Mycobiology 38(4) : 295-301 (2010)
DOI:10.4489/MYCO.2010.38.4.295

Antioxidant Activities and Tyrosinase Inhibitory Effects of Different Extracts from *Pleurotus ostreatus* Fruiting Bodies

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(Received October 13, 2010. Accepted October 26, 2010)

We evaluated the antioxidant activity and tyrosinase inhibitory effects of *Pleurotus ostreatus* fruiting bodies extracted with acetone, methanol, and hot water. The antioxidant activities were tested against β-carotene-linoleic acid, reducing power, 1,1-diphenyl-2-picrylhydrazyl free radical scavenging activity, and ferrous chelating ability. Furthermore, phenolic acid and flavonoid contents were also analyzed. The methanol extract showed the strongest β-carotene-linoleic acid inhibition as compared to the other exracts. The acetone extract (8 mg/mL) showed a significantly high reducing power of 1.54 than the other extracts. The acetone extract was more effective than other extracts for scavenging on 1,1-diphenyl-2-picrylhydrazyl radicals. The strongest chelating effect (85.66%) was obtained from the acetone extract at 1.0 mg/mL. The antioxidant activities of the extracts from the *P. ostreatus* fruiting bodies increased with increasing concentration. A high performance liquid chromatography analysis detected seven phenolic compounds, including gallic acid, protocatechuic acid, chlorogenic acid, naringenin, hesperetin, formononetin, and biochanin-A in an acetonitrile and 0.1 N hydrochloric acid (5 : 1) solvent extract. The total phenolic compound concentration was 188 μg/g. Tyrosinase inhibition of the acetone, methanol, and hot water *P. ostreatus* extracts increased with increasing concentration. The results revealed that the methanol extract had good tyrosinase inhibitory ability, whereas the acetone and hot water extracts showed moderate activity at the concentrations tested. The results suggested that *P. ostreatus* may have potential as a natural antioxidant.

KEYWORDS : Antioxidant, Phenolic compounds, *Pleurotus ostreatus*, Tyrosinase inhibition

Pleurotus ostreatus, commonly known as oyster mushroom, is an important edible fungus in Korea. It grows in a wide range of temperatures, utilizing various lignocelluloses, and is becoming more popular throughout the world [1]. *P. ostreatus* is a good source of dietary fiber and other valuable nutrients [2]. This mushroom contains a number of biologically active compounds with various therapeutic activities, including the ability to modulate the immune system, decrease blood lipid concentrations, prevent high blood pressure and atherosclerosis, as well as hypoglycemic and antithrombotic activities [3].

Oxidative damage caused by free radicals may be related to aging and diseases, such as atherosclerosis, diabetes, cancer, and cirrhosis [4]. Although almost all organisms possess antioxidant defense and repair systems that have evolved to protect them against oxidative damage, these systems are insufficient to entirely prevent damage [5]. However, antioxidant supplements or foods containing antioxidants may be useful to help the human body reduce oxidative damage. Mushrooms produce a variety of secondary metabolites, including phenolic compounds, polyketides, terpenes, and steroids [6]. Recently, mushrooms were considered a good source of protein and phenolic antioxidants, such as variegatic acid and gallic acid [7].

Tyrosinase, also called polyphenol oxidase [8], is a copper-containing mono-oxygenase present in a diverse range of organisms and is responsible for melanization in animals and enzymatic browning of fruit. This enzyme catalyzes two distinct reactions involving molecular oxygen: the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones [9]. The nutritional value and taste components of *P. ostreatus* have been thoroughly studied. Mushroom research has focused on nutritional and therapeutic effects, and little information is available on the antioxidant properties of mushrooms. Therefore, our objective was to evaluate the antioxidant activities of *P. ostreatus*, including β-carotene-linoleic acid, reducing power, scavenging effects on radicals, and chelating effects on ferrous ions. The potential phenolic and flavonoid compound contents and tyrosinase inhibition properties of *P. ostreatus* were also analyzed.

