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Antioxidant and free radical scavenging activities of *Phellinus merrillii* extracts

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ABSTRACT. This study aimed to investigate possible antioxidant activity of various extracts of *Phellinus merrillii* (PM). The explored items include: ABTS free radical scavenging assay, determination of total phenolics contents (TPC), ferric reducing antioxidant power assay (FRAP), rapid screening of antioxidant by dot-blot DPPH (1, 1-diphenyl-2-picrylhydrazyl) staining, DPPH radical-scavenging activities and reducing power measurement. In the ABTS free radical scavenging assay, the n-BuOH fraction displayed the highest total antioxidant activity (17.13 ± 0.04 mM). In the determination of total phenolics contents (TPC) and ferric reducing antioxidant power assay (FRAP), the EtOAc fraction had the highest phenolics contents (46.21 ± 0.02 mM) and reducing antioxidant power (19.09 ± 0.03 mM). In the rapid screening of antioxidant by dot-blot DPPH staining, the n-BuOH fraction showed the highest strong dot-blot staining. In the reducing power measurement, the crude extract had the highest reducing power at 2 mg/ml concentration. In the DPPH radical-scavenging activities, the EtOAc fraction had the highest antioxidant activity (IC_{50} = 0.66 ± 0.01 mg/ml). As regard the correlation coefficients among ABTS assay, FRAP assay, and total phenolics contents, it can be seen that correlation coefficients in each case were significant. Among all extracts, the highest amount of total phenolics contents were found in the EtOAc fractiont. It is suggested that the PM might contribute its antioxidant activities on EtOAc and n-BuOH fraction. In high-performance liquid chromatography tandem mass (LC/MS/MS) analysis for hispolon, the daughter ion scanned chromatograms of PM was established. Both hispolon and PM showed similar daughter ion spectrum at the retention time of 4.7 min and had more lobes in m/z 219 and m/z 135. This indicated that PM did contain the active ingredient hispolon. Both the IC_{50} of DPPH radical scavenging activity for hispolon and BHT were 42.4 ± 2.9 and 81.2 ± 3.2 μM, respectively. These findings mean that hispolon was most important in antiradical activities. It was suggested that hispolon might contribute to its antioxidant activities in PM.

Keywords: 1,1-diphenyl-2-picrylhydrazyl (DPPH); Ferric reducing antioxidant power assay (FRAP); Free radicals; Glutathione reduced form (GSH); High-performance liquid chromatography tandem mass (LC/MS/MS); *Phellinus merrillii* (PM); Scavenging effect; Total antioxidant capacity; Total phenolics contents (TPC); Trolox equivalent antioxidant capacity (TEAC).

INTRODUCTION

It is commonly accepted that, in a situation of oxidative stress, reactive oxygen species, such as superoxide ($O^{\cdot}_2$), hydroxyl ($OH^{\cdot}$) and peroxyl ($^{\cdot}OOH$, $ROO^{\cdot}$) radicals, are generated. The reactive oxygen species play an important role related to the degenerative or pathological processes of various serious diseases, such as aging (Burns et al., 2001), cancer, coronary heart disease, Alzheimer's disease (Smith et al., 1996; Diaz et al., 1997), neurodegenerative disorders, atherosclerosis, cataracts, and inflammation (Aruoma, 1998). Traditional medicine is widespread and plants still presents a large source of natural antioxidants that might serve as leads for the development of novel drugs. Several anti-inflammatory, digestive, anti-necrotic, neuroprotective, and hepatoprotective drugs have recently

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been shown to have an antioxidant and/or anti-radical scavenging mechanism as part of their activity (Lin and Huang, 2002; Repetto and Llesuy, 2002). In the search for sources of natural antioxidants and compounds with radical scavenging activity during recent years, some have been found, such as echinacoside in *Echinaceae* root (Hu and Kitts, 2000), anthocyanin (Espin et al., 2000), phenolic compounds (Rice-Evans et al., 1997), water extracts of roasted *Cassia tora* (Yen and Chuang, 2000), whey proteins (Tong et al., 2000), and thioredoxin *h* protein from sweet potato (Huang et al., 2004).

The antioxidants present in dietary mushrooms are of great interest as possible protective agents to help the human body reduces oxidative damage without any interference (Adams and Wermuth, 1999). Now they are recognized as functional foods and as a source of physiologically beneficial components (Wasser and Weis, 1999). Mushrooms have been shown to boost heart health; lower the risk of cancer; promote immune function; ward off viruses, bacteria, and fungi; reduce inflammation; combat allergies; and help balance blood sugar levels and support the body's detoxification mechanism (Ada et al., 2005). Mushrooms have also been shown to accumulate a variety of secondary metabolites including phenolic compounds, polypeptides, terpenes, steroids, etc. Mushroom phenolics have been found to be an excellent antioxidant and synergist (Li et al., 2005). Furthermore, several companies are developing capsules from combinations of mushrooms, and these capsules, although expensive, have been shown to be health beneficial, including fighting against cancer (Mau et al., 2005).

