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Antioxidant Properties of *Antrodia camphorata* in Submerged Culture

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The biologically active compounds, antioxidant activities, and free radical scavenging effects of dry matter of cultural medium (DMCM), filtrate (DMF), and different solvent extracts of mycelia from *Antrodia camphorata* in submerged culture (ACSC) were investigated. DMF showed the strongest inhibition of lipid peroxidation as a function of its concentration, and was comparable to the antioxidant activity of BHA at the same concentration of 0.2 mg/mL. The hexane extract of mycelia had the weakest antioxidant ability, whereas other mycelial extracts exhibited a modest inhibition of lipid peroxidation. DMF and water extract of mycelia (WEM) showed marked activity in free radical scavenging. The antioxidant activities of filtrate and mycelial extracts were correlated with the presence of total polyphenols, the crude triterpenoids, and the protein/polysaccharide ratio of the crude polysaccharides. It was found that DMCM had lower antioxidant ability than DMF in different model systems, indicating that the major antioxidant components in DMF must be derived from the secondary metabolites of mycelia. The results presented herein indicate that DMF could possibly act as a chemopreventing agent with respect to free radical-related diseases.

KEYWORDS: ***Antrodia camphorata*; antioxidant; polysaccharide; polyphenol; triterpenoid**

INTRODUCTION

A growing amount of research in biology and medicine has been devoted to reactive oxygen species (ROS). There now is considerable evidence that ROS induce oxidative damage in biomolecules. This damage causes atherosclerosis, aging, cancer, and several other diseases. Some of the relevant ROS are as follows: hydroxyl ($OH^{\bullet}$), superoxide anion radical ($O_2^{\bullet -}$), nitric oxide ($NO^{\bullet}$), peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$), hydrogen peroxide (H_2O_2), and hypochloride (HOCl) (*1*). Antioxidants, which scavenge free radicals, are known to play important roles in preventing ROS-induced diseases. Fortunately, dietary foods contain a wide variety of ROS-scavenging antioxidants, for example, flavonoids and antioxidative vitamins such as ascorbic acid and α-tocopherol (*2*). Epidemiological studies have shown that higher intake of fresh vegetables, fruits, tea, and wine is associated with reduced risk of heart disease (*3*). This is the reason for the current strong interest in natural antioxidants and their roles in human health and nutrition.

Medicinal mushrooms have been used in China for two thousand years to improve health and achieve longevity. Recently, they have received more and more attention in many studies which have used modern scientific methods to analyze their bioactive components (*4*). The most important components of mushrooms are water-soluble polysaccharides in fruiting bodies (*5*). The range of molecular weights for polysaccharides is large and their bioactive characteristics vary greatly (*6*). Investigations also have indicated that water extract or polysaccharide from mushroom may possess superoxide dismutase (SOD)-like antioxidant activity (*7*) and radical scavenging properties (*8*).

Antrodia camphorata is also well-known as niu-chang-chih or niu-chang-ku. Niu-chang is the Chinese common name for *Cinnamomum kanehirai*, which is one of the endangered species in Taiwan; "ku" in Chinese means mushroom; and "chih" means *Ganoderma*-like fungus. It is a new species of the genus *Antrodia* (family Polyporaceae, Aphyllophorales) parasitic on the inner cavity of the endemic species *Cinnamomum kanehirai* Hay (*9*). *Antrodia camphorata* was identified as a new *Ganoderma* species, *Ganoderma camphoratum*, in 1990 on the basis of their similar characteristics (*10*). Traditionally, it has been used as a remedy for food, alcohol, and drug intoxication, diarrhea, abdominal pain, hypertension, skin itching, and liver cancer among Chinese (*11*). Preliminary pharmacological studies revealed that zhankuic acid (a type of steroid acid) of fruiting bodies showed cytotoxicity against P388 murine leukemia at 4 μg/mL, and was anticholinergic as well as antiserotonergic as tested on the guinea pig ileum preparation at 10 μg/mL (*12*). In addition, methanol extracts from dry mycelium and fresh fruiting bodies had similar antioxidant activity, whereas those from dry fruiting bodies did not have antioxidant ability (*13*). The growth rate of natural *Antrodia camphorata* in the wild is very slow, and it is difficult to cultivate in a green house, thus, it is expensive to obtain fruiting bodies. Therefore, using a

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submerged culture method to obtain useful cellular materials, or to produce effective substances from cultured mycelia, might be a possible way to overcome the disadvantage of the retarded growth of fruiting bodies (*14*). However, the antioxidant abilities of *Antrodia camphorata* in submerged culture (ACSC) have not yet been studied. The objectives of this study were to investigate the antioxidant activity and the free radical scavenging activity of ACSC; to compare them with those of known antioxidants, such as Trolox, BHA, and gallic acid; and to elucidate their active antioxidant compounds.

