Antioxidant Small Phenolic Ingredients in *Inonotus obliquus* (persoon) Pilat (Chaga)

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Inonotus obliquus **(persoon) Pilat (Chaga, in Russia, kabanoanatake in Japan) is a fungus having been used as a folk medicine in Russia and said to have many health beneficial functions such as immune modulating and anti-cancer activities. In the present study, the antioxidant activity of hot water extract (decoction) of Chaga was precisely compared with those of other medicinal fungi (*Agaricus blazei Mycelia*, *Ganoderma lucidum* and *Phellinus linteus*) showing Chaga had the strongest antioxidant activity among fungi examined in terms of both superoxide and hydroxyl radicals scavenging activities. Further determination of the antioxidant potential of isolated fruiting body (brown part) and Sclerotium (black part) revealed the 80% MeOH extract of fruiting body had the highest potential as high as that of Chaga decoction. Finally, seven antioxidant components were isolated and purified from the 80% MeOH extract of Chaga fruiting body, and their chemical structures were determined as small phenolics as follows: 4-hydroxy-3,5-dimethoxy benzoic acid 2-hydroxy-1-hydroxymethyl ethyl ester (BAEE), protocatechic acid (PCA), caffeic acid (CA), 3,4-dihybenzaladehyde (DB), 2,5-dihydroxyterephtalic acid (DTA), syringic acid (SA) and 3,4-dihydroxybenzalacetone (DBL). Notably, BAEE was assigned as the new compound firstly identified from the natural source in the present study.**

Key words *Inonotus obliquus* (persoon) Pilat; Chaga; fungus; small phenolic; antioxidant ingredient

Inonotus obliquus (persoon) Pilat (Chaga in Russia, Kabanoanatake or Chaga in Japan) is a fungus habiting as parasitism on birch in the cold latitudes of Europe, Japan and Korea. In Russia, the black shapeless overgrowth of fungus after 10—15 years of parasitism on trunks, mostly of birch, is usually called Chaga and has been used for medicinal preparations.[1] Recently, many reports have been published concerning the health promoting functions of Chaga, for example, protection of DNA damage from oxidative stress,[2] anti-inflammatory,[3] anti-nociceptive[3] and anti-tumor activities.[4,5] However, no report has been published on the chemical structures of active ingredients from Chaga except some terpenoids until now.[6]

On the other hand, it is known oxidative stress is involved in variety of disorders[7—9] such as cancer, hypertension, neurodegenerative (Alzheimer's and Parkinson's disease) and autoimmune diseases, and thus many antioxidant ingredients from foods or other natural sources are being challenged for diseases protection and treatment.[10,11] In this context, antioxidant property of Chaga attracts much attention. Although it was reported that polyphenolic fraction of Chaga extract showed antioxidant activity, the structures of the antioxidant principle were left unclear.[12]

Chaga is comprised of two eye-distinguishable parts, black (mainly outside) and brown (mainly inside) that were previously classified as Sclerotium (ST) and Fruiting body (FB), respectively, by T. Mizuno *et al.*,[13] but most of Chaga products in Japan were processed without distinguish them. Therefore, in the present study, the antioxidant potential of hot water extract (decoction) of Chaga was compared first with those of other medicinal fungi[14] (*Agaricus blazei Mycelia*, *Ganoderma lucidum* and *Phellinus linteus*). Then, ST and FB parts were separated and precisely examined their antioxidant property to identify the antioxidant ingredients.

Experimental

Chemicals 1,1-Diphenyl-2-picrylhydrazyl (DPPH), BHT, protocatechuic acid, syringic acid diethylene triamine-*N*,*N*,*N*′,*N*″,*N*″-penta acetic acid (DTPA) and 2-morpholinpethanesulfonic acid (MES) were purchased from Wako Pure Chemical Industries, Ltd. 6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), butyl hydroxy toluene (BHT), 2,5-dihydroxyterephtalic acid, 3,4-dihydroxybenzaldehyde and caffeic acid from Sigma-Aldrich. Dimethylpyroline oxide (DMPO) and xsanthine oxidase (XOD, 20 U/ml, from cow milk) were from LABOTEC Co., Ltd., and Boehringer Mannheim Co., Germany, respectively.

