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Full Length Research Paper

Antioxidant activity of aqueous extract of a *Tolypocladium* sp. fungus isolated from wild *Cordyceps sinensis*

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***Tolypocladium* sp. Ts-1 was isolated from the fruiting body of a wild *Cordyceps sinensis*, one of the best known traditional Chinese medicine and health foods. The antioxidant activities of hot-water extracts from cultured mycelia of *Tolypocladium* sp. were assessed in different *in vitro* systems. The extracts showed superoxide dismutase (SOD) activity of 35.6 U/mg protein and are effective in scavenging superoxide radical in a concentration dependent fashion with IC_{50} value of 1.3 mg/mL. As a reinforcement of the action, similar radical scavenging effects of the extracts were also discerned with both site-specific and non site-specific hydroxyl radical using the deoxyribose assay method. 1,1-Diphenyl-2-picrylhydracyl (DPPH) radical scavenging activities of the extracts reached more than 75.2% at the concentrations of 3-6 mg/mL. The extracts showed moderate reducing power and ferrous ion chelating activity. Moreover, the protective effects of the extracts against hydrogen peroxide (H_2O_2)-induced lesion to rat PC12 cell (pheochromocytoma cell line) was observed in a dose-dependent manner from 0.25 to 2.0 mg/mL. Our results suggest that the aqueous extract of *Tolypocladium* sp. mycelium has strong antioxidant activities and is a potential source of natural antioxidant products. This is the first report on antioxidant activity of *Tolypocladium* fungus isolated from wild *C. sinensis*, an endangered species.**

Key words: Aqueous extracts, antioxidant activity, free radicals, PC12 cells, *Tolypocladium* sp.

INTRODUCTION

Cordyceps sinensis (Berk.) Sac, known as Dong-Chong-Xia-Cao (winter worm-summer grass) in Chinese, has been considered as a tonic food and herbal medicine in China for many centuries. This precious medicinal mushroom formed on an insect larva has been used in traditional Chinese medicine to treat kidney ailments, to relieve pain, to restore general health and appetite, and to promote longevity (Zhu et al., 1998a). Nowadays, numerous pharmacological activities have been found in this medicinal fungus including antitumour, anti-inflammation and antiatherosclerosis (Zhu et al., 1998b). The free radical scavenging and antioxidant activity of natural *Cordyceps* species and cultivated fungal mycelia, and fruiting bodies, for example, have been observed in several studies (Chen et al., 2006; Yu et al., 2006; Zhan et al., 2006).

Tolypocladium sp. is one of the numerous anamorphic fungal species isolated from wild *C. sinensis* (Li, 1988). The aqueous extract and exopolysaccharide from *Tolypocladium* fungus have been found possessing anti-tumor and immunomodulatory activities (Yang et al., 2004; Leung et al., 2006). Some reports indicated that the tumor development has a close relationship with oxidative stress (Sun, 1990). With respect to antioxidant activity in *Cordyceps*, little is known about the antioxidant action of *Tolypocladium* fungus. As a follow-up to our previous characterization of natural antioxidants from fungal compounds (Sun et al., 2004), the antioxidant potential of hot water extract from *Tolypocladium* sp. Ts-1

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was evaluated by DPPH/superoxide/hydroxyl radical scavenging, reducing power and metal chelating activity in the present study. The superoxide dismutase (SOD) activity in mycelia extracts was also investigated. Finally, the oxidative injury protective effect of the extracts was examined on PC12 cells (pheochromocytoma cell line) treated with peroxide hydrogen (H_2O_2).

MATERIALS AND METHODS

Cultivation and extraction of mycelia of *Tolypocladium* sp.

The fungus used in this study was isolated from the fruiting body of a wild *C. sinensis* collected on the high plateaus (altitude over 4000–4500 m) in Sichuan, China. It was identified as one of the anamorph strains phylogenetically related to *C. sinensis* and belongs to *Tolypocladium* genus (Chen et al., 2006) and was deposited in our lab with a voucher number Ts-1. The Ts-1 mycelium was cultivated on liquid medium containing (per liter) 40 g glucose, 10 g yeast extract, 5 g peptone, 1 g KH_2PO_4 and 0.5 g $MgSO_4$ in shake-flasks at 150 rpm and 28°C. After 7 days of shaking incubation, mycelium biomass was harvested from the liquid culture by filtration and dried at 50°C. The dry mycelium was pulverized and 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 lyophilized to dryness in vacuum. The lyophilized power was stored in dark at 4°C before being used for the bioactivity tests.