MATERIALS AND METHODS

Materials. Mycelia from *Antrodia camphorata* in submerged culture was obtained from the biotechnology center of Grape King Inc., Chungli, Taiwan. Linoleic acid, nitro blue tetrazolium (NBT), α,α-diphenyl-β-picrylhdrazyl (DPPH), horseradish peroxidase (HRPase), linoleic acid (99%), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate (ABTS), ferric chloride, and ferrozine (3-(2-pyridyl)-5,6-bis (4-phenylsulfonic acid)-1,2,4-triazine) were purchased from Sigma Chemical Co. (St. Louis, MO). Tween 20 (polyoxyethylenesorbitan monolaurate), and phenazine methosulfate (PMS) were purchased from E. Merck Co. (Darmstadt, Germany). Hydrogen peroxide, sodium dihydrogen phosphate, anhydrous dipotassium hydrogen phosphate, potassium dihydrogen phosphate, boric acid, and disodium hydrogen phosphate were purchased from Shimakyu Co. (Japan). Trolox (Hoffman-La Roche) and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Dimethyl sulfoxide (DMSO) was obtained from Fluka Chemie (Buchs, Switzerland).

Culture Conditions of *Antrodia camphorata*. *Antrodia camphorata* hyphae were separated from the fruiting bodies and inoculated into a cultural medium which was composed of 2.5% corn starch, 2% sucrose, 0.5% yeast extract, 0.1% KH_2PO_4, 0.3% $MgSO_4$, 0.3% $(NH_4)_2SO_4$, and 0.05% citric acid in distilled water, and adjusted to initial pH range 5.3−5.5. Each shaking-flask culture was carried out in 2-L Erlenmeyer flasks containing 1 L of medium, and incubated at 27−30 °C for 7 days. Thereafter, 3.5 L of shaking-flask cultures was inoculated into a 500-L fermenting tank containing 350 L of cultural medium, and then cultured at 27−30 °C for 7 days with aeration (0.5 vvm) and shaken at 50 rpm/min with a rotary shaker to obtain a mucilaginous medium containing mycelia. Residue sugar concentration was about 0.1 g/L after cultivating for 7 days. The mycelia were collected by means of centrifugation (4 °C, 8000 rpm for 15 min) and then washed with distilled water. Finally, the mycelia were freeze-dried to a powder form. The yield of mycelia in submerged culture was 1.1 g dry weight of mycelia/100 g ACSC.

Preparation of Filtrate (or Cultural Medium) from ACSC. One liter of mycelial extracellular medium (or cultural medium) was filtered, concentrated 6-fold under vacuum, and freeze-dried to a powder form. The yields of dry matter of cultural medium (DMCM) and filtrate (DMF) powder were 11.95 and 5.17 g, respectively.

Preparation of Mycelial Extracts from ACSC. The freeze-dried mycelia (about 10 g) from the submerged culture (1 L) was homogenized at 7000 rpm for 2 min at room temperature (homogenizer polytron PT 3000; kinematica Switzerland), then extracted with distilled water (2 L) at 30 °C for 24 h. The extracts were filtered through Whatman No. 2 filter paper, concentrated 6-fold under vacuum, and then freeze-dried to a powder form. The freeze-dried mycelia (10 g) was extracted 3 times with 2 L of methanol, ethyl acetate, and hexane, respectively, under 30 °C. The extracts were filtered through Whatman No. 2 filter paper and then concentrated to dryness. The yields of water (WEM), methanol (MEM), ethyl acetate (EAEM), and hexane (HEM) extracts of mycelia were 4.5, 4.6, 0.98, and 0.56 g, respectively.