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Materials and Methods

Chemicals and reagents. β-carotene, linoleic acid, chloroform, polyoxyethylene sorbitan monopalmitate (Tween40), butylated hydroxytoluene (BHT), α-tocopherol (TOC), 1,1-diphenyl-2-picrylhydrazyl (DPPH), L-ascorbic acid, potassium ferricyanide, trichloroacetic acid, ferrous chloride, ferric chloride, ferrozine, Folin-Ciocalteu reagent, gallic acid, methanol, 3,4-dihydroxy-L-phenylalanine (L-DOPA), mushroom tyrosinase, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals and solvents used were high performance liquid chromatography (HPLC) or analytical grade.

Mushroom and extraction. Fresh, mature fruiting bodies of *P. ostreatus* (cultivar Chun Chu 2) were obtained from Hanultari mushroom farm, Korea. A pure culture was deposited in the Culture Collection DNA Bank of Mushroom (CCDBM), Division of Life Sciences, University of Incheon, Korea and acquired accession number, IUM-4143. Fresh fruiting bodies were dried with hot air at 40°C for 48 hr and finely pulverized. Five grams of powdered samples were extracted with 100 mL of 60% acetone and 80% methanol with stirring at 150 rpm for 24 hr at 25°C to obtain acetone and methanol extracts. The mixture was filtered through two layers of Whatman no. 1 filter paper. The same quantity of sample was boiled at 100°C for 3 hr with 100 mL deionized distilled water to obtain a hot water extract. The mixture was cooled to room temperature and filtered through Whatman no. 1 filter paper. The residues were then extracted with two additional 100 mL aliquots of acetone, methanol, and deionized water, as described above. The combined extracts were evaporated with a rotary evaporator (Eyela, Saitama, Japan) at 40°C, and the remaining solvent was removed with a freeze-drier (Optizen, Daejeon, Korea). The yields from the acetone, methanol, and hot water extracts of *P. ostreatus* were 22.50, 13.78, and 22.16% (w/w), respectively.

Antioxidant activity by β-carotene-linoleic acid. Antioxidant activity was determined by measuring the inhibition of volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation [10]. A stock solution of a β-carotene-linoleic acid mixture was prepared as follows: 0.5 mg β-carotene was dissolved in 1 mL of chloroform, and 25 μL of linoleic acid and 200 mg Tween40 was added. The chloroform was removed completely using a vacuum evaporator. Then, 100 mL of oxygenated distilled water was added with vigorous shaking; 2.5 mL of this reaction mixture was dispensed to test tubes, 0.5 mL of various concentrations (0.5~20.0 mg/mL) of the extracts in methanol was added, and the reaction mixture was incubated for up to 2 hr at 50°C. The same procedure was repeated with the positive controls BHT and TOC, and a blank. After the incubation, the absorbance of the mixtures was measured at 490 nm using a spectrophotometer (Optizen POP; Mecasys Co. Ltd., Daejeon, Korea). The absorbance was measured until the β-carotene color disappeared. The β-carotene bleaching rate (R) was calculated according to Eq. (1).

$$R = \ln(a/b)/t \quad (1)$$

where, ln = natural log, a = absorbance at time t (0), and b = absorbance at time t (120 min). The antioxidant activity (AA) was calculated as the percent inhibition relative to the control using Eq. (2).

$$AA = [(R_{control} - R_{sample})/R_{control}] \times 100 \quad (2)$$

AAs of the extracts were compared with those of BHT and TOC at 0.5 mg/mL and with a blank consisting of 0.5 mL methanol.

Reducing power. Reducing power was determined according to the method of Gulcin *et al*. [11]. Each extract (1~8 mg/mL) in methanol (2.5 mL) was mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide, and the mixture was incubated at 50°C for 20 min. Then, 2.5 mL of 10% trichloroacetic acid was added, and the mixture was centrifuged at 200 ×g (6K 15; Sigma, Mannheim, Germany) for 10 min. The upper layer (2.5 mL) was mixed with 2.5 mL of deionized water and 0.5 mL of 0.1% ferric chloride. Finally, the absorbance was measured at 700 nm against a blank. BHT and TOC were used as positive controls.