Macrofungi was commonly used as a nutrition supplements to a variety of diseases in Asia (Jong and Birmingham, 1992; Chen et al., 2006a). In Taiwan, several different species of *Phellinus* were widely applied for anticancer, antioxidant purposes and hepatoprotective effects. *Phellinus linteus* demonstrated anti-tumor activity in several studies (Lin et al., 2003; Kim et al., 2003; Li et al., 2004; Bae et al., 2005). Also, there were several reports about the antioxidant effects from *Phellinus.* The methanolic extract of the basidiocarps of *Phellinus linteus* demonstrated antioxidative effect (Chung et al., 1998) and antimutagenic activities (Sohn and Nam, 2001). Studies indicated that *Phellinus linteus* could protect primary cultured rat hepatocytes against hepatotoxins (Kim et al., 2004). Also *Phellinus rimosus* (Berk) Pilat possess antioxidant and antihepatotoxic activities (Ajith and Janardhanan, 2002).

Phytochemicals, especially phenolics in fruits and vegetables, are suggested to be the major bioactive compounds for health benefits. Phenolics are one of the groups of nonessential dietary components that have been associated with the inhibition of atherosclerosis and cancer. The bioactivity of phenolics may be related to their ability to chelate metals, inhibit lipoxygenase, and scavenge free radicals (Mallavadhani et al., 2006; Lin et al., 2005).

Hispolon and hispolon derivatives were isolated from the fungus *Phellinus igniarius* (Mo et al., 2004). Hispolon, a yellow pigment was first found in *Inonotus hispidus* in 1996 (Ali et al., 1996b). Hispolon has been reported to exhibit apoptosis effect on human epidermoid KB cells (Chen et al., 2006b) and antivirus activities (Awadh et al., 2003). Hispolon also inhibit chemiluminescence response of human mononuclear cells and suppress mitogen-induced proliferation of spleen lymphocytes of mice (Ali et al., 1996a).

No report on the antioxidant activities of PM was presently available. In this work, we reported that PM displayed antioxidant activities in a series of *in vitro* tests such as total antioxidant activity, determination of total phenolics contents (TPC), ferric reducing antioxidant power assay (FRAP), DPPH (1, 1-diphenyl-2-picrylhydrazyl) staining, DPPH radical-scavenging activity and reducing power method.

MATERIALS AND METHODS

Materials

1, 1-Diphenyl-2-picrylhydrazyl(DPPH), potassium peroxodisulfate ($K_2S_2O_8$), tris (hydroxylmethyl) aminomethane, glutathione reduced form (GSH), potassium ferricyanide ($K_3Fe(CN)_6$), trichloroacetic acid (TCA), ferric chloride ($FeCl_3$), (+)-catechin, aluminum chloride hexahydrate ($AlCl_3 \cdot 6H_2O$), rutin, sodium bicarbonate (Na_2CO_3), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), 2, 4, 6-tris(2-pyridyl)-*s*-triazine (TPTZ) were purchased from Sigma Chemical Co. (St. Louis, MO USA). Folin-Ciocalteu solution, methanol and ethanol were purchased from Merck. Trolox (6-hydroxy-2, 5, 7, 8-tetramethychroman-2-carboxylic acid), ABTS [2, 2′-azinobis (3-ethylbenzothiazoline)-6-sulfonic acid] diammonium salt were purchased from Roche. Hispolon was purchased from BJYM Pharm. & Chem. Co. Ltd. (Beijing, China). *Phellinus merrillii* was purchased from the Ji Pin mushroom store (Nantou, Taiwan) and identified by Dr. Yu-Cheng Dai (Institute of Applied Ecology, Chinese Academy of Science, China).

Extraction of *Phellinus merrillii*

1.5 kg of PM was soaked with 5 l of 70% ethanol at room temperature. The samples were filtered with filter paper (Advantec No. 1, Japan) while the residue was further extracted under the same conditions three times. The filtrates collected from these separate extratcions were combined and evaporated to dryness under vacuum. The crude extract (60 g) was dissolved into water and formed as suspension. Hexane 250 ml was added into the suspension and mixed thoroughly. Then, hexane and water layers were separated. EtOAc added into the water layer was mixed well and were separated. Finally, n-BuOH was mixed with water layer and formed n-BuOH, water soluble and water insoluble fractions (Figure 1).

ABTS free radical scavenging assay

Total antioxidant status of the PM was measured using 2, 2′-azinobis[3-ethylbenzthiazoline]-6-sulfonic acid (ABTS) assay (Re et al., 1999). ABTS was dissolved in deionized water to 7 mM concentration, and potassium persulphate added to a concentration of 2.45 mM. The reaction mixture was left to stand at room temperature overnight (12~16 h) in the dark before use. The resultant intensely-coloured $ABTS^{\bullet+}$ radical cation was diluted with 0.01 M PBS (phosphate buffered saline), pH 7.4, to give an absorbance value of ~0.70 at 734 nm. The test compound was diluted 100 × with the ABTS solution to a total volume of 1 ml. Absorbance was measured spectrophotometrically at time intervals of 1 min after addition of each extract. The assay was performed at least in triplicate. Controls containing 990 µl of PBS, to replace ABTS, were used to measure absorbance of the extract themselves. The assay relies on the antioxidant capability of the samples to inhibit the oxidation of ABTS to $ABTS^{\bullet+}$ radical cation. The total antioxidant activities were expressed as mM trolox equivalent antioxidant capacity (TEAC).

