Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Antioxidant polyphenols from the mycelial culture of the medicinal fungi *Inonotus xeranticus* and *Phellinus linteus*

J.-Y. Jung[1], I.-K. Lee[1], S.-J. Seok[2], H.-J. Lee[1], Y.-H. Kim[3] and B.-S. Yun[1]

1 Functional Metabolites Research Center, KRIBB, Yuseong, Daejeon, Korea
2 National Institute of Agricultural Science and Technology, RDA, Suwon, Korea
3 College of Pharmacy, Chungnam National University, Daejeon, Korea

Keywords
1,1-distyrylpyrylethan, 3,14′-bihispidinyl, antioxidant, hispidin, hypholomine B, *Inonotus xeranticus*, *Phellinus linteus*.

Correspondence
Bong-Sik Yun, Functional Metabolomics Research Center, KRIBB, Yuseong, Daejeon 305-333, Korea. E-mail: ybs@kribb.re.kr

2007/0816: received 25 May 2007, revised 24 August 2007 and accepted 26 November 2007

doi:10.1111/j.1365-2672.2008.03737.x

Abstract

Aims: The medicinal fungi *Inonotus xeranticus* and *Phellinus linteus* in the family Hymenochaetaceae have been used as traditional medicines for the treatment of various diseases. However, the compound responsible for the antioxidant activity is still unknown. Therefore, this study was conducted to characterize the antioxidant substances present in cultured broths made from these fungi.

Methods and Results: Antioxidant fractions of the cultured broths obtained from *I. xeranticus* and *P. linteus* were analysed using reversed-phase HPLC, which revealed several peaks that exhibited a potent free radical scavenging activity. To identify these antioxidant peaks, an *I. xeranticus* strain was mass-cultured, and the cultured broth was separated using antioxidant activity-guided fractionation. Four major active substances were purified and identified as hispidin and its dimers, 3,14′-bihispidinyl, hypholomine B, and 1,1-distyrylpyrylethan based on spectroscopic analyses. All compounds exhibited a significant scavenging activity against these radical species in a concentration-dependent manner.

Conclusions: Antioxidant substances found in the cultured broths of the medicinal fungi *I. xeranticus* and *P. linteus* were identified as hispidin and its dimers, 3,14′-bihispidinyl, hypholomine B, and 1,1-distyrylpyrylethan.

Significance and Impact of the Study: Polyphenol antioxidants were isolated from the cultured broth of the medicinal fungi *I. xeranticus* and *P. linteus* and identified based on extensive spectroscopic analyses. These compounds exhibited a strong antioxidant activity.

Introduction

Free radicals have been implicated in the pathogenesis of various diseases, such as myocardial and cerebral ischemia, arteriosclerosis, diabetes, rheumatoid arthritis, inflammation and cancer-initiation, and they have also been implicated in the ageing process (Coyle and Puttfarcken 1993; Margaill *et al.* 2005). There is considerable evidence that antioxidants may help prevent illnesses caused by oxidative stress because they have the capacity to quench free radicals, thereby protecting cells and tissues from oxidative damage (Diolock *et al.* 1998). Some synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been shown to exert the toxicological effects in comparison with natural antioxidants (Saito *et al.* 2003; Stefanidou *et al.* 2003). Thus, the demand for alternative and safe antioxidants from natural sources is gradually growing.

Several mushrooms belonging to the genera *Inonotus* and *Phellinus* have been used as traditional medicines for treatment of gastrointestinal cancer, liver or heart diseases, and stomach ailments (Zhu *et al.* 2007). Although they produce a large and diverse variety of secondary metabolites, polysaccharides have been considered to be

responsible for their biological effect, and the anticancer and immunomodulating activities of β-glucans derived from these mushrooms have been well documented (Jeong *et al.* 2004; Zaidman *et al.* 2005). A recent investigation of the chemical constituents of the fruiting body of these fungi resulted in isolation of hispidin class compounds with a novel carbon skeleton, such as inoscavins A–D (Lee *et al.* 2006; Lee and Yun 2007), phelligridins A–G (Mo *et al.* 2004), and tetrameric hispidin phelligridimer A (Wang *et al.* 2005). Although cultured broths made using these medicinal fungi are known to exhibit a significant antioxidant activity, the mechanisms responsible for this activity remain unknown. In this study, cultured broths made from the fungi *Inonotus xeranticus* and *Phellinus linteus* were analysed using reversed-phase HPLC, and four major antioxidant substances were purified through various column chromatographies. Based on spectroscopic analysis, the antioxidant substances were identified.

Materials and methods

Chemicals and fungi

All chemicals were purchased from Sigma-Aldrich Korea (Yongin, Korea). All solvents used for extraction and separation were of analytical grade (SK Chemicals, Ulsan, Korea) and HPLC solvents were of HPLC grade obtained from SK Chemicals. Thin-layer chromatography (TLC) and reversed-phase TLC plates were purchased from Merck Ltd. (Seoul, Korea) and Sephadex LH-20 was obtained from Amersham Biosciences (Uppsala, Sweden). The fresh mushroom *Inonotus xeranticus* was collected at Chonan-si, Korea, in April 2005, and identified by mycologist Soon-Ja Seok, a staff of the National Institute of Agricultural Science and Technology, Korea. The fungal strain *I. xeranticus* BS064 was obtained by single spore germination method from the collected mushroom. In brief, a small piece of fresh mushroom was fixed at the inner side of the cap of a Petri-dish containing potato dextrose agar medium. After incubation at 28°C for 5 days, monospore culture was used to obtain axenic culture of *I. xeranticus* BS064. Three strains (52404, 50385, 50073) of *P. linteus* were obtained from the Rural Development Administration (RDA) in Korea.