Scavenging effect on 1,1-diphenyl-2-picrylhydrazyl radicals. The hydrogen atom or electron donation ability of the corresponding extracts and some pure compounds were measured from the bleaching of the purple colored DPPH methanol solution [12]. Four mL of various concentrations (0.125~2.0 mg/mL) of the extracts in methanol was added to 1 mL of DPPH radical solution in methanol (final concentration of DPPH was 0.2 mM). The mixture was shaken vigorously and allowed to stand for 30 min, and the absorbance of the resulting solution was measured at 517 nm using a spectrophotometer. Inhibition of the DPPH free radical in percent (I%) was calculated as:

$$I\% = [(A_{control} - A_{sample})/A_{control}] \times 100$$

where, $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. BHT, TOC, and L-ascorbic acid were used as positive controls.

Chelating effects on ferrous ions. The chelating effect was determined according to the method of Dinis *et al*. [13]. Briefly, 2 mL of various concentrations (0.063~1.0

mg/mL) of the extracts in methanol was added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by adding 5 mM ferrozine (0.2 mL). The total volume was adjusted to 5 mL with methanol, and the mixture was shaken vigorously and left at room temperature for 10 min. The absorbance of the solution was measured spectrophotometrically at 562 nm. The inhibition percentage of the ferrozine-Fe^{2+} complex formation was calculated using the following formula:

$$\text{Metal chelating effect (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

where, $A_{control}$ is the absorbance of the control (control contained $FeCl_2$ and ferrozine; complex formation molecules), and A_{sample} is the absorbance of the test compound. BHT and TOC were used as positive controls.

Analysis of phenolic compounds. Fifteen standard phenolic compounds, including gallic acid, pyrogallol, homogentisic acid, protocatechuic acid, (+)-catechin, chlorogenic acid, caffeic acid, vanillin, ferulic acid, naringin, resveratrol, naringenin, hesperetin, formononetin, and biochanin-A were purchased from Sigma Aldrich and used for calibration curves. The standard stock solutions (50, 100, 250, and 500 ppm) were prepared in DMSO. Sample compounds were identified based on retention times of authentic standards and were quantified by comparing their peak areas with those of the standard curves.

Sample preparation for the phenolic compound analysis followed Kim *et al*. [14]. Two grams of dried mushroom powder were mixed with 10 mL of acetonitrile and 2 mL of 0.1 N hydrochloric acid and stirred at 150 rpm for 2 hr at room temperature. The suspension was filtered through Whatman no. 42 filter paper. The extract was freeze-dried, and the residues were redissolved in 10 mL of 80% aqueous methanol (HPLC grade) and filtered through a 0.45 μm nylon membrane filter (Titan, Rockwood, TN, USA). The 20 μL filtrate was loaded onto an Agilent-1100 series liquid chromatography HPLC system (Agilent Technologies, Waldbronn, Germany). Separation was achieved on a 250 nm × 4.6 mm i.d., 5 μm, YMC-Pack ODS AM (YMC Co. Ltd., Kyoto, Japan) column. The mobile phase was distilled water with 0.1% glacial acetic acid (solvent A) and acetonitrile with 0.1% glacial acetic acid (solvent B). The gradient was 0 min, 92% A; 0~2 min, 90% A; 2~27 min, 70% A; 27~50 min, 10% A; 50~51 min, 0% A; 51~60 min, 0% A; 60~63 min, 92% A. The run time was 60 min using a flow rate of 1 mL/min. Detection was performed with a diode array detector at a wavelength of 280 nm.