Determination of polysaccharide, protein, mannitol and total phenolic contents

The aqueous extract (5 g) was submerged in 400 ml hot water. The supernatant was condensed to 1/3 volume and centrifuged at 3000 r/min for 10 min to discard undissolved substances, followed by adding three times of its volume of absolute ethanol, stirred vigorously and kept overnight at 4°C. The precipitate was collected by centrifugation and washed with absolute ethanol, and then was redissolved, dialyzed with a dialysis tube of 3.3 cm in diameter to remove small molecules (<14,000 Da), and finally lyophilized to obtain the polysaccharide powder (PS). The polysaccharide content in extracts was determined using the phenol-sulfuric acid method (Dubois et al., 1956) and the protein content by the Bradford method (Bradford, 1976).

The mannitol content in extracts was analysed using the colorimetric method reported by Zhan et al. (2006). Total phenolic content of the extract was quantified by the Folin–Ciocalteau's reagent according to the method of Yuan et al. (2005). The content of total phenols was calculated on the basis of the calibration curve of gallic acid (Sigma, USA).

Superoxide dismutase (SOD) activity assay

Mycelia were freeze-dried and then ground using liquid nitrogen. After a fine powder was obtained, the sample (0.2 g) was suspended in 2 mL extraction buffer at pH 7.0 containing 100 mM potassium phosphate, 10 mM sodium ascorbate and 5 mM EDTA with 0.4 g polyvinylpolypyrrolidone (PVP). Cell debris was removed by centrifugation at 15,000 g, for 15 min at 4°C. Supernatants were used as samples for measurement of SOD activity. SOD activity assay system was based on the inhibitory effect of SOD on the spontaneous autoxidation of pyrogallol (Marklund and Marklund, 1974). One unit was defined as the amount of SOD required to inhibiting the initial rate of pyrogallol autoxidation by 50% and was expressed as U per mg protein (U/mg protein). Protein was determined according to Bradford method (Bradford, 1976), by using bovine serum albumin (BSA) as the standard.

1,1-Diphenyl-2-picrylhydracyl radical scavenging assay

1,1-Diphenyl-2-picrylhydracyl (DPPH) radical scavenging activity was done according to the method of Hatano et al. (1989) with some modifications. Briefly, 1 ml of DPPH (Sigma, USA) solution (0.2 mmol/L in 95% ethanol (v/v)) was incubated with different concentrations of the extract. The reaction mixture was shaken and incubated at room temperature in dark for 30 min. The decrease in absorbance at 517 nm was then measured. α-Tocopherol was used as positive control 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 calculated using the following equation:

$$\text{Scavenging effect (\%)} = [A_b - (A_s - A_{sb})]/A_b \times 100$$

Where A_b, A_s and A_{sb} are the absorbances at 517 nm of the blank, extract or control, and sample blank, respectively.

Superoxide radical scavenging assay

The superoxide radical scavenging ability of the extracts was measured as described in our pervious work (Sun et al., 2004). The reaction mixture contained different concentrations of the extracts, 10 μM phenazine methosulphate (PMS, Sigma, USA), 78 mM NADH (Sigma, USA) and 50 μM nitroblue tetrazolium (NBT, Shanghai Biochemical Factory, Shanghai, China) in Tris-HCl buffer (16 mM, pH 8.0), was incubated at room temperature for 5 min and the color reaction of the rest superoxide radical with NBT was detected at 560 nm. Butylated hydroxytoluene (BHT, Shanghai Biochemical Factory, Shanghai, China) was used as positive controls. The scavenging activity of superoxide radical (%) was thus calculated with the equation described as in the case of DPPH.