Spectroscopic analysis

Ultraviolet (UV) and infrared (IR) spectra were recorded using a Shimadzu UV-300 (Shimadzu Co., Kyoto, Japan) and a FT-IR Equinox 55 (Bruker Optics Co., Karlsruhe, Germany) spectrometers, respectively. Electrospray ionization (ESI) mass spectra were taken using a Navigator mass spectrometer (Thermo Finnigan, San Jose, CA, USA) in positive and negative modes, and high resolution electrospray ionization (HRESI) mass spectra were obtained using an ABI Mariner mass spectrometer (Perseptive Biosystem, Boston, MA, USA) with polyethylene glycol as internal standard. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained using a Varian UNITY Inova NMR spectrometer (Varian Korea Ltd., Pyeongtaek, Korea) with CD_3OD as the solvent, and chemical shifts were given in ppm (δ) using tetramethylsilane as the internal standard. ^{1}H NMR was performed at 400 MHz, and the ^{1}H-^{1}H chemical shift correlated spectroscopy (COSY) technique was employed to assign ^{1}H shifts and couplings. ^{13}C NMR was at 100 MHz with proton decoupling. ^{1}H-detected heteronuclear multiple-quantum coherence (HMQC) and heteronuclear multiple bond connectivity (HMBC) techniques were used to assign correlations between ^{1}H and ^{13}C signals.

HPLC analysis of antioxidant compounds

To detect antioxidant constituents, the antioxidative fractions of the cultured broths of fungi *I. xeranticus* and *P. linteus* were analysed by analytical reversed-phase HPLC. One strain of *I. xeranticus* and three strains of *P. linteus* were grown in potato dextrose broth medium (10 ml) at 28°C for 10 days, and then ethyl acetate (10 ml) was added into each cultured broth to extract antioxidant compounds. The ethyl acetate-soluble portion was concentrated under reduced pressure and the concentrates were dissolved in methanol. These samples were analysed by analytical HPLC (Waters Millennium System, Milford, MA, USA) that consisted of an autosampler (717), a pump (515), a photodiode array detector (2996), and a reversed-phase C18 column (150 × 4·6 mm i.d.; Cosmosil, Nacalai tesque, Japan). A linear gradient using water acidified with 0·04% TFA (v/v) and methanol, at a flow rate of 1 ml min^{-1}, was used, following 5 min at 30% methanol and reaching 90% methanol in 23 min. The column was washed with 90% methanol for 3 min and then equilibrated at 30% methanol for 4 min. The UV absorbance of the peaks with antioxidant activity was monitored by a photodiode array detector.

Isolation of antioxidant compounds

The strain *I. xeranticus* BS064, which was chosen for the isolation of active compounds **1–4**, was grown in potato dextrose broth medium (10 l) at 28°C for 10 days. Five liters of ethyl acetate was added into the cultured broth and extracted for 1 h by vigorously shaking. The ethyl acetate-soluble portion was concentrated under reduced pressure and then subjected to a column of Sephadex

LH-20 eluting with MeOH to give two antioxidant fractions. One was rechromatographed on a column of Sephadex LH-20 with 70% aqueous MeOH and then, finally purified by reversed-phase TLC developing with 70% aqueous MeOH to give **1** (hispidin, 9·4 mg) and **2** (3,14′-bihispidinyl, 2·2 mg). The other was purified by Sephadex LH-20 column chromatography with 70% aqueous MeOH, followed by preparative reversed-phase HPLC with 60% aqueous MeOH (0·04% of TFA, v/v) to afford **3** (hypholomine B, 3·5 mg) and **4** (1,1-distyrylpyrylethan, 5·5 mg).

DPPH radical scavenging activity assay

The ability of the samples to scavenge DPPH (1,1-diphenyl-2-picrylhydrazyl) radical was determined by the reported method (Blois 1958). Five milliliters of each sample, along with positive control (BHA, trolox, caffeic acid) prepared in methanol (0·08–10 mmol l^{-1}), was combined with 95 μl of 150 μmol l^{-1} methanolic DPPH in triplicate. Following incubation at room temperature for 30 min, the absorbance at 517 nm was read using a Molecular Devices Spectromax microplate reader (Sunnyvale, CA, USA).

ABTS radical cation decolorization assay

ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate)] radical scavenging activity was carried out by using ABTS radical cation decolorization assay with minor modifications (Re *et al.* 1999). ABTS was dissolved in water to a concentration of 7 mmol l^{-1}. The ABTS cation radical was produced by reacting ABTS stock solution with 2·45 mmol l^{-1} potassium persulfate and by allowing the mixture to stand in the dark for 12 h. After adding 95 μl of the ABTS radical cation solution to 5 μl of antioxidant compounds in methanol, the absorbance was measured by microplate reader at 734 nm after mixing up to 6 min.

Superoxide radical anion scavenging activity assay

Superoxide radical anion scavenging activity was evaluated by the xanthine/xanthine oxidase method (Beauchamp and Fridovich 1971). In brief, each well of a 96-well plate contained 100 μl of the following reagents: 50 mmol l^{-1} potassium phosphate buffer (pH 7·8), 1 mmol l^{-1} EDTA, 0·04 mmol l^{-1} NBT (nitroblue tetrazolium), 0·18 mmol l^{-1} xanthine, 250 mU ml^{-1} xanthine oxidase, and each concentration of samples was incubated for 30 min at 37°C in the dark. The xanthine oxidase catalyzes the oxidation of xanthine to uric acid and superoxide, and the superoxide reduces NBT to blue formazan. The reduction of NBT to blue formazan was measured at 560 nm in a microplate reader.