Tyrosinase inhibition. Tyrosinase inhibition activity was determined using the modified dopachrome method with L-DOPA as the substrate [15]. A 96-well microtiter plate was used to measure absorbance at 475 nm with 700 nm as a reference. Extract fractions were dissolved in 50% DMSO. Each well contained 40 μL of sample with 80 μL of phosphate buffer (0.1 M, pH 6.8), 40 μL of tyrosinase (31 units/mL), and 40 μL of L-DOPA (2.5 mM). The mixture was incubated for 10 min at 37°C, and absorbance was measured at 475 nm using a UVM 340 microplate reader (Asys, Eugendorf, Austria). Each sample was accompanied by a blank containing all components except L-DOPA. L-ascorbic acid and kojic acid were used as positive controls. The results were compared with a control consisting of 50% DMSO in place of the sample. The percentage of tyrosinase inhibition was calculated as follows:

$$[(A_{control} - A_{sample})/A_{control}] \times 100\%$$

Statistical analysis. Data are expressed as means ± SDs of three replicate determinations and were analyzed by SPSS ver. 13 (SPSS Inc., Chicago, IL, USA). A one way analysis of variance and Duncan's new multiple-range test were used to determine the differences among the means.

Results and Discussion

Aantioxidant activity against β-carotene-linoleic acid. The acetone, methanol, and hot water extracts of *P. ostreatus* showed different patterns of antioxidant activi-

Table 1. Antioxidant activity against β-carotene-linoleic acid of different concentrations of various extracts from the fruiting bodies of *Pleurotus ostreatus*

Solvent and control	Sample concentration (mg/mL)			
	0.5	2.0	8.0	20.0
Acetone	68.73 ± 0.18	89.94 ± 0.19	91.02 ± 0.22	94.56 ± 0.21
Methanol	65.89 ± 0.13	84.25 ± 0.22	91.12 ± 0.28	95.06 ± 0.16
Hot water	65.32 ± 0.22	58.92 ± 0.35	85.62 ± 0.17	94.12 ± 0.15
BHT	95.21 ± 0.17	–	–	–
TOC	96.02 ± 0.18	–	–	–

Values expressed as means ± SD (n = 3).
–, not analyzed; BHT, butylated hydroxytoluene; TOC, α-tocopherol.

Table 2. Reducing power of different concentrations of various extracts from the fruiting bodies of *Pleurotus ostreatus*

Solvent and control	Sample concentration (mg/mL)			
	1.0	2.0	4.0	8.0
Acetone	0.361 ± 0.07	0.611 ± 0.10	0.969 ± 0.11	1.540 ± 0.17
Methanol	0.541 ± 0.03	0.712 ± 0.05	0.996 ± 0.05	1.252 ± 0.06
Hot water	0.362 ± 0.07	0.473 ± 0.11	0.705 ± 0.15	1.262 ± 0.23
BHT	3.212 ± 0.49	–	–	–
TOC	2.162 ± 0.32	–	–	–

Values expressed as means ± SD (n = 3).
–, not analyzed; BHT, butylated hydroxytoluene; TOC, α-tocopherol.

ties against β-carotene-linoleic acid. The methanol extract showed the strongest linoleic acid inhibition capacity (95.06%) at 20 mg/mL concentration as compared to the acetone and hot water extracts (Table 1). Comparable antioxidant activity results of volatile solvent extracts from several commercial and medicinal mushrooms have been reported [16]. It is likely that the antioxidative components in the mushroom extracts reduced β-carotene destruction by neutralizing the linoleate free radical and other free radicals formed.

Reducing power. The reducing power of the *P. ostreatus* acetone, methanol, and hot water extracts as a function of their concentration is shown in Table 2. The reducing power increased with increasing concentration. The strongest reducing power inhibition was identified in the acetone extract at a concentration of 8 mg/mL. A value of 1.54 and the lowest inhibition of reducing power (1.25) was exhibited by the methanol extract. The reducing power values of BHT and TOC at 1.0 mg/mL were 3.21 and 2.16, respectively (Table 2). Reducing power properties are generally associated with the presence of reductones, which exert antioxidant action by breaking the free radical chain and donating a hydrogen atom [17, 18].

