흰목이 버섯 배양액의 항산화 활성 규명

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In Vitro Evaluation for Antioxidant Activities of Culture Broth of *Tremella fuciformis*

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Abstract To investigate antioxidant activity of the culture broth from submerged culture of *Tremella fuciformis*, we preferentially analyzed the chemical composition of culture broth, which was mainly composed of carbohydrate (296.39 mg/g) and protein (9.24 mg/g), respectively. Also, contents of polyphenols, flavonoids and flavonols were 16.63 mg/g, 9.19 mg/g and 83.74 μg/g, respectively. Next, we examined the scavenging abilities on DPPH (1,1-diphenyl-2-picrylhydrazyl) radical, ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) radical, the reducing power, and chelating ability on ferrous ions. All antioxidant activities of the culture broth were increased in proportion to its concentrations. The IC_{50} values were in order as follows ABTS radical scavenging activity < DPPH radical scavenging activity < chelating power. Accordingly, these results suggest that pharmacological function of *T. fuciformis* might be due to, at least partially, their protective effects against oxidation and the culture broth of *T. fuciformis* was free radical inhibitors or scavengers at low concentration, involving possibly in termination of free radical reaction as primary antioxidants.

Keywords: *Tremella fuciformis*, antioxidant, scavenging activity, reducing power

Introduction

Free radicals have been implicated in the pathogenesis of various diseases, including myocardial and cerebral ischemia, arteriosclerosis, diabetes, rheumatoid arthritis, inflammation, and cancer-initiation, as well as in the aging process [1,2]. There is considerable evidence that antioxidants could help to prevent these diseases because they have the capacity to quench free radicals [3]. Although some synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), exhibit potent free radical scavenging effects, they have been demonstrated to exert toxicological effects as compared with natural antioxidants [4,5]. Thus, the demand for alternative and safe antioxidants from natural sources has gradually grown.

In contrast, medicinal mushrooms produce various classes of secondary metabolites with potent antioxidant activity. The search of new products with antioxidative properties is very active field of recent research. Mushrooms have been used as food and food-flavoring material in soups and sauces for centuries, due to their unique and subtle flavor. Recently, they have become attractive as functional foods and as a source of physiologically beneficial medicines, while being devoid of undesirable side-effects [6]. The nutritional values and taste components of the commercial mushrooms have been thoroughly studied [7].

Mushrooms are known to have high values in nutrition and disease prevention. Several researchers [8-11] have reported that mushrooms possess strong antioxidant and free radical scavenging activity. Potential active components with therapeutic effects such as polysaccharides, triterpenoids, and steroids have also been isolated from medicinal

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mushrooms. Polysaccharides extracted from fruiting bodies of medicinal mushrooms and their submerged cultures showed similar biological responses in antioxidant and free radical scavenging activities [12,13] and cytokine stimulating activities on macrophages [14].

T. fuciformis, belonging to the order of the *Tremellales* and the family of the *Tremellaceae*, has been appreciated as an edible mushroom. It also has been used for medicinal purposes due to its diverse physiological activities such as improving immunodeficiency and preventing senile degradation of microvessels [15-17].

In this study, the antioxidant activities of the culture broth from submerged culture of *T. fuciformis* were investigated *in vitro* and evaluated by scavenging abilities on DPPH and ABTS, the reducing power, and the chelating ability on ferrous ions.

MATERIALS AND METHODS

Chemicals

Folin-Ciocalteu reagent, gallic acid, (+)-catechin, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and potassium persulfate were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and all other reagents were of analytical grade.

Cultivation of *T. fuciformis* and Sample Preparation

T. fuciformis was used in the experiment. The medium used for a liquid culture contains the followings (per liter): 20 g of glucose, 2 g of tryptone, 0.46 g of KH_2PO_4, 1 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, and pH was adjusted to 8.0. The medium was sterilized and the incubation was carried out in a 5 L fermenter containing 3 L medium, with a stirring rate of 200 rpm and an aeration rate of 2 vvm at 25℃ for 5 days. After centrifugation (2,800 × g for 15 min), the supernatant was concentrated with a rotary evaporator at 60℃ (5 times) and the concentrates were used for assay of antioxidant activity.

