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Article

Evaluation of the Antioxidant Activity of the Fruiting Body of *Phellinus linteus* Using the On-line HPLC-DPPH Method

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Phellinus linteus **has traditionally been used as a medicinal mushroom in Oriental countries. Bioactive phytochemicals from the fruiting body of *P. linteus* were investigated using the on-line HPLC-DPPH system. The system was used to separate 10 antioxidants: protocatechuic acid (1) protocatechualdehyde, (2) caffeic acid (3), ellagic acid (4), hispidin (5), davalliallactone (6), interfungins A (7), hypholomine B (8), inoscavin A (9), and methyldavallialactone (10). Of these, compounds 6 and 7 showed the strongest inhibitory effect against the DPPH radical and superoxide anion radical-scavenging capacity. On comparison with quercetin (IC_{50}, 44.0 μM), the IC_{50} value of compounds 6 and 7 for DPPH radical-scavenging capacity were 19.6 μM and 18.5 μM. Moreover, the antioxidative capacity of compound 6 at 40 mM was 2.95 nmol Trolox equivalents per mg dry wt. These results indicated that hispidin dimers such as hispidin with hispidin or hispolon had a stronger effect on radical-scavenging capacity than hispidin itself.**

Key words: *anti-oxidants, DPPH radicals, on-line RP-HPLC-DPPH,* Phellinus linteus, *superoxide anion radical-scavenging capacity*

Reactive oxygen species (ROS) are among the major sources of primary catalysts initiating oxidation *in vivo* and *in vitro*. Oxygen-derived free radicals such as the superoxide anion radical and hydroxyl radical are thought to be linked to the onset and progression of various pathological conditions, such as cancer and diabetes as well as to the aging process [Halliwell, 1997; Evans *et al.*, 2002]. Recently, the capacity of antioxidants has been assessed using model systems such as the luminal chemiluminescence, 2,2-diphenyl-1-picrylhydrazyl, 3-ethylbenzothiazoline-6-sulfonate radical anion, ferric thiocyanate, and methyl linoleate peroxidation methods [Koleva *et al.*, 2000]. These systems facilitate the screening, identification, and comparison of primary antioxidants. However, due to their batch-like characteristics, they are unsuitable for identifying which individual compounds are responsible for the overall effect. Also, it is difficult to quantitatively assay the antioxidant capacity because of the short lifespan of these radicals. Additionally, the capacity of natural antioxidants often decreases during their isolation and purification due to decomposition. A method that combines antioxidant separation and the evaluation of their capacity would present major advantages. Details of a new and rapid on-

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Abbreviations: DPPH, 2,2-dipheny-1-picrylhydrazyl; ESI, electospray ionization; HMBC, heteronuclear multiple bond coherence; HMQC, heteronuclear multiple quantum coherence; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; ROS, reactive oxygen species; RP, reverse phase

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line method for screening complex mixtures for their radical-scavenging components has previously been published; a methanolic solution of the DPPH stable free radical was used and an optimized instrumental setup was presented [Dapkevicius *et al.,* 2001]. The greatest benefit of the method is that, besides the quantification by UV detection, the radical-scavenging capacity of a single substance can be measured, and its contribution to the overall capacity of a mixture of antioxidants can be calculated. Thus, it is no longer necessary to purify every single constituent for offline assays to obtain results; leading to very significant cost and time reductions [Koleva *et al.*, 2000]. However, there are few reports concerning on-line separation and antioxidant capacity assessment, or the quantitative analysis of radical-scavenging compounds in complex samples.