Deoxyribose assay for site-specific and non site-specific hydroxyl radical scavenging activity

Non site-specific hydroxyl radical scavenging activity of the extracts was measured using the deoxyribose assay (Halliwell et al., 1987). Every 0.5 mL solution carrying the extracts at varying concentrations was added to 1.0 mL solution of 20 mM potassium phosphate buffer (pH 7.4) containing 2.8 mM 2-deoxy-*D*-ribose (Sigma, USA), 104 μM EDTA, 100 μM $FeCl_3$, 100 μM ascorbic acid and 1 mM hydrogen peroxide. The obtained mixtures were incubated at 37°C for 1 h followed by addition of an equal volume of 10% trichloroacetic acid containing 0.5% thiobarbituric acid. The afforded solutions were subsequently boiled at 100°C for 15 min, cooled in ice and then measured for the absorbance at 532 nm. Reduced glutathione was used as a positive control. The scavenging activity of hydroxyl radical (%) was thus calculated with the equation described as in the case of DPPH.

The procedure for measuring site-specific hydroxyl radical scavenging activity was identical to that detailed above but with ethylenediamine tetra-acetic acid (EDTA, Sigma, USA) replaced by an equal volume of buffer as reported previously (Kitts et al., 2000).

Determination of reducing power

The reducing power of the extracts was determined by the method

of Oyaizu (1986) with some modification. Extracts (0–10 mg/mL) in phosphate buffer (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. Then trichloroacetic acid (2.5 ml, 10 mg/ml) was added to the mixture, which was then centrifuged at 1000 g for 10 min. The supernatant (2.5 ml) was mixed with 2.5 ml of deionized water and ferric chloride (0.5 ml, 1.0 mg/mL), and then the absorbance was read at 700 nm. Higher absorbance of the reaction mixture indicated greater reducing power. α-Tocopherol was used as a positive control.

Metal ion chelating assay

The binding activity of ferrous ions by the extract was estimated in terms of the decrease in the maximal absorbance of the iron-ferrozine complex (Lopes et al., 1999). Briefly, the extract (0–10 mg/mL) was incubated with 2 mM $FeCl_2$ for 10 min followed by mingling with 5 mM ferrozine. As soon as the equilibrium was reached the absorbance of the mixture at 562 nm was detected. EDTA was used as positive control. The ability of the extract to chelate ferrous ion was thus calculated with the equation described as in the case of DPPH, with *Ab* as the aborbance of the blank without extract or EDTA and *As* as the absorbance in the presence of the extract or EDTA.

Culture of PC12 cells and viability assay

PC12 cells (pheochromocytoma cell line) were cultured in RPMI1640 supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 100 U/mL penicillin, 80 U/mL streptomycin and 10 mM Hepes. All experiments were carried out 24–48 h after cells were seeded. H_2O_2 solution (8.8 M) was stored at 4°C prior to use. And H_2O_2 stock solution at 100 mM was freshly prepared in phosphate buffer (pH 7.4). PC12 cells, after a 24 h pre-incubation with the testing sample, was subjected to a 1 h hydrogen peroxide injury by adding the stock solution to the culture media with H_2O_2 finally at 150 μM. The damage was halted by removal of the H_2O_2-containing liquor followed immediately by a re-cultivation in the fresh medium. Assays for cell viability was performed 24 h after the PC12 cells were cultured in fresh medium (Sun et al., 2005). Cell survival, expressed as the percentage of viable cells among all counted cells, was evaluated by morphological inspection under phase-contrast microscope (Nikon), and cell counting under a hemocytometer helped by the Trypan blue dye (0.5%) exclusion protocol.

RESULTS

Mycelia biomass and SOD activity of Ts-1 fungus in liquid cultures

Figure 1 shows the typical time courses of mycelia growth and SOD activity of Ts-1 fungus in shake-flask cultures. The mycelium biomass showed a lag-phase in the first 1–2 days and exponential growth in the next 2–4 days. The mycelium biomass reached a maximum concentration of 30.2 g dw/L on day 7. Mycelia of the Ts-1 fungi were used to analyze SOD activity. The intracellular SOD activities in Ts-1 mycelia exhibited a biphasic time course during the culture period, reaching the first peak on day 3 (13.4 U/mg protein) at the early exponential phase, and the second and much higher peak on day 8 (35.6 U/mg protein) at the late stationary phase.