Chaga (*Inonotus obliquus* (persoon) Pilat) (Russian product) and other fungi were provided by Isukra Industry Co., Ltd., Tokyo.

Chaga Decoction and Extracts Preparation For preparing decoctions, 1 g of dry Fungi samples (*Agaricus blazei Mycelia*, *Ganoderma lucidum*, and *Phellinus linteus* and Chaga) was gently boiled in 10 ml of H_2O for 1 h. After cooling down to the room temperature, the solution was diluted with H_2O to get the volume back to the original. Then the extract was centrifuged at 3400 rpm for 10 min. The supernatant thus obtained was stored in a freezer at −20 °C until use.

Both decoctions and extracts were prepared from fruiting body (FB) and sclerotium (ST) of Chaga, respectively. The extracts were prepared as follows; The powders (1 g) prepared from ST and FB portions of Chaga were extracted by 10 ml of H_2O or aqueous MeOH solutions (20%, 50%, 80% MeOH) overnight at R.T. The solutions were centrifuged at 3400 rpm for 10 min and the supernatants were freeze-dried and stored at −20 °C.

Isolation and Identification of Antioxidant Ingredients from the FB of Chaga The steps for active ingredients purification were summarized in Chart 1. The FB powder of Chaga (dry weight 320 g) was extracted with 80% aqueous MeOH (1 l×5 times) at room temperature (R.T.). The extract was dried up under reduced pressure to give the 80% MeOH extract (22.8 g). The 80% MeOH extract was then partitioned between ethyl acetate (EtOAc) and H_2O to afford EtOAc soluble fraction (1-a) (12.6 g) and H_2O soluble fraction (1-b) (10.2 g). The 1-a fraction was subjected to Diaion HP-20 column and eluted stepwisely with the following solvents to obtain 5 fractions (2-a—e); 30% MeOH (2-a), 50% MeOH (2-b), 75% MeOH (2-c), 100% MeOH (2-d) and EtOAc (2-e) fractions. The 2-a and 2-b fractions recovered were further separated by HPLC using a reverse phase C-18 preparative column with 4% CH_3CN (0.01% TFA) as the elution solution to purify compound B (20 mg), C (11 mg) and G (11 mg), and then compound A (9 mg), D (4 mg) and E (10 mg) with 12% CH_3CN (0.01% TFA). F (53 mg) was recovered from 2-b in the fraction eluted with 12% CH_3CN (0.01% TFA). The chemical structures of purified compounds were determined by MS and NMR as shown below. Assignment of compounds B, C, D, E, F and G were further made by HPLC using respective authentic standards commercially available. NMR data are given here only for compound A as the new compound from natural source.

^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded by Bruker DPX-400 spectrometer in CD_3OD. Standard pulse sequence and pa-

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rameters were used for the measurement. ESI-MS (positive mode) was measured by a Micromass LCT Spectral data of identified compounds are given below.

Compound A. 4-Hydroxy-3,5-dimethoxy-benzoic acid 2-hydroxy-1-hydroxymethyl Ethyl Ester (BAEE): Yellow needles, $C_{12}H_{16}O_7$ HR-ESI-MS *m/z* 295.0817 $[M+Na]^+$ (Calcd for $C_{12}H_{16}O_7Na$ 295.0794); ^{1}H-NMR δ: 7.37 (2H, s, H-2, H-6), 4.14 (1H, dd, J=4.9, 4.9 Hz, H-8), 3.88 (4H, d, J=4.9 Hz, H-9, H-10), 3.89 (6H, s, 3-OCH_3, 6-OCH_3); ^{13}C-NMR δ 127.4 (C-1), 108.2 (C-2, C-6), 154.5 (C-3, C-5), 141.4 (C-4), 169.3 (C-7), 84.9 (C-8), 62.2 (C-9, C-10), 3.89 (3-OCH_3, 5-OCH_3).