Scavenging effect on DPPH. The scavenging effects of the acetone, methanol, and hot water extracts from the fruiting bodies of *P. ostreatus* on DPPH radicals increased with increasing concentration. At 0.125~2.0 mg/mL, the scavenging abilities of the acetone, methanol, and hot water extracts on the DPPH radical ranged from 33.62~91.52%, 49.33~85.13%, and 36.35~82.81%, respectively (Fig. 1). The results indicated that the acetone extract possessed good activity, whereas the methanol and hot water extracts showed moderate and poor activity, respectively, at the concentrations tested. However, at 0.125~2.0 mg/mL, BHT, TOC, and L-ascorbic acid showed excellent scavenging ability of 85.25~98.74%, 67.37~97.78%, and 96.74~98.23%, respectively. Lee *et al*. [19] reported that the ethanol extracts of *Hypsizygus marmoreus*, *Agaricus bisporus*, and *P. citrinopileatus* fruiting bodies scavenged DPPH radicals by 46.6~68.4% at 5 mg/mL. The scavenging abilities of the fruiting bodies, mycelia, and filtrate of the cold and hot water extracts at 20 mg/mL were 20.7~52.3%, 37.6~48.3%, and 19.6~23.3%, respectively. It seems that the scavenging ability of *P. ostreatus* fruiting bodies was more effective than those mentioned above. Various extracts might react with free radicals, particularly the peroxy radicals, which are the major propagators of the fat autoxidation chain, thereby terminating the chain reaction [20]. The antioxidant activity of natural antioxidants is due to the termination of the free radical reaction [17].

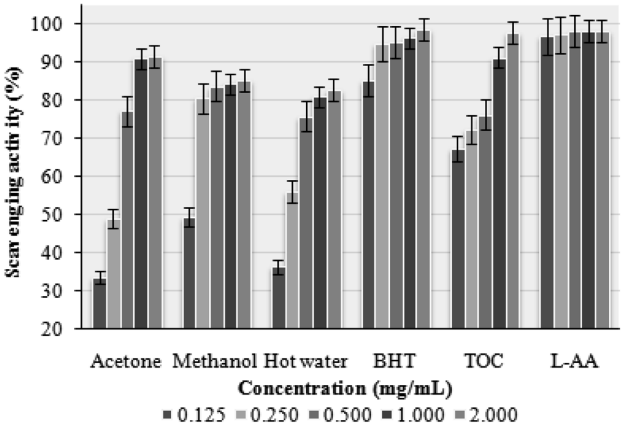

Fig. 1. Scavenging activity of various extracts from the fruiting bodies of *Pleurotus ostreatus* against 1,1-diphenyl-2-picrylhydrazyl. Values expressed as means ± SE (n = 3). BHT, butylated hydroxytoluene; TOC, α-tocopherol; L-AA, L-ascorbic acid.

Chelating effects on ferrous ions. The chelating ability of the acetone, methanol, and hot water extracts at five different concentrations (0.063, 0.125, 0.250, 0.500, and 1.000 mg/mL) from the *P. ostreatus* fruiting bodies toward ferrous ions was investigated. BHT and TOC were used as ferrous ion standards. As shown in Fig. 2, the chelating capacity of the extracts increased with increasing concentration. The strongest chelating effect (85.66%) was obtained with the acetone extract at 1.0 mg/mL. At this concentration, the lowest chelating effect was exhibited by the hot water extract (82.29%). All of the extracts evalu-