Effect of ACSC on Trolox Equivalent Antioxidant Capacity (TEAC) Assay. The total antioxidant activity of ACSC was measured using the TEAC assay as described by Miller et al. (*15*), with minor modifications. The TEAC value is based on the ability of the antioxidant to scavenge the blue-green 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate ($ABTS^{\bullet+}$) radical cation relative to the $ABTS^{\bullet+}$ scavenging ability of the water-soluble vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). $ABTS^{\bullet+}$ radical cation can be generated by the interaction of ABTS (100 μM), H_2O_2 (50 μM), and peroxidase (4.4 unit/mL). To measure antioxidant capacity, 0.25 mL of ACSC (0.2 mg/mL) was mixed with an equal volume of ABTS, H_2O_2, peroxidase, and deionized water. Absorbance was monitored at 734 nm for 10 min. The decrease in absorption at 734 nm after the addition of reactant was used to calculate the TEAC value. TEAC value is expressed as millimolar concentration of Trolox solution having the antioxidant equivalent to a 1000 ppm solution of the sample under investigation. The higher the TEAC value of the sample means the stronger the antioxidant ability.

Effect of ACSC on Hydrogen-Donating Activity. The hydrogen-donating activity of ACSC was expressed by the scavenging effect of DPPH and measured according to the method of Williams et al. (*16*). A reaction mixture containing 4 mL of ACSC extracts and 1 mL of 0.2 μM DPPH was allowed to stand at room temperature for 30 min, and the absorbance was read at 517 nm against blank samples.

Effect of ACSC on Scavenging of Superoxide Anion. The ability of ACSC to scavenge superoxide anion was determined through spectrophotometric measurement of the reduction of nitro blue tetrazolium (*17*). Superoxide anion was generated in a nonenzymatic system. The reaction mixture, which contained the same volume of ACSC extracts, 60 μM PMS, 468 μM NADH, and 150 μM NBT in 0.1 M pH 7.4 of phosphate buffer, was incubated at ambient temperature for 5 min, and the absorbance was read at 560 nm against blank samples.

Effect of ACSC on Scavenging of Nitric Oxide. The scavenging effect of ACSC on nitric oxide was measured according to the method of Marcocci et al. (*18*). Aliquots of 4 mL of extract solutions (0.2 mg/mL) were added to 1 mL of sodium nitroprusside solution (25 mM) in a test tube and then incubated at 37 °C for 2 h. An 0.5-mL portion of the incubation solution was removed and diluted with 0.3 mL of Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotination of nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride was immediately read at 570 nm and referred to the absorbance of standard solutions of sodium nitrite salt treated in the same way with Griess reagent.

Effect of ACSC on Lipid Peroxidation. The inhibition of lipid peroxidation was determined using the method of Liegeois et al. (*19*). An aqueous solution of linleic acid and AAPH solution was prepared as described by Liegeois et al. (*19*). A 30-μL aliquot of 16 mM linoleic acid dispersion was added to a UV cuvette containing 2.81 mL of 0.05 M phosphate buffer, pH 7.4, pre-thermostated at 40 °C. The oxidation reaction was initiated at 37 °C under air by adding 150 μL of 40 mM AAPH solution. Oxidation was carried out in the presence of aliquots (20 μL) of ACSC (0.2 mg/mL). The rate of peroxidation at 37 °C was monitored by recording the increase in absorbance at 234 nm caused by conjugated diene hydroperoxides.

Polysaccharide and Protein Contents. Polysaccharide is usually associated with protein as complexes. The contents of polysaccharide and protein in ACSC polysaccharide extracts were determined using the phenol−sulfuric acid method (*20*) and Bio-Rad protein assay kit, respectively.

Determination of Total Polyphenol. The total polyphenol was determined colorimetrically using the Folin−Ciocalteau method (*21*). Samples of ACSC (100 mg) were extracted with 250 mL of methanol/water (60:40, v/v; 0.3% HCl) and then filtered through a 0.45-μm Millipore filter. To the filtrate, 100 μL of the extract, 100 μL of 50% Folin−Ciocalteau reagent, and 2 mL of 2% sodium carbonate were added and mixed completely. After 2 h, the absorbance of the solution at 750 nm was measured with a spectrophotometer. Quantitation was based on the standard curve of gallic acid (0∼0.5 mg/mL), which was dissolved in methanol/water (60:40, v/v; 0.3% HCl).