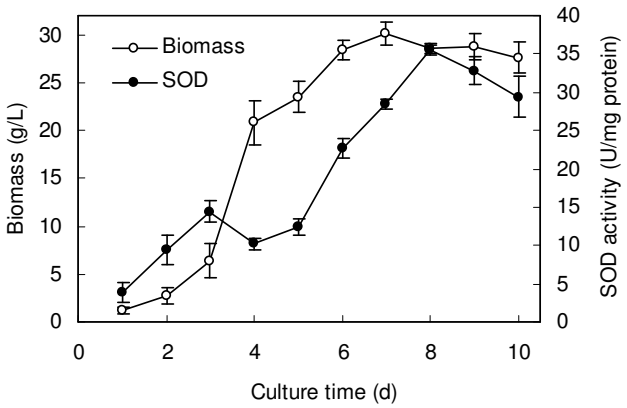

Figure 1. Time courses of mycelium biomass and SOD activity in Ts-1 fungus liquid cultures at 28°C on a rotary shaker at 150 rpm. Values represent the mean ± S.D. of triplicate samples.

Table 1. Extraction yield and contents of polysaccharides, protein, mannitol and total phenols of hot water extracts from Ts-1 fungus.

Parameter	Content (%, w/w)
Extraction yield [a]	25.4 ± 3.5
Protein	4.2 ± 0.2
Polysaccharides	16.3 ± 1.2
Mannitol	5.8 ± 0.6
Total phenols	1.3 ± 0.2

[a]Extracted from lyophilized mycelium (10.0 g). Others were quantified from hot water extracts. Each value is expressed as mean ± standard deviation (n = 3).

In the hot water extraction, the cultured mycelia produced a high total extract yield (25.4%, w/w). The aqueous extract with 16.3% polysaccharides (w/w), 4.2% proteins (w/w), 5.8% mannitol (w/w) and 1.3% total phenols (w/w) in the raw powder (Table 1), was used in further study to determine the antioxidant activities.

Scavenging effect on DPPH radicals

To investigate the effects of the Ts-1 fungus extract on the antioxidant activity *in vitro*, the DPPH scavenging rate of the aqueous extracts was examined. When the aqueous extract concentration was increased from 0.5 to 4.5 mg/mL, the DPPH scavenging rate increased from 10.7 to 80.1% (Figure 2). However, no increase in DPPH scanvenging rate was observed above 5 mg/mL of the extract.

Scavenging effect on superoxide anion and hydroxyl radicals

The superoxide radical was generated in a PMS/NADH

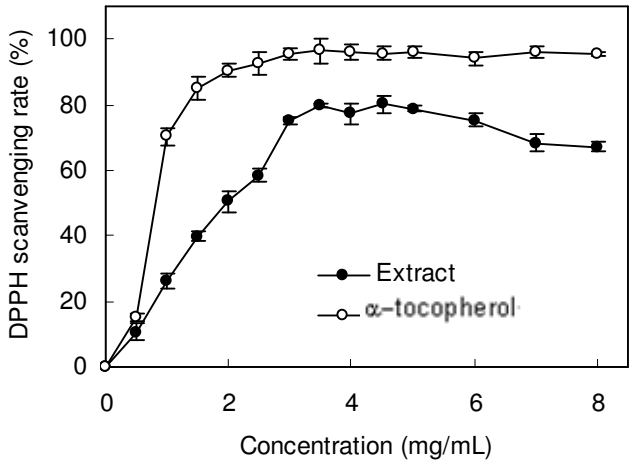

Figure 2. Scavenging effect on DPPH radicals by aqueous extracts from Ts-1 fungus. α-Tocopherol was used as positive control and the sample solution without DPPH was used as sample blank. Values represent the mean ± S.D. of triplicate samples.

