, Huang, & Mau, 2006). Although the DPPH• scavenging ability of hot water extracts from *C. sinensis* was some what lower than that of BHT and α-tocopherol, the commercial antioxidants, it still reached more than 80% at the concentrations of 4–8 mg/ml as shown in Fig. 2 and more effective than that of the extract from the mushrooms mentioned above.

Reducing powers of hot-water extracts from mature and baby Ling chih, mycelia and filtrate were 0.48, 0.44, 0.23 and 0.42 at 1 mg/ml and 1.08, 1.04, 0.95 and 1.12 at 20 mg/ml, respectively (Mau et al., 2005). At 1 and 20 mg/ml, hot-water extracts from *A. cylindracea* fruit bodies, mycelia and filtrate showed reducing powers of 0.22, 0.20 and 0.33 at 1 mg/ml, and 1.02, 0.86 and 1.14, respectively (Tsai et al., 2006). In the present study, the extracts from *C. sinensis* have moderate abilities of reducing power on Fe^{3+}–Fe^{2+} transformation (Fig. 2).

The hot-water extracts from *C. sinensis* also showed moderate ferrous ion chelating abilities (Fig. 3), just similar with the water extract from *A. cylindracea* fruitbodies and mycelia (Tsai et al., 2006) and further purification of the extracts may improve the ability.

Some of the compounds in the natural and cultured mycelia of *C. sinensis* were reported to be the effective components of scavenging free radicals, e.g. polysaccharides (Li et al., 2002, 2003) and mannitol and cordycepin (Zhang et al., 2003). However, the contents of polysaccharides, mannitol and protein were determined in this study (Table 1) and there was no evident correlation between the contents of any compounds and the antioxidant activity. Mannitol, a polyol, is believed to be able to scavenge ROS (Jennings, Ehrenshaft, Pharr, & Williamson, 1998), whilst mannitol solution showed scavenging activity on hydroxyl radical only but not on superoxide radical in the present study (Fig. 1). Further, the fact that the scavenging rate of hydroxyl radical by mannitol solution was significantly lower than that by the extracts from *C. sinensis* ($p < 0.05$) at the same concentration (Fig. 1) suggested that mannitol was only partially in charge of the scavenging activity of extracts from *C. sinensis* on hydroxyl radical. The cordycepin in the natural and cultured mycelia of *C. sinensis* has been reported as often too low to be detectable by HPLC (Guo, Zhu, Zhang, & Zhang, 1998; Li et al., 2004). Therefore, it cannot be concluded here that any certain compound has a main effect on the antioxidant activity of *C. sinensis*. It may be possible that the antioxidant activity is determined by a combination of effects of some of the compounds.

Both the extracts from cultured and natural mycelia have direct and potent antioxidant activities and the extracts from cultured mycelia of *C. sinensis* had stronger activities, shown as lower IC50, than that from natural mycelia in all the assays conducted in this study except the linoleic acid peroxidation test (Table 3). These results suggest that pharmacological functions of *C. sinensis* are due to, at least partially, their protective effects against oxidation and that it is a promising way to use the cultured mycelia of the fungus for the antioxidant activity to reduce the human demands on the natural resources of *C. sinensis*, an endangered species.

Acknowledgments

The authors are grateful to Professor Peter J. Houghton, King's College, University of London, for his critical review of the manuscript and also for his valuable comments and suggestions. This study was supported by the National Science Fund for Distinguished Young Scholars (30025002) from National Natural Science Foundation of China, and by the Key Research Direction of Renovation Programme (KSCX2-SW-101C) and the scheme of Introduction of Overseas Outstanding Talents, operated by the Chinese Academy of Sciences.

References

Balaban, R. S., Nemoto, S., & Finkel, T. (2005). Mitochondria, oxidants and aging. *Cell*, *120*, 483–495.

Blois, M. S. (1958). Antioxidants determination by the use of a stable free radical. *Nature*, *181*, 1199–1200.

Buenz, E. J., Bauer, B. A., Osmundson, T. W., & Motley, T. J. (2005). The traditional Chinese medicine *Cordyceps sinensis* and its effects on apoptotic homeostasis. *Journal of Ethnopharmacology*, *96*, 19–29.

Buenz, E. J., Weaver, J. G., Bauer, B. A., Chalpin, S. D., & Badley, A. D. (2004). *Cordyceps sinensis* extracts do not prevent Fas-receptor and

hydrogen peroxide-induced T-cell apoptosis. *Journal of Ethnopharmacology*, *90*, 57–62.

Cai, Z.-J., Chen, S.-J., Yin, D.-H., & Zhang, Y. (2004). Scavenging effect of *Cordyceps* growing in different environment on hydroxyl radical. *Chinese Traditional and Herbal Drugs*, *35*, 57–59 (in Chinese).