Determination of Crude Triterpenoid. The triterpenoid was determined using the method of Chen et al. (*22*). ACSC extracts (0.1 g) were extracted with 50% (50 mL) ethanol for 1 h. The mixture was filtered, and the filtrate was evaporated to dryness with a rotary evaporator. The residue was extracted with $CHCl_3/H_2O$ three times. The $CHCl_3$ layer was further extracted with saturated $NaHCO_3$ (50 mL) three times, and the alkali solution was collected and acidified with 6

Table 1. Antioxidant Activities of the Extracts from *Antrodia camphorata* in Submerged Culture (ACSC)

ACSC	Trolox equivalent antioxidant capacity (TEAC, mM)[a]
filtrate	
DMF[b]	0.74 ± 0.01a
mycelia	
WEM	0.63 ± 0.02b
MEM	0.40 ± 0.01c
EAEM	0.32 ± 0.02d
HEM	0.21 ± 0.01e
medium	
DMCM	0.01 ± 0.00f

[a] TEAC expressed as millimolar concentration of Trolox solution having the antioxidant equivalent to a 1000 ppm solution of the sample under investigation. Values in column with the different letters are significantly different ($p < 0.05$). Results are mean ± SD for $n = 3$. [b] DMF: dry matter of filtrate; WEM, MEM, EAEM, HEM: water, methanol, ethyl acetate, and hexane extracts, respectively, of mycelia; DMCM: dry matter of cultural medium.

N HCl to a pH of 3−4. Then, the solution was extracted with ethyl acetate three times. The solvent was evaporated, and a pale yellow solid material was obtained. The solid was dried in an oven to yield an acidic-ethyl acetate-soluble material containing fractions (crude triterpenoids).

Statsistical Analysis. The values of means and standard deviation (mean ± SD) and the 95% confidence intervals (CI) of means for verifying the statistical significance of all the parameters were calculated. When necessary, data were tested using 2-way ANOVA. p values < 0.05 were considered statistically significant. All data were means of three measurements.

RESULTS

Antioxidant Activity of ACSC by TEAC Assay. The antioxidant activities of filtrate and mycelia extracts from ACSC were evaluated by means of TEAC assay, and the results are shown in **Table 1**. The antioxidant activities of filtrate and mycelia extracts were found to be in the order of DMF > WEM > MEM > EAEM > HEM. DMF and WEM had the highest TEAC values (0.74 and 0.63 mM Trolox equivalent, respectively), and the other extracts had lower TEAC values (0.21−0.40 mM Trolox equivalent). The results indicated that the higher the polarity of the extracts of ACSC, the stronger the antioxidant activity. The results also indicated that unknown active components with water-soluble characteristics might exist in the filtrate and mycelia extract. To understand whether the antioxidant ability of DMF was derived from the original culture medium of mycelia, we also investigated the antioxidant ability of the dry matter of culture medium (DMCM). DMCM did not show any antioxidant activity in scavenging $ABTS^{+\bullet}$ radical. This result suggested that the antioxidant effect of the filtrate was not derived from the culture medium of mycelia.

Effect of ACSC on Lipid Peroxidation. Figure 1 shows the antioxidant activities of ACSC extracts measured based on conjugated diene formation in the AAPH/linoleic acid system and compares them with those of commercial antioxidants BHA and Trolox. DMF and WEM exhibited good antioxidant activity in linoleic acid peroxidation. The inhibitory effects of DMF and WEM on lipid peroxidation were approximately equivalent to that of BHA (50%) but less than that of Trolox (>80%) at a concentration of 0.2 mg/mL. Although the inhibitory effects of MEM and EAEM on lipid peroxidation were less than those of DMF and WEM, MEM and EAEM also had obvious antioxidant activity (40%). This means that nonaqueous extracts of mycelia also had active components which increased the inhibitory