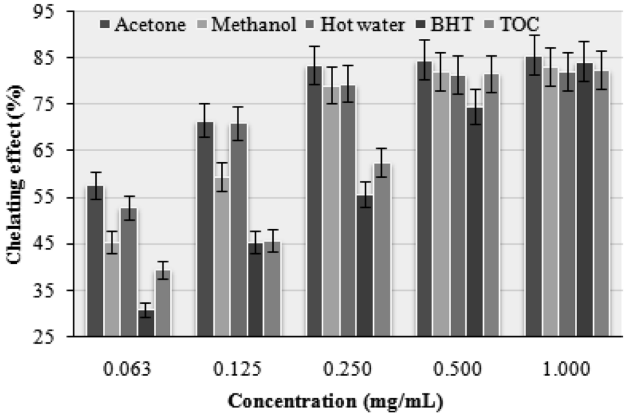

Fig. 2. Chelating effect of various *Pleurotus ostreatus* extracts. Values expressed as means ± SE (n = 3). BHT, butylated hydroxytoluene; TOC, α-tocopherol.

ated here showed significantly higher chelating effects on ferrous ions than those of the standards, BHT, or TOC at concentrations of 0.063, 0.125, 0.250, and 0.5 mg/mL, respectively. *Ganoderma tsugae* and *Agrocybe cylindracea* hot water extracts at 20 mg/mL chelated ferrous ions by 39.5~42.6% and 45.8%, respectively [21, 22]. At 1~5 mg/mL, the chelating abilities of *H. marmoreus* and *P. citrinopileatus* were 75.6~92.6% [19]. It seems that the chelating ability of *P. ostreatus* on ferrous ions was similar to that of *H. marmoreus* and *P. citrinopileatus* but more effective than that of *G. tsugae* and *A. cylindracea*. Chelating agents may serve as secondary antioxidants, because they reduce the redox potential thereby stabilizing the oxidized form of the metal ions. As ferrous ions are the most effective pro-oxidants in food systems [23], the high ferrous-ion chelating ability of the various extracts from the fruiting bodies of *P. ostreatus* could be beneficial.

Analysis of phenolic compounds. Gallic acid, pyrogallol, homogentisic acid, protocatechuic acid, (+) catechin, chlorogenic acid, caffeic acid, vanillin, ferulic acid, naringin, resveratrol, naringenin, hesperetin, formononetin, and biochanin-A were used as standards to detect phenolic compounds in the *P. ostreatus* extracts. Seven phenolic compounds, gallic acid, protocatechuic acid, chlorogenic acid, naringenin, hesperetin, formononetin, and biochanin-A were identified in the *P. ostreatus* extract (Fig. 3). The total phenolic compound content was 188 μg/g. The highest phenolic compound concentration was recorded as protocatechuic acid (81 μg/g), followed by gallic acid (36 μg/g), chlorogenic acid (27 μg/g), formononetin (14 μg/g), naringenin (10 μg/g), hesperetin (10 μg/g), and biochanin-A (10 μg/g). Thus, the phenolic compound content could be used as an important indicator of antioxidant capacity. Several reports have convincingly shown a close relationship between antioxidant activity and phenolic content [24]. Mushroom extracts have high levels of phenolic