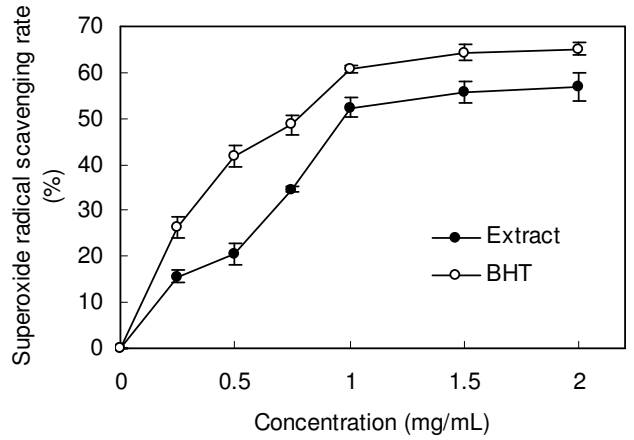

Figure 3. Scavenging effect on superoxide radicals by aqueous extracts from Ts-1 fungus. Butylated hydroxytoluene was used as positive controls. Values represent the mean ± S.D. of triplicate samples.

system and assayed by the reduction of NBT. As shown in Figure 3, the extracts scavenged superoxide radical in a concentration-dependent manner. At a concentration of 1.0 mg/mL, as much as 50.4% of superoxide radical was scavenged by the aqueous extracts from Ts-1 fungus. The IC_{50} value of the extracts for eliminating superoxide radical was disclosed to be 1.3 mg/mL. The IC_{50} of BHT as a positive control detected in the same experimental procedure was 0.8 mg/mL.

The site-specific and non site-specific hydroxyl radical scavenging activity of the aqueous extracts was assessed using deoxyribose assays. The extract exhibited both site-specific and non site specific scavenging activity on hydroxyl radical in a dose- dependent manner (0.25 - 2.0

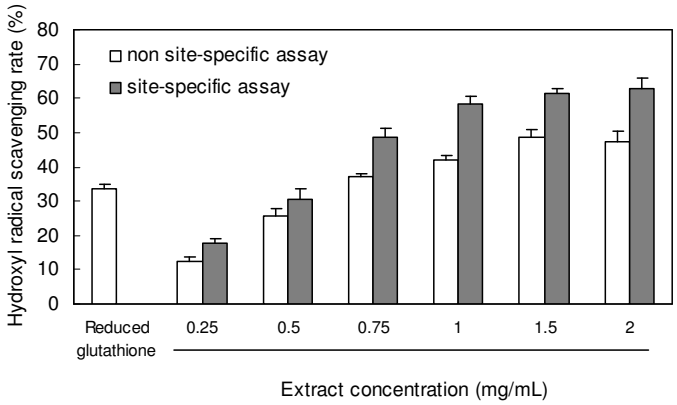

Figure 4. Scavenging effect on the site-specific and non-site-specific hydroxyl radicals b by aqueous extracts from Ts-1 fungus. Reduced glutathione served as a positive control in non-site-specific assay. Values represent the mean ± S.D. of triplicate samples.

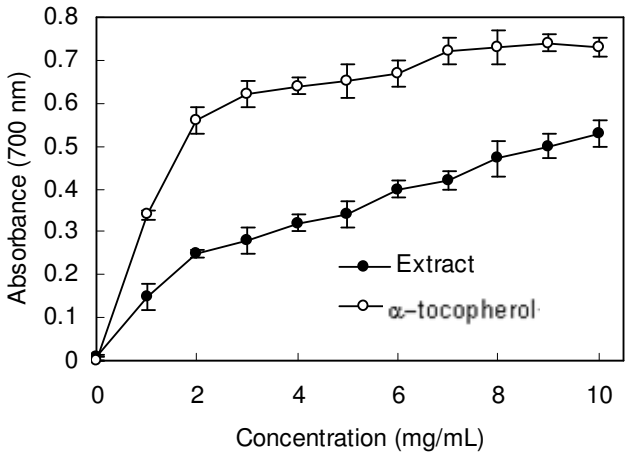

Figure 5. The reducing power of aqueous extracts from Ts-1 fungus. α-Tocopherol served as a positive control. Values represent the mean ± S.D. of triplicate samples.

mg/mL, Figure 4). Regarding the magnitude of the action, the suppressive effect of the extract on deoxyribose damage was greater in the site-specific protocol than that in the non site-specific. At the concentration of 100 mg/mL, the scavenging activity of the extract against hydroxyl radical discerned in site-specific and non site-specific assays was 66.9 and 45.2%, respectively.

Reducing power

The reducing power of the aqueous extracts from Ts-1 fungus was shown in Figure 5. Like the other antioxidant activities, the reductive potential of the extracts exhibited a dose-dependent activity within a concentration range of 0 – 10 mg/mL.

