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Free radical-scavenging and inhibition of nitric oxide production by four grades of pine mushroom (*Tricholoma matsutake* Sing.)

Hyun-Woo Lim [a], Jung-Hye Yoon [a], Young-Suk Kim [b], Min-Won Lee [a], So-Young Park [c], Hyung-Kyoon Choi [a,*]

[a] *College of Pharmacy, Chung-Ang University, Seoul 156-756, South Korea*
[b] *Department of Food Science and Technology, Ewha Womans University, Seoul 120-750, South Korea*
[c] *Department of Cell and Molecular Biology, Northwestern University, Chicago IL 60611, USA*

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Abstract

Fractions from methanol extracts of four grades of pine mushroom (*Tricholoma matsutake* Sing.) were evaluated for their free radical-scavenging and inhibition of nitric oxide production, and the underlying mechanisms were elucidated. The fractions from first-grade pine mushroom exhibited the highest degree of free radical-scavenging and inhibition of nitric oxide production among the various grades of mushroom tested, and free radical-scavenging and inhibition of nitric oxide production by the second-, third-, and fourth-grade mushroom fractions were successively lower. The degree of free radical-scavenging by each fraction decreased in the order ethyl acetate > butanol > diethyl ether > water. Inhibition of nitric oxide production by each fraction decreased in the order ethyl acetate > butanol > water. The ethyl acetate and butanol fractions of methanol-extracted pine mushroom samples exhibited potential anti-oxidative and anti-inflammatory effects that might be attributable to phenolics or flavonoids.

Keywords: Pine mushroom; *Tricholoma matsutake* sing.; Radical-scavenging activity; Inhibition of nitric oxide production; Total phenolic content; Total flavonoid content

1. Introduction

Mushrooms have been widely used since ancient times, not only as foods or food-flavouring materials but also for medicinal or functional purposes. Pine mushroom (*Tricholoma matsutake* Sing.) is the most valuable species throughout the world, exhibiting a characteristic and delicate flavour as well as several biological activities, such as cholesterol lowering, anti-oxidant, immunomodulating, and anti-tumor effects in humans (Ebina, Kubota, Ogamo, & Matsunaga, 2002; Hoshi et al., 2005; Mau, Lin, & Song, 2002). In particular, pine mushrooms cultivated in the pine forests of South Korea are the most highly valued, mainly due to the unique environment and climate of South Korea. Pine mushrooms are generally categorized into four grades, based on their quality, and these have been sold in commercial markets for use in Korean cuisine. The price typically varies from $US 50/kg for fourth-grade mushrooms to more than $US 400/kg for first-grade mushrooms. Pine mushrooms of first grade are of the highest quality, and are over 8 cm long with an unopened pileus. Pine mushrooms of second grade are generally 6–8 cm long, whereas their widths are irregular and their pilei are about one-third opened. Pine mushrooms of third grade are less than 6 cm long and pine mushrooms of fourth grade have completely opened pilei (http://www.pinemushroom.or.kr/eng/life/select.html, 2005). It has been reported that the pine mushrooms contain alkaloids, amides, steroids, and sterols (Lee, Choi, Ka, & Park, 2003; Ohnuma et al., 2000). The anti-oxidative activities of various species of mushroom have been investigated (Cheung & Cheung,

* Corresponding author. Tel.: +82 2 820 5605; fax: +82 2 816 7338.
E-mail address: hykychoi@cau.ac.kr (H.-K. Choi).

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2005; Cheung, Cheung, & Ooi, 2003; Choi, Ku, Chang, & Lee, 2005; Mau, Chang, Huang, & Chen, 2004; Mau et al., 2002; Mau, Tsai, Tseng, & Huang, 2005). However, the scavenging of free radicals and the inhibition of nitric oxide by different grades of pine mushroom have not been investigated. In this study, we examined the free radical-scavenging and inhibition of nitric oxide production by different fractions of extracts obtained from four grades of pine mushroom. In addition, we measured the total phenolics and flavonoid content of each fraction.

2. Materials and methods

2.1. Pine mushrooms

We used four grades of pine mushroom that were cultivated in Inje-eup, Gangwon-do, South Korea, in 2004. The pine mushrooms were divided into four grades according to the morphological guidance (http://www.pinemushroom.or.kr/eng/life/select.html, 2006) and the voucher specimens were deposited at the herbarium of the College of Pharmacy, Chung-Ang University. Fresh mushrooms were wrapped in low-density polyethylene film and stored at −70 °C until used. Immediately before use, the mushrooms were thawed at 4 °C for 3 h before being sliced using a cutter (model SFS-102, Shinomura, Sanjŏ, Niigata, Japan). The mushrooms were placed in a stainless steel container, frozen in liquid nitrogen, and then ground in a blender (model HMC-400 T, Hanil Electric, Seoul, Korea).

