

# Evaluation of Antioxidant, Anti-cholinesterase, and Anti-inflammatory Effects of Culinary Mushroom *Pleurotus pulmonarius*

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**Abstract** Culinary mushroom *Pleurotus pulmonarius* has been popular in Asian countries. In this study, the anti-oxidant, cholinesterase, and inflammation inhibitory activities of methanol extract (ME) of fruiting bodies of *P. pulmonarius* were evaluated. The 1,1-diphenyl-2-picryl-hydrazyl free radical scavenging activity of ME at 2.0 mg/mL was comparable to that of butylated hydroxytoluene, the standard reference. The ME exhibited significantly higher hydroxyl radical scavenging activity than butylated hydroxytoluene. ME showed slightly lower but moderate inhibitory activity against acetylcholinesterase (AChE) and butyrylcholinesterase than galantamine, a standard AChE inhibitor. It also exhibited protective effect against cytotoxicity to PC-12 cells induced by glutamate (10–100 µg/mL), inhibitory effect on nitric oxide (NO) production and inducible nitric oxide synthase protein expression in lipopolysaccharide-stimulated RAW 264.7 macrophages, and carrageenan-induced paw edema in a rat model. High-performance liquid chromatography analysis revealed the ME of *P. pulmonarius* contained at least 10 phenolic compounds and some of them were identified by the comparison with known standard phenolics. Taken together, our results demonstrate that fruiting bodies of *P. pulmonarius* possess antioxidant, anti-cholinesterase, and inflammation inhibitory activities.

**Keywords** Anti-cholinesterase, Anti-inflammation, Antioxidant, Cytotoxicity, *Pleurotus pulmonarius*

The metabolic processes in the human body will produce free radicals or reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical, and hydrogen peroxide. These ROS are capable of oxidizing bio-molecules including lipids, proteins, DNA, and RNA. Furthermore, they play crucial roles in the development of degenerative diseases such as arthritis, asthma, atherosclerosis, cancer, cirrhosis, dementia, and Parkinson's disease. Although all living organisms possess defense systems to mitigate oxidative damage, these defense systems are not sufficient enough to protect the body against oxidative damages [1]. Thus,

screening new antioxidants from natural sources including mushrooms is crucial. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) is a free radical that is stable at room temperature. It has a purple color in methanol solution. DPPH is reduced in the presence of antioxidant molecule, giving rise to a yellowish color. Antioxidants are molecules that can inhibit or quench free radical reactions and delay or inhibit cellular damage. Antioxidants can be categorized as enzymatic and non-enzymatic antioxidants. Enzymatic antioxidant works by breaking down and removing free radicals and converts dangerous oxidative products to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and then to water in a multi-step process, while non-enzymatic antioxidant works by interrupting free radical chain reactions and donating hydrogen atom to a radical, thereby scavenging free radicals [2]. Lipid peroxidation is an oxidative degradation process of lipids. It is mainly generated by ROS such as hydroxyl radical, hydrogen peroxide, and singlet oxygen. These ROS can readily attack polyunsaturated fatty acids and initiate a self-propagating chain reaction by stealing electrons from lipids in cell membranes, resulting in cell damage and deterioration of biological systems. Lipid peroxidation is a critical step in the pathogenesis of several diseases including atherosclerosis, asthma, Parkinson's disease, and kidney failure [3]. Hydroxyl radicals are considered as one of the rapid initiators of the lipid peroxidation process. They can eliminate hydrogen

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atoms from polyunsaturated fatty acid or from each carbon atom of the sugar moiety of DNA, thus causing oxidative damage to DNA. These effects have been implicated in mutagenesis, carcinogenesis, and aging [4].

Acetylcholinesterase (AChE) is one of the main cholinesterase in the body. It catalyzes the breakdown of acetylcholine and other choline esters functioning as neurotransmitters. AChE is mainly found at neuromuscular junctions where it can terminate synaptic transmission. High levels of AChE in neuromuscular junctions can cause neurological disorders [5]. An AChE inhibitor is a chemical that prevents acetylcholine from breaking down by AChE enzyme, thereby increasing both the level and the duration of action of neurotransmitter acetylcholine [6]. AChE inhibitors are the most important prescription drugs that medicate early symptoms of Alzheimer's disease (AD). To improve cognitive symptoms of AD, AChE inhibitors such as donepezil, galantamine, huperzine A, physostigmine, and tacrine have been developed. However, these medicines have side effects, including vomiting, diarrhea, loss of body weight, insomnia, and nausea [7]. Thus, it is important to search for new AChE inhibitors from natural products without causing side effect.

Inflammation is considered localized complex biological response as a result of infection from pathogens, irritation caused by thermal heat, UV light, or ionizing radiation, and tissue injury. Recently, non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin, aspirin, piroxicam, ibuprofen, and diclofenac are widely used for the treatment of inflammatory symptoms. However, administration of NSAIDs in a long-term may give rise to significant side effects [8]. Therefore, it is necessary to develop good and effective anti-inflammatory products from natural sources. Carrageenan-induced acute rat paw edema assay has been widely used to screen *in vivo* anti-inflammatory activity. The time course of carrageenan-induced paw edema development in rats is generally characterized by biphasic step [9]. The first phase of inflammation take places within half an hour after carrageenan administration is mainly due to the release of histamine and serotonin, whereas the second phase of inflammation involves kinin and bradykinin mediated by prostaglandins. The first phase of inflammation is characterized by an increasing in outward movement of vascular permeability and cellular infiltration of fluid and proteins into extracellular spaces, whereas prostaglandin release is responsible for edema formation in the second phase [10].

Mushrooms have been used as good nutritious foods since they are rich in carbohydrates, proteins, free amino acids, vitamins, and different essential mineral elements [11, 12]. In the literature, more than 300 species of mushrooms with various therapeutic activities have been listed as folk medicines. They are rich in many bioactive metabolites with high medicinal values, including polysaccharides, polyphenols, flavonoids, terpenoids, ergosterols, and volatile organic compounds [13, 14]. Therefore, mushrooms have shown

various biological activities including immunity-stimulating, antitumor, antimicrobial, antioxidant, anti-diabetic, anti-hyperlipidemic, anti-hypercholesterolemic, hepatoprotective, and anti-inflammatory activities [15, 16]. Among mushrooms belonging to genus *Pleurotus*, several species including *P. ostreatus*, *P. eryngii*, *P. ferulae*, and *P. citrinopileatus* have been studied for their therapeutic potentials due to their antimicrobial, antitumor, hypoglycemic, hypotensive, and anti-inflammatory activities [17].