Determination of total phenolics contents (TPC)

Total phenolics contents were determined using the Folin-Ciocalteu method (Ragazzi and Veronese, 1973). One mL of the extract was added to 10.0 ml distilled water and 2.0 ml of Folin-Ciocalteu phenol reagent (Merck-Schuchardt, Hohenbrun, Germany). The mixture was allowed to stand at room temperature for 5 min and then 2.0 ml sodium carbonate was added to the mixture. The resulting blue complex was then measured at 680 nm. The contents of phenolics contents were expressed as mM trolox equivalent antioxidant capacity (TEAC) (Hou et al., 2004).

Ferric reducing antioxidant power assay (FRAP)

The ferric reducing antioxidant power assay (FRAP) of each standard solution was measured according to a modified protocol developed by Benzie and Strain, 1996. To prepare the FRAP reagent, a mixture of 0.1 M acetate buffer (pH 3.6), 10 mM TPTZ, and 20 mM ferric chloride (10:1:1, v/v/v) was made. To 1.9 ml of reagent was added 0.1 ml of extract. Readings at the absorption maximum (593 nm) were taken every 15 s using a Shimadzu UV-visible 2501 spectrophotometer, and the reaction was monitored for up to 10 min. Trolox solution was used to perform the calibration curves. The reducing antioxidant power were expressed by the method of Benzie and Strain, 1996, as mM trolox equivalent antioxidant capacity (TEAC).

Rapid screening of antioxidant by dot-blot and DPPH staining

Each diluted sample of the PM was carefully loaded onto a 20 cm × 20 cm TLC layer (silica gel 60 F254; Merck) and allowed to dry (3 min). Drops of each sample were loaded, in order of decreasing concentration (2, 1, 0.5, 0.25, and 0.125 mg/ml), along the row. The staining of the silica plate was based on the procedure of Soler-Rivas et al., 2000. The sheet bearing the dry spots was placed upside down for 10 s in a 0.4 mM DPPH solution. Then the excess of solution was removed with a tissue paper and the layer was dried with a hair-dryer blowing cold air. Stained silica layer revealed a purple background with white spots at the location where radical-scavenger capacity presented. The intensity of the white color depends upon the amount and nature of radical scavenger present in the sample (Huang et al., 2004).

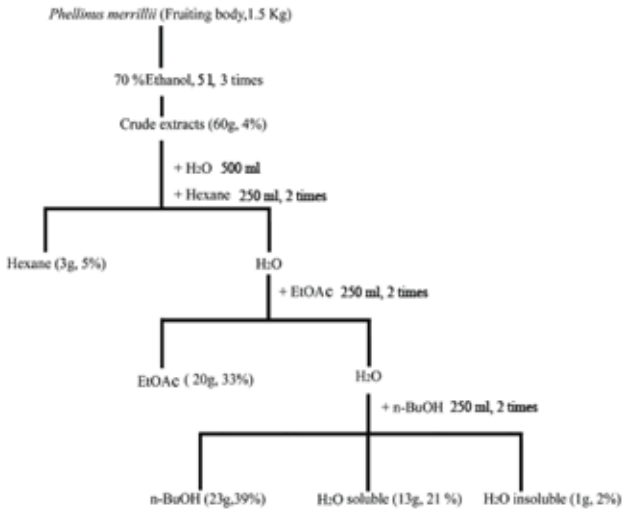

Figure 1. Extraction and fractionation scheme of *Phellinus merrillii.*

Determination of antioxidant activity by reducing power measurement

The reducing powers of the PM and glutathione were determined according to the method of Yen and Chen, 1995. The PM (0, 0.125, 0.25, 0.5, 1.0, and 2.0 mg/ml) and glutathione (0, 0.125, 0.25, 0.5, 1.0, and 2.0 mg/ml) were mixed with an equal volume of 0.2 M phosphate buffer, pH 6.6, and 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min, during which period ferricyanide was reduced to ferrocyanide. Then an equal volume of 1% trichloroacetic acid was added to the mixture, which was then centrifuged at 5,000 *g* for 10 min. The upper layer of the solution was mixed with distilled water and 0.1% $FeCl_3$ at a ratio of 1:1:2, and the absorbance at 700 nm was measured to determine the amount of ferric ferrocyanide (prussian blue) formed. Increased absorbance of the reaction mixture indicated increased reducing power of the sample (Huang et al., 2005).

Scavenging activity against DPPH radical

The effect of crude extracts on the DPPH radical was estimated according to the method of Yamaguchi et al., 1998. An aliquot of crude extract (30 µl) and

glutathione (GSH) (0.5 mg/ml, 30 µl) were mixed with 100 mM Tris-HCl buffer (120 µl, pH 7.4) and then with 150 µl of the DPPH in ethanol to a final concentration of 250 µM. The mixture was shaken vigorously and left to stand at room temperature for 20 min in the dark. The absorbance at 517 nm of the reaction solution was measured spectrophometrically. The percentage of DPPH decolourization of the sample was calculated according to the following equation as: % decolourization = [1 - ABS sample / ABS control] × 100. The 50% inhibition (IC_{50}) of antioxidant activity was calculated as the concentrations of samples that inhibited 50% of scavenging activity of DPPH radicals activity under these conditions (Huang et al., 2007).