Cytotoxicity

The cell viability was determined by measurement of the reduction product of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Mosmann 1983). In brief, A549, HT-1080, and SW-620 cells were pre-cultured in 96-well plates with 180 μl of DMEM containing 5% FBS for 24 h. This was then added to diluted sample solution to a final volume of 200 μl and cultured under 5% CO_2 at 37°C for 48 h. MTT was dissolved in phosphate-buffered saline (PBS) and added to the cell culture. After 2 h, the medium was removed and the remaining MTT crystals were dissolved in 100 μl of DMSO. The absorbance was measured at 570 nm with a background correction at 690 nm.

Results

HPLC analysis of antioxidant fraction

It has been known that the cultured broths of the medicinal fungi *I. xeranticus* and *P. linteus* exhibit potent antioxidant activity. To find out antioxidant substances, the ethyl acetate extract, responsible to activity, of cultured broths of one *I. xeranticus* and three *P. linteus* strains were analysed by the reversed-phase HPLC equipped with a photodiode array detector. Several antioxidant peaks appeared, and their UV absorption maxima were very similar to each other, suggesting that these compounds had similar chromophore in their structure. Among them, four peaks with retention times of 12·6, 13·6, 15·2 and 21·1 min were prominent and common as shown in Fig. 1. To identify these major substances, the fungus *I. xeranticus* BS064 was mass-cultured.

Structure determination of antioxidant compounds

To isolate and characterize the compounds **1–4**, the fungus *I. xeranticus* BS064 was grown and ethyl acetate was added into the cultured broth to extract active compounds. The ethyl acetate-soluble portion was concentrated and subjected to Sephadex LH-20 column chromatography twice, followed by reversed-phase TLC or preparative reversed-phase HPLC to give compounds **1–4**.

Compound **1** was obtained as a yellow powder and its molecular weight was determined to be 246 daltons by the ESI-mass measurements, which provided the quasi-molecular ion peaks at *m/z* 245 $[M-H]^-$ in negative ion mode and at *m/z* 269 $[M+Na]^+$ in positive ion mode. The ^1H NMR spectrum exhibited the signals because of a 1,2,4-trisubstituted benzene at δ 7·00 (d, J = 1·8 Hz), 6·90 (dd, J = 8·4, 1·8 Hz) and 6·76 (dd, J = 8·4 Hz), two

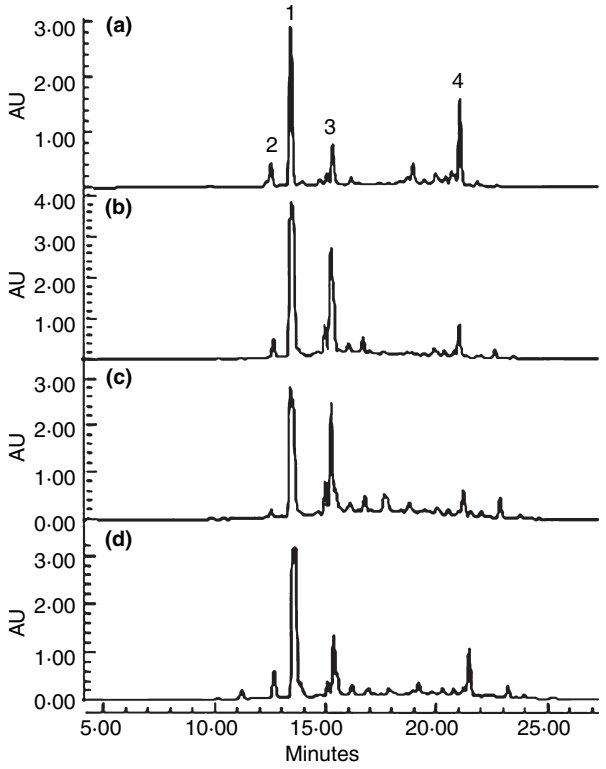

Figure 1 HPLC chromatogram of antioxidant fraction of the cultured broths of *Inonotus xeranticus* BS064 (a) and *Phellinus linteus* 52404 (b), 50385 (c), and 50073 (d) strains.

olefinic methines attributable to a *trans*-1,2-disubstituted double bond unit at δ 7·26 (d, J = 16·0 Hz) and 6·53 (d, J = 16·0 Hz), and a sp^2 singlet methine at δ 6·06. These spectroscopic properties suggested that **1** resembled to hispidin, which was ubiquitous in mushroom metabolite. Thus, the retention time of **1** was directly compared with that of commercialized hispidin by analytical HPLC. Consequently, **1** was identical with that of hispidin. The ^{1}H NMR data of **1** are presented in Table 1 and its structure is shown in Fig. 2.

Compound **2** was obtained as small amount of yellow powder and its molecular weight was determined to be 490 daltons by the ESI-mass measurements, which gave the quasi-molecular ion peaks at *m/z* 489 $[M-H]^-$ in negative ion mode and at *m/z* 513 $[M+Na]^+$ in positive ion mode. In the ^{1}H NMR spectrum, three aromatic methine protons assignable to a 1,2,4-trisubstituted benzene moiety at δ 7·05 (d, J = 1·8 Hz), 6·97 (dd, J = 8·4, 1·8 Hz), 6·78 (d, J = 8·4 Hz), four olefinic methine protons attributable to two *trans*-1,2-disubstituted double bonds at δ 7·36 (d, J = 16·0 Hz), 7·22 (d, J = 16·0 Hz), 6·64 (d, J = 16·0 Hz), and 6·56 (d, J = 16·0 Hz), and four singlet methine protons at δ 7·23, 6·66, 6·25 and 6·09 were evident. Above spectral data implied that **2** was a dimeric structure of hispidin. In extensive literature survey, **2** was identified as 3,14′-bihispidinyl (Fig. 2), which was previously reported from the fruiting body of *Phellinus pomaceus* (Klaar and Steglich 1977). The mass and ^{1}H NMR data were consistent with 3,14′-bihispidinyl, and ^{1}H resonances were assigned in comparison with those reported in the literature (Klaar and Steglich 1977).