Analysis of Chemical Components

Total polyphenol content was determined using the Folin-Ciocalteu method [18], adapted to a micro scale. In a 1.5-mL eppendorf tube, 0.79 mL distilled water, 0.01 mL culture broth appropriately diluted, and 0.05 mL Folin-Ciocalteu reagent was added and mixed. After exactly 1 min, 0.15 mL of sodium carbonate (20%) was added, mixed and allowed to stand at room temperature in obscurity, for 120 min. The absorbance was read at 750 nm, and the total polyphenol concentration was calculated from a calibration curve, using gallic acid as standard.

Total flavonoid contents in extract were determined by the method of Woisky and Salatino [19] with minor modifications. To 0.5 mL of the extract solution, 0.5 mL of 20 mg/mL $AlCl_3$ in ethanol solution was added. After 1 h at room temperature, the absorbance was measured at 420 nm. Extract samples were evaluated at the final concentration of 20 mg/mL. Total flavonoid contents were calculated as catechin (mg/g) from a calibration curve.

The total flavonol content was estimated using the p-dimethylaminocinnamaldehyde (DMACA) method [20]. Culture broth (0.2 mL), diluted 1:100 with MeOH, was put into a 1.5-mL eppendorf tube and added 1 mL DMACA solution (0.1% in 1 N HCl in MeOH). The mixture was mixed with vortex and allowed to react at room temperature for 10 min. Then, the absorbance at 640 nm was read against blank prepared similarly without DMACA. The concentration of total flavonol was estimated from a calibration curve, constructed by plotting known solutions of catechin (1-16 mg/L).

Total sugar and protein contents were determined in culture broth by the phenolsulphuric acid method [21] and Bradford method [22], using glucose and bovine serum albumin as a standard, respectively.

DPPH Radical Scavenging Activity

The scavenging activity of the free and bound extracts on DPPH radical was measured according to the method of Cheung *et al.* [23] with some modifications. Aliquots (0.8 mL) of 0.2 mM DPPH ethanolic solution was mixed with 0.2 mL of the broth. The mixture was vigorously shaken and left to stand for 10 min under subdued light. The absorbance was measured at 520 nm. The DPPH radical scavenging activity (%) was calculated by the following equation: Radical scavenging activity (%) = $(1 - A_{sample}/A_{control}) \times 100$, where A_{sample} is the absorbance in the presence of sample and $A_{control}$ is the absorbance in the absence of sample, respectively. All extracts were analyzed in triplicate.

ABTS Radical Scavenging Activity

The scavenging activity of the culture broth from *T. fuciformis* on ABTS radical cation was measured according to the method of Re *et al.* [24] with some modifications. ABTS radical cation was generated by adding 7 mM ABTS to 2.45 mM potassium persulfate solution and the mixture was left to stand overnight in the dark at room temperature. The ABTS radical cation solution was diluted with distilled water to obtain an

absorbance of 1.4-1.5 at 414 nm (molar extinction coefficient, $\varepsilon = 3.6 \times 10^4$ mol^{-1} 1 cm^{-1}) [25]. Diluted ABTS radical cation solution (1 mL) was added to 50 μL of the brothor ascorbic acid standard solution or distilled water. After 90 min, the absorbance was measured at 414 nm.

Measurement of Reducing Power

The reducing power of the culture broth was determined by the method of Yen and Chen [11] with some modification. Extracts (2-10 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℃ for 20 min. After 2.5 mL of 10 mg/mL trichloroacetic acid was added, the mixture was centrifuged at 1,160 × 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.

Assay of Ferrous ion Chelating activity

The chelating activity of extracts on ferrous ion was measured as reported by Decker and Welch [26]. One mL of culture broth (0.125-8 mg/mL) was mixed with 3.7 mL of deionized 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. Ethylendiaminetetraacetate (EDTA) was used as positive control and chelating activity on ferrous ion was calculated using the same equation given in DPPH radical scavenging activity, with $A_{control}$ as the absorbance of the blank without extract or EDTA and A_{sample} as the absorbance in the presence of the culture broth or EDTA.

RESULTS AND DISCUSSION

Chemical Composition of Culture Broth

We examined the chemical composition of culture broth as shown in Table 1. The total polysaccharide and protein contents were found 296.39 and 9.24 mg/g, respectively. Culture broth was mainly composed with carbohydrate. Polyphenols, flavonoids and flavonols contents showed 16.63, 9.19 mg/g and 83.74 μg/g, respectively.