Analysis of hispolon and PM by LC/MS/MS

LC/MS/MS was conducted to analyze both the standard (hispolon) and the 70% ethanol crude extract of PM according to Chen et al., 2006a, b; Lai et al., 2006. Its purity was more than 95% based on reversed phase LC/MS/MS analysis (Instrument: Micromass Quattro Ultima tandem mass; HPLC: Waters 2695 Alliance LC & 996 PDA with Automatic Liquid Sampler and Injector; Data processor: MassLynx NT Quattro Data Acquisition).

Analyses were carried out to using negative mode electrospray ionization of LC/MS/MS analysis. Chromatographic separation was performed on a C_{18} column (Cosmosil 5-C_{18}, 150 × 4.6 mm, i.d., 5 µm) under an isocratic elution of a mixed solvent system in a composition of each 70% of methanol and 30% of water at flow rate of 0.5 ml/min. A full UV spectrum was scanned from 200 to 400 nm. The source and desolvation temperature was set at 120°C and 350°C, respectively. All processes of the capillary voltage setting 3kV, cone voltage setting 100V and collision energy setting 15eV for hispolon $[M-H]^-$ fragment (m/z=219.8) daughter ion scan. The daughter ion spectrum obtained will be compared with the LC/MS/MS library database.

Statistical analysis

Data are expressed as mean ± S.E.M. Means of triplicate analyses were calculated. The Student's *t* test was used for comparison between two treatments. A difference was considered to be statistically significant when $p < 0.05$.

RESULTS

Extraction and fractionation of *Phellinus merrillii*

From 1.5 kg of PM, 60 g of 70% ethanol extract was obtained. The yield was 4%. The crude extract (60 g) was suspended in water and partitioned with hexane, EtOAc, n-BuOH sequentially to yield 3 g hexane fraction (5%), 20 g EtOAc fraction (33%), 23 g n-BuOH fraction (39%), 13 g water soluble fraction (21%) and 1 g water insoluble fraction (2%) (Figure 1).

Effect of the *Phellinus merrillii* on the total antioxidant capacity

The antioxidant capacity of crude extract, hexane, EtOAc, n-BuOH, soluble water and water insoluble fractions of PM were evaluated according to the ABTS decoloration method and the FRAP assay. Their total phenoplics contents were also determined. The results were shown in Figure 2. Inhibition of generation of the $ABTS^{\cdot+}$ radical cation was the basis of the spectrophotometric methods that had been applied to the measurement of the total antioxidant activities of solutions of pure substances, aqueous mixtures and beverages. Total antioxidant activities of several different fractions of PM (0.1 mg/ml) were measured using the $ABTS^{\cdot+}$ method. Trolox was used as positive control. The total antioxidant activity was expressed as Trolox mM by reference to the Trolox standard calibration curve. The n-BuOH fraction displayed the highest total antioxidant activity (17.13 ± 0.04 mM TEAC) (Figure 2). Total antioxidant capacity of crude and subfractions of PM were evaluated as 16.29 ± 0.35 mM for crude extract, 16.10 ± 0.31 mM for EtOAc fraction, 13.88 ± 0.19 mM for water insoluble fraction and 13.78 ± 0.39 mM for water soluble fraction. The hexane fraction had the lowest total antioxidant capacity of 12.36 ± 0.11 mM.

Total Phenolics contents of fractions of *Phellinus merrillii*

The total phenolics contents of crude extract and hexane, EtOAc, n-BuOH, soluble water and water insoluble fractions were expressed as mM of trolox equivalent. The EtOAc fraction had the highest phenolics contents of 46.21 ± 0.02 mM, crude extract and n-BuOH fractions had the phenolics contents of 36.32 ± 0.01 mM and 44.95 ± 0.01 mM, water insoluble fraction and hexane fraction had the phenolics contents of 8.19 ± 0.01 mM and 9.71 ± 0.01 mM. The water fraction had the lowest phenolics contents of 2.26 ± 0.01 mM (Figure 2).

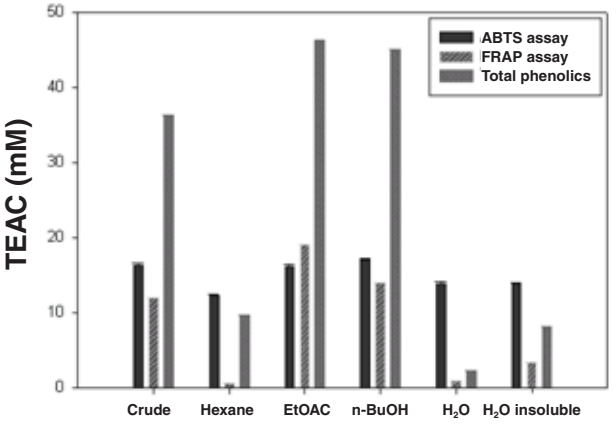

Figure 2. TPC, ABTS, and FRAP assays of various fractions including crude extrat, hexane, EtOAc, n-BuOH, water soluble and water insoluble fractions. Each absorbance value represented the average of triplicates of different samples analyzed.