Compound **3** was isolated as yellow powder, and its UV absorption maxima at 257 and 388 nm indicated that **3** was also a hispidin derivative. The molecular weight was determined to be 490 daltons by the ESI-mass measurements, which provided an intense quasi-molecular ion peak at *m/z* 489 $[M-H]^-$ in negative ion mode, while in positive ion mode, mass peak was not detectable. The molecular formula of **3** was deduced as $C_{26}H_{18}O_{10}$ from the combination of molecular weight and ^{1}H and ^{13}C NMR spectra. In the ^{1}H NMR spectrum in CD_3OD, six aromatic methine signals assignable to two 1,2,4-trisubstituted benzene moieties at δ 7·05 (d, J = 2·0 Hz), 6·96 (dd, J = 8·4, 2·0 Hz), 6·78 (d, J = 8·4 Hz) and 6·80 (d, J = 2·0 Hz), 6·72 (dd, J = 8·0, 2·0 Hz), 6·79 (d, J = 8·0 Hz), two olefinic methine peaks attributable to a *trans*-1,2-disubstituted double bond unit at δ 7·38 (d, J = 16·0 Hz) and 6·67 (d, J = 16·0 Hz), two sp^3 methine doublets at δ 5·77 (d, J = 6·4 Hz) and 4·30 (d, J = 6·4 Hz), and two sp^2 methine singlets at δ 6·40 and 6·08 were observed. In the ^{13}C NMR spectrum, 25 carbons comprised of two sp^3 methine carbons, 10 sp^2 methine carbons, 12 sp^2 quaternary carbons including two ester carbonyls, and eight oxygenated carbons were appeared. However, the proton and carbon peaks of 3′-methine were not detectable in CD_3OD because of hydrogen-deuterium exchange through keto-enol tautomerism. In the literature survey using the above spectral data, we found that **3** was related to hypholomine B, a hispidin dimer previously isolated from the fungi *Flammula* spp. *Hypholoma* spp. and *Pholiota* spp. To confirm the chemical structure and complete the proton and carbon chemical shift assignments, two dimensional NMR spectra including ^{1}H-^{1}H COSY, HMQC and HMBC were obtained. All proton-bearing carbons were established with the aid of an HMQC spectrum and four partial structures were elucidated by the ^{1}H-^{1}H COSY spectrum, as shown in Fig. 3. The structure of **3** was finally determined by the HMBC spectrum. The hispidin moiety was assigned by the long-range correlations from H-5 to C-3, C-4, C-6 and C-7, from H-7 to C-5, C-6 and C-9, from H-8 to C-6, C-10 and C-14, from H-10 to C-8, C-12 and C-14, from H-13 to C-9 and C-11, and from H-14 to C-8, C-10 and C-12, and these chemical shift values were in good agreement with the corresponding protons and carbons of hispidin. Other long-range correlations from H-5′ at δ 6·08 to C-3′, C-4′, C-6′ and C-4′, from H-7′ at δ

Table 1 ^{1}H and ^{13}C NMR spectral data of hispidin (**1**), 3,14′-bihispidinyl (**2**), hypholomine B (**3**), and 1,1-distyrylpyrylethan (**4**) in CD_3OD

	1	**2**	**3**		**4**	
No.	δ_H	δ_H	δ_C	δ_H	δ_C	δ_H
2			162·5		168·9	
3	Quenched		99·7		106·7	
4			174·5		169·7	
5	6·06 (s)*	6·25 (s)	96·0	6·40 (s)	102·7	6·13 (s)
6			165·6		159·7	
7	6·53 (d, *J* = 16·0)	6·64 (d, *J* = 16·0)	116·9	6·67 (d, *J* = 16)	116·5	6·51 (d, *J* = 15·8)
8	7·26 (d, *J* = 16·0)	7·36 (d, *J* = 16·0)	138·8	7·38 (d, *J* = 16)	136·9	7·24 (d, *J* = 15·8)
9			128·6		128·9	
10	7·00 (d, *J* = 1·8)	7·05 (d, *J* = 1·8)	115·0	7·05 (d, *J* = 2·0)	114·8	7·00 (br s)
11			146·8		146·7	
12			149·1		148·5	
13	6·76 (d, *J* = 8·4)	6·78 (d, *J* = 8·4)	116·6	6·78 (d, *J* = 8·4)	116·5	6·75 (d, *J* = 8·2)
14	6·90 (dd, *J* = 1·8, 8·4)	6·97 (dd, *J* = 1·8, 8·4)	122·4	6·96 (dd, *J* = 8·4, 2·0)	121·9	6·89 (br d, *J* = 8·2)
2′			167·8		168·9	
3′			91·0	Quenched	106·7	
4′			173·2		169·7	
5′		6·09 (s)	103·5	6·08 (s)	102·7	6·13 (s)
6′			163·6		159·7	
7′		6·56 (d, *J* = 16·0)	53·4	4·30 (d, *J* = 6·4)	116·5	6·51 (d, *J* = 15·8)
8′		7·22 (d, *J* = 16·0)	92·8	5·77 (d, *J* = 6·4)	136·9	7·24 (d, *J* = 15·8)
9′			131·5		128·9	
10′		7·23 (s)	113·8	6·80 (d, *J* = 2·0)	114·8	7·00 (br s)
11′			146·9		146·7	
12′			147·6		148·5	
13′		6·66 (s)	116·6	6·79 (d, *J* = 8·0)	116·5	6·75 (d, *J* = 8·2)
14′			118·9	6·72 (dd, *J* = 8·0, 2·0)	121·9	6·89 (br d, *J* = 8·2)
1″					26·4	4·79 (q, *J* = 7·6)
2″					16·1	1·58 (d, *J* = 7·6)

All spectra were recorded at 400 MHz for proton and at 100 MHz for carbon.
*Proton resonance multiplicity and coupling constant (*J* = Hz) in parenthesis.