Cheung, L. M., Cheung, P. C. K., & Ooi, V. E. C. (2003). Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chemistry*, *81*, 249–255.

CITES Management Authority of China and China Customers (2000). Announcement of adjustment for catalogue of exporting and importing wild animals and plants (in Chinese).

Committee of Pharmacopeia, Chinese Ministry of Health. (1964). *Chinese pharmacopeia (1963 edition): Part one*. Beijing: The People's Medical Publishing House (in Chinese).

Committee of Pharmacopeia, Chinese Ministry of Health. (2005). *Chinese pharmacopeia (2005 edition): Part one*. Beijing: Chemical Industry Press (in Chinese).

Decker, E. A., & Welch, B. (1990). Role of ferritin as a lipid oxidation catalyst in muscle food. *Journal of Agricultural and Food Chemistry*, *38*, 674–677.

Dong, C.-H., & Yao, Y.-J. (2005). Nutritional requirements of mycelial growth of *Cordyceps sinensis* in submerged culture. *Journal of Applied Microbiology*, *99*, 483–492.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Guo, C., Zhu, J., Zhang, C., & Zhang, L.-J. (1998). Determination of adenosine and 3′-deoxyadenosine in *Cordyceps* by HPLC. *China Journal of Chinese Materia Mdeica*, *23*, 236–237 (in Chinese).

Halliwell, B., Gutteridge, J. M. C., & Arugma, O. I. (1987). The deoxyribose method: A simple 'Test-Tube' assay for determination of rate constants for reactions of hydroxyl radicals. *Analytical Biochemistry*, *165*, 215–219.

Huang, D.-J., Ou, B.-X., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry*, *53*, 1841–1856.

Jennings, D. B., Ehrenshaft, M., Pharr, D. M., & Williamson, J. D. (1998). Roles for mannitol and mannitol dehydrogenase in active oxygen-mediated plant defense. *Proceedings of the National Academy of Sciences of the United States of America*, *95*, 15129–15133.

Jiang, Y., & Yao, Y.-J. (2002). Names related to *Cordyceps sinensis* anamorph. *Mycotaxon*, *84*, 245–254.

Jiang, Y., & Yao, Y.-J. (2003). Anamorphic fungi related to *Cordyceps sinensis*. *Mycosystema*, *22*, 161–176 (in Chinese).

Jung, C. H., Seog, H. M., Choi, I. W., Park, M. W., & Cho, H. Y. (2006). Antioxidant properties of various solvent extracts from wild ginseng leaves. *LWT—Food Science and Technology*, *39*, 266–274.

Ke, C.-K. (2005). *Study on Cordyceps sinensis in China*. Beijing: China Science and Technology Press (in Chinese).

Klaunig, J. E., & Kamendulis, L. M. (2004). The role of oxidative stress in carcinogenesis. *Annual Review of Pharmacology and Toxicology*, *44*, 239–267.

Li, S.-P., Li, P., Dong, T. T.-X., & Tsim, K. W.-K. (2001). Anti-oxidation activity of different types of natural *Cordyceps sinensis* and cultured *Cordyceps* mycelia. *Phytomedicine*, *8*, 207–212.

Li, S.-P., Li, P., Lai, C.-M., Gong, Y.-X., Kan, K. K.-W., Dong, T. T.-X., et al. (2004). Simultaneous determination of ergosterol, nucleosides and their bases from natural and cultured *Cordyceps* by pressurised liquid extraction and high-performance liquid chromatography. *Journal of Chromatography A*, *1036*, 239–243.

Li, S.-P., Su, Z.-R., Dong, T. T.-X., & Tsim, K. W.-K. (2002). The fruiting body and its caterpillar host of *Cordyceps sinensis* show close resemblance in main constituents and anti-oxidation activity. *Phytomedicine*, *9*, 319–324.

Li, S-. P., Zhao, K.-J., Ji, Z.-N., Song, Z.-H., Dong, T. T.-X., Lo, C.-K., et al. (2003). A polysaccharide isolated from *Cordyceps sinensis*, a traditional Chinese medicine, protects PC12 cells against hydrogen peroxide-induced injury. *Life Sciences*, *73*, 2503–2513.

Li, X.-Q., Bao, T.-T., & Wang, Y. (1999). Determination of mannitol in *Cordyceps sinensis* by colorimetric method. *Chinese Traditional and Herbal Drugs*, *30*, 19–21 (in Chinese).