Ferric reducing antioxidant power assay (FRAP)

The ferric reducing antioxidant power of crude extract and hexane, EtOAc, n-BuOH, water soluble and water insoluble fractions were expressed as mM of trolox equivalent. The EtOAc fraction had the highest reducing antioxidant power of 19.09 ± 0.02 mM, crude extract and n-BuOH fraction had the reducing antioxidant power of 12.00 ± 0.01 mM and 13.98 ± 0.02 mM, water insoluble fraction and water fraction had the reducing antioxidant power of 3.37 ± 0.01 mM and 1.01 ± 0.01 mM. The hexane fraction had the lowest reducing antioxidant power of 0.68 ± 0.01 mM (Figure 2).

Relationship between total antioxidant activity (TEAC), total phenolics contents (TPC) and FRAP assay

The Correlation cofficients (R^2) of antioxidant capacity (TEAC), total phenolics contents, and FRAP assay of crude extract, hexane, EtOAc, n-BuOH, water soluble and water insoluble fractions of PM were shown in Figure 3A and 3B.

Relationship between ABTS assay (TEAC) and TPC in crude extract, hexane, EtOAc, n-BuOH, soluble water and water insoluble fractions were shown in Figure 3A (R^2 = 0.8145, $p < 0.05$). Relationship between ABTS assay (TEAC) and FRAP assay in crude extract, hexane, EtOAc, n-BuOH, soluble water and insoluble water fractions were shown in Figure 3B (R^2 = 0.7878, $p < 0.05$).

The correlation coefficients (R^2) values of TEAC and total phenolics contents showed higher correlation. The higher the total phenolics contents, the higher the antioxidant activity the fractions exhibited.

Rapid screening of antioxidant by dot-blot and DPPH staining

Antioxidant capacity of the PM was eye-detected semi-quantitatively by a rapid DPPH staining TLC method. Each diluted crude extract and hexane, EtOAc, n-BuOH, water soluble and water insoluble fractions were applied as a dot on a TLC layer that was then stained with DPPH solution (Figure 4). This method was typically based on the inhibition of the accumulation of oxidized products. The generation of free radicals was inhibited by the addition of antioxidants and scavenging of the free radicals shifted the end point. The reduced glutathione was used as a positive control. Initial faint spots appeared, and 1 h later weak spots could be observed in sample row. This white spots with strong intensity appeared quickly at the concentration of 0.25 mg/ml of PM per application, and down to the dilution at 0.125 mg/ml of the PM (Figure 4). In the DPPH staining, the n-BuOH fracton showed the highest strong dot-blot staining.

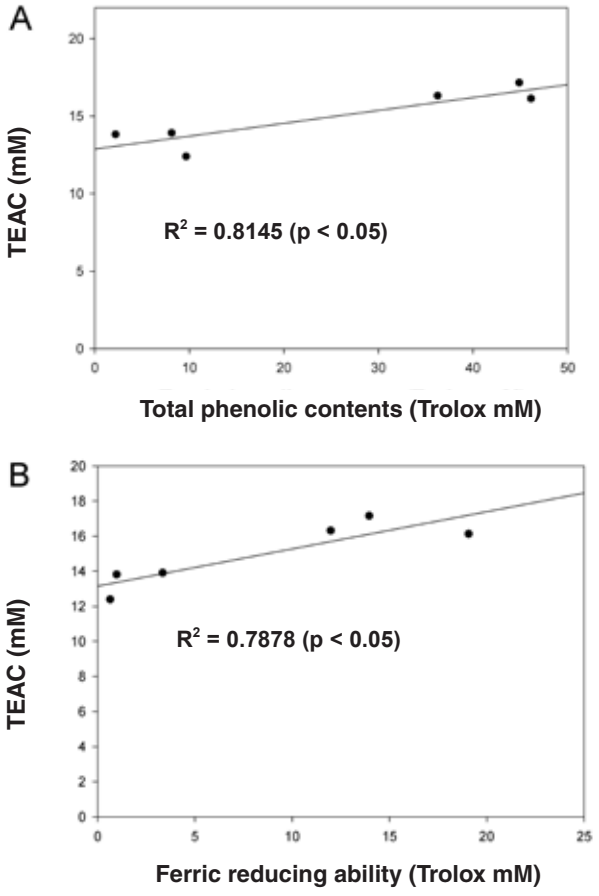

Figure 3. The antioxidant capacity of various extracts including crude extract, hexane, EtOAc, n-BuOH, water soluble and water insoluble fractions of *Phellinus merrillii* were evaluated according to the ABTS decoloration method and the FRAP assay. (A) correlation coefficient between TPC and ABTS assay; (B) correlation coefficient between FRAP assay and ABTS.