4·30 to C-3, C-4, C-5′, C-6′ and C-9′, from H-8′ at δ 5·77 to C-4, C-6′, C-10′ and C-14′, from H-10′ to C-8′, C-12′ and C-14′, from H-13′ to C-9′ and C-11′, and from H-14′ to C-10′ and C-12′ unambiguously established the structure of **3**. Thus, **3** was identified as hypholomine B (Fiasson *et al.* 1977) and in this study, proton and carbon chemical shift assignments of hypholomine B were completed for the first time.

Compound **4**, a yellow powder, showed the UV maxima at 251 and 376 nm, which indicated that **4** was also a hispidin class compound. Its molecular weight was determined to be 518 daltons by the ESI-mass measurements, which provided a peak of very low intensity at *m/z* 519 $[M+H]^+$ in positive ion mode but in negative ion mode, an intense peak at *m/z* 517 $[M-H]^-$. The molecular formula of **4** was determined to be $C_{28}H_{22}O_{10}$ by high resolution ESI-mass providing a peak at *m/z* 519·1287 $[M+H]^+$ (calcd for $C_{28}H_{23}O_{10}$, 519·1285). The IR spectrum showed absorption bands for hydroxyl (3430 cm^{-1}), conjugated carbonyl (1650 cm^{-1}), and aromatic rings (1550 cm^{-1}). On the basis of its molecular formula and other physicochemical properties, we found that **4** was very similar to 1,1-distyrylpyrylethan, a hispidin dimer reported as a fungal pigment (Gill and Steglich 1987). To the best of our knowledge, however, any information about 1,1-distyrylpyrylethan including detailed structural elucidation and ^{1}H and ^{13}C peaks assignments has not appeared in the literature. Therefore, we carried out the complete ^{1}H and ^{13}C chemical shift assignments and structure elucidation by two-dimensional NMR experiments. The ^{1}H NMR spectrum in CD_3OD showed three aromatic methine signals assignable to a 1,2,4-trisubstituted benzene moiety at δ 7·00 (br s), 6·89 (br d, J = 8·2 Hz), and 6·75 (d, J = 8·2 Hz), two olefinic methine peaks attributable to a *trans*-1,2-disubstituted double bond unit at δ 7·24 (d, J = 15·8 Hz) and 6·51 (d, J = 15·8 Hz), and a sp^2 methine singlet at δ 6·13. In addition, a methine quartet at δ 4·79 and a methyl doublet at δ 1·58 with small intensity as compared to other peaks were observed. The

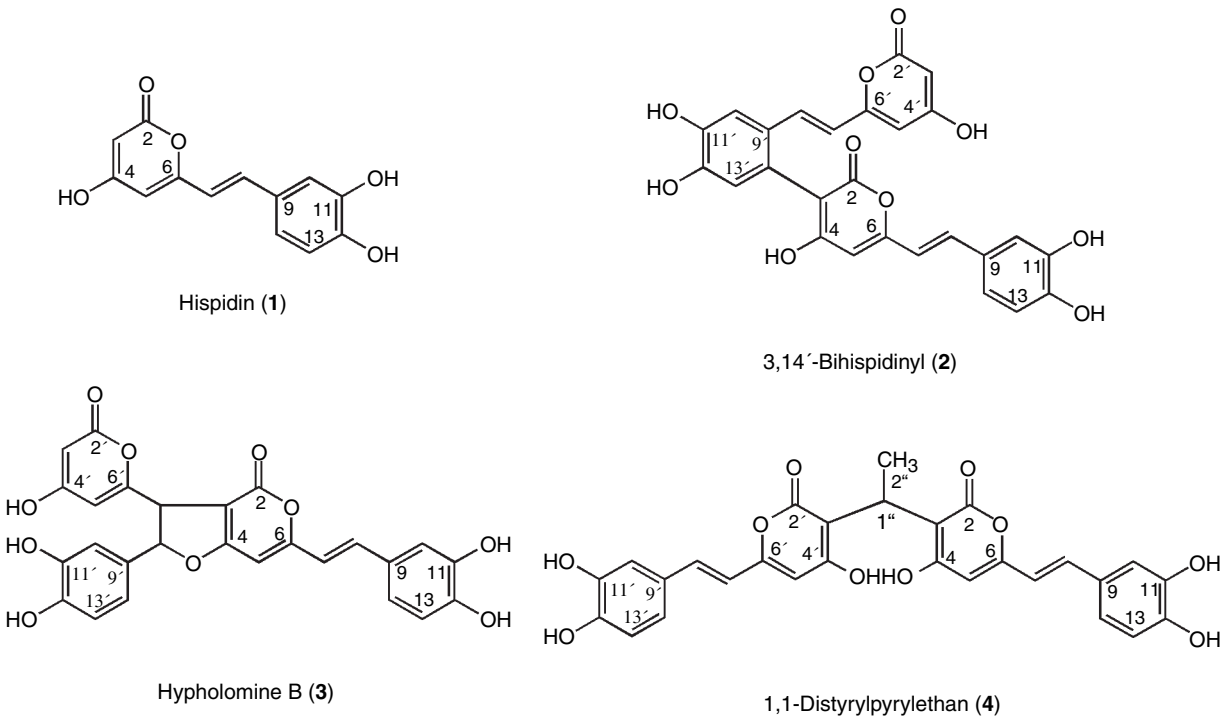

Figure 2 Structures of hispidin (**1**) and its dimers, 3,14′-bihispidinyl (**2**), hypholonine B (**3**), and 1,1-distyrylpyrylethan (**4**).