Culinary mushroom *P. pulmonarius* is commonly known as Indian oyster or lung oyster mushroom prefers to grow in warm weather. It utilizes various lignocellulosic materials, and it is very popular in Asian countries [18]. Recently, *P. pulmonarius* has become commercially available as an important culinary mushroom in Korea. Although *P. pulmonarius* possesses good sources of dietary nutrients and other valuable medicinal components [19, 20], the therapeutically beneficial effects of *P. pulmonarius* have not been thoroughly studied. Therefore, the objective of this study was to investigate the antioxidant, anti-cholinesterase, and anti-inflammation activities of methanol extract (ME) of *P. pulmonarius* fruiting bodies. In addition, its protection against cytotoxicity of PC-12 cells induced by glutamate was determined in this study. Moreover, the profile of phenolic compounds present in ME of fruiting bodies of this mushroom was analyzed.

## MATERIALS AND METHODS

**Chemicals and reagents.** Anti-inducible nitric oxide synthase (iNOS) antibody and enhanced chemiluminescence kit were obtained from Santa Cruz Biotechnology Co. (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Amersham Bioscience Co. (Buckinghamshire, UK), respectively. All other chemicals and solvents used for experiments were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

**Animals.** Sprague Dawley female rats (5-week-old, 155~165 g) were obtained from Daehan-Biolink Inc. (Eumseong, Korea). Rats were kept in polypropylene cages at  $23 \pm 2^\circ\text{C}$  and 50~60% relative humidity with 12 hr light and dark cycles. They were provided free access to water and food (standard rat chow). Rats were acclimated in the animal house for 1 wk before experiments. The experimental design and protocols were approved by the Animal Ethics Committee of the Incheon National University.

**Mushroom and extract preparation.** The fruiting bodies of *P. pulmonarius* were obtained from Mushroom Research Institute, Geonggi Agricultural Research and Extension Service, Korea. Air dried ( $45^\circ\text{C}$  for 48 hr) fresh fruiting bodies were finely pulverized. In this study, ME of *P. pulmonarius* was used for evaluating physiologically beneficial activities because ME from other mushrooms contained higher concentration of phenolic compounds and exhibited significantly higher antioxidant, xanthine

oxidase and tyrosinase inhibitory effects compared with the hot water extract [21]. To prepare mushroom extract, 15 g of the sample powder in 300 mL of methanol (80%) were kept in shaker (120 rpm) for 24 hr at 25°C. The powder mixture in methanol solution was filtered. The residue was extracted with 300 mL of methanol (80%) two more times as described above. Methanol in the extract solution was removed using a rotary evaporator under reduced pressure at 45°C. Water remained in the extract was evaporated by freeze-dry.

#### Antioxidant assay.

**DPPH radical scavenging activity:** Antioxidant effect of ME of *P. pulmonarius* fruiting bodies was assessed by using DPPH assay [22] with slight modifications. Briefly, 1 mL of DPPH (0.1 mM) in methanol was mixed with 1 mL of various ME concentrations (0.125, 0.25, 0.5, 1.0, and 2.0 mg/mL). The mixture was vortexed and incubated for 30 min at room temperature in the dark. The absorbance of the mixture was determined at wavelength of 517 nm using a UV-vis spectrophotometer. The following formula was employed to determine the DPPH radical scavenging activity:

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

where  $A_{\text{control}}$  was the absorbance value of the control (containing all reagents without the test sample) and  $A_{\text{sample}}$  was the absorbance value of the test sample. Butylated hydroxytoluene (BHT) was used as the positive control.

**Inhibitory activity against lipid peroxidation:** The inhibitory activity of ME of *P. pulmonarius* fruiting bodies against lipid peroxidation was determined using a previously described method [23] with minor modification. Briefly, egg yolk homogenate (250  $\mu$ L of 10%, v/v) and 50  $\mu$ L of ME were added to a test tube. The final volume was adjusted to 500  $\mu$ L with distilled water. Twenty-five microliters of  $\text{FeSO}_4$  (0.07 M) was supplemented to the above mixture and incubated at room temperature for 30 min. After the incubation, 750  $\mu$ L of acetic acid (20%, pH 3.5) and equal volume of thiobarbituric acid (TBA, 0.8%) in sodium dodecyl sulphate (1.1%) with 25  $\mu$ L of trichloroacetic acid (TCA, 20%) were added. The mixture was vortexed and incubated in 100°C water bath for 60 min. After cooling down to room temperature, 3.0 mL of 1-butanol was added to the each tube to extract the organic phase. The solution was centrifuged at 3,000 rpm for 10 min. The absorbance value of the supernatant was measured at wavelength of 532 nm with a spectrophotometer. The percentage of lipid peroxidation inhibition was determined using the following formula:

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

where  $A_{\text{control}}$  was the absorbance value of the control without any test sample and  $A_{\text{sample}}$  was the absorbance value of the test sample. BHT was used as positive control.

**Hydroxyl radical ( $\text{OH}^\cdot$ ) scavenging activity:** Hydroxyl radical ( $\text{OH}^\cdot$ ) scavenging effect of the ME *P. pulmonarius* fruiting bodies was investigated using published method [24] with minor modifications. Briefly, 500  $\mu$ L of various concentrations of ME (0.125, 0.25, 0.5, 1.0, and 2.0 mg/mL) were incubated with a solution containing 100  $\mu$ L of 2.8 mM 2-deoxyribose dissolved in phosphate buffer (10 mM, pH 7.4), 200  $\mu$ L of  $\text{FeCl}_3$  (200  $\mu$ M), 1.04  $\mu$ M ethylenediaminetetraacetic acid (1 : 1 v/v), 100  $\mu$ L of  $\text{H}_2\text{O}_2$  (1.0 mM), and 100  $\mu$ L of ascorbic acid (1.0 mM). The extent of deoxyribose degradation was measured after adding 1.0 mL of 1% TBA and 1.0 mL of 1% TCA followed by incubation at 100°C for 20 min. The absorbance value was measured on an UV-Vis spectrophotometer at wavelength of 532 nm. The percent of inhibition on hydroxyl radical was calculated using the following formula:

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

where  $A_{\text{control}}$  was the absorbance value of the control without any test sample and  $A_{\text{sample}}$  was the absorbance value of the test sample. BHT was used as positive control.