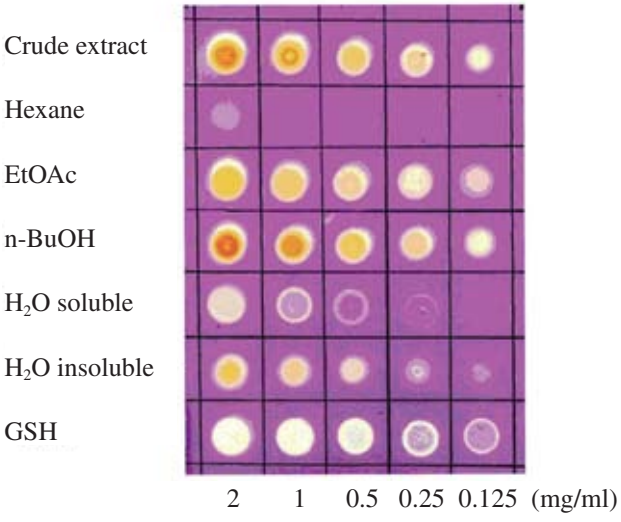

Figure 4. Dot blot assay of the crude and various fractions of *Phellinus merrillii* on a silica sheet stained with a DPPH solution in methanol. The crude and fractions of PM (2, 1, 0.5, 0.25, and 0.125 mg/ml) were applied from left to right in sample row; GSH (2, 1, 0.5, 0.25, and 0.125 mg/ml) were applied from left to right in control row.

Measurement of reducing power

We investigated the Fe^{3+}-Fe^{2+} transition to measure the PM*'s* reducing capacity. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Meir et al., 1995). The antioxidant activities of putative antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, and radical scavenging (Diplock, 1997). The reducing power of PM was shown in Figure 5, with GSH as a positive control. PM exhibited a dose dependent reducing power activity within the applied concentrations (0, 0.125, 0.25, 0.5, 1.0, and 2.0 mg/ml). The reducing power of crude extract and hexane, EtOAc, n-BuOH, water soluble, water insoluble fraction and GSH were compared. The crude extract had the highest reducing power at 2 mg/ml concentration. However, hexane fraction showed lowest reducing power (Figure 5).

Scavenging activity against DPPH radical

The DPPH radical had been used widely in the model system to investigate the scavenging activities of several natural compounds such as phenolic compounds, anthocyanins, or crude extracts of plants. DPPH radical was scavenged by antioxidants through the donation of hydrogen, forming the reduced DPPH-H$^{\bullet}$. The color changed from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517 nm. Table 1 showed the radical-scavenging activity of the different fractions of PM and GSH, using the DPPH coloring method. It was found in 0.5 mg/ml of EtOAc fraction of PM had the highest radical-scavenging activity (IC_{50} = 0.66 ± 0.01 mg/ml), followed by n-BuOH fraction (0.78 ± 0.01 mg/ml), crude extract (0.81 ± 0.02 mg/ml), water fraction (0.83 ± 0.05 mg/ml) and water insoluble fraction (0.93 ± 0.06 mg/ml). Hexane fraction had the lowest radical-scavenging activity (IC_{50} = 3.79 ± 0.01 mg/ml).

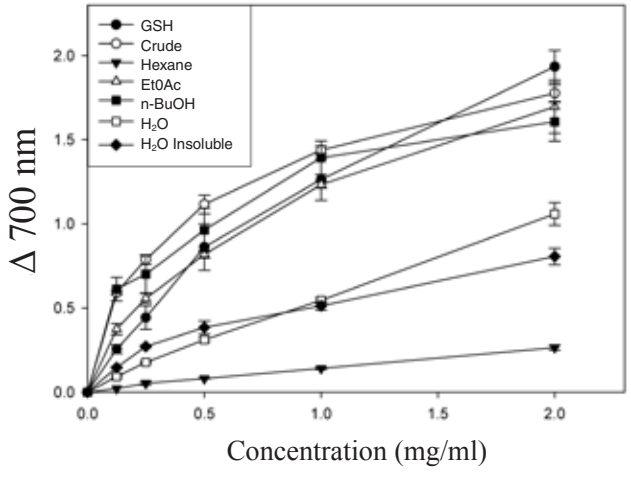

Figure 5. Antioxidative activities of the crude extract and various fractions of *Phellinus merrillii* (0, 0.125, 0.25, 0.5, 1.0, and 2.0 mg/ml) were measured by the reducing power method. GSH was used as a positive control. Each absorbance value represented the average of triplicates of different samples analyzed.

Table 1. DPPH radical scavenging activity of various fractions of *Phellinus merrillii.*

	DPPH radical scavenging activity IC_{50} (mg/ml)
GSH	0.42 ± 0.04
H_2O	0.83 ± 0.05
Crude	0.81 ± 0.02
Hexane	3.79 ± 0.01
EtOAc	0.66 ± 0.01
n-BuOH	0.78 ± 0.01
H_2O Insoluble	0.93 ± 0.06

Table 2. Antioxidant activity of hispolon.

	Scavenging activity of DPPH radicals (%), IC_{50} (μM)
BHT (control)	81.2 ± 3.2
Hispolon	42.4 ± 2.9

Compositional analysis of hispolon and PM by LC/MS/MS

In high-performance liquid chromatography tandem mass (LC/MS/MS) analysis for hispolon, the daughter ion scanned chromatograms of PM was established. Both hispolon and PM showed similar daughter ion spectrum at the retention time of 4.7 min and had more lobes in m/z 219 and m/z 135 (Figure 6). This indicated that PM did contain the active ingredient of hispolon.