Mau, J.-L., Tsai, S.-Y., Tseng, Y.-H., & Huang, S.-J. (2005). Antioxidant properties of hot water extracts from *Ganoderma tsugae* Murrill. *LWT—Food Science and Technology*, *38*, 589–597.

Mokbel, M. S., & Hashinaga, F. (2006). Evaluation of the antioxidant activity of extracts from buntan (*Citrus grandis* Osbeck) fruit tissues. *Food Chemistry*, *94*, 529–534.

Park, S.-W., Kim, S.-H., Park, K.-H., Kim, S.-D., Kim, J.-Y., Baek, S.-Y., et al. (2004). Preventive effect of antioxidants in MPTP-induced mouse model of Parkinson's disease. *Neuroscience Letters*, *363*, 243–246.

Pegler, D. N., Yao, Y.-J., & Li, Y. (1994). The Chinese 'Caterpillar Fungus'. *Mycologist*, *8*, 3–5.

Sharma, S. (2004). Trade of *Cordyceps sinensis* from high altitudes of the Indian Himalaya: Conservation and Biotechnological priorities. *Current Science*, *86*, 1614–1619.

Stevenson, C. S., Koch, L. G., & Britton, S. L. (2006). Aerobic capacity, oxidant stress, and chronic obstructive pulmonary disease—A new take on an old hypothesis. *Pharmacology and Therapeutics*, *110*, 71–82.

Tsai, S. Y., Huang, S. J., & Mau, J. L. (2006). Antioxidant properties of hot water extracts from *Agrocybe cylindracea*. *Food Chemistry*, *98*, 670–677.

Vinning, G., & Tobgay, S. (2004). *Cordyceps*: A market analysis. Ministry of Agriculture, Thimphu, Kingdom of Bhutan, 〈www.moa.gov.bt/reports/Cordyceps.doc〉.

Wang, A. (1694). *Synopsis of Materia Media*. Reproduced in 1955 by the Commercial Press, Shanghai (in Chinese).

Wang, G.-D. (1995). *Cordyceps species: Ecology, cultivation and application*. Beijing: Science and Technology Reference Press (in Chinese).

Willcox, J. K., Ash, S. L., & Catignani, G. L. (2004). Antioxidants and prevention of chronic disease. *Critical Reviews in Food Science and Nutrition*, *44*, 275–295.

Winters, A. L., & Minchin, F. R. (2005). Modification of the Lowry assay to measure proteins and phenols in covalently bound complexes. *Analytical Biochemistry*, *346*, 43–48.

Yamaguchi, Y., Kagota, S., Nakamura, K., Shinozuka, K., & Kunitomo, M. (2000a). Inhibitory effects of water extracts from fruiting bodies of cultured *Cordyceps sinensis* on raised serum lipid peroxide levels and aortic cholesterol deposition in atherosclerotic mice. *Phytotherapy Research*, *14*, 650–652.

Yamaguchi, Y., Kagota, S., Nakamura, K., Shinozuka, K., & Kunitomo, M. (2000b). Antioxidant activity of the extracts from fruiting bodies of cultured *Cordyceps sinensis*. *Phytotherapy Research*, *14*, 647–649.

Yao, Y.-J. (2004). Conservation and rational use of the natural resources of *Cordyceps sinensis*. *Science News*, No.15, 28–29 (in Chinese).

Yen, G.-C., Chang, Y.-C., & Su, S.-W. (2003). Antioxidant activity and active compounds of rice koji fermented with *Aspergillus candidus*. *Food Chemistry*, *83*, 49–54.

Yen, G.-C., & Chen, H.-Y. (1995). Antioxidant activity of various tea extract in relation to their antimutagenicity. *Journal of Agricultural and Food Chemistry*, *43*, 27–32.

Zhan, Y., Dong, C.-H., & Yao, Y.-J. (2006). Antioxidant activities of aqueous extract from cultivated fruit-bodies of *Cordyceps militaris* in vitro. *Journal of Integrative Biology*, *48*(10), 1–5.

Zhang, X.-Q., Pu, Y.-P., Yin, L.-H., & Zhong, W.-J. (2003). Studies on the scavenging effect on superoxide anion free radical and hydroxyl free radical of *Cordyceps sinensis*. *Chinese Journal of Gerontology*, *23*, 773–775 (in Chinese).

Zhao, X.-M. (1765). *Supplementary to the compendium of materia medica*. Reproduced in 1955 by the Commercial Press, Shanghai (in Chinese).

Zhu, J. S., Halpern, G. M., & Jones, K. (1998). The scientific rediscovery of an ancient Chinese herbal medicine: *Cordyceps sinensis*. *Journal of Alternative and Complementary Medicine*, *4*, 289–303 (Part I); 4, 429–457 (Part II).