2.2. Reagents

L-Ascorbic acid, 1,1-diphenyl-2-picrylhydrzyl (DPPH), RPMI 1640 medium, lipopolysaccharide (LPS), N^G-monomethyl-L-arginine (L-NMMA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), Griess reagent (0.1% naphthylethylenediamine and 1% sulfanilamide in 5% H_3PO_4 solution), reagent-grade methanol, diethyl ether, ethyl acetate, butanol, and dimethyl sulfoxide (Me_2SO) were obtained from Sigma (St. Louis, MO, USA). Penicillin (100 units/ml) streptomycin (0.1 g/l), and 10% fetal bovine serum were purchased from Gibco-BRL (Gaithersburg, MD, USA).

2.3. Extraction and fractionation of mushrooms

A 1 kg sample (15–20 individual mushrooms according to grades) of each of the four grades of pine mushroom was extracted with 2 l of dichloromethane at room temperature for 12 h (repeated three times) and was then filtered through Whatman No. 4 filter paper (Whatman International Ltd., Maidstone England). The residue was then extracted with 2 l of methanol as described above. The methanol was evaporated under reduced pressure and the remaining residue was dissolved in distilled water before being fractionated sequentially with diethyl ether, ethyl acetate, and butanol at room temperature (repeated three times). After removal of each solvent, four fractions were obtained, which are hereafter referred to as the diethyl ether, ethyl acetate, butanol, and water fractions.

2.4. Free radical-scavenging activity

Anti-oxidant activity was measured, based on scavenging of the stable free radical, DPPH, using a modified version of a previously described method (Shimada, Fujikawa, Yahara, & Nakamura, 1992). A sample of each fraction (20 μl volume; 1 and 2 g/l concentration) in 80% methanol was added to 180 μl of a solution that contained DPPH. After 30 min, the absorbance was measured at a wavelength of 492 nm, using an enzyme-linked immunosorbent assay (ELISA) plate reader (GENios Pro, TECAN, Austria). The free radical-scavenging activity was expressed as follows:

$$\text{DPPH scavenging activity } (\%) = [(A_c - A_s)/A_c] \times 100$$

where A_c is the absorbance of the control sample and A_s is the absorbance of the test sample. L-Ascorbic acid (0.1 g/l) was used as a positive control.

2.5. Inhibition of nitric oxide production

RAW 264.7 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured at 37 °C in a humidified atmosphere (5% CO_2) in RPMI 1640 medium (Sigma) supplemented with penicillin (100 units/ml), streptomycin (0.1 g/l), and 10% fetal bovine serum (Gibco-BRL).

Cytotoxicity was measured by measuring mitochondrial-dependent reduction of MTT to formazan (Mosmann, 1983). Cells were seeded at a density of 1×10^5 cells/ml on 96-well plates. After incubation of the cells for 2 h, the cells were treated with samples of each fraction (1 and 2 g/l). The cells were incubated for a further 24 h, after which the medium was replaced with fresh medium that contained MTT (final concentration = 0.25 g/l). The cells were incubated for 1 h at 37 °C. The medium was then removed, and the residue was dissolved in 150 μl ME_2SO. The reduction of MTT to formazan by the cells was quantified by measuring the absorbance at 540 nm, using an ELISA plate reader (GENios Pro).

Nitric oxide concentrations were measured using a modification of the Griess reaction (Ding, Nathan, & Stuehr, 1988). RAW 264.7 cells, plated as described above, were preincubated for 2 h at 37 °C in a humidified atmosphere (5% CO_2). The cells were then incubated with 20 μl LPS (1 mg/l) and 20 μl each of the test samples with LPS (1 mg/l) and 20 μl each of the test samples (1 and 2 g/l) for 24 h. Thereafter, the concentration of nitrites that had accumulated in the medium was quantified as an index of nitric oxide production. Griess reagent was added to 100 μl of each of the supernatants from the cells treated with the test samples. Absorbance was measured at 540 nm and the concentration of nitric oxide in each

sample was calculated from a sodium nitrite standard curve.