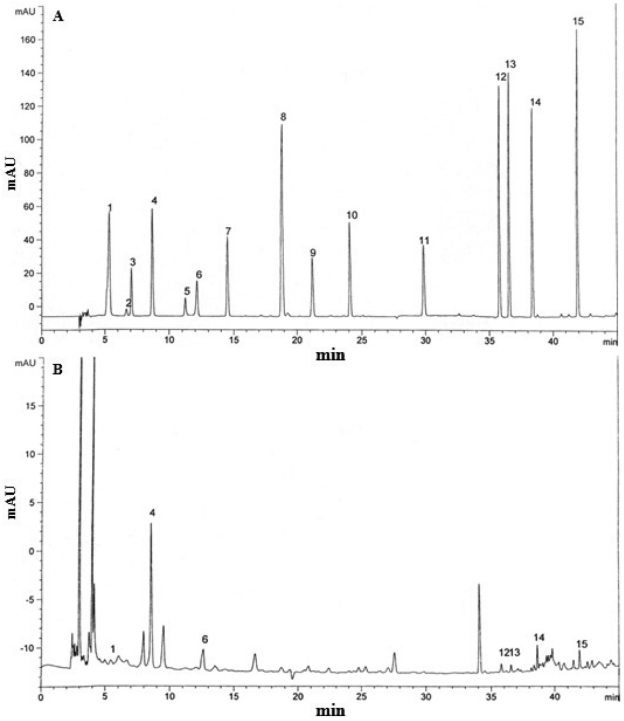

Fig. 3. High performance liquid chromatography of phenolic compounds. A, Standard mixture of 15 phenolic compounds; B, *Pleurotus ostreatus* extract. 1, gallic acid; 2, pyrogallol; 3, homogentisic acid; 4, protocatechuic acid; 5, (+) catechin; 6, chlorogenic acid; 7, caffeic acid; 8, vanillin; 9, ferulic acid; 10, naringin; 11, resveratrol; 12, naringenin; 13, hesperetin; 14, formononetin; 15, biochanin-A.

compounds, which are composed of one or more aromatic rings bearing one or more hydroxyl groups, and exhibit extensive free radical-scavenging activities as hydrogen donors or electron-donating agents, as well as metal ion-chelating properties. The greater numbers of hydroxyl groups in the phenolics may result in higher antioxidant activity [25, 26].

Tyrosinase inhibition. Tyrosinase inhibitory activities of the acetone, methanol, and hot water extracts from the *P. ostreatus* fruiting bodies increased with increasing concentration. At 0.125~1.0 mg/mL, the tyrosinase inhibition of the acetone, methanol, and hot water extracts were in the range of 11.37~52.05%, 11.36~59.56%, and 9.60~49.60%, respectively (Fig. 4). These results showed that the methanol extract had good tyrosinase inhibitory activity, whereas the acetone and hot water extracts showed moderate and poor activity, respectively, at the concentrations tested. However, at 0.125~1.0 mg/mL, L-ascorbic acid and kojic acid showed excellent scavenging abilities of 75.12~92.74% and 91.23~99.00%, respectively. The inhibition of tyrosinase activity might depend on the hydroxyl groups on the phenolic compounds of the mushroom extracts,

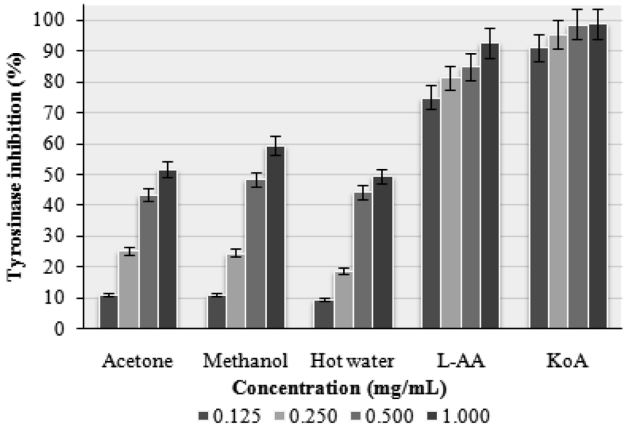

Fig. 4. Tyrosinase inhibition activity of various *Pleurotus ostreatus* extracts. Values expressed as means ± SE (n = 3). L-AA, L-ascorbic acid; KoA, kojic acid.

which may form hydrogen bonds with an enzyme site, leading to lower enzymatic activity. Some tyrosinase inhibitors act through hydroxyl groups that bind to the tyrosinase active site, resulting in steric hindrance or altered conformation [27, 28]. Gallic acid is an effective tyrosinase activity inhibitor [29, 30]. The antioxidant activity mechanism may also be an important reason for tyrosinase inhibition activity [28]. Therefore, high level of phenols and good antioxidant and antityrosinase activities indicated that the *P. ostreatus* fruiting bodies could be used as a natural food source of antioxidants.

Acknowledgements

This research was supported by a mutual research grant from the Rural Development Administration (Agenda 9-27-63; No. 200901OFT092763229).

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