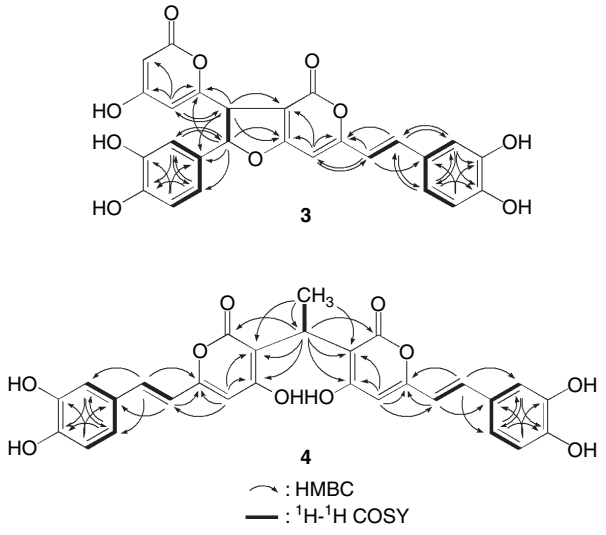

Figure 3 Structure elucidation by two-dimensional NMR experiments.

^{13}C NMR spectrum revealed the presence of only 15 carbons comprising one methyl carbon, one sp^3 methine carbon, six sp^2 methine carbons, seven quaternary carbons including an ester carbonyl, and four oxygenated sp^2 carbons. All proton-bearing carbons were assigned with the aid of an HMQC spectrum. The above spectroscopic data suggested that **4** was a symmetrical dimer of hispidin. The structure of **4** was unambiguously established by the HMBC spectrum. HMBC correlations from H-5 to C-3, C-6 and C-7, from H-7 to C-6 and C-9, from H-8 to C-6, C-10 and C-14, from H-10 to C-8, C-12 and C-14, from H-13 to C-9 and C-11, and from H-14 to C-8, C-10 and C-12 revealed the presence of hispidin moiety, as shown in Fig. 3. Additional long-range correlations from H-1″ at δ 4·79 to C-2, C-3 and C-4, and H-2″ at δ 1·58 to C-1″ and C-3 suggested that ethyl moiety was attached to C-3. From the molecular formula $C_{28}H_{22}O_{10}$, 18 degrees of unsaturation, and the proton integral ratio of 3 : 2 between methyl protons of H-2″ and methine protons derived from hispidin moiety revealed that **1** was a symmetrical dimer by an axis of ethyl group as shown. Specific rotation value of zero proposed **4** to be a mixture of enantiomers.

Evaluation of the antioxidant capacity of compounds 1–4

A major property of an antioxidant is its ability to scavenge free radicals. Therefore, we assessed the free radical scavenging efficacy of compounds **1–4** by using DPPH radical, ABTS radical cation, and superoxide radical anion scavenging assay methods. The DPPH and ABTS radical scavenging activity results were expressed in terms of trolox equivalent antioxidant capacity

Table 2 Free radical scavenging activity

Compounds	TEAC*,†		Superoxide‡ IC_{50} (μmol l^{-1})†
	DPPH	ABTS	
Hispidin (**1**)	1·31 ± 0·81	2·27 ± 0·71	34·9 ± 4·0
3,14′-bihispidinyl (**2**)	0·90 ± 0·61	0·55 ± 0·17	61·6 ± 3·0
Hypholomine B (**3**)	0·31 ± 0·22	0·24 ± 0·52	55·2 ± 3·1
1,1-distyrylpyrylethan (**4**)	0·37 ± 0·15	0·18 ± 0·20	32·0 ± 1·1
Caffeic acid	0·11 ± 0·23	0·18 ± 0·35	16·5 ± 2·3
BHA	0·34 ± 0·11	0·12 ± 0·26	>500

DPPH, α,α-Diphenyl-β-picrylhydrazyl; ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid).
*Expressed as IC_{50} of μmol l^{-1} compound/IC_{50} of μmol l^{-1} trolox.
†Results presented as the mean (n = 3) ± SD.
‡Xanthine/xanthine oxidase.

(TEAC, IC_{50} of μmol l^{-1} compound/IC_{50} of μmol l^{-1} trolox).

The synthetic nitrogen-centreed species DPPH radical serves as both the oxidizable substrate, which can be reduced by an electron- or hydrogen-donating compound to its hydrazine derivative, and the reaction indicator molecule. As seen in Table 2, the results of the DPPH radical scavenging assay demonstrate that the compounds were capable of scavenging DPPH free radicals in the following order of activity: caffeic acid > hypholomine B > 1,1-distyrylpyrylethan > 3,14′-bihispidinyl > trolox > hispidin.

Generation of the ABTS radical cation forms the basis of one of the spectrophotometric methods. In the ABTS radical scavenging assay, all the compounds were able to scavenge the ABTS radical cation (Fig. 4) in a concentration-dependent manner and, with the exception of hispidin, their activities were found to be approximately two to three times greater than that of trolox (Table 2).

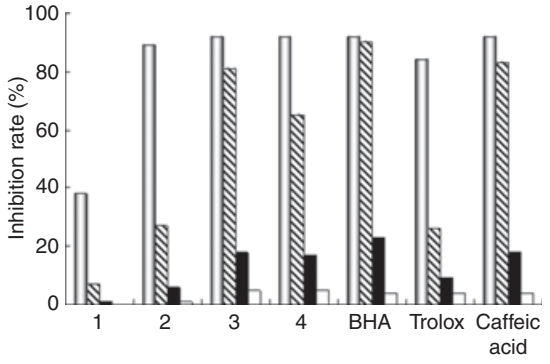

Figure 4 Antioxidant capacity of hispidin (**1**), 3,14′-bihispidinyl (**2**), hypholomine B (**3**), 1,1-distyrylpyrylethan (**4**), BHA, trolox, and caffeic acid measured by the ABTS radical scavenging assay. Values are means ± SD, n = 3 (P < 0.01). (▥), 100 μmol l^{-1}; (▨), 20 μmol l^{-1}; ■, 4 μmol l^{-1}; □, 0.8 μmol l^{-1}.