#### High-performance liquid chromatography (HPLC) identification and quantification of phenolic compounds.

Twenty phenolic compound standards were purchased from Sigma-Aldrich Co.. The preparation of samples for analysis of phenolic compounds followed the procedures of Kim *et al.* [25]. Briefly, HPLC system 2695 of Alliance (Waters, Milford, MA, USA) was employed for analyzing phenolic compounds present in the ME of *P. pulmonarius* fruiting bodies. Reverse phase column (XSELECT CSH; 3.5  $\mu$ m  $\times$  150 mm  $\times$  4.6 mm i.d.) with temperature kept at 40°C was used for chromatographic separation. Injection volume was 20  $\mu$ L. Mobile phase and gradient followed those of Im *et al.* [26]. Photodiode array detector (Waters 2988) was employed for identifying and quantifying phenolic compounds present in the extract of *P. pulmonarius* fruiting bodies at wavelength of 280 nm.

#### Cholinesterase inhibitory activity.

**AChE inhibitory activity:** Anti-AChE activity of ME was investigated using the method of Ellman *et al.* [27]. Acetylthiocholine iodide was used as the substrate while electric eel AChE (Sigma-Aldrich Co.) was employed as the reference enzyme. Briefly, 120  $\mu$ L of sodium phosphate buffer (0.1 mM, pH 8.0), 30  $\mu$ L of various concentration of ME (0.063, 0.125, 0.25, 0.5, and 1.0 mg/mL) dissolved in methanol, and 30  $\mu$ L of AChE (3 U/mL) were supplemented and kept for 30 min at 25°C. Then 10  $\mu$ L of 0.5 mM 5,5'-dithio(bis-2-nitrobenzoic) acid (DTNB) was added. The reaction was started by the addition of 10  $\mu$ L of acetylthiocholine iodide (0.71 mM). As a result of the reaction, acetylthiocholine iodide was hydrolyzed to thiocholine and acetate by the AChE enzyme. Thiocholine then reacted with DTNB to form yellow colored 5-thio-2-nitrobenzoate anion. The yellow color intensity was measured

at wavelength of 412 nm in 96-well microplate reader. The AChE inhibitory activity (%) was calculated using the following formula:

$$\text{AChE inhibitory activity (\%)} = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100,$$

where  $A_{\text{control}}$  was the enzyme activity of the control and  $A_{\text{sample}}$  was the enzyme activity of the test sample. Galantamine was used as the positive control.

**Butyrylcholinesterase (BChE) inhibitory activity:** BChE inhibition activity was performed using the method of Orhan *et al.* [28] with slight modifications. Butyrylcholine iodide was used as a substrate while horse serum BChE was employed as the reference enzyme. Briefly, 120  $\mu\text{L}$  of  $\text{Na}_2\text{HPO}_4$  (100 mM, pH 8.0), 30  $\mu\text{L}$  of varying concentration of ME dissolved in methanol, and 30  $\mu\text{L}$  of BChE (0.35 U/mL) was supplemented and kept at 25°C for 30 min. After adding 10  $\mu\text{L}$  of DTNB (0.5 mM), the reaction was started by adding 10  $\mu\text{L}$  of substrate (0.2 mM). The absorbance value was obtained at wavelength of 412 nm on a microplate reader. BChE inhibitory activity (%) was calculated using the following formula:

$$\text{BChE inhibitory activity (\%)} = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100,$$

where  $A_{\text{control}}$  was the enzyme activity of the control and  $A_{\text{sample}}$  was the enzyme activity of the test sample. Galantamine was used as the positive control.

**Protective activity of ME for PC-12 cells against glutamate-induced cytotoxicity.** The protective activity of ME for PC-12 cells against glutamate-induced cytotoxicity was performed using the method of Ma *et al.* [29] with slight modifications. Briefly, PC-12 cells were cultured in RPMI 1640 media supplemented with 5% (v/v) fetal bovine serum, 10% (v/v) horse serum, 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C in 5%  $\text{CO}_2$  atmosphere. Cells were seeded into 96-well culture plate at a density of  $1 \times 10^5$  cells/well and incubated for 24 hr at 37°C. The medium was then replaced with fresh media containing 10 mM of glutamate. After 12 hr of culturing, PC-12 cells were treated with various concentrations of ME (5, 10, 20, 40, and 100  $\mu\text{g}/\text{mL}$ ) followed by incubation for 24 hr. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [28] was employed to determine PC-12 cell viability. Briefly, MTT (10  $\mu\text{L}$  of 5 mg/mL) was added into each well and PC-12 cells were incubated for another 4 hr. The upper layer of the medium was then removed. Dark blue formazan crystals precipitated in the cell culture plate were dissolved in dimethyl sulfoxide (200  $\mu\text{L}$ ) and the absorbance was measured at wavelength of 570 nm on a microplate reader.

#### Inflammation-inhibiting activity.

**Inhibitory effect on NO production:** The inhibitory effect of the ME on NO production was determined using

published method [30] with minor modifications. Briefly, RAW 264.7 murine macrophages were seeded into 96-well culture plate ( $5 \times 10^5$  cells/well) and incubated for 12 hr at 37°C with humidified 5%  $\text{CO}_2$  atmosphere. The medium was then changed to fresh medium (0.2 mL) and incubated for an additional 60 min. The RAW 264.7 cells were then stimulated with lipopolysaccharide (LPS, 1  $\mu\text{g}/\text{mL}$ ) for 24 hr without or with ME (0.5, 1.0, and 2.0  $\mu\text{g}/\text{mL}$ ). For the analysis of NO production, 50  $\mu\text{L}$  of the supernatant was used. NO production in the media of RAW 264.7 cells was determined with Griess assay. Briefly, the cell culture medium (120  $\mu\text{L}$ ) was mixed with an equal volume of Griess reagent. The reaction mixture was then incubated at 25°C for 30 min. The absorbance of each well was measured at 540 nm on a micro-titer plate reader. The amount of NO produced in the medium was calculated using standard curve of sodium nitrite.