2.6. Total phenolics content

The total phenolic content was determined by a modified Folin–Ciocalteu method (Singleton & Rossi, 1965). Each test sample (200 μl) was added to a test tube (10 ml volume) that contained 2.6 ml of distilled water. After vortexing the tubes, 200 μl of Folin–Ciocalteu's phenol reagent (Sigma) was added to each tube. The tubes were vortexed and, 6 min later, 2 ml of 7% Na_2CO_3 were added to each tube. The tubes were vortexed again and then allowed to stand for 90 min at room temperature. Thereafter, the absorbance of each sample was measured against a blank at 750 nm, using a spectrophotometer (Optizeu 2120UV, Mecasys, Korea). A calibration curve was constructed, using 0.0125, 0.0250, 0.0500, and 0.1000 g/l of gallic acid (Sigma) as a standard. The total phenolics content is expressed as milligrams of gallic acid per gram of dry extract.

2.7. Total flavonoid content

The total flavonoid content was determined, using a modified version of the method described by Zhishen, Mengcheng, and Jianming (1999). Each test sample (1 ml) and 4 ml of distilled water were added to a volumetric flask (10 ml volume). Five minutes after adding 0.3 ml of 5% $NaNO_2$, 0.3 ml of 10% $AlCl_3$ was added. After 6 min, 2 ml of 1 M NaOH were added, and the volume was made up to 10 ml with distilled water. The absorbance of the solution was measured against a blank at 510 nm using a spectrophotometer (Optizeu 2120UV). A calibration curve was constructed using 0.125, 0.250, 0.500, and 1.000 g/l of catechin (Sigma) as a standard. The total flavonoid content is expressed as milligrams of catechin per gram of dry extract. All measurements in this study were made in triplicate and the data are reported as the mean values ± one standard deviation.

3. Results and discussion

3.1. Free radical-scavenging by different grades of pine mushroom

The dichloromethane extract showed a severe cytotoxicity by MTT assay (data not shown), so further experiments were performed using the methanol extract only. The yieldS (percentage of the dry weight of mushroom) of the methanol extracts and the diethyl ether, ethyl acetate, butanol and water fractions, for each of the four grades of pine mushroom, are listed in Table 1. Even though the data were obtained from one methanol extract (repeated three times extraction), we used 15–20 individual mushrooms (1 kg) in each grade of pine mushroom to obtain the extract. Therefore, it can be suggested that the data described in this paper are representative of each sample.

Table 1
Yields (%) of the methanol extract fractions obtained from four grades of pine mushroom

	First grade	Second grade	Third grade	Fourth grade
Methanol extract	7.56[a]	7.38[a]	7.40[a]	7.36[a]
Diethyl ether fraction	11.52[b]	10.76[b]	11.22[b]	12.14[b]
Ethyl acetate fraction	3.98[b]	3.74[b]	3.23[b]	2.73[b]
Butanol fraction	22.67[b]	21.70[b]	21.57[b]	19.01[b]
Water fraction	38.02[b]	39.79[b]	40.20[b]	41.91[b]

[a] Percentage of the dried mushroom.
[b] Percentage of the dried methanol extract.

The yields of the methanol extracts were similar for each of the four grades of mushroom and ranged from 7.36% to 7.56%. The water fraction (38.0–41.9% of the methanol extract) was the largest of the four fractions, followed in decreasing order by the butanol, diethyl ether, and ethyl acetate fractions. The aforementioned yield order was similar for each of the four grades of mushroom.

The scavenging of free radicals by the diethyl ether, ethyl acetate, butanol, and water fractions was tested by measuring scavenging of the stable free radical, $DPPH^{\cdot}$. The first-grade pine mushroom extract exhibited the highest degree of free radical-scavenging activity among the four grades of mushroom. Free radical-scavenging by the 2 g/l fractions of first-grade mushrooms ranged from 61.0% (ethyl acetate fraction) to 30.5% (water fraction) (Table 2). Free radical-scavengings by the second-, third-, and fourth-grade mushroom extracts were successively lower. The ethyl acetate and butanol fractions of the first-grade mushroom extract exhibited the highest degree of free radical-scavenging ($61.0 \pm 0.94\%$ and $57.3 \pm 1.91\%$ for the ethyl acetate and butanol fractions, respectively, at a concentration of 2 g/l).

The commercial price of pine mushrooms varies substantially with their grade, from $US 50 to 400 per kilogram. This variation in price is due mainly to differences in flavour and texture; obviously, first-grade pine mushrooms, which are the most expensive grade, have the best flavour and texture. In the present study, extracts obtained from first-grade pine mushrooms exhibited higher degrees of free radical-scavenging than the other grades, which exhibited sequentially lower degrees of radical-scavenging according to the grade of mushroom.