Phellinus linteus, which grows well on trees such as *Morus alba*, is a well-known fungus of the genus *Phellinus*, from the Hymenochaetaceae family, and has been used as a traditional herbal medicine in Oriental countries for years. Phenolic compounds, including hispidin and hispidin derivatives, from *P. llinteus* have been isolated and reported [Gonindard *et al.*, 1997; Park *et al.*, 2004; Chen *et al.*, 2006; Hwang *et al.*, 2006; Min *et al.*, 2006; Lee *et al.*, 2008a]. Recent studies have shown that these phenolic compounds have antioxidant, antitumor, and anticomplement capacities, as well as β-secretase, protein kinase C, and aldose reductase inhibitory capacities [Gonindard *et al.*, 1997; Park *et al.*, 2004; Chen *et al.*, 2006; Hwang *et al.*, 2006; Min *et al.*, 2006; Lee *et al.*, 2008a]. Furthermore, recent investigations into various hispidin derivatives and polyphenols have been conducted and a bundle of yellow antioxidant pigments have been isolated and identified as inoscavins A-C, phelligridins A-G, phelligridimer A, and interfungins A-C [Lee and Yun, 2007]. There are several reports on the antioxidant principles of medicinal mushrooms such as *P. igniarius* and *Inonotus xeranticus* [Wang *et al.,* 2005; Lee and Yun, 2007], but there have been no detailed reports on *P. linteus.* Our preliminary study found that crude extracts of *P. linteus* exhibited high DPPH radical-scavenging capacity, implying that it might be a good candidate for further development as an antioxidant source.

As the first study of this kind, we investigated the antioxidant capacities from the fruiting body of *P. linteus* using a rapid screening method -the on-line HPLC-DPPH method- and identified the active compounds by LC-MS and NMR analyses. Furthermore, the structure-capacity relationships of the phytochemicals were also investigated.

Materials and Methods

Chemicals. DPPH, Folin-Ciocalteu's phenol reagent, quercetin dihydrate, gallate, and (+)-catechin were purchased from Sigma (St. Louis, MO). DPPH solutions were freshly prepared in MeOH daily and were protected from light. The solvents used in the HPLC analysis and extraction were of the highest quality available.

Extraction and purification of antioxidative compounds from *P. linteus* (Fig. 1). Fresh *P. linteus* mushrooms were obtained from Samsung Herb Medicine Co. Ltd., Chuncheon, Korea and a voucher specimen (No. RIC-021) was deposited and maintained at the Herbarium of the Regional Innovation Center, Hallym University, Chuncheon, Korea. Compounds were isolated from the *P. linteus* mushrooms by the method described in the previous paper [Lee *et al.*, 2008a; 2008b]. Their structures were shown in Fig. 1.

DPPH radical-scavenging capacity. The free radical-scavenging capacities of the *P. linteus* extract and its fractions were assessed by DPPH assay [Cao *et al.,* 1997]. Fifty microliters of the flower extract of *P. linteus* in MeOH -yielding a series of extract concentrations of 10, 25, 50, and 100 μg/mL in each reaction- were mixed with 1,000 μL of 0.1 mM DPPH-MeOH solution. MeOH (50 μL) was used as the control in this experiment. After 30 min of incubation at room temperature, the reduction in DPPH free radicals was measured as reading the absorbance at 517 nm. Quercetin dehydrate was used as the positive control.

Superoxide anion radical-scavenging assay. A photo-chemiluminescence assay was used for the determination of the integral antioxidative capacity of the extracted and fractionated substances from *P. linteus*. The antioxidative capacity of the putative antioxidative substances was estimated using the standard Photochem® system (Analytik Jena AG, Jena, Germany) kit, where the luminal plays a double role as a photosensitizer as well as the oxygen radical detection agent. The anti-oxidative capacity of the sample was quantified by comparison with the calibration curve created using Trolox standards for antioxidants, and according to the manufacturer's instructions [Apati *et al.,* 2003; Jung *et al.,* 2007].

Determination of total phenolic content. The total phenolic content was determined using the Folin-Ciocalteu method [Sakthivelu *et al.,* 2008]. The MeOH extract and its fractions (5 mg) of *P. linteus* were dissolved in 5 mL of dimethylsulfoxide/water (50:50, v/v). The solution (500 μL) at various concentrations (0.1-2 mg/mL) was mixed with 500 μL of 50% Folin-Ciocalteu