**Western blot analysis of iNOS level:** Western blot analysis for iNOS level was performed using published method [31] with slight modifications. Briefly, RAW 264.7 cells were treated as described in the section of inhibitory effect on NO production. Cell extracts were prepared and used for western blot analysis using anti-iNOS antibody (Santa Cruz Biotechnology). Equivalent amounts of proteins were verified by re-probing the blot with anti- $\beta$ -actin antibody (Santa Cruz Biotechnology).  $\beta$ -Actin expression was used as loading control. The expression of each protein and  $\beta$ -actin was detected using ECL western blotting detection system (Amersham Biosciences).

**Carrageenan-induced acute inflammatory model:** Carrageenan-induced hind paw edema assay [32] was performed to determine the *in vivo* anti-inflammation effect of ME. Rats were divided into five groups (five rats per group). For the edema assay, 50  $\mu\text{L}$  of saline containing different concentrations of ME (5, 15, and 50 mg/kg per body weight), and indomethacin (5 mg/mL), the positive control, was injected into sub-plantar of rat hind-paw. After 30 min of administration of ME or indomethacin, 1% of 0.1 mL carrageenan (carrageenan, type IV; Sigma) was injected into the right hind paw of rats. Paw volumes were determined using a plethysmometer (MK-101P; Muromachi Kikai, Tokyo, Japan) at times just before injection of carrageenan and at 2, 4, and 6 hr after the administration of carrageenan. Paw volume was measured by the difference in paw volume at pre-injection volume and paw volume at different time points. The inhibitory effect of ME on inflammation was calculated using the following formula:

$$\text{Increase of paw volume (\%)} = [(V_t - V_o)/V_o] \times 100,$$

where  $V_t$  was the final paw volume of each rat at different time point after the injection and  $V_o$  was the paw volume of each rat at pre-injection time.

**Statistical analysis.** Data are presented as means  $\pm$  standard deviations (SD). Statistical differences between control and treatment groups were tested by one-way

analysis of variance (ANOVA) followed by Tukey's *post-hoc* test using SPSS ver. 13.0 (SPSS Inc., Chicago, IL, USA). A *p*-value of less than 0.05 was considered as statistically significant.

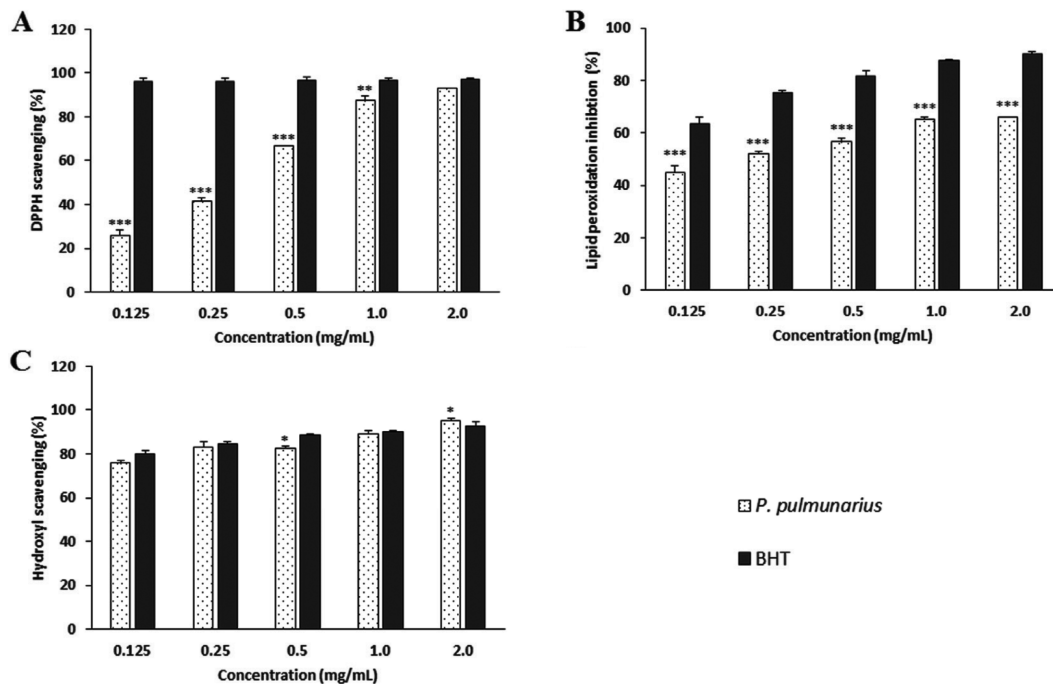
## RESULTS AND DISCUSSION

### Antioxidant activity of *Pleurotus pulminarius* extracts.

**DPPH free radical scavenging activity:** The DPPH free radical scavenging activity of the ME from fruiting bodies from *P. pulmonarius* was increased as the extract concentration was increased. The DPPH radical scavenging activity of ME at concentration range of 0.125~2.0 mg/mL was 25.67~92.73%. However, BHT, the positive control, showed excellent scavenging ability (from 96.19% to 96.97%) at the same concentration range (Fig. 1A). The scavenging activities of ME at all concentrations tested were significantly lower than those of BHT at similar concentrations. It has been reported that the DPPH radical scavenging activities of ME and hot water extract of fruiting bodies of mushroom *Lentinula edodes* are 3.39~29.4% and 38.3~40.04%, respectively, at concentration range of 1.5~9.0 mg/mL [33]. The DPPH radical scavenging activities of ME and hot water extract of fruiting bodies of mushroom *Volvariella volvacea* have been reported to be 17.8~57.8% and 20.2~37.9%, respectively, at similar concentration range (1.5~9.0 mg/mL) [33]. The DPPH scavenging activities of ethanol extracts of wild mushrooms such as *Termitomyces robustus*, *Termitomyces clypeatus*, *Lenzites* species, and *Lentinus subnidus* have been reported to be ranging from 29.8~

75.2% at 2.0 mg/mL [34]. The DPPH scavenging activities of the ME of *P. pulmonarius* fruiting bodies obtained in this study were relatively higher than those of mushrooms mentioned above. Therefore, it has the potential of being used for promoting good health.