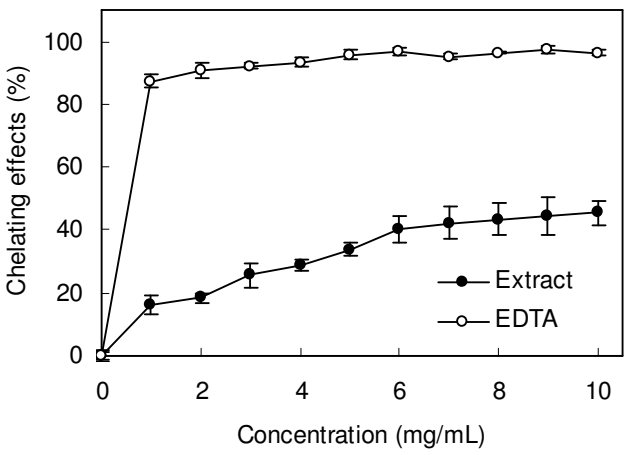

Figure 6. The Fe^{2+} chelating activity of the aqueous extracts from Ts-1 fungus. EDTA was used as positive control. Values represent the mean ± S.D. of triplicate samples.

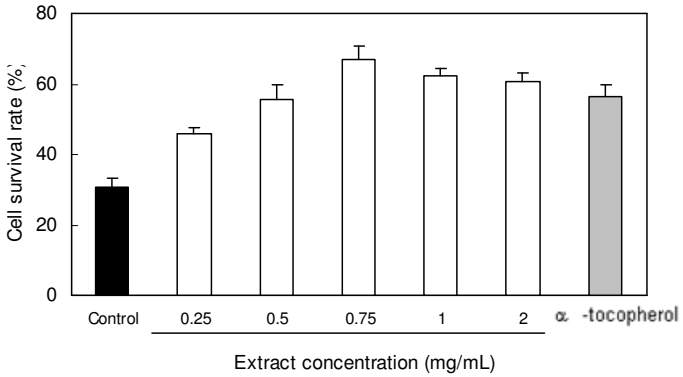

Figure 7. Effects of the aqueous extracts from Ts-1 fungus on the toxicity induced by H_2O_2 in PC12 cells. Cells were incubated with 150 μM H_2O_2 for 1 h. The extract was added to the culture 24 h prior to H_2O_2 addition. α-Tocopherol (1 mM) served as a positive control. The data (expressed as percent of blank control (without H_2O_2 injury) value) are means ± S.D. of triplicate samples.

Metal chelating activity

The extracts exhibited a moderate chelating activity to Fe^{2+}. As shown in Figure 6, 1.0 mg/mL EDTA chelated almost 87.4% of Fe^{2+} whereas the extracts chelated only 45.5% of Fe^{2+} at a concentration of 10 mg/mL. This indicated that the extracts possess a weaker Fe^{2+}-chelating ability than that of EDTA.

Effects on viability of H_2O_2-treated PC12 cells

As shown in Figure 7, the treatment of PC12 cells with the H_2O_2 (150 μM) resulted in notable cell death or decrease in cell viability, about 30.6% after 1 h H_2O_2 damage. The extracts possessed protective effects against H_2O_2 insult, and the protective effect of the extracts was in a dose-dependent manner from 0.25 to 2.0 mg/mL. The pre-incubation of the cells with the extracts at 0.75 mg/mL could let over 67.1% of the H_2O_2-induced cells survive whereas the viability discerned with α-tocopherol (at 1 mM) group was 56.4% only.