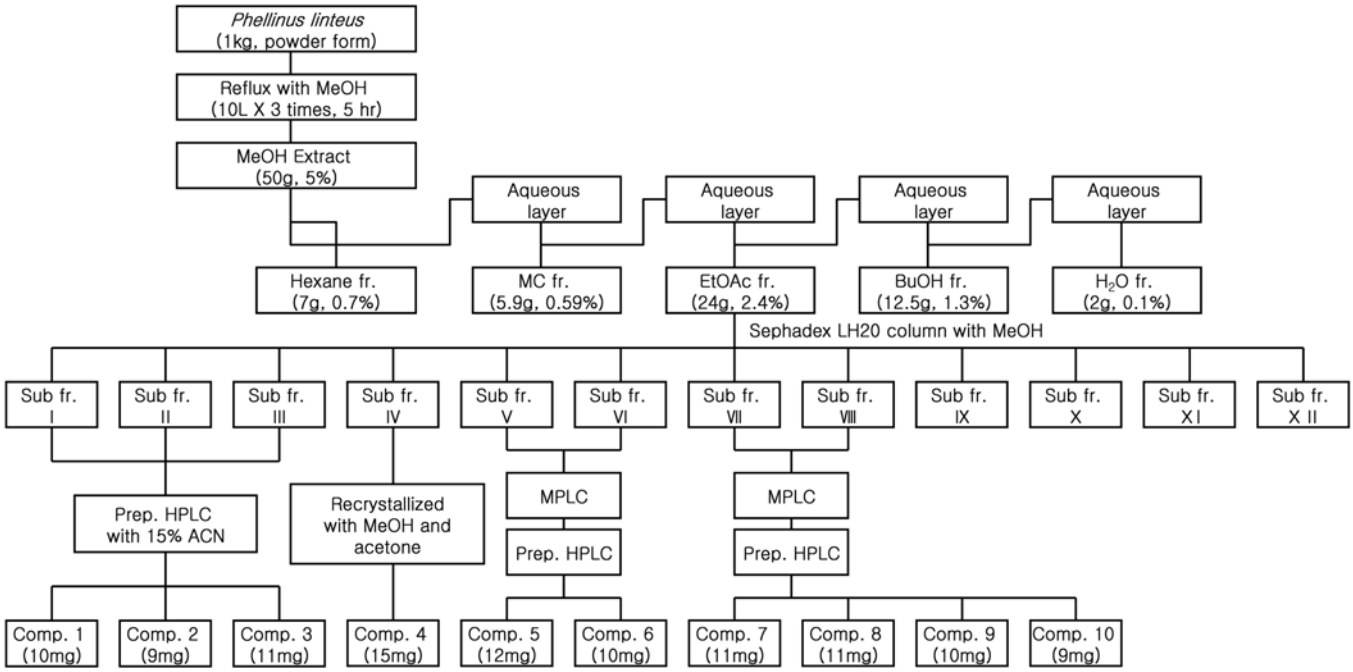

Fig. 1. The scheme of extraction and isolation of antioxidant compounds from *P. linteus*

reagent. After incubation for 2-5 min, 1.0 mL of 20% sodium carbonate was added. After 10 min of incubation at room temperature, the mixture was centrifuged at 150 ×g for 8 min, and the absorbance of the supernatant was measured at 730 nm on a UNIKON×1 UV/visible spectrophotometer (SECOMAN, Domont, France). The total phenolic content was expressed as mg of gallic acid per gram of dried extract or fraction, using (+)-gallic acid as a calibration standard.

Determination of total flavonoid content. The total flavonoid contents of the MeOH extract and its fractions were measured by colorimetric method [Xu and Chang, 2009]. Briefly, 1 mL of each sample at various concentrations (0.1-2 mg/mL) was diluted with 4 mL of deionized H_2O in a volumetric flask. Initially, 0.3 mL of 5% $NaNO_2$ was added to each volumetric flask. The flasks and their contents were then stored at ambient conditions for 5 min. Next, 0.3 mL of 10% aluminum chloride solution was added and kept for 6 min, followed by the addition of 2 mL of 1 M NaOH. Each reaction flask was then immediately diluted and mixed with 2.4 mL of deionized H_2O. Finally, the absorbance at 510 nm was measured on a UNIKON×1 UV/visible spectrophotometer (SECOMAN, Domont, France). The total flavonoid content was expressed as mg of (+)-catechin equivalent per g of dried extract or fraction, using (+)-catechin as a calibration standard.