**Inhibition activity against lipid peroxidation:** The inhibitory activity of ME of *P. pulmonarius* fruiting bodies against lipid peroxidation was investigated at five different concentrations. Its inhibitory activities against lipid peroxidation ranged from 44.99~65.87% at concentration of 0.125 to 2.0 mg/mL (Fig. 1B), demonstrating increased inhibition activity against lipid peroxidation with increasing concentration of the ME. However, the inhibitory activity of BHT against lipid peroxidation was 90.30%, which was significantly ( $p < 0.001$ ) higher than that of the ME. The  $IC_{50}$  values of ME of wild and cultivated mushrooms such as *Pleurotustuber-regium*, *Termitomycesrobustus*, *Lentinus squarrosulus*, *Pleurotus ostreatus*, *Pleurotus sajor-caju*, and *Auricularia auricula* against lipid peroxidation have been reported to be 1.04, 0.43, 1.00, 0.15, 0.75, and 1.51 mg/mL, respectively [35]. The inhibitory activities of hot water extracts of 14 different mushrooms collected from Malaysia at 10 mg/mL against lipid peroxidation have been reported to be from 33.33% to 58.18% [36]. In this study, the inhibitory ability of ME of *P. pulmonarius* against lipid peroxidation was found to be 65.87% at concentration of 2.0 mg/mL and its  $IC_{50}$  value was 0.21 mg/mL. These results indicate that the ME of *P. pulmonarius* fruiting bodies has relatively good inhibitory activity against lipid peroxidation compared to all mushrooms mentioned above. Therefore, *P. pulmonarius*

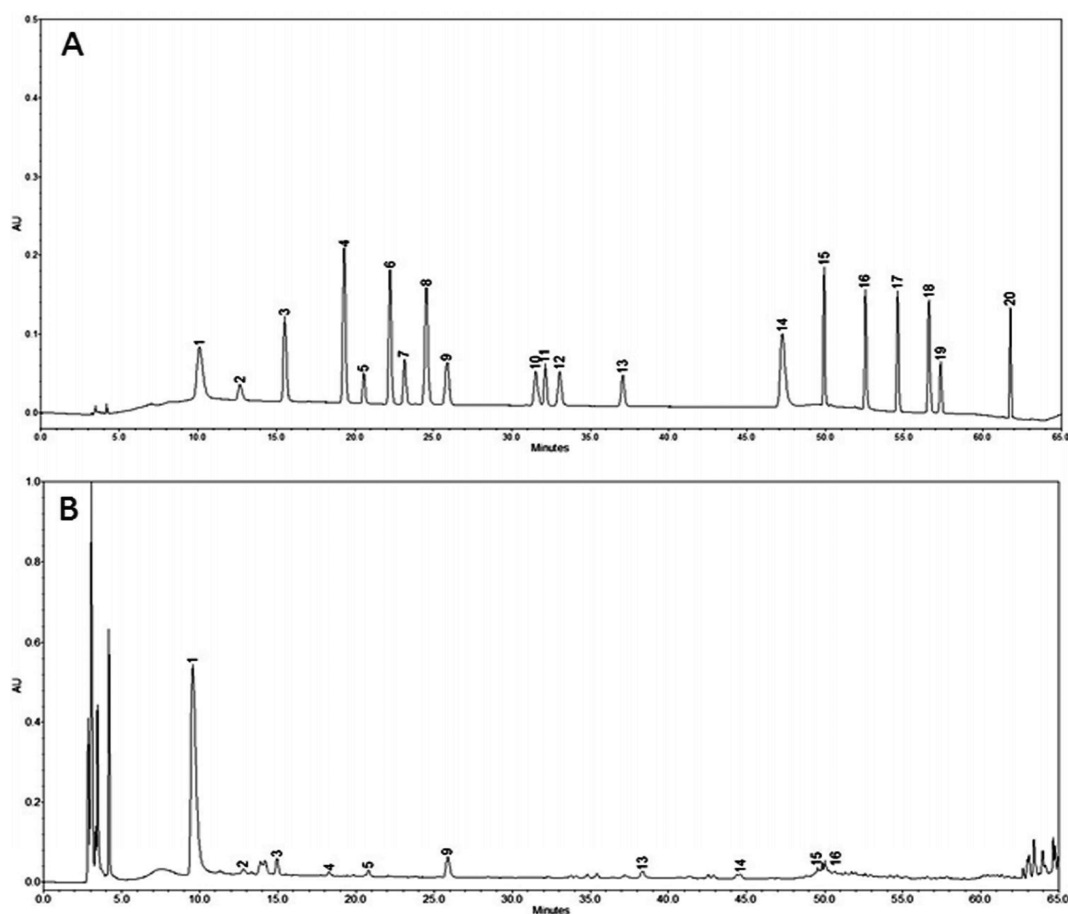


**Fig. 1.** Antioxidant activities of methanol extract from fruiting bodies of *Pleurotus pulmonarius*. A, DPPH radical scavenging activity; B, Lipid peroxidation inhibition activity; C, Hydroxyl radical ( $OH^{\cdot}$ ) scavenging activity. Values are means  $\pm$  SD ( $n = 3$ ). BHT, butylated hydroxytoluene; DPPH, 1,1-Diphenyl-2-picryl-hydrazyl. \*\*\*  $p \leq 0.001$ , \*\*  $p \leq 0.01$ , \*  $p \leq 0.05$  vs. BHT group.

fruiting bodies could be used as natural antioxidant agents. **Hydroxyl radical (OH<sup>•</sup>) scavenging activities:** The hydroxyl radical scavenging activity of ME of *P. pulmonarius* at various concentrations (0.125~2.0 mg/mL) ranged from 75.60~95.13%, whereas those of BHT ranged from 79.87~92.66% (Fig. 1C). Therefore, the ME of *P. pulmonarius* had potent hydroxyl radical scavenging activities at the concentration tested. In this study, it was found that various ME concentrations of *P. pulmonarius* prevented the degradation of 2-deoxyribose by eliminating hydroxyl radicals in the solution. The ME exhibited a concentration-dependent scavenging activity against hydroxyl radicals. The observed IC<sub>50</sub> value of ME of *P. pulmonarius* was 0.116 mg/mL, whereas the IC<sub>50</sub> value of BHT was 0.111 mg/mL, indicating that the hydroxyl radical scavenging activity of the ME of *P. pulmonarius* was comparable to BHT. It has been reported that a ME of fruiting bodies of *Pleurotus florida* has 59% of hydroxyl radical scavenging activity at concentration of 1.0 mg/mL [37], which is lower than that (89.04%) of ME of *P. pulmonarius* tested in this study at the same concentration. It has been documented that the hydroxyl scavenging ability of hot water extract of mushroom

*Vovariella vovacea* fruiting bodies is 69.34%, whereas its ME has activity of 62.45% at concentration of 0.25 mg/mL [33]. Taken together, these results suggest that the scavenging effect of ME of *P. pulmonarius* is better than the extracts of the above-mentioned mushrooms. Therefore, the ME of *P. pulmonarius* is a powerful OH<sup>•</sup> radical scavenger that can be used to prevent OH<sup>•</sup> radical related disorders.