Mau et al. (2004) reported that free radical-scavenging by methanol extracts obtained from various species of mushroom (*Grifola frondosa*, *Morchella esculenta* and *Termitomyces albuminosus*) ranged from 10% to 20% at a concentration of 2 g/l (Mau et al., 2004). It has also been reported that the free radical-scavenging by the water extract obtained from shiitake mushrooms (*Lentinus edodes*) was 55.4% at a concentration of 6 g/l (Cheung et al., 2003). The scavenging of free radicals by the various fractions of the extract of first-grade pine mushrooms in the present study ranged from 30.5% (for the water fraction) to 61.0% (for the ethyl acetate fraction) at a concentration of 2 g/l. These results suggest that pine mushrooms

Table 2
Free radical[a]-scavenging (%) by the diethyl ether, ethyl acetate, butanol and water fractions (1 and 2 g/l concentration) of four grades of pine mushroom

Fraction (g/l)		First grade	Second grade	Third grade	Fourth grade
Diethyl ether	1	34.8 ± 3.56	29.1 ± 0.19	28.1 ± 2.19	22.5 ± 1.38
	2	46.9 ± 0.77	37.8 ± 0.86	34.6 ± 2.31	28.7 ± 0.97
Ethyl acetate	1	51.4 ± 2.12	44.7 ± 1.91	41.8 ± 2.90	33.9 ± 3.67
	2	61.0 ± 0.94	52.4 ± 1.58	48.1 ± 1.46	38.5 ± 3.76
Butanol	1	47.6 ± 0.80	44.2 ± 2.23	39.6 ± 2.19	36.8 ± 2.10
	2	57.3 ± 1.91	50.8 ± 2.26	44.0 ± 1.57	39.8 ± 2.19
Water	1	25.6 ± 0.57	17.5 ± 1.24	16.6 ± 1.52	9.17 ± 0.75
	2	30.5 ± 0.64	25.0 ± 0.66	22.3 ± 0.78	16.6 ± 0.75
L-Ascorbic acid[b]	0.1		80.4 ± 0.27		

[a] The stable free radical, 1,1-diphenyl-2-picrylhydrzyl.
[b] Positive control.

may be a good source of the anti-oxidants that have been implicated in inhibiting aging or cancer.

3.2. Inhibition of nitric oxide production

To investigate the effects of the various fractions on nitric oxide production, the cytotoxicity of each fraction was measured using the MTT assay. The ethyl acetate, butanol and water fractions did not cause any cytotoxicity, whereas the diethyl ether fraction was cytotoxic (data not shown). Therefore, the effects of the ethyl acetate, butanol and water fraction on nitric oxide production were investigated.

As shown in Table 3, inhibition of nitric oxide production by each fraction decreased in the order ethyl acetate > butanol > water. In addition, nitric oxide concentrations, in each of the fractions mentioned above, decreased in the order first > second > third > fourth grade. For example, nitric oxide production was inhibited by 61.6%, 55.8%, 53.4%, and 46.7% by the 2 g/l ethyl acetate fraction from first-, second-, third-, and fourth-grade mushrooms, respectively. Therefore, the degree to which nitric oxide production was inhibited by the ethyl acetate fraction from the first-grade mushrooms (61.6%) was similar to the degree of inhibition caused by treatment with 0.1 g/l L of NMMA (67.0%).

Nitric oxide is formed by nitric oxide synthase; in inflammatory cells, nitric oxide is produced by an inducible isoform of nitric oxide synthase. Because high concentrations of nitric oxide can be toxic, inhibition of the overproduction of nitric oxide is an important goal (Wang, Chen, Liang, & Duh, 2005). Our findings suggest that the ethyl acetate and butanol fractions of pine mushroom, both of which substantially inhibited nitric oxide production, could be used to inhibit inflammation.

3.3. Correlation of radical-scavenging activity and inhibition of nitric oxide production with phenolic and flavonoid contents

The total phenolic and flavonoid contents of each fraction, for each of the four grades of mushroom, are listed in Table 4. The total phenolic and flavonoid contents of different grades of pine mushroom decreased in the order first > second > third > fourth grade. The ethyl acetate fraction had a higher phenolics content than had the butanol fraction in each of the four grades of mushroom (Table 4). The ethyl acetate fraction from the first-grade mushrooms had the highest phenolics content among all fractions, even though the yield of ethyl acetate was the smallest among the fractions. The phenolic content in the ethyl acetate fraction ranged from 92.4 to 47.3 mg gallic acid/g of dried fraction, and the flavonoid contents ranged from 37.4 to 22.3 mg catechin/g of dried fraction. The phenolic contents in the butanol fraction ranged from