On-line DPPH radical-scavenging analysis. Antioxidant capacity of the EtOAc fraction of *P. linteus* was further monitored by the on-line HPLC-DPPH method. The instrumental setup used is depicted in Fig. 2. The separation of the antioxidative components was carried out by analytic Thermo HPLC on a model P2000 Gradient Pump (Thermo Separation Products, San Jose, CA), a model AS3000-021 thermostatted autosampler, and a model SCM 1000-022 degasser with a Union UK-C8 column (250×4.6 mm, 3.5 μm, Imtakt Co., Kyoto, Japan). The mobile phase was solvent A (0.1% formate in ultrapure water) and B (100% ACN). The elution conditions were 0-85 min of 10-40% B (linear gradient) and 85-100 min of 40-100% B at a flow rate of 0.5 mL/min using a Thermo 6000LP detector at a wavelength of 220 nm. The HPLC-separated analytes reacted post-column with the DPPH at a concentration of 25 mg/L in MeOH. The DPPH reagent was continuously pumped via a Pinnacle PCX (Pickering Lab, LCTech, Dorfen, Germany) and set at 0.3 mL/min. The induced bleaching was detected photometrically as a negative peak at 517 nm by a Thermo 2000LP detector (Thermo-Electron Corp., Waltham, MA) [Bandoniene and Murkovic, 2002b].

Statistical analysis. All of the experiments were done in triplicate and the total phenolic and flavonoid contents were assessed using the correlation and regression program of Microsoft Excel (Version 2003). The results were presented as the mean±standard deviation.

Results and Discussion

DPPH and superoxide radical-scavenging capacity of *P. linteus* extract and its fractions. The present study

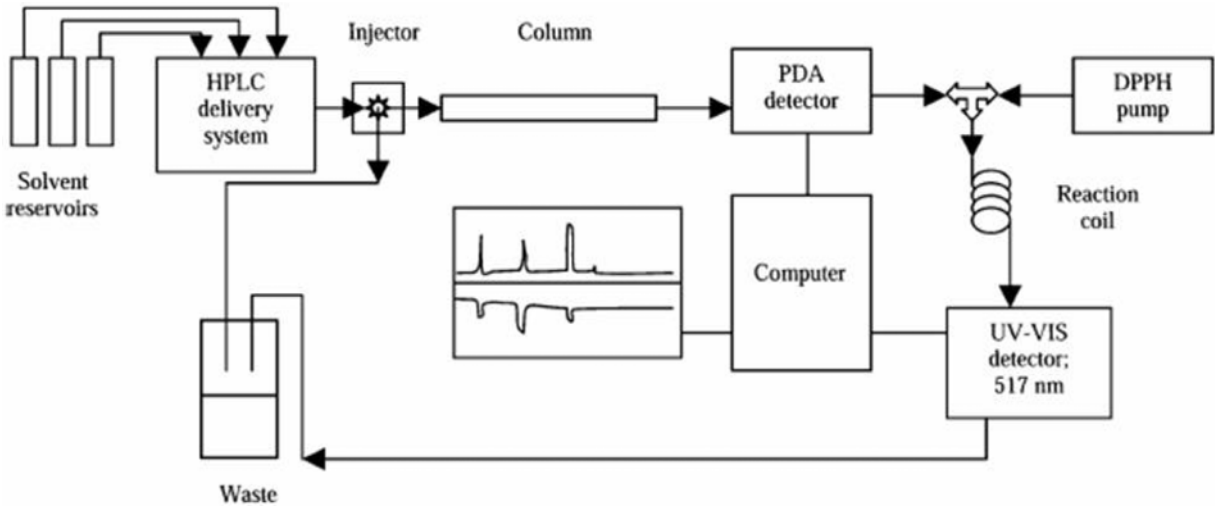

Fig. 2. The scheme of instrumental set-up for the on-line HPLC-DPPH detection of free radical-scavenging compounds.