Xanthine oxidase (XO) is a key enzyme that catalyzes oxidation of hypoxanthine or xanthine to uric acid. During the reoxidation of XO, oxygen acts as an electron acceptor, producing superoxide radicals and hydrogen peroxide. Table 2 shows the superoxide radical scavenging activity of polyphenols **1–4**. Hypholomine B and 1,1-distyrylpyrylethan of the tested compounds showed potent activity comparable to that of the positive control, caffeic acid and the other compounds also exhibited significant scavenging activity against superoxide radicals.

Cytotoxicities of antioxidant polyphenols

The *in vitro* cytotoxicities of polyphenols **1–4** were determined by MTT assay. These compounds did not show any significant cytotoxicity up to 200 μmol l^{-1} against the cancer cell lines A549 (lung adenocarcinoma), HT-1080 (fibrosarcoma) and SW 620 (colorectal adenocarcinoma).

Discussion

Mushrooms are ubiquitous in nature, and some of them are nutritionally functional foods and important sources of physiologically beneficial medicines. Several mushrooms belonging to the genera *Inonotus* and *Phellinus*, such as *Inonotus obliquus, I. xeranticus*, *P. linteus*, and *Phellinus ignarius*, have been used as traditional medicines for the treatment of gastrointestinal cancer, diabetes, and stomach ailments. It is believed that polysaccharides and proteoglycans are responsible for the biological effects of these medicinal mushrooms, and a great deal of literature regarding the immunological response of β-glucans isolated from these medicinal mushrooms exists (Zaidman *et al.* 2005). β-Glucans enhance immune system activity by stimulating B-lymphocytes, T-lymphocytes, and macrophages, consequently inhibiting tumor cell growth and metastasis without toxicity. In addition to having immunomodulating properties, they are known to cause antiallergic, anti-inflammatory, and hypoglycemic effects. These medicinal mushrooms also produce a large and diverse variety of secondary metabolites, including a cluster of yellow pigment. Recent evaluation of the chemical constituents of the yellow pigment present in the fruiting bodies of *I. xeranticus* and *P. igniarius* led to isolation of hispidin (styrylpyrone) class compounds with a novel carbon skeleton, including inoscavins A–D (Lee *et al.* 2006; Lee and Yun 2007), phelligridins A–G (Mo *et al.* 2004), and a tetrameric hispidin, phelligridimer A (Wang *et al.* 2005). It has also been reported that these compounds had a significant free radical scavenging activity.

Conversely, the fermented mycelium of these medicinal fungi has been used to make an anticancer agent. Poly-

saccharides and protein-bound polysaccharide extracts from the cultured mycelium of medicinal fungi have also been clinically used for the treatment of cancer. Specifically, krestin from cultured *Coriolus versicolor*, mesima from cultured *P. linteus*, lentinan from cultured *Lentinus edodes*, and schizophylan from cultured *Schizophyllum commune* are developed and sold as anticancer agents. Additionally, fermentation broth from *P. linteus* is used as an antioxidant ingredient to make functional beverages and cosmetics in Korea. Although the antioxidant activity of *P. linteus* is well known, its antioxidant substance is yet to be determined. In this study, we focused on the secondary metabolites with antioxidant activity found in the cultured broths made from *I. xeranticus* and *P. linteus* and found four major antioxidants, hispidin and its dimers, 3,14′-bihispidinyl, hypholomine B, and 1,1-distyrylpyrylethan. Hispidin is reportedly a selective PKC-β inhibitor found in *Phellinus pomaceus* (Klaar and Steglich 1977; Gonindard *et al.* 1997). The other components are known fungal pigments (Fiasson 1982); however, the antioxidative properties of these metabolites are reported here for the first time. Their antioxidant effects may originate from the catechol moiety. It has been proposed that a good antioxidant must have a low O-H bond dissociation enthalpy to facilitate the H-abstraction from a radical and that the radical generated by the hydrogen-transfer process must be stable (Zhang *et al.* 2003). Wright *et al.* (2001) showed that a -OH group in the *o*-diphenol moiety tends to lower the bond dissociation enthalpy value by stabilizing the radical formed, thereby enhancing the antioxidant capacity. Antioxidant capacity is also affected by the intra-molecular hydrogen bonds that can exist in the catechol moiety and the inter-molecular hydrogen bonds between these functional groups that contain polar protic solvents (H_2O, CH_3OH). In the case of DPPH and ABTS radicals, compounds **2–4**, which have two catechol moieties in their structure, exhibited more potent scavenging activity than hispidin, which has only one catechol moiety. Therefore, it is proposed that the antioxidant effects of compounds **1–4** against DPPH and ABTS radicals may have originated from its catechol moiety. However, their ability to scavenge superoxide radicals seems to be dependent on another part of the structure.

Diverse polyphenol antioxidants that contain a styrylpyrone moiety have been isolated from the fruiting body of *I. xeranticus* (Lee *et al.* 2006; Lee and Yun 2007). In comparison with the metabolites isolated from the fruiting body and mycelial culture, we found that metabolites isolated from the fruiting body were more complex and had a greater structural diversity when compared with metabolites isolated from mycelial culture. It has been proposed that the polyphenolic compounds from the fruiting body were biosynthesized via oxidative coupling of the precursor hispidin and 6-(3,4-dihydroxyphenyl)-4-hydroxy-3,5-hexadien-2-one (hispolon) or 3,4-dihydroxyphenylpropanoids, such as 4-(3,4-dihydroxyphenyl)-3-buten-2-one. This reaction is probably catalysed by mushroom peroxidase. Interestingly, the compounds isolated from the mycelial culture are hispidin and dimeric hispidins, which would be biogenerated by the oxidative coupling of two moles of hispidin, specifically by the condensation of dehydrohispidin by the ligninolytic enzyme (Klaar and Steglich 1977; Fiasson *et al.* 1977). Although 3,4-dihydroxyphenylpropenoids, such as caffeic acid, were highly accumulated in the cultured broth, the condensation products of phenylpropenoids and hispidin shown in the fruiting body were not isolated or were not the major component. This result suggests that the enzymatic systems that produce polyphenolic compounds differ in the fruiting body and the cultured mycelium.