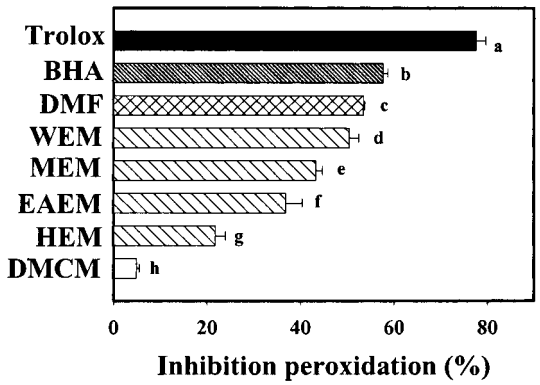

Figure 1. Comparison of antioxidant activities of extracts (0.2 mg/mL) from cultural medium and *Antrodia camphorata* in submerged culture (ACSC) as measured by conjugated diene formation in AAPH/linoleic acid system at 20 min incubation. Each value is the mean ± standard deviation ($n = 3$). DMF: dry matter of filtrate; WEM, MEM, EAEM, HEM: water, methanol, ethyl acetate, and hexane extracts, respectively, of mycelia; DMCM: dry matter of cultural medium.

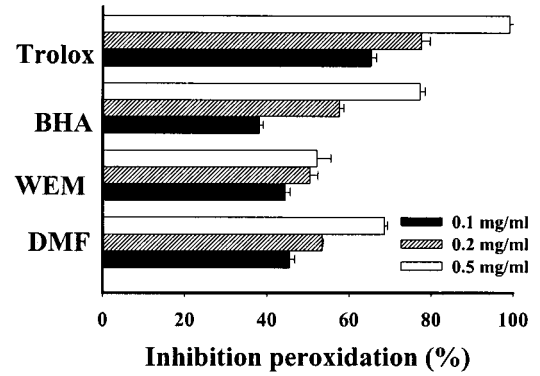

Figure 2. Antioxidant activities of Trolox, BHA, dry matter of filtrate (DMF), and water extract of mycelia extracts (WEM) from *Antrodia camphorata* in submerged culture (ACSC) as measured by conjugated diene formation in AAPH/linoleic acid system at 20 min incubation. Each value is the mean ± standard deviation ($n = 3$).

ability in the AAPH/linoleic acid system. The inhibitory effect of DMCM was weaker than that of the filtrate, indicating that the antioxidant ability of the filtrate might not have been derived from the culture medium. **Figure 2** shows that WEM had the maximum ability to inhibit lipid peroxidation (45%) at a concentration of 0.2 mg/mL, but that its ability did not increase with increasing concentration. In contrast, a concentration-dependent increase in the antioxidant activity of DMF was found up to a concentration of 0.5 mg/mL. The inhibitory activity was 70% at a concentration of 0.5 mg/mL. The antioxidant ability of DMF was similar to that of BHA and about 0.7-fold smaller than that of Trolox.

Free Radical Scavenging Activities of ACSC Extracts. Reactive oxygen species (ROS) are thought to induce cellular damage and play pathological roles in several human diseases, such as atheroscleroisis, cancer, aging, and central nervous system injury. ROS can attack lipids, carbohydrates, proteins, and DNA, causing cellular damage (*2*). Thus, we aimed to investigate the free radical scavenging effects of ACSC extracts to determine whether ACSC extracts could have the potential ability to prevent free-radical-related diseases.

As the results show in **Figure 3**, the DPPH scavenging effect of DMF (63%) was better than that of mycelial extracts (<55%) at a concentration of 0.2 mg/mL, but less than those of Trolox

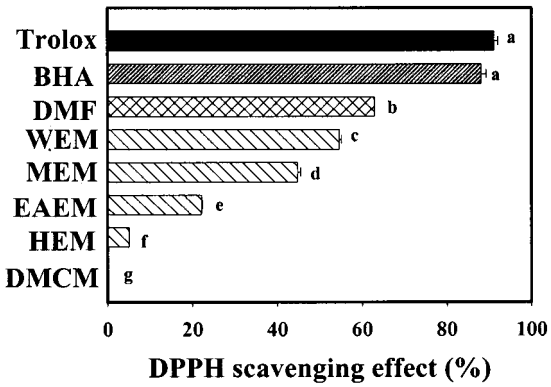

Figure 3. DPPH scavenging effects of extracts (0.2 mg/mL) from *Antrodia camphorata* in submerged culture. Each value is the mean ± standard deviation ($n = 3$).