Table 3
Inhibition of nitric oxide production (%) by the ethyl acetate, butanol and water fractions of four grades of pine mushroom in cultured RAW 264.7 cells stimulated by lipopolysaccharide

Fraction (g/l)		First grade	Second grade	Third grade	Fourth grade
Ethyl acetate	1	51.7 ± 1.62	48.8 ± 0.77	46.3 ± 0.12	37.2 ± 2.16
	2	61.6 ± 1.97	55.8 ± 0.53	53.4 ± 0.60	46.7 ± 0.76
Butanol	1	32.8 ± 3.32	25.9 ± 0.08	24.0 ± 0.53	17.9 ± 0.11
	2	46.6 ± 2.53	42.3 ± 0.18	37.2 ± 0.58	30.4 ± 0.90
Water	1	23.4 ± 0.53	18.0 ± 0.01	17.9 ± 2.09	15.0 ± 2.38
	2	28.8 ± 1.60	24.6 ± 0.87	22.7 ± 2.14	16.7 ± 1.40
L-NMMA	0.1		67.0 ± 0.03		

L-NMMA, N^G-monomethyl-L-arginine.

Table 4
Total phenolic and flavonoid contents of the ethyl acetate and butanol fraction of extracts from four grades of pine mushroom

	Fraction	First grade	Second grade	Third grade	Fourth grade
Total phenolics content[a]	Ethyl acetate	92.4 ± 3.22	70.9 ± 4.61	64.7 ± 1.65	47.3 ± 0.47
	Butanol	68.0 ± 5.54	64.3 ± 3.18	58.7 ± 1.32	42.9 ± 2.47
Total flavonoid content[b]	Ethyl acetate	37.4 ± 2.33	28.7 ± 1.56	25.7 ± 7.88	22.3 ± 1.67
	Butanol	25.3 ± 4.22	24.2 ± 4.67	21 ± 4.56	18.2 ± 5.33

[a] mg gallic acid/g dried fraction.
[b] mg catechin/g dried fraction.

Table 5
Correlation coefficients for correlations of free radical-scavenging activity and inhibition of nitric oxide production versus the total phenolic and flavonoid contents of the ethyl acetate and butanol fractions (2 g/l concentration)

	Fraction	Free radical-scavenging	Inhibition of nitric oxide production
Total phenolics content	Ethyl acetate	0.9855	0.9877
	Butanol	0.8230	0.9475
Total flavonoid content	Ethyl acetate	0.9250	0.9184
	Butanol	0.9416	0.9849

68.0 to 42.9 mg gallic acid/g of dried fraction, and the flavonoid content in the same fraction ranged from 23.3 to 18.2 mg catechin/g of dried fraction.

The correlation coefficients for the correlations among free radical-scavenging activity and inhibition of nitric oxide production versus the phenolics and flavonoid contents are listed in Table 5. The correlation coefficients ranged from 0.8230 to 0.9877, which suggested that there is a strong correlation between free radical-scavenging and inhibition of nitric oxide production and the contents of phenolics and flavonoids.

It has been reported that the anti-oxidant activity of plant materials is correlated closely with their content of phenolic compounds (Velioglu, Mazza, Gao, & Oomah, 1998). Choi et al. (2005) reported that the total phenolic and flavonoid contents of ethanol extracts, from various species of mushroom, were correlated with anti-oxidant activity. In addition, flavonoids have been shown to have anti-inflammatory effects (Manthey, Guthrie, & Grohmann, 2001). In the present study, the ethyl acetate fraction had a higher flavonoid content than had the butanol fraction, for each of the four grades of pine mushroom, and the ethyl acetate fraction of first-grade mushrooms had a particularly high flavonoid content (Table 4). Therefore, the higher free radical-scavenging and inhibition of nitric oxide production of the ethyl acetate fraction may be due to larger amounts of phenolics and flavonoids in this fraction than in to the other fractions.

4. Conclusions

The extract of first-grade pine mushrooms exhibited higher degree of free radical-scavenging and inhibition of nitric oxide production than did extracts of lower grades of the same species of mushroom. In addition, the ethyl acetate and butanol fraction exhibited relatively higher degrees of free radical-scavenging and inhibition of nitric oxide production than did the other fractions. This study revealed that the high-grade pine mushroom, which has been known as a good material for special taste and flavour is also good for potential anti-oxidative and anti-inflammatory effects.

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