Acknowledgements

This work was supported by the grants from the BioGreen 21 Program (20050401-034-645-196) and from the On-Site Cooperative Agriculture Research Project, Rural Development Administration (RDA), Republic of Korea.

References

Beauchamp, C. and Fridovich, I. (1971) Superoxide dismutase: improved assay and an assay applicable to acrylamide gels. *Anal Biochem* **44**, 276–287.

Blois, M.S. (1958) Antioxidant determinations by the use of a stable free radical. *Nature* **181**, 1199–1200.

Coyle, J.T. and Puttfarcken, P. (1993) Oxidative stress, glutamate, and neurodegenerative disorders. *Science* **262**, 689–695.

Diolock, A.T., Charleux, J.L., Crozier-Willi, G., Kok, F.J., Rice-Evans, C., Roberfroid, M., Stahl, W. and Vina-Ribes, J. (1998) Functional food science and defense against reactive oxidative species. *Br J Nutr* **80**(Suppl. 1), 77–112.

Fiasson, J.L. (1982) Distribution of styrylpyrones in the basidiocarps of various Hymenochaetaceae. *Biochem Syst Ecol* **10**, 289–296.

Fiasson, J.L., Gluchoff-Fiasson, K. and Steglich, W. (1977) Pigments and fluorescent compounds from Hypholoma fasciculare (Agaricales). *Chem Ber* **110**, 1047–1057.

Gill, M. and Steglich, W. (1987) *Pigments of Fungi*. Wien, New York: Springer, pp. 88–97.

Gonindard, C., Bergonzi, C., Denier, C., Sergheraert, C., Klaebe, A., Chavant, L. and Hollande, E. (1997) Synthetic hispidin, a PKC inhibitor, is more cytotoxic toward cancer cells than normal cells in vitro. *Cell Biol Toxicol* **13**, 141–153.

Jeong, S.C., Cho, S.P., Yang, B.K., Jeong, Y.T., Ra, K.S. and Song, C.H. (2004) Immunomodulating activity of the exopolymer from submerged mycelial culture of Phellinus pini. *J Microbiol Biotechnol* **14**, 15–21.

Klaar, M. and Steglich, W. (1977) Isolation of hispidin and 3,14′-bihispidinyl from *Phellinus pomaceus*. *Chem Ber* **110**, 1058–1062.

Lee, I.K. and Yun, B.S. (2007) Highly oxygenated and unsaturated metabolites providing a diversity of hispidin class antioxidants in the medicinal mushrooms *Inonotus* and *Phellinus*. *Bioorg Med Chem* **15**, 3309–3314.

Lee, I.K., Seok, S.J., Kim, W.K. and Yun, B.S. (2006) Hispidin derivatives from the *Inonotus xeranticus* and their antioxidant activity. *J Nat Prod* **69**, 299–301.

Margaill, I., Plotkine, M. and Lerouet, D. (2005) Antioxidant strategies in the treatment of stroke. *Free Radic Biol Med* **39**, 429–443.

Mo, S.Y., Wang, S.J., Yang, Y.C., Chen, X.G. and Shi, J.G. (2004) Phelligridins C-F: Cytotoxic pyrano[4,3-c][2]benzopyran-1,6-dion and Furo[3,2-c]pyran-4-one derivatives from the fungus *Phellinus igniarius*. *J Nat Prod* **67**, 823–828.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**, 55–63.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A. and Yang, M. (1999) Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radcal Biol Med* **26**, 1231–1237.

Saito, M., Sakagami, H. and Fujisawa, S. (2003) Cytotoxicity and apoptosis induction by butylated hydroxyanisol (BHA) and butylated hydroxytoluene (BHT). *Anticancer Res* **23**, 4693–4701.

Stefanidou, M., Alevisopoulos, G., Chatziioannou, A. and Kouteslinis, A. (2003) Assessing food additive toxicity using a cell model. *Vet Hum Toxicol* **45**, 103–105.

Wang, Y., Wang, S.J., Mo, S.Y., Li, S., Yang, Y.C. and Shi, J.G. (2005) Phelligridimer A, a highly oxygenated and unsaturated 26-membered macrocyclic metabolite with antioxidant activity from the fungus *Phellinus igniarius*. *Org Lett* **7**, 4733–4736.

Wright, J.S., Johnson, E.R. and DiLabio, G.A. (2001) Predicting the activity of phenolic antioxidants: theoretical method, analysis of substituent effects, and application to major families of antioxidants. *J Am Chem Soc* **123**, 1173–1183.

Zaidman, B.Z., Tassin, M., Mahajna, J. and Wasser, S.P. (2005) Medicinal mushroom modulators of molecular targets as cancer therapeutics. *Appl Microbiol Biotechnol* **67**, 453–468.

Zhang, H.Y., Sun, Y.M. and Wang, X.L. (2003) Substituent effects on O-H bond dissociation enthalpies and ionization potentials of catechols: A DFT study and its implications in the rational design of phenolic antioxidants and elucidation of structure-activity relationships for flavonoid antioxidants. *Chem Eur J* **9**, 502–508.

Zhu, T., Guo, J., Collins, L., Kelly, J., Xiao, Z.J., Kim, S.H. and Chen, C.Y. (2007) *Phellinus linteus* activates different pathways to induce apoptosis in prostate cancer cells. *Br J Cancer* **96**, 583–590.