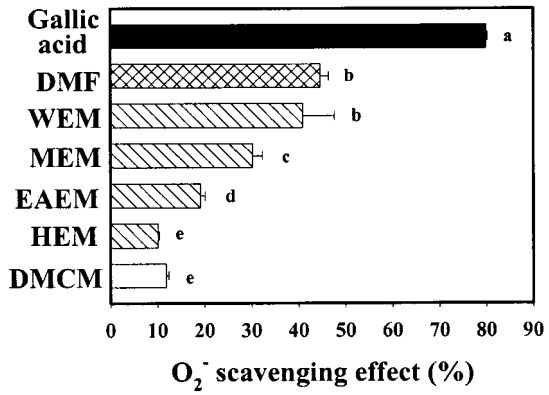

Figure 4. Superoxide scavenging effects of extracts (0.2 mg/mL) from *Antrodia camphorata* in submerged culture. Each value is the mean ± standard deviation ($n = 3$).

and BHA (90%) at the same concentration. In contrast, DMCM did not show any scavenging effect on DPPH radicals, which meant that the antioxidant activity of the filtrate was not derived from the original cultural medium.

Figure 4 shows that the superoxide scavenging effects of DMF and WEM were about 50%, followed by those of MEM (30%) and EAEM (20%) at a concentration of 0.2 mg/mL. The superoxide scavenging effects of HEM and DMCM were less than 20%. Aruoma et al. (*23*) found that gallic acid had a potential ability to scavenge superoxide. Thus, the scavenging effect of gallic acid on superoxide was compared with those of ACSC extracts at the same concentration (0.2 mg/mL). The results indicated that the scavenging effect of DMF on superoxide was 0.63-fold smaller than that of gallic acid.

As shown in **Figure 5**, the NO scavenging effect of WEM (60%) was higher than those of the other extracts at a concentration of 0.2 mg/mL, but was significantly lower than that of Trolox (70%) at the same concentration ($p < 0.05$). The NO scavenging effect of DMF was equal to that of BHA (40%). MEM and EAEM exhibited modest scavenging effects on NO (30%), whereas HEM and DMCM had the weakest scavenging effects (<20%).

Active Components in ACSC. Several components, such as polyphenol, triterpenoid, and polysaccharide, have been isolated from mushrooms. They have been proved to possess effective antioxidant activities (*8*, *24*). To determine the main antioxidant compounds in ACSC extracts, their contents of total polyphenol, crude triterpeniod, and polysaccharide were assayed. As the results show in **Table 2**, the yield of polysaccharide extracts in DMF (23.2%) was about 2-fold greater than that of

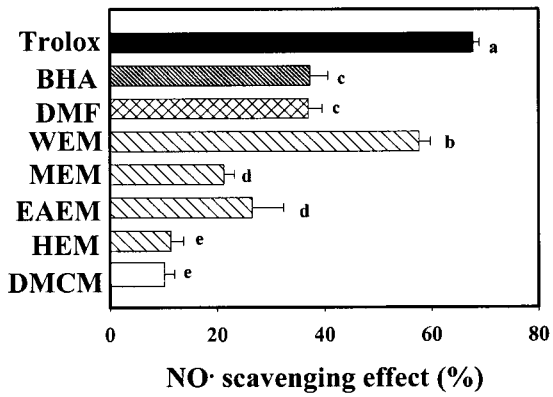

Figure 5. NO· scavenging effects of extracts (0.2 mg/mL) from *Antrodia camphorata* in submerged culture. Each value is the mean ± standard deviation ($n = 3$).

Table 2. Yield and Protein/Polysaccharide Ratio of Polysaccharide in the Dry Matter of Filtrate (DMF) and Water Extract of Mycelia (WEM) from *Antrodia camphorata* in Submerged Culture (ACSC)

ACSC	polysaccharide yield %	protein/polysaccharide ratio (%)
DMF	23.2	1.9
WEM	12.2	23.6

Table 3. Polyphenol and Triterpenoid Contents of Extracts from Cultural Medium and *Antrodia camphorata* in Submerged Culture (ACSC)