was conducted to evaluate the potential antioxidative capacity of the fruiting body of *P. linteus* according to various fractions, including Hex, MC, EtOAc, *n*-BuOH, and H_2O fractions, using two different measurements; DPPH and superoxide anion radical-scavenging assays (Table 1). Of these, the EtOAc fraction showed the strongest capacity in the DPPH (IC_{50}, 24.8 μg/mL) and superoxide anion radical-scavenging (3.01±0.05 nmol Trolox equivalent per mg dry wt.) assays. The results were in good agreement with the DPPH radical-scavenging capacities reported in the paper by Chang *et al.* [2007]. Moreover, the *n*-BuOH fraction also showed a good inhibitory capacity (30.3±0.7 μg/mL) against the DPPH radical, but it was two times weaker than the EtOAc fraction in terms of superoxide radical-scavenging capacity.

Total phenolic and flavonoid contents of *P. linteus* extract and its derived soluble fractions. Determinations of the total phenolic and flavonoid contents of the different fractions of *P. linteus* were carried out using the Folin-Ciocalteu colorimetric and aluminum chloride methods, respectively. As a result, the highest amounts of total phenolic and flavonoid compounds were found in the EtOAc fraction. The total phenolic contents were in the following order: EtOAc (661.2 mg/g)>*n*-BuOH (297.4) >CH_2Cl_2 (127.0)>Hex (57.7), H_2O (55.9) (Table 1). Moreover, the flavonoid contents had the following order: EtOAc (556.9 mg/g)>*n*-BuOH (243.5)>CH_2Cl_2 (124.0)>Hex (78.9), H_2O (15.4). These results indicate that the antioxidant capacity of the EtOAc fraction may be strongly correlated to its total phenolic or flavonoid content. In many studies, correlation between phenolic compounds and antioxidative capacities has been described [Velioglu *et al.,* 1998]. The antioxidant property of many plant extracts has been attributed to their phenolic contents and/or structures [Rice-Evans *et al.,* 1997]. The

Table 1. Total phenolic and flavonoid contents and radical-scavenging capacities of crude methanolic extract and its fractions (Fr.) from *P. linteus* measured by DPPH and superoxide anion radical-scavenging assays

Samples	Total phenolics[1)]	Total flavonoids[2)]	DPPH	Superoxide anion radical-scavenging capacity[3)]
			(IC_{50}, μg/mL)	(nmol Trolox equivalent per mg dw.)
MeOH extract	297.0±1.34	280.2±1.05	35.2	2.96±0.03
Hex Fr.	57.7±0.13	78.9±0.29	>142.9	n.d.[4)]
CH_2Cl_2 Fr.	127.0±0.56	124.0±0.8	116.6	n.d.
EtOAc Fr.	661.2±2.98	556.9±1.97	24.8	3.01±0.05
n-BuOH Fr.	297.4±1.04	243.5±0.98	30.3	1.51±0.04
H_2O Fr.	55.9±0.07	15.4±0.08	n.d.	2.27±0.03

Data are mean±SD values (n=3).
[1)]Gallic acid equivalents (mg/g dw. of each fraction), [2)](+)-Catechin equivalents (mg/g dw. of each fraction), [3)]At a concentration of 0.04 μg/mL, [4)]n.d.: not detected.