Polyphenolic compounds in plants are powerful free radical scavengers which can inhibit lipid peroxidation by neutralizing peroxy radicals generated during the oxidation of lipids [27]. Since mushrooms (edible fungi) also possess polyphenolic compounds [23,28], it is interesting to investigate the antioxidant activity of mushroom in relation to their total polyphenolic contents. Generally, flavonoids are a large group of natural polyphenolic compounds, consisting mainly of flavonols, flavanols and anthocyanidins. It was reported that antioxidants with hydrogen donating and lipid peroxidation inhibiting properties in *Agrocybe aegerita* were selectively extracted into the ethyl acetate fraction [29]. Since a strong positive correlation was found between the total polyphenolic content and trolox equivalent antioxidant capacity value, as well as the inhibition of lipid peroxidation at 0.5 mg/mL for the methanol crude extract and its fractions, the strong antioxidant potency of the ethyl acetate fraction might be highly contributed by the abundant polyphenolic compounds that it contained. Huang [30] reported that methanolic extracts from a medicinal mushroom *Antrodia camphorate* (Changchih) showed excellent antioxidant activities as evidenced by 5.32-5.78% of lipid peroxidation at 1.0 mg/mL. Accordingly, antioxidant activity of the culture broth containing polyphenolic compounds from *Tremella fuciformis* was investigated.

Table 1. Total contents of polysaccharides, protein and polyphenols, and flavonols of culture broth from submerged culture of *T. fuciformis*

Dry weight (mg/mL)	Carbohydrate (mg/g)	Protein (mg/g)	Polyphenols (mg/g)	Flavonoids (mg/g)	Flavonols (μg/g)
61.3	296.39 ± 10.21	9.24 ± 0.28	16.63 ± 0.21	9.19 ± 0.68	83.74 ± 24.68

DPPH Radical Scavenging Activity

It is well known that antioxidants can seize the free radical chain of oxidation and form stable free radicals, which would not initiate or propagate further oxidation. DPPH has been used extensively as free radical to evaluate reducing substances. As shown in Fig. 1, the culture broth of *T. fuciformis* showed 50.1% of the DPPH radical scavenging ability at 10.0 mg/mL and 60.7% at 14.0 mg/mL, respectively. The percentage of inhibition sharply increased according to the concentrations of the broth from 2 mg/mL of the beginning point. The scavenging activity on DPPH, a stable free radical, is a widely used index and a quick method to evaluate antioxidant activity [31]. Cheung *et al.* [23] reported that the highest scavenging activities on DPPH radical were 55.4% for the water extract of *Lentinus edodes* (Berk.) Singer and 37.9% for *Volvariella volvacea* (Bull.) Singer at the concentration of 1-9 mg/mL. Also, it is presented that 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 [27]. And, other previous papers reported that the culture broth of mushrooms showed a

similar DPPH radical scavenging activity [23,27]. These results revealed that the culture broth of *T. fuciformis* was free radical inhibitors or scavengers, acting possibly as primary antioxidants.

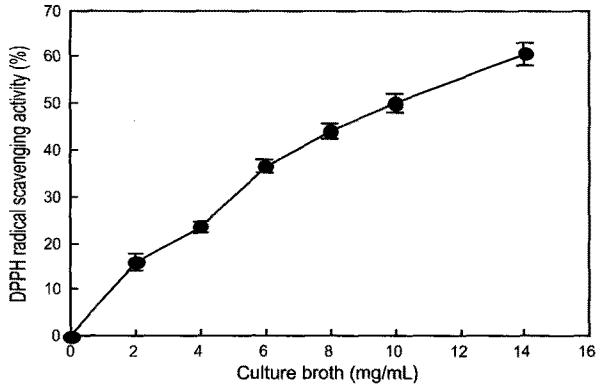

Fig. 1. DPPH radical scavenging activity of the culture broth from submerged culture of *T. fuciformis*.