**Identification and quantification of phenolic compounds in *Pleurotus pulmonarius* extracts.** To identify and quantify the phenolic compounds present in the ME of *P. pulmonarius* fruiting bodies, HPLC analysis was performed. Ten phenolic compounds were identified from the ME of *P. pulmonarius* fruiting bodies (Fig. 2). Their total concentration was 135.89 µg/g. Phenolic compounds detected in the ME of *P. pulmonarius* fruiting bodies included gallic acid (84.85 µg/g), homogentisic acid (10.82 µg/g), procatechuic acid (4.99 µg/g), (+)-catechin (1.15 µg/g), chlorogenic acid (8.12 µg/g), vanillin (12.19), naringin (4.71 µg/g), myricetin (1.29 µg/g), resveratrol (2.29 µg/g), and quercetin (5.48 µg/g) (Fig. 2B). The phenolic compounds with the lowest and highest concentrations in the ME of *P. pulmonarius* fruiting



**Fig. 2.** High-performance liquid chromatography analysis of phenolic compounds. A, Standard compounds; B, *Pleurotus pulmonarius*. 1, gallic acid; 2, homonogentisic acid; 3, procatechuic acid; 4, (+)-catechin; 5, chlorogenic acid; 6, (–)-epicatechin; 7, (–)-epigallocatechin gallate; 8, caffeic acid; 9, vanillin; 10, rutin hydrate; 11, p-coumaric acid; 12, ferullic acid; 13, naringin; 14, myricetin; 15, resveratrol; 16, quercetin; 17, naringenin; 18, kaempferol; 19, formonoentin; 20, biochanin-A.



bodies were (+)-catechin and gallic acid, respectively. The ME of *P. pulmonarius* fruiting bodies possessed different numbers of phenolic compounds, ranging from 3 to 15, with gallic acid and protocatechuic acid being the most common compounds present in this mushroom. The profiles of phenolic compounds and their concentrations are good indicators of the antioxidant potential of mushrooms [38]. Phenolic compounds are regarded as the most important antioxidant components in mushrooms. The correlation between the concentration of phenolic compounds and the total antioxidant capability of mushroom has been reported previously [39, 40]. Consistent with earlier reports, our results also showed that phenolic compounds present in the ME of *P. pulmonarius* fruiting bodies corresponded to its DPPH radical scavenging, lipid peroxidation inhibition, and hydroxyl radical scavenging of activities.

#### Anti-cholinesterase activity of *Pleurotus pulmonarius* extracts.

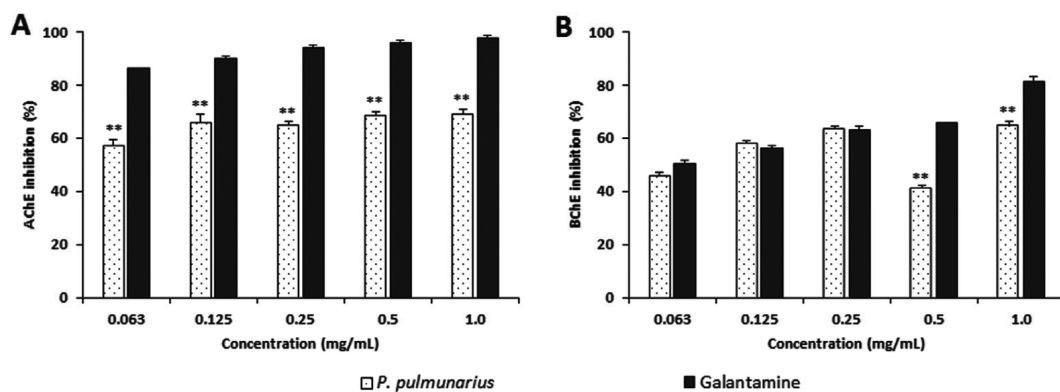
**AChE inhibitory activity:** The AChE inhibitory effects of the ME of *P. pulmonarius* fruiting bodies ranged from 57.24~69.05% at concentrations of 0.063~1.0 mg/mL (Fig. 3A). It also exhibited inhibitory activities toward AChE in a concentration-dependent manner. However, the inhibitory activity of the ME of *P. pulmonarius* fruiting bodies toward AChE was significantly ( $p < 0.001$ ) lower than that of galanthamine, the positive control, at all concentration tested. A previous study has reported that the AChE inhibitory activities of 7 wild mushroom species belonging to genus *Polyporus* and 3 other mushroom species (*Cantharellus cibarius*, *Lactarius deliciosus*, and *Trametes versicolor*) are ranged from 6.81~37.61% at concentration of 0.5 mg/mL [41], which are significantly lower than the AChE inhibitory activity (68.60%) of the ME of *P. pulmonarius* fruiting bodies in this study. Phenolic acids and flavonoid derivatives have been reported to be potent inhibitors of AChE [42]. The HPLC results of this study showed that the ME of *P. pulmonarius* fruiting bodies possessed 10 phenolic compounds, including gallic acid (84.85  $\mu\text{g/g}$ ), chlorogenic

acid (8.12  $\mu\text{g/g}$ ), resveratrol (2.29  $\mu\text{g/g}$ ), and quercetin (5.48  $\mu\text{g/g}$ ) (Fig. 2B). These compounds have been reported to possess strong AChE inhibitory potential [43]. Therefore, that AChE inhibitory activity found in the ME of *P. pulmonarius* fruiting bodies might be due to its contents of phenolic compounds.