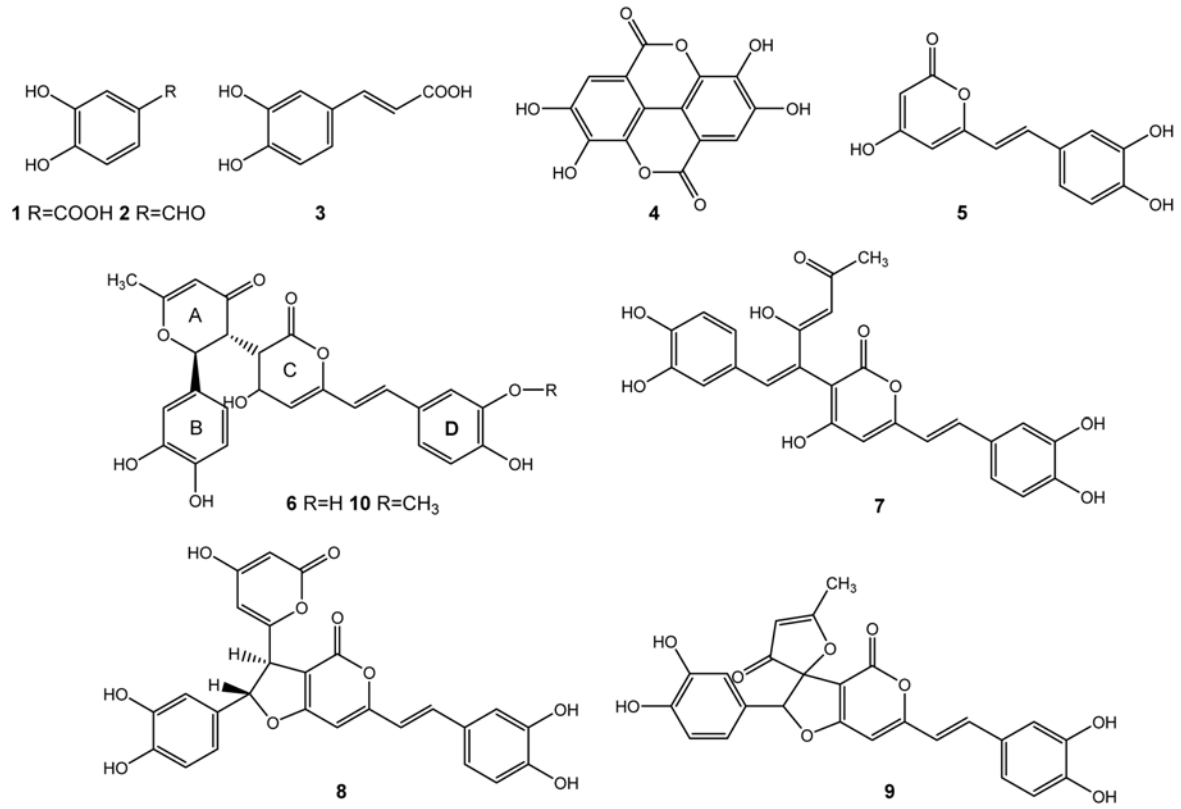

Fig. 3. Phytochemicals identified from *P. linteus*. 1, protocatechuic acid; 2, protocatechualdehyde; 3, caffeic acid; 4, ellagic acid; 5, hispdidin; 6, davallialactone; 7, interfungins A; 8, hypholomine B; 9, inoscavin A; 10, methyldavallaialactone.

antioxidative capacity of phenolic compounds is mainly because of their redox properties, which can play an important role in neutralizing free radicals, quenching singlet and triplet oxygen species, or decomposing peroxides.

Identification of antioxidative compounds from *P. linteus*. Bioassay-guided fractionation of the EtOAc fraction from the MeOH extract of *P. linteus* led to the isolation of antioxidative compounds of polyphenol-type hispidin derivatives. The structures of antioxidative compounds **1-10** were elucidated based on 1-dimensional (^{1}H- and ^{13}C- NMR) and 2-dimensional NMR (HMQC and HMBC) spectral data and all spectral data were consistent with the literature [Cui *et al.*, 1990; Wang *et al.*, 2005; Lee and Yun, 2007]. The antioxidant compounds were identified as protocatechuic acid [(**1**), 3,4-dihydroxybenzoic acid], protocatechualdehyde [(**2**), 3,4-dihydroxybenzaldehyde], caffeic acid [(**3**), 3,4-dihydroxycinnamic acid], ellagic acid [(**4**), 2,3,7,8-tetrahydroxy-[1]benzopyrano[5,4,3-cde][1]benzopyran-5,10-dione], hispidin [(**5**), 6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone)], davallialactone [(**6**), 3-(3,4-dihydroxyphenyl)-6-(2-(3,4-dihydroxyphenyl)ethenyl)-3a,4-dihydro-8-hydroxy-4-(2-oxopropylidene)-1H,3H-Furo(3,4-c)oxepin-1-one], interfungins A (**7**), hypholomine B (**8**), inoscavin A (**9**), and methyldavallialactone [(**10**), 3-(3,4-dihydroxyphenyl)-6-(2-(3-methyl-4-hydroxyphenyl) ethenyl)-3a,4-dihydro-8-hydroxy-4-(2-oxopropylidene)-1H,3H-Furo(3,4-c)oxepin-1-one] (Fig. 3). Compounds **1-4** were identified as phenolic compounds, and compounds **5-10** were hispidin derivatives