Determination of the antioxidative activity of hispolon

We used hispolon to measure antioxidant activity and BHT was used as positive control. Both IC_{50} values of hispolon and BHT were 42.4 ± 2.9 and 81.2 ± 3.2 mM, respectively, when scavenging activity of DPPH radicals (%) was measured. These results demonstrated that hispolon exhibited better antioxidative activity than BHT (Table 2).

DISCUSSION

Mushrooms are nutritionally functional foods and important sources of physiologically beneficial medicines. They produce various classes of secondary metabolites with interesting biological activities and, thus, have the potential to be used as valuable chemical resources for drug discovery (Zjawiony, 2004). Several mushrooms belonging to the genera *Inonotus* and *Phellinus*, such

as *Inonotus obliquua*, *Phellinus linteus*, *Phellinus ribis* and *Phellinus igniarius* have been used as traditional medicines for the treatment of gastrointestinal cancer, cardiovascular disease, tuberculosis, liver or heart diseases, fester, bellyache, bloody gonorrhea, stomach ailments, and diabetes (Nakamura et al., 2004). Polysaccharides, especially β-glucan, are considered to be responsible for their biological activity. The isolation of polysaccharides derived from medicinal mushrooms and their biological activity had been reported (Vinogradov and Wasser, 2005). Interestingly, these mushrooms often contain a bundle of yellow antioxidant pigments which are composed of hispidin derivatives and polyphenols.

The antioxidant activity of mushroom extracts with stronger inhibition of lipid peroxidation occurring at high concentrations of the extracts in most cases (Cheung and Peter, 2005). The possible mechanism of antioxidant activity of mushroom extracts includes scavenging of free radicals possibly through hydrogen-holding capacity and oxidation by peroxy radicals (Ali et al., 1996b).

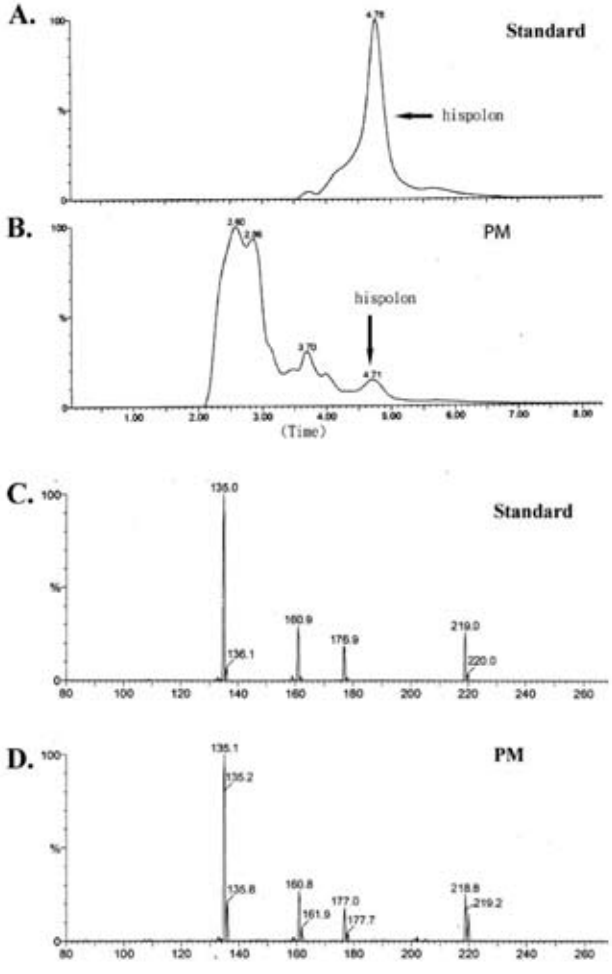

Figure 6. Comparison of daughter ion scanned (ES-D219.8) chromatograms (A & B) of hispolon standard vs. sample and each of daughter ion spectrum (C & D), it indicated that sample contains hispolon.

In this paper, the results from *in vitro* experiments demonstrated that crude extract, n-BuOH and EtOAc fractions had better antioxidant activity in reducing power method. The crude extract had the highest reducing power at 2 mg/ml concentration. In the DPPH staining, the EtOAc and n-BuOH fractions shared the same patterns with the crude extract and appeared as white spots when they were diluted from 2.0 to 0.125 mg/ml. In the DPPH staining, the n-BuOH fraction showed the highest strong dot-blot staining. In the DPPH colorimetric method, it was found that EtOAc fraction of PM had the highest radical-scavenging activities. In the ABTS free radical scavenging assay, the n-BuOH fraction displayed the highest total antioxidant activity. Total phenolics contents demonstrated that the EtOAc fraction had the highest amounts of total phenolic contents than other fractions. In ferric reducing antioxidant power assay (FRAP), the EtOAc fraction exhibited the highest reducing power. ABTS with high correlation were established between total phenolics contents and total antioxidant activity (TEAC) assay. This data indicated that the correlation coefficients (R^2) values of TEAC and total phenolics contents showed higher correlation. The higher the total phenolics contents the higher the antioxidant activity of the fractions. The *ex vivo* or *in vivo* antioxidant activity of PM should be studied in the near future.