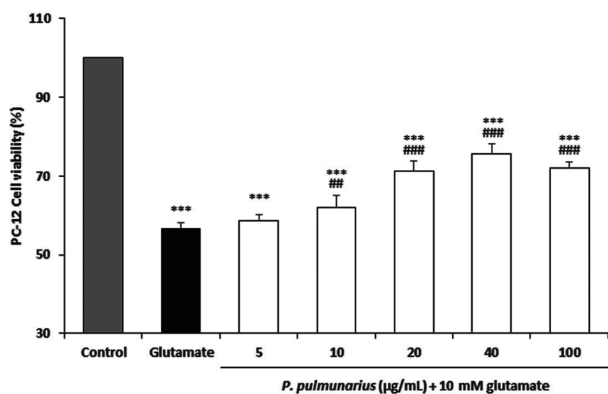
**Butyrylcholinesterase inhibitory activity:** The inhibitory effect of the ME of *P. pulmonarius* fruiting bodies on BChE was analyzed in this study. The inhibitory activities of the ME of *P. pulmonarius* fruiting bodies toward BChE were 45.67~64.56% at concentrations of 0.063~1.0 mg/mL. These activities were significantly ( $p < 0.001$ ) lower than that of positive control galanthamine (50.55~81.12%). The BChE inhibitory activity of the ME of *P. pulmonarius* fruiting bodies was increased with increasing concentration of the extract (Fig. 3B). It has been reported that phenolic acids (such as chlorogenic and gallic acids) and flavonoid derivatives (such as quercetin, genistein, lucolin-7-O-galactoside, naringin, silibinin, and silymarin) possess BChE inhibitory activities [44]. In this experiment, two of these phenolic acids (gallic acid and chlorogenic acid) and one flavonoid compound (quercetin) were detected in the ME of *P. pulmonarius* fruiting bodies. These compounds might have contributed to the moderate inhibitory effect of the ME of *P. pulmonarius* fruiting bodies toward BChE.

#### Glutamate-Induced cytotoxicity of *Pleurotus pulmonarius* extracts.

Glutamate-induced cytotoxicity was evaluated by using PC-12 cells. The viability of PC-12 cells in the medium supplemented with glutamate (10 mM) was found to be 56.65% compared to that of control PC-12 cells without glutamate treatment. The viabilities of PC-12 cells after treatment with 5, 10, 20, 40, and 100  $\mu\text{g/mL}$  of the ME of *P. pulmonarius* fruiting bodies were 58.67%, 62.08%, 71.19%, 75.59%, and 71.94%, respectively (Fig. 4). These results showed that the cytotoxicity of PC-12 cells caused by glutamate treatment was significantly lessened by supplementation of the ME at concentrations of 10~100  $\mu\text{g/mL}$ . PC-12 cells treated with (-)-epigallocatechingallate,



**Fig. 3.** Cholinesterase inhibitory activities of methanol extract from fruiting bodies of *Pleurotus pulmonarius*. A, Acetylcholinesterase inhibitory activity; B, Butyrylcholinesterase inhibitory activity. AChE, acetylcholinesterase; BChE, butyrylcholinesterase. Values are means  $\pm$  SD ( $n = 4$ ). \*\* $p \leq 0.01$  vs. galanthamine group.

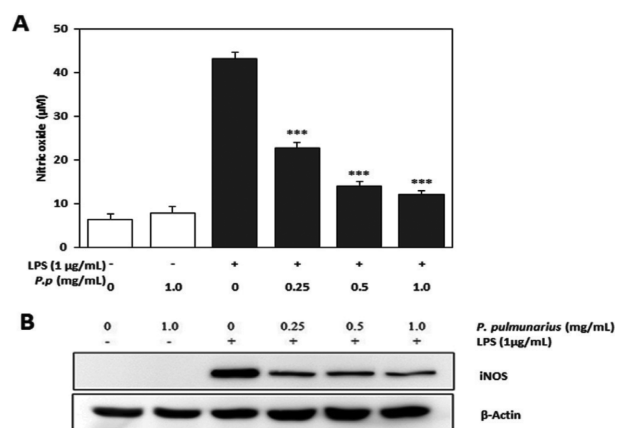


**Fig. 4.** Glutamate-induced cytotoxicity activity of methanol extracts from fruiting body of *Pleurotus pulmonarius* against PC-12 cells. Values are means  $\pm$  SD ( $n = 3$ ). \*\*\* $p \leq 0.001$  vs. control group; \*\*\* $p \leq 0.001$ , \*\* $p \leq 0.01$  vs. glutamate group.

a polyphenolic compound isolated from green tea leaves, have mitigated glutamate-induced  $[Ca^{2+}]_i$  increase, while the viability of PC-12 cells caused by glutamate-induced toxicity is increased [44]. Varying concentrations of biochanin A also reduced the cytotoxic effect of glutamate treatment on PC-12 cells [45]. Our experimental results suggest that the cytotoxicity caused by glutamate treatment on PC-12 cells can be attenuated by increasing the concentrations of ME of *P. pulmonarius* fruiting bodies.

#### Anti-inflammatory activity of *Pleurotus pulmonarius* extracts.

**Inhibition on NO production:** To determine the NO concentration in the media of LPS-stimulated RAW 264.7 cells, Griess assay [46] was employed. NO concentration in the media of RAW 264.7 cells after 24 hr of LPS treatment was increased ~6.80-fold from 6.35 to 43.17  $\mu$ M, whereas the NO concentration produced in the media of RAW 264.7 cells treated with various concentrations of ME of *P. pulmonarius* fruiting bodies was decreased significantly in a concentration-dependent manner. RAW 264.7 cells treated with 1 mg/mL of ME of *P. pulmonarius* fruiting bodies produced 7.83  $\mu$ M of NO, which was 1.23-fold higher compared to that by control RAW 264.7 cells with LPS treatment (Fig. 5A). Therefore, the ME of *P. pulmonarius* fruiting bodies showed significantly higher inhibition activity against NO production in LPS-stimulated RAW 264.7 cells ( $p \leq 0.001$ ). No cytotoxic effect of the ME was observed based on MTT test (data not shown). The reduced production of NO in ME treated RAW 264.7 macrophages might be due to the inhibition of ME on iNOS protein expression. It has been reported that ethanol extracts of mycelia and fruiting bodies of mushroom *Cordyceps militaris* can inhibit the production of NO in a concentration-dependent manner upon stimulation by LPS in RAW 264.7 macrophages [47]. Furthermore, MEs from fruiting bodies of edible wild mushrooms such as *Agaricus bisporus*, *Cantharellus cibarius*, *Lactarius deliciosus*, and *Craterellus*