On-line HPLC-DPPH. The on-line HPLC-DPPH method can be used for the rapid assessment of antioxidant compounds or, more precisely, for the radical-scavenging capacity of compounds in complex mixtures, particularly plant extracts. Combined UV (positive signals) and DPPH• quenching (negative signals) chromatograms under gradient conditions of the EtOAc fraction from the fruiting body of *P. linteus* are presented in Fig. 3. Several eluted phytochemicals in the EtOAc fraction were detected and gave positive peaks on the UV detector at 220 nm. All of the detected peaks from compounds **1-10** showed a hydrogen-donating capacity (negative peak) toward the DPPH radical at the applied concentration. It is well-known that DPPH• absorbs 517 nm light. The more rapidly the absorbance decreases, the more potent the antioxidant capacity of the compound is in terms of its hydrogen-donating ability [Bandoniene and Murkovic, 2002a; 2002b]. The results confirmed that this on-line assay can be useful for rapid screening antioxidant

Table 2. Contents of major phytochemicals and radical-scavenging effect of compounds 1-10 isolated from the ethylacetate fraction of *P. linteus*

Compounds[1)]	Contents[2]	DPPH (IC_{50}, μM)	Superoxide anion radical-scavenging capacity[3)] (nmol Trolox equivalent per mg dw)
1	0.63±0.01	154.6	2.33
2	1.63±0.01	37.7	<0.79
3	4.42±0.02	55.7	3.39
4	12.77±0.03	25.8	1.69
5	5.96±0.01	109.8	2.08
6	10.93±0.02	19.6	2.95
7	6.24±0.01	18.5	1.61
8	21.91±0.05	51.7	2.73
9	4.11±0.01	76.5	1.61
10	4.79±0.01	52.2	2.51
Quercetin[4)]	N.D.[5)]	44.0	N.D.

Data are mean±SD values (n=3).
[1)]Their names and structures are shown in Fig. 1, [2)]mg/g methanolic extract, [3)]Compounds 1-4 at 100 mM; compounds 5-10 at 40 mM, [4)]Reference control in this study, [5)]N.D.: not determined

compounds, without the need to isolate and purify non-target photochemicals.

Contents and antioxidant capacities of the major active compounds in the extract of *P. linteus*. According to the screening results from the on-line HPLC-DPPH system, three major phytochemicals, compounds **4**, **6**, and **8**, were found in the EtOAc fraction, and their amounts were determined to be 12.77±0.03, 10.93±0.02, and 21.91±0.05 mg/g of MeOH extract dry wt., respectively, by recalculating with their relative percentage of individual area to the total area of MeOH extract from the HPLC (at 220 nm) profiles (Table 2). To determine the antioxidant capacities of compounds **1-10** from the EtOAc fraction, DPPH and superoxide anion radical-scavenging assays were performed (Table 2). Quercetin, a well-known antioxidant compound, was used as a positive control. The results revealed that compounds **2**, **4**, **6**, and **7** showed significant inhibitory capacities (IC_{50} values: 37.7±0.9, 25.8±0.1, 19.6±0.2, and 18.5±0.3 μM, respectively) against the DPPH radical, that were stronger than that of quercetin (44.0±0.8 μM), a well-known antioxidant. The data was consistent with previously reported results from hispidin derivatives isolated from other medicinal mushrooms and how their exhibition of strong antioxidant capacity [Wang *et al.*, 2005; Lee and Yun, 2007]. Moreover, based on the superoxide anion radical-scavenging capacity, compounds **1-4** were determined at 100 mM because those phenolic compounds could not be measured at 40 mM due to relatively low capacity. Compound **6** showed the largest inhibitory capacity (2.95±0.11 nmol Trolox equivalent per mg dry wt.) at 40 mM followed by compounds **8** and **10**, accounting for 2.73±0.12 and 2.51±0.04 nmol, respectively.