ABTS Radical Scavenging Activity

This method measures the relative antioxidant ability of culture broth to scavenge the radical $ABTS^{\cdot+}$ in the aqueous phase. The $ABTS^{\cdot+}$, generated by potassium persulfate, is presented as an excellent tool for determining the antioxidant activity of hydrogen-donating antioxidants (scavengers of aqueous phase radicals) and of chain-breaking antioxidants (scavengers of lipid peroxyl radicals). As shown in Fig. 2, the ABTS radical scavenging activity of culture broth was enhanced with increasing its concentrations. At 8 mg/mL concentration of the culture broth, the radicals were scavenged with approximately 70% of inhibiting activity. This showed higher inhibiting activity at same concentration compared to that of DPPH radicals. Antioxidant activity of the natural antioxidants has been shown to be involved in termination of free radical reaction [32].

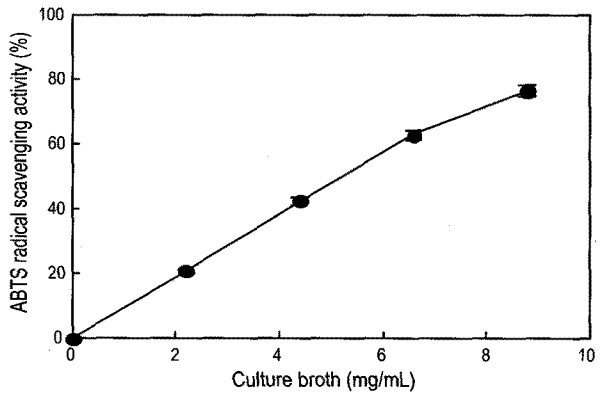

Fig. 2. ABTS radical scavenging activity of the culture broth from submerged culture of *T. fuciformis*.

Reducing Power

The reducing power of the culture broth from *T. fuciformis* was shown in Fig. 3. In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. The presence of reducers (i.e. antioxidants) causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, measuring the formation of Perl's Prussian blue at 700 nm can monitor the Fe^{2+} concentration [33]. Like other antioxidant activity, the reductive potential of culture broth exhibited a dose dependent activity within a concentration range of 0-10 mg/mL. The reducing power of *T. fuciformis* increased linearly with concentrations of the culture broth. Mau *et al.* [34] reported that 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. Tsai *et al.* [27] mentioned that 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 at 20 mg/mL, respectively. In the present study, the culture broth from *T. fuciformis* was proved to be moderate ability of reducing power on Fe^{3+}-Fe^{2+} transformation (Fig. 3) and the reducing power might be due to their hydrogen-donating ability as described by Shimada *et al.* [32]. Accordingly, *T. fuciformis* might contain higher amount of reductone, which could react with free radicals to stabilize and block redical chain reaction.

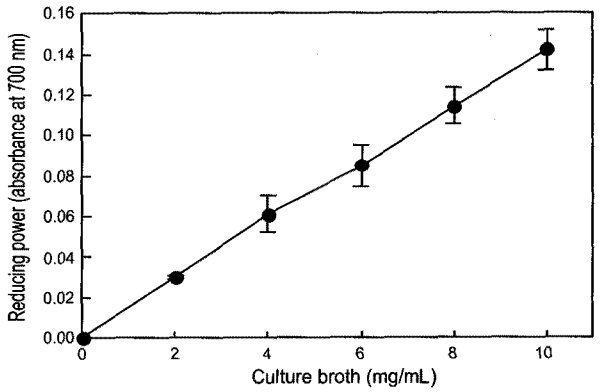

Fig. 3. Reducing power of the culture broth from submerged culture of *T. fuciformis*.

Fe^{2+} Chelating Activity

Antioxidants can be explained as reductants, and inactivation of oxidants by reductants can be described as redox reactions in which one reaction species (oxidant) is reduced at the expense of the oxidation of another antioxidant. The Fe^{2+} chelating activity measures the

antioxidant effect of any substance in the reaction medium as reducing ability. The culture broth showed a moderate ferrous ion chelating ability and reached 54.2% at 48 mg/mL (Fig. 4). The chelating effect increased with increasing concentration of culture broth. However, EDTA showed an excellent chelating ability of 95% at a concentration as low as 0.15 mg/mL (data not shown). The culture broth from *T. fuciformis* also showed slightly weak Fe^{2+} chelating ability, just similar to that of the water extract from *A. cylindracea* fruit bodies and mycelia [27]. On the contrary, the hot water extracts from *Pleutotus citrinopileatus* fruit bodies and mycelia chelated 82.1% and 87.9% of ferrous ion at 5 mg/mL, respectively [35]. Accordingly, further purification of the culture broth may improve the chelating ability. However, since ferrous ions are the most effective pro-oxidants in the food system, the weak ferrous ion chelating abilityof *T. fuciformis* would be somewhat beneficial.