In LC/MS/MS analysis, the finger print chromatogram of PM was established. Both PM and hispolon showed similar peak at the retention time of 4.7 min and had more lobes in m/z 219 and m/z 135. This implied that PM did contain the active ingredient hispolon. Hispolon was better than BHT in the IC_{50} of DPPH radical scavenging activity. These findings imply that hispolon was most important in antiradical activities. It was suggested that hispolon might contribute to the antioxidant activities of PM.

The relatively higher contents of phenolics in PM might explain the high antioxidant property (Bimla and Punita, 2006). Positive correlations were established between total phenolics contents in the mushroom extract and their antioxidant activities, similar to those observed in *Lentinus edodes* and *Volvariella volvacea* (Cheung et al., 2003).

Polyphenolic compounds have an important role in stabilizing lipid oxidation and are associated with antioxidant activity (Yen et al., 1993). The phenolic compounds may contribute directly to antioxidative action (Duh et al., 1999). It is suggested that polyphenolic compounds may exhibit inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1.0 g is ingested daily from a diet rich in fruits and vegetables sources (Tanaka et al., 1998). The antioxidative activities could be ascribed to the different mechanisms exerted by different phenolic compounds and due to the synergistic effects of different compounds. The antioxidants present in six different fractions may have different functional properties, such as reactive oxygen species scavenging (quercetin and catechin) (Hatano et al., 1989), inhibition of the generation of free radicals and chain-breaking activity,

e.g. *p*-coumaric acids (Laranjinha et al., 1995) and metal chelation (Van-Acker et al., 1998). These compounds were normally phenolic compounds, which were effective hydrogen donors, such as tocopherols, flavonoids, and derivatives of cinnamic acid, phosphatidic and other organic acids.

Although ethanol extract of *Phellinus linteus* was shown to scavenge directly the stable DPPH radical over a concentration range of 10 μg/ml (30.1 ± 2.72% inhibition) to 300 μg/ml (85.5 ± 4.2% inhibition). It scavenged the stable radical DPPH in a concentraction-dependent manner. The EC_{50} value of *Phellinus linteus* was calculated to be 22.07 μg/ml, whereas that of Vitamin C, used as a positive control, was 5.11 μg/ml (Song et al., 2003). They demonstrated that ethyl acetate extract of *P. rimosus* exhibited significant *in vitro* antioxidant activity. The ethyl acetate extract of *P. rimosus* also showed potent anthepatotoxic activity against CCl_4-induced acute toxicity in rat liver (Ajith and Janardhanan, 2002). Our previous studies also showed the similar results.

In conclusion, PM possessed antioxidant and free radical scavenging activities. Above all, there are many total phenolic compounds in PM including hispolon. PM might be an agent with anti-cancer, hepatoprotective and ant-inflammatory potentials and should be further investigated in the future.

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梅里爾針層孔菌抽出物之抗氧化與自由基清除活性

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本研究主要的目的是研究梅里爾針層孔菌不同溶劑分層的萃取物之抗氧化活性。探討的方法如下：ABTS 自由基清除測定、測定總酚類含量、FRAP 測試、快速 DPPH（1, 1 二苯基 2 picrylhydrazyl）呈色反應、DPPH 清除自由基活性和還原力等幾項。ABTS 自由基清除測定中，在 n-BuOH 分層中，表現出最高的總抗氧化活性（17.13 ± 0.04 mM）。測定總酚類含量和 FRAP 分析中，在 EtOAc 分層中，有最高的酚類含量（46.21 ± 0.02 mM）和還原性抗氧化力（19.09 ± 0.03 mM）。在快速 DPPH 呈色反應中，在 n-BuOH 分層中，表現出最強的呈色反應。在 DPPH 清除自由基活性中，EtOAc 分層中，有最強的抗氧化活性，其 IC_{50} 為（0.66 ± 0.01 mg/ml）。在還原力的測試中，粗抽出物中，在 2 mg/ml 濃度中有最高的還原力。在 ABTS 測試、 FRAP 測試和總酚類含量相互之間的比較時其相關係數是有意義的。在所有不同溶劑分層之萃取物之中，在 EtOAc 有最高的總酚類含量。由結果可顯示梅里爾針層孔菌在 EtOAc、n-BuOH 分層中具有較高的抗氧化活性。在高效液相層析質譜儀分析法中，梅里爾針層孔菌的抽出物的指紋圖譜被建立，hispolon 與梅里爾針層孔菌的抽出物顯示出有相似的波峰與相同的 4.7 分鐘的滯留時間，均獲得 m/z 219 與 m/z 135 較大的裂片，這表示梅里爾針層孔菌的乙醇抽出物含有活性成份 hispolon。而 hispolon 和 BHT 的 DPPH 自由基清除之 IC_{50} 為 42.4 ± 2.9 和 81.2 ± 3.2 μM，由此發現 hispolon 對於抗氧化活性是非常重要的，因此表示 hispolon 在梅里爾針層孔菌中有助於抗氧化活性。

關鍵詞：1, 1 二苯基 2 picrylhydrazyl (DPPH)；三價鐵還原性抗氧化力測試；自由基；谷胱甘肽；高效液相層析串聯式質譜分析法；梅里爾針層孔菌；清除活性；總抗氧化力；總酚類含量；Trolox 相等性的抗氧化力。