Compounds **6-10** are highly oxygenated and functionalized aromatic compounds that possess a unique basic structural unit, namely, the 6-[2-(3,4-dihydroxyphenyl) ethenyl]-4-hydroxy-2H-pyran-2-one (hispidin, **5**) moiety. From the viewpoint of biogenesis, it was reported that compound **8**, which has an unusual structure comprising 2,3-dihydro-4-H-furo[3,2-c]pyran-4-one, may be biosynthesized by the oxidation coupling of two hispidin moieties [Wang *et al.*, 2005], while compounds **6** and **9** may be biosynthesized by rearrangement of compound **7,** which is biosynthesized by the condensation of hispidin and 6-(3,4-dihoxryophenyl)-4-hydroxy-3,5-hexadiene-2-one (hispolon) [Lee and Yun, 2007]. These results indicated that hispidin dimers, such as hispidin with hispidin or hispolon, had a stronger negative effect on radical-scavenging capacity than hispidin alone. In relation to inhibitory capacities, the number of hydroxyl groups exhibited a positive relationship and the presence of methyl groups on the D-ring exhibited a negative relationship.

In conclusion, this is the first report to address the bioactive compounds from the fruiting body of *P. linteus*. Accordingly, ten specific antioxidants were isolated and identified from the extract of *P. linteus*. In particular, compounds **4**, **6**, and **8** were found to be three major bioactive phytochemicals. Especially, compound **6** exhibited excellent inhibitory capacity against free radicals. Thus, given the antioxidant capacity of the fruiting body of *P. linteus,* it might have good potential for use in natural health products.

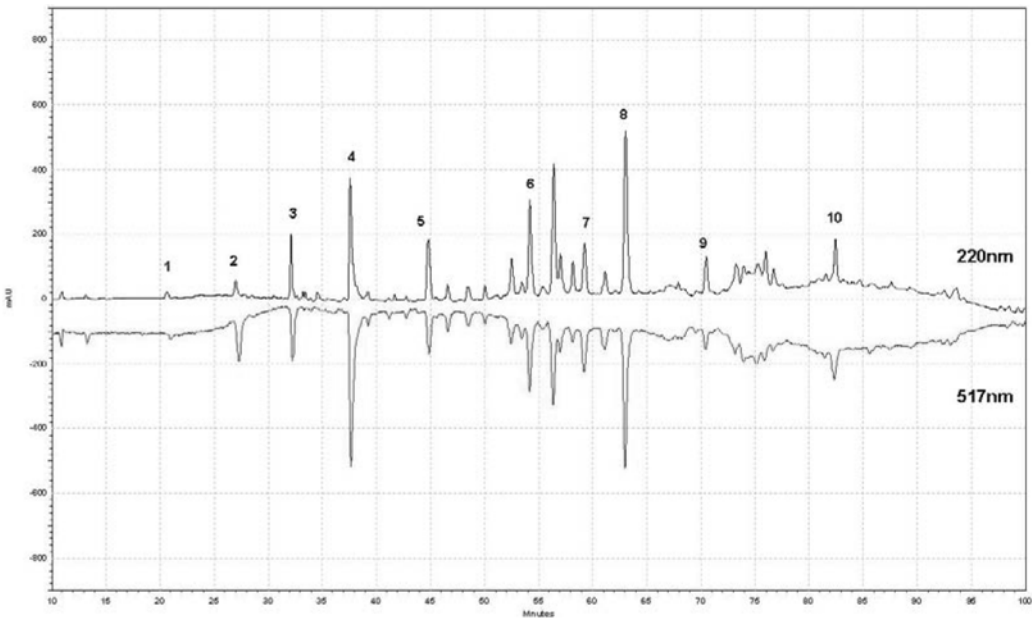

Fig. 4. UV and DPPH radical quenching chromatograms of the EtOAc fraction extracted from the fruiting body of *P. linteus*. The names and structures of peak no. 1-10 are shown in Fig. 3.

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