ACSC	total polyphenols content (mg/g extract)	crude triterpenoids (mg/g extract)
filtrate		
DMF[a]	66. 8 ± 2.5a[b]	47.0 ± 0.1b
mycelia		
WEM	70.5 ± 2.0a	ND
MEM	38.0 ± 0.7b	107.9 ± 1.3a
EAEM	16.2 ± 1.4c	47.1 ± 0.1b
HEM	12.2 ± 0.6d	21.4 ± 0.2c
medium		
DMCM	ND[c]	ND

[a] DMF: dry matter of filtrate; WEM, MEM, EAEM, HEM: water, methanol, ethyl acetate, and hexane extracts, respectively, of mycelia; DMCM: dry matter of cultural medium. [b] Each value is the mean ± standard deviation ($n = 3$). [c] Not detectable.

WEM (12.2%). However, DMF and WEM showed no significant difference ($p > 0.05$) in their ability to scavenge superoxide. At a concentration of 0.2 mg/mL, they scavenged about 50% of the sueroxide (**Figure 4**). This finding suggests that polysaccharide was not a major compound in DMF and WEM as far as scavenging superoxide as concerned.

Table 3 shows that WEM had a higher amount of total polyphenol (71 mg/g) than the other extracts from mycelia, in which the total polyphenol content was lower than 40 mg/g. However, DMF had 67 mg/g of total polyphenol, and no total polyphenol was detected in DMCM. These results are in agreement with the finding that the higher the total polyphenol content, the stronger the antioxidant activity and free radical scavenging activity. Therefore, total polyphenol might be the main active component responsible for the antioxidant activity of ACSC.

The crude triterpenoid contents in the ACSC extracts are shown in **Table 3**. As the results show, all the ACSC extracts contained triterpenoids; however, the crude triterpeniods content

varied in the extracts. The crude triterpenoid content of the ACSC extracts was in the order of MEM > EAEM = DMF > HEM > WEM, DMCM. MEM had higher crude triterpenoids content (108 mg/g) than the other extracts, and EAEM and DMF (47 mg/g) were next highest in content. It is worth noting that no crude triterpenoids could be detected in WEM. The results suggest that crude triterpenoid was not the main antioxidant responsible for WEM.

DISCUSSION

It was well established that lipid peroxidation was one of the reactions resulting in the formation of free radicals in cells and tissues. The one-electron reduction products of O_2 and ROS actively participated in the initiation of lipid peroxidation. The present work showed that the ACSC extracts acted as good radical scavengers in a variety of systems and inhibited lipid peroxidation. Thus, it can be concluded that the antioxidant activity of ACSC extracts was positively correlated with their ability to scavenge radicals, especially for both DMF and WEM, which showed potential antioxidant activity. On the other hand, DMCM had a lower free radical scavenging effect, indicating that the source of antioxidant in the filtrate (DMF) was not the original cultural medium. Huang (*13*) reported that the DPPH and superoxide scavenging effects of fresh fruiting bodies methanol extracts of *Antrodia camphorata* were 31 and 66%, respectively, at a concentration of 0.5 mg/mL. Yen and Wu (*25*) indicated that the antioxidant ability of methanol extracts of *Ganoderma tsugae* on DPPH scavenging effect was about 42% at the concentration of 0.2 mg/mL. On the other hand, the DPPH and superoxide scavenging effect of DMF were 60 and 45%, respectively, at the same concentration. Thus, the antioxidant activity of DMF is comparable to those of fruiting body of *Antrodia camphorata* and *Ganoderma tsugae*.

DMF and WEM showed the same efficacy in scavenging superoxide (**Figure 4**). However, we found that the scavenging abilities of DMF and WEM on superoxide were not correlated with their polysaccharide contents. These findings suggested that the polysaccharide content in DMF and WEM was not a major factor contributing to the effectiveness of antioxidative activity. It has been reported that the functional activity of polysaccharide could be influenced by many factors such as, molecular weight, branched degree, water solubility, structure, and configuration (*26*−*28*). Liu et al. (*8*) found that polysaccharide extracts of mushrooms had scavenging effects on superoxide that appeared to be dependent on the amount of protein present in the protein−polysaccharide complexes. Our results indicated that the polysaccharide in WEM had a higher protein/polysaccharide ratio than did that in DMF (**Table 2**). This might explain why the polysaccharide content in WEM was obviously less than that in DMF, but no significant difference was observed ($p > 0.05$) in their superoxide-scavenging effects (**Figure 4**). The mechanism of free radical scavenging by polysaccharides is still not fully understood. It is only known that glycolated-protein may scavenge ROS by one-electron transfer or a hydrogen abstraction mechanism (*29*). Thus, the protein/polysaccharide ratio might be an important factor in ROS-scavenging ability in polysaccharide extracts.