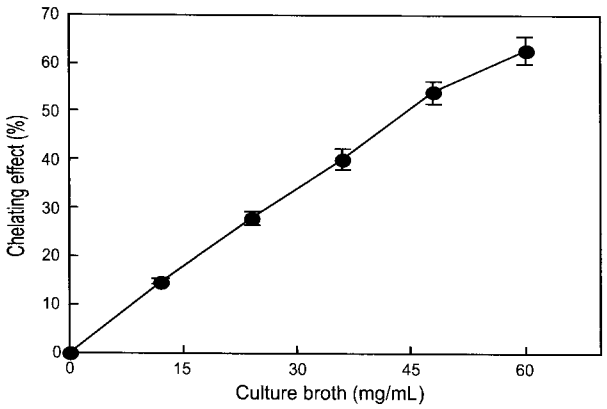

Fig. 4. Ferrous ion chelating activity of the culture broth from submerged culture of *T. fuciformis*.

IC_{50} Value in Antioxidant Properties

The antioxidant properties assayed herein were summarized in Table 2, except for reducing power, and the results were normalized and expressed as IC_{50} value (mg of hot water extract/mL) for comparison. For the culture broth from submerged culture of *T. fuciformis*, IC_{50} value followed the ascending order ABTS radical scavenging activity < DPPH radical scavenging activity < chelating power. With regard to IC_{50} value, effectiveness in antioxidant properties inversely correlated with the value. Accordingly, the culture broth showed high radical scavenging activities compared to the chelating power.

Antioxidant activity has become one of the focuses for the study on mechanisms of the nutraceutical and therapeutical effects of traditional Chinese medicines [9] and there are numerous antioxidant methods and modifications for evaluation of antioxidant activity [36]. 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 the culture broth from submerged culture of *T. fuciformis* was demonstrated by using a range of testing systems *in vitro*. The results suggest that the culture broth has direct and potent antioxidant activities.

Table 2. IC_{50} values of the culture broth obtained from submerged culture of *T. fuciformis* on antioxidant properties

	IC_{50} value[a] (mg/mL)		
	DPPH·	ABTS·	Chelating power
Culture broth	10.26 ± 0.30	5.46 ± 0.10	45.87 ± 1.31

[a] IC_{50} value is the effective concentration at which DPPH or ABTS radicals were scavenged and chelating effect was inhibited by 50%.

Mushrooms have long constituted an integral part of the normal human diet; these are reported to contain relatively large amounts of vitamins A, C and β-carotene, all of which have protective effects because of their antioxidant properties [37]. Mushrooms also contain many polyphenols, which are very efficient scavengers of peroxy radicals [38]. The extract of the mushroom, *Ganoderma lucidum*, has been reported to remove the hydroperoxide radical, the main factor in the human aging process [13]. *Pleurotus ostreatus* is reported to contain higher concentrations of cystine, methionine and aspartic acid than other edible mushrooms, such as *Agaricus bisporus* (brown), *A. bisporus* (white) and *Lentines edodes* [38]. Levostatin, a cholesterol-lowering drug derived from *Pleurotus* species, and its analogues are reported to be the best therapeutic agents for correcting hypercholesterolemia [39]. Ethyl acetate and methanol extracts of *P. florida* have been found to exhibit potent scavenging of hydroxyl radicals and inhibition of lipid peroxidation activities [40]. In addition, *Plerotus ostreatus*has also been reported to possess excellent reducing power on ferric ions [41]. Hitherto, research has tended to focus on the dietary value of edible mushrooms.

These results suggest that pharmacological functions of *T. fuciformis* are due to, at least partially, their protective effects against oxidation and that it is a promising way to use the culture broth of the fungus for the antioxidant activity to reduce the human demands on the natural resources of *T. fuciformis*, an endangered species.

There is no study so far concerning characterization of the active antioxidative components in *T. fuciformis*. Since polyphenolic compounds might be a possible class of antioxidant in this mushroom, future work on the isolation and structural characterization of the active components is needed. Also, the antioxidant activity of these components,

in regard to the mechanisms for radical scavenging and protection against lipid peroxidation, will be the primary objective of further investigation.

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