Compound B. Protocatechuic Acid (PCA): White powder. $C_7H_6O_4$ HR-ESI-MS *m/z* 155.0348 $[M+H]^+$ (Calcd for $C_7H_7O_4$ 155.0344).

Compound C. 3,4-Dihydroxybenzaldehyde (DB): White needles $C_7H_6O_3$ HR-ESI-MS *m/z* 139.0413 $[M+H]^+$ (Calcd for $C_7H_7O_3$ 139.0395).

Compound D. Caffeic Acid (CA): Yellow needles $C_9H_8O_4$ HR-ESI-MS *m/z* 181.0422 $[M+H]^+$ (Calcd for $C_9H_9O_4$ 181.0417).

Compound E. Syringic Acid (SA): White powder $C_9H_{10}O_5$ HR-ESI-MS *m/z* 199.0588 $[M+H]^+$ (Calcd for $C_9H_{11}O_5$ 199.0606).

Compound F. 3,4-Dihydroxybenzalacetone (DBL): Yellow needles $C_{10}H_{10}O_3$ HR-ESI-MS *m/z* 179.0727 $[M+H]^+$ (Calcd for $C_{10}H_{11}O_3$ 179.0708).

Compound G. 2,5-Dihydroxyterpatalic Acid (DTA): Yellow powder $C_8H_6O_6$ HR-ESI-MS *m/z* 199.0243 $[M+H]^+$ (Calcd for $C_8H_7O_6$ 199.0243).

Measurement of Antioxidant Activity by ESR Scavenging activity toward hydroxyl radical (·OH) was measured for the decoctions of fungi by spin trapping ESR method reported previously.[15] Ultraviolet light (UV)–H_2O_2 system was used as ·OH generator.[16] Reaction solution containing an aliquot of sample solution (100 μl), 150 mM DMPO (80 μl) and 50 mM H_2O_2 (120 μl) in total volume of 300 μl was taken into a hematocrit caprillary tube and then irradiated with UV light (254 nm) for 5 min. DMPO–OH signal was measured by the ESR at just 1 min after UV illumination.

Scavenging activity toward Superoxide anion radical ($O_2^{\cdot-}$) was determined according to the method reported elsewhere.[17] Hypoxanthin/xanthine oxdase system was used as $O_2^{\cdot-}$ generator. To pH 7.4 PBS buffer (170 μl), 2 mM hypoxanthine (160 μl), 10 mM DTPA (60 μl), 9.2 M DMPO (20 μl) and various concentration of the sample solution was added under stirring condition and then followed by the addition of 0.4 U/ml xanthine oxidase (160 μl). The mixed solution was further stirred for 60 s at R.T., taken up in a hematocrit capillary tube and was subjected to ESR spectra measurements.

ESR spectra were determined using JEOL LES-TE 200 ESR spectrometer (X-band Microwave Unit) under the condition as follows: microwave power; 8 mV/MHz, microwave frequency; 9.18 GHz, modulation amplitude; 0.1—0.2 mT, time constant; 0.03 s, sweep time; 0.5 min, fields; 314.0±10 mT, sweep width ±10 mT.

Measurement of DPPH Scavenging Activity DPPH scavenging activity was measured as reported previously.[15] To the mixure containing 0.5 mM DPPH (80 μl) in MeOH solutions and 0.1 M MES buffer (80 μl) in 50% MeOH (pH 6.0) in microplates, an aliquot of sample aqueous solution was added to make the total volume of 200 μl. After 10 min of the reaction, the optical density at 570 nm was measured with microplate reader (BIO-RAD Model 550).