The TEAC assay has been developed and used to determine the total antioxidant capacities of various samples, and it has usually been used to provide a ranking order of antioxidants (*30*). Comparing the results of the TEAC assay (**Table 1**) with those of the total polyphenol content (**Table 3**), it is found that the antioxidant activities of the ACSC extracts were significantly correlated with the total polyphenol contents ($r = 0.91$). Rice-Evans et al. (*31*) reported that the antioxidant activity of phenolics was mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and single oxygen quenchers. Hence, it is possible that the total polyphenols existing in the ACSC extracts were the major component affecting antioxidant activity. It is still not clear what kind of nutrients in the medium are transformed to polypenol. It is only known that polyphenol is derived from phenylalanine, which in turn is formed via the classical shikimate pathway starting from phosphenol pyruvate and erythrose 4-phosphate (*32*).

It was reported that one-third of 150 mushroom species showed significant antioxidant activity in the inhibition of lipid peroxidation (*33*). In the present study, the correlation between the inhibition of lipid peroxidation (**Figure 1**) and the total polyphenol content (**Table 3**) demonstrated a linear relationship ($r = 0.87$). Moreover, we also found high correlation between the inhibition of lipid peroxidation and the crude triterpenoids content of nonaqueous mycelial extracts (MEM, EAEM, and HEM) ($r = 0.89$). These results indicated that the total polyphenols in the ACSC extracts were an active component involved in the inhibition of lipid peroxidation. However, triterpenoids also played a role in the nonaqueous ACSC extracts. Zhu et al. (*24*) reported that the greater inhibitory activity of triterpenoids in lipid peroxidation could be attributed to their high lipid solubility. We propose that crude triterpenoids are another type of antioxidant in the methanol and ethyl acetate extracts of mycelia. As shown in **Table 3**, WEM had higher total polyphenols content than the DMF; however, no crude triterpenoids was detected in WEM. To confirm that the higher TEAC value of DMF might be contributed from crude triterpenoids, the $ABTS^{\bullet+}$ scavenging effect of crude triterpenoids in DMF was also measured. The results indicated that the scavenging effect of crude triterpenoid was dose-dependent, and its TEAC value was about 3.0 mM. It may explain why the WEM had higher total polyphenols content than the DMF but a lower TEAC value. No polysaccharides, total polyphenols, or crude triterpenoids were detected in DMCM. In contrast, the filtrate (DMF) contained large amounts of these components. Therefore, *Antrodia camphorata* could metabolize culture medium into active components, such as polysaccharides, total polyphenols, and crude triterpenoids, during the fermentation process of the submerged culture.

In conclusion, the antioxidant abilities of the ACSC extracts were correlated with their total polyphenols content based on the evaluation of different antioxidant test systems. Thus, the antioxidant activities of the ACSC extracts might mostly be attributed to the total polyphenols. The crude triterpenoid contents in the methanol and ethyl acetate extracts of mycelia showed positive correlation with the inhibitory effect on lipid peroxidation, indicating that the crude triterpenoids might have played an important role in the antioxidant activity of the methanol and ethyl acetate extracts of mycelia. In addition, the superoxide-scavenging activity of the polysaccharide−protein complex in DMF and WEM was found to be related to its protein ratio. DMF had higher contents of total polyphenols and triterpenoids, making it the most effective antioxidant fraction in the ACSC extracts. Therefore, DMF could possibly act as a chemopreventing agent with respect to free-radical-related diseases. Further investigation on the in vivo antioxidant ability of DMF is underway in our laboratory.

ABBREVIATIONS USED

ACSC, *Antrodia camphorata* in submerged culture; DMF, dry matter of filtrate; WEM, water extract of mycelia; MEM,

methanol extract of mycelia; EAEM, ethyl acetate extract of mycelia; HEM, hexane extract of mycelia; DMCM, dry matter of culture medium.

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