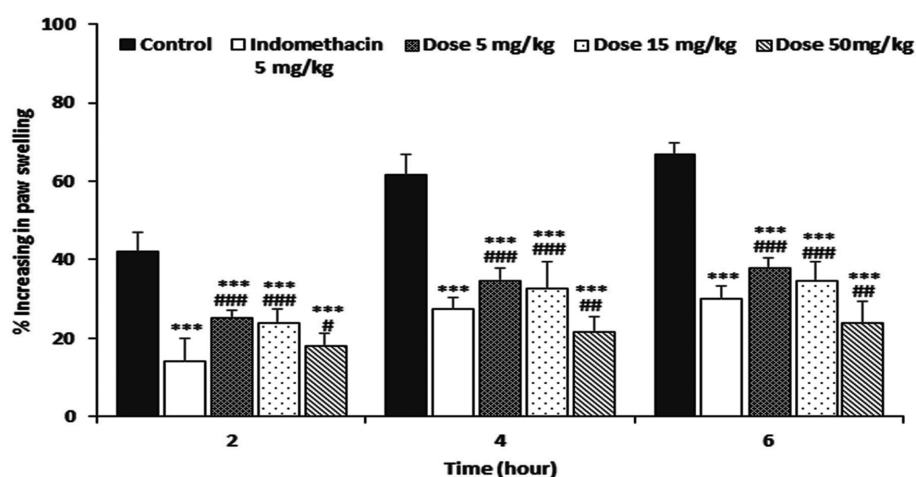
**Fig. 5.** Inhibitory effect of *Pleurotus pulmonarius* methanol extract on lipopolysaccharide (LPS)-induced nitric oxide production and expression of inducible nitric oxide synthase (iNOS) in RAW 264.7 cells. A, Nitric oxide production; B, Expression of iNOS.  $\beta$ -Actin was used as an internal control. Accumulated nitric oxide in the culture medium was determined by the Griess method. The values are means  $\pm$  SD ( $n = 3$ ). \*\*\* $p \leq 0.001$  vs. LPS treated group.

*cornucopioides* can also suppress the NO production in LPS-induced RAW 264.7 macrophages. The strong inhibition effect on NO production by these mushrooms are related to their phenolic compounds such as caffeic acid, gallic acid, homogentisic acid, protocatechuic acid, myricetin, and pyrogallol [48]. Therefore, the inhibitory effect of the ME on LPS-stimulated production of NO in RAW 264.7 macrophages might be partly due to its phenolics and other compounds.

**Western blot analysis:** NO production of LPS-stimulated RAW264.7 cells was decreased with a dose-dependently after treatment with ME of *P. pulmonarius* fruiting bodies at different concentrations. Western blot analysis was performed under the assumption that the previous experimental results on iNOS expression might be responsible for the effect of ME in reducing NO production. The amount of iNOS protein was reduced by ME of *P. pulmonarius* fruiting bodies in a dose-dependent manner. However,  $\beta$ -actin protein expression was not changed by ME treatment, indicating that the ME of *P. pulmonarius* fruiting bodies only inhibited the expression of iNOS protein (Fig. 5B). It has been reported water extract of *Ganoderma lucidum* fruiting bodies can inhibit NO production and the expression of iNOS in LPS-induced RAW 264.7 cells in a dose-dependent manner [49]. Ethyl acetate extract of fruiting bodies of *P. baumii* can also inhibit the production of NO and iNOS protein expression in LPS-induced RAW 264.7 cells [31]. These results revealed that the ME of *P. pulmonarius* fruiting bodies could decrease the production of NO and the expression of iNOS protein.

**Carrageenan-induced paw edema of rats:** The carrageenan-stimulated hind-paw edema in the control group was developed progressively as time went by, whereas the





**Fig. 6.** Effect of methanol extract from fruiting bodies of *Pleurotus pulmonarius* on carrageenan-induced paw edema. The values are means  $\pm$  SD ( $n = 5$ ).  $***p < 0.001$  vs. control group;  $###p < 0.001$ ,  $##p < 0.01$ ,  $*p < 0.05$  vs. indomethacin group.

positive control administered with indomethacin (5 mg/kg) reduced the edema volume of rats significantly at 2, 4, and 6 hr after the administration by 68.02%, 55.26%, and 55.09%, respectively, compared to the control group. The administration of various concentrations of ME of *P. pulmonarius* fruiting bodies (5, 15, and 50 mg/kg body weight) also significantly ( $p < 0.001$ ) inhibited edema development at 2~6 hr after the injection. The highest inhibition (66.16%) was observed at 4 hr after injection with 50 mg/kg of ME (Fig. 6). The edema inhibitory activity of the ME was dose-dependent. Although the ant-edema effect of the ME at 50 mg/kg was significantly higher ( $p < 0.01$ ) than that of indomethacin at 5 mg/kg, the edema inhibitory effects of the ME at 5 mg/kg was significantly lower ( $p < 0.01$ ) than that of indomethacin at the same concentration of 5 mg/kg. The results of this study showed that the ME of *P. pulmonarius* fruiting bodies could significantly inhibit rat paw edema development from the first phase to the second phase (2~6 hr), suggesting that ME of *P. pulmonarius* fruiting bodies might play a crucial role in inhibiting the release and action of histamine, serotonin, and prostaglandin. Since the ME of *P. pulmonarius* fruiting bodies possessed high anti-inflammatory potential, it might be useful for treating oxidative stress-induced inflammatory disorders.

In conclusion, the ME of *P. pulmonarius* fruiting bodies possessed moderate DPPH scavenging and lipid peroxidation inhibiting activities, while its hydroxyl radical scavenging activity was higher than that of BHT. The ME of *P. pulmonarius* fruiting bodies also showed moderate cholinesterase inhibitory effect. It also exhibited protective effect against glutamate-induced cytotoxicity to PC-12 cells at 10~100  $\mu\text{g}/\text{mL}$ . The ME of *P. pulmonarius* fruiting bodies also showed high inflammation inhibitory activities by inhibiting NO production, iNOS expression, and carrageenan-induced hind paw edema in rats. Its high phenolic contents might have contributed to its good antioxidant, anti-

cholinesterase, and anti-inflammatory activities. Our results suggest that *P. pulmonarius* might be used as a natural product to promote human health through its antioxidant, anti-cholinesterase, and anti-inflammation activities.

## ACKNOWLEDGEMENTS

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