Measurement of Ferric Reducing Power (FRP) FRP potential of the Chaga extracts and ingredients were determined according to the method of Gow-Chin Yen *et al.*[18] The reaction solutions (40 μl) containing different concentrations of sample in 0.2 M PBS (pH 6.6) were mixed with 30 mM aqueous potassium hexacyanoferrate $K_3[Fe(CN)_6]$ solution (100 μl). After incubating for 20 min at 50 °C, 10% trichloroacetic acid (100 μl) was added and centrifuged at 3000 rpm for 10 min. The supernatant (50 μl) was mixed with 1.7 mM aqueous $FeCl_3$ (150 μl) and absorbance at 655 nm was determined by microplate reader. The data are presented as BHT equivalent of sample solution at 1 mg/ml.

Results

Antioxidant Activity of Fungal Extracts Superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical (·OH) scavenging activities were compared for the decoctions of four different fungi samples (Chaga, *Agaricus blazei Mycelia*, *Phellinus linteus* and *Ganoderma lucidum*) by ESR spin trapping method. Results revealed that Chaga extract has the strongest antioxidant activity among fungi extracts examined, especially toward $O_2^{\cdot-}$ scavenging. For example, IC_{50} of $O_2^{\cdot-}$ scavenging activity of Chaga (*Inonotus obliquus*) was 35 μg dry weight/ml but was 1306 μg dry weight/ml for the next strongest *Phellinus linteus*. The $O_2^{\cdot-}$ scavenging activity of Chaga was thus approximately 60 times stronger than *Agaricus blazei Mycelia* (IC_{50} 2106 μg dry weight/ml) when compared by IC_{50} (Table 1). Chaga also showed the strongest ·OH scavenging activity (IC_{50} 140 μg dry weight/ml) among the four fungus samples examined, and then *Phellinus linteus* (IC_{50} 272 μg dry weight/ml) followed.

Table 1. Superoxiside and Hydroxyl Radical Scavenging Activities of Decoctions from Medical Mushrooms, IC_{50} [μg Dry Fungus/ml]

Fungi sample	$O_2^{\cdot-}$ scavenging activity IC_{50} [μg/ml]	·OH scavenging activity IC_{50} [μg/ml]
Inonotus	35	1432
Ganoderma	1702	11300
Phellinus	1306	2720
Agaricus	2106	3461

Antioxidant Activity of Chaga Extracts from ST and FB Parts Decoctions and solvent extracts (H_2O and increased concentrations of MeOH as extraction solvent) were prepared from ST and FB parts, respectively. Resultant extracts and decoctions were determined their antioxidant potential by DPPH scavenging and FRP activities. In these antioxidant assays, both decoctions and 80% MeOH extracts both from ST and FB parts showed relatively high activity among the preparations examined. In the MeOH extracts, both DPPH scavenging and FRP activities were increased depending on MeOH concentrations so that the strongest activity was seen in 80% MeOH extracts among the aqueous MeOH extracts obtained both ST and FB parts (Fig. 1). Among ST part preparations (both extracts and decoction), decoction sample (DPPH IC_{50}; 60.2±2.18 μg dry residue/ml, FRP; 1.49±0.05 mM BHT as equivalent) showed the highest activity in both DPPH scavenging and FRP assays and then 80% MeOH extract followed (DPPH IC_{50}; 84.3±13.7 μg dry residue/ml, FRP; 1.10±0.08 mM as BHT equivalent). On the other hand, the 80% MeOH extract showed the highest activity (DPPH IC_{50}; 65.5±3.43 μg dry residue/ml, FRP; 1.38±0.04 mM as BHT equivalent) among the FB part preparations. The activity profile of FRP was almost the same as DPPH scavenging activity among the preparations both from ST and FB parts. However, it was notified that the activity profile of DPPH scavenging and FRP was not same for the aqueous MeOH extracts from ST and FB parts. The DPPH activity recovered in the 0 to 50% MeOH extracts was almost same for both FB and ST parts but the recovery of FRP activity was low in the fractions from ST compared to FB (Fig. 1). When DPPH scavenging and FRP activities were compared among all the aqueous MeOH extracts, the 80% MeOH extract of FB part showed the strongest activity and the activity was almost same as the decoctions. Therefore, farther study to identify the active ingredients was carried out using the 80% MeOH extract of FB part.