DISCUSSION

Free radicals such as superoxide radical (O_2·), hydroxyl radical (·OH) and other reactive oxygen species (ROS) are associated with cellular necrosis and a variety of pathological conditions such as cancer, degenerative disease in neurons, hepatopathies, atherosclerosis, and even aging (Pryor, 1986). Supplementation with antioxidants could represent an important therapeutic potential to minimize the damage. Gradually growing attention has been paid to the discovery and development of efficient and safe antioxidants from natural resources. As a rich reservoir of bioactive resources, some medicinal fungi have demonstrated to be excellent producers of antioxidant metabolites (Sun et al., 2005; Lee et al., 2007; Lee and Yun, 2007). It was reported that aqueous extracts from wild *C. sinensis* could scavenge hydroxyl radicals and that methnol extracts could do the same on hydroxyl and superoxide anion radicals (Yu et al., 2006). Leung et al. (2006) reported that the hot water extracts of the mycelium of *Tolypocladium* sp. fungus isolated from wild *C. sinensis* showed low cytotoxic effect on B16 melanoma cells in culture (about 25% inhibition) but significant anti-tumour effect in animal tests, causing 50% inhibition of B16 cell-induced tumour growth in mice. However, little is known about the effects of the other biological activities of *Tolypocladium* sp., a common fungal species living on the insect host of *C. sinensis*. In the present study, we first demonstrated that the hot water extract of cultured *Tolypocladium* sp. mycelia have direct and potent antioxidant activities.

As shown in Figures 1 and 2, the extracts showed SOD activity and are effective in scavenging superoxide radical in a concentration dependent fashion with IC_{50} value of 1.3 mg/mL. This was much higher than the superoxide scavenging activities of hot water extracts from natural host fungus *C. sinensis* with the IC_{50} values of 8.6 mg/mL (Zhang et al., 2003). After ascertaining a moderate ferrous ion chelating ability of the extracts (Figure 6), the site-specific (e.g., Fe^{2+} + H_2O_2) and non-site-specific (e.g., Fe^{2+} + EDTA + H_2O_2) HO· scavenging actions were assessed utilizing the deoxyribose assay (Figure 4). In the site-specific assay, Fe^{2+} can be directly chelated by the extracts before the generation of HO· from Fenton reactants. However, in the non-site-specific assay the chelation of Fe^{2+} with the extracts was diminished by EDTA. This observation could be explained by assuming that the HO· scavenging activity of the extracts was directly related to its affinity to the radical in a non-site-specific manner. The IC_{50} values (1.8 mg/mL) of non site-specific hydroxyl radical scavenging of the extracts was

also much lower than that of hot water extracts from natural mycelia of *C. sinensis*, and cultured mycelia of *Paecilomyces sinensis*, *Cephalosporium sinensis* and *Mortierella hepialid* reported by Zhang et al. (2003) as 7.5 - 10.3 mg/mL. Furthermore, a widely used index and quick method on DPPH scavenging was employed to evaluate the antioxidant activity (Mokbel and Hashinaga, 2006). Although the DPPH scavenging ability of the extracts was lower than that of the commercial antioxidant, α-tocopherol, it still reached more than 75.2% at the concentrations of 3 - 6 mg/mL (Figure 2). The results suggest that the extract from *Tolypocladium* sp. mycelia is a promising resource of natural antioxidants.

Although it is not a free radical, H_2O_2 is very harmful to cells because it may cross biological membranes and the highly reactive hydroxyl radical can be synthesized from it by a Fenton reaction (Pryor, 1986). H_2O_2 was frequently used as a generator of *in vitro* oxidative stress model. Our present studies confirmed that H_2O_2 treatment on the PC12 cells, which displays phenotypic characteristics of both adrenal chromaffin cells and sympathetic neurons (Greene and Tischler, 1976), caused a marked decrease in cell survival (Figure 7). However, when PC12 cells were pre-incubated with the extract, the H_2O_2-induced reduction in cell survival was remarkably attenuated, suggesting a protective role of the extract as an antioxidant.

Some of the compounds in the natural and cultured myclia of *C. sinensis* were reported to be the effective components of scavenging free radicals, e.g. polysaccharides, mannitol and cordycepin (Zhan et al., 2006; Zhang et al., 2003). The polysaccharides, proteins, mannitol and total phenols were found in the extract of this study. It may be possible that the antioxidant activity of the extract is determined by a combination of effects of some of these compounds. In addition to determining the bioactive compounds from *Tolypocladium* sp. fungus it is also desired to ascertain whether the extracts can exert such activities *in vivo*, especially to assess the potential to attenuate or avoid the damages of free radicals in pathophysiological conditions such as carcinogenic or earlier atherogenic events. While adding new information valuable for a better understanding of *Tolypocladium* fungus, these findings are of significance to use the cultured mycelia of the fungus for the antioxidant activity to reduce the human demands on the natural *C. sinensis* endangered species.

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