Isolation and Determination of Active Principles and Their Antioxidant Activity The 80% MeOH extract (23.8 g) was farther fractionated into two fractions (1-a (12.6 g), 1-b (11.2 g)) by solvent extraction (see Chart 1). The fraction 1-a showed approximately 4.4 times stronger

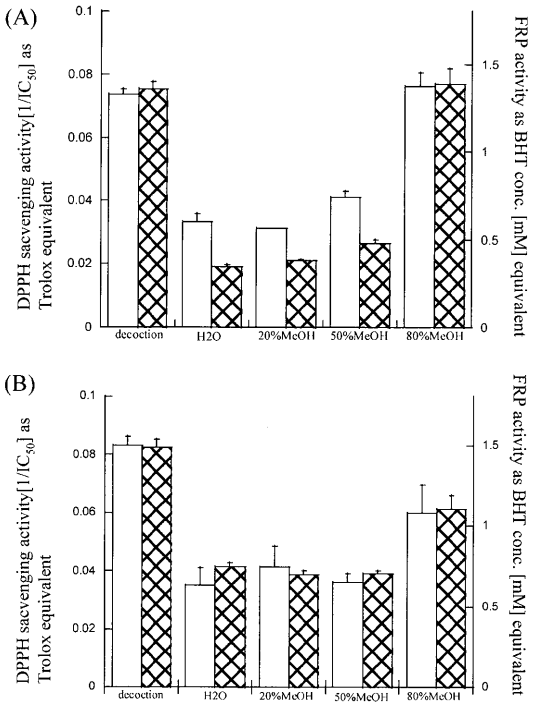

Fig. 1. Antioxidant Activities of Chaga Extracts Prepared by Various Solvents

□: DPPH scavenging activity was showed Trolox equivalent $1/IC_{50}$ [μg/ml]$^{-1}$, ▨: FRP activity expressed as BHT equivalent [mg/ml] was determined at 1 mg/ml concentration. (A) FB part extracts, (B), ST part extracts.

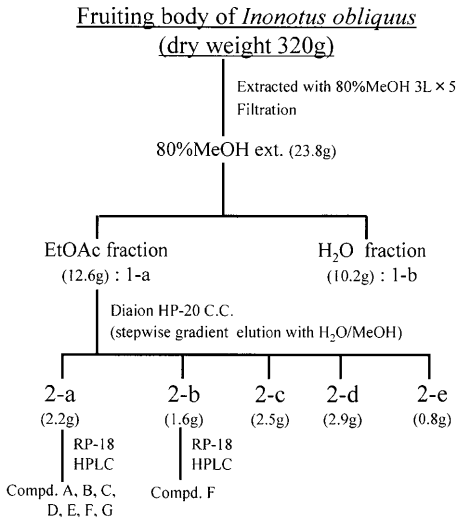

Chart 1. Isolation Flow Chart of Active Ingredients from 80% MeOH Extract of FB Part of Chaga

DPPH scavenging activity than 1-b (IC_{50}: 1-a; 51.3±0.03 μg dry residue/ml, 1-b; 226.2±9.51 μg dry residue/ml). Therefore, 1-a was farther separated into 5 fractions (2-a (2.2 g), b (1.6 g), c (2.5 g), d (2.9 g) and e (0.8 g)) by Diaion HP-20 column chromatography. As the results, 2-a and 2-b showed relatively high activity in both DPPH (IC_{50}: 2-a; 29.3± 2.05 μg dry residue/ml, 2-b; 41.6±18.07 μg dry residue/ml) and FRP (2-a; 4.43±0.45 mM as BHT equivalent, 2-b; 4.29± 0.36 mM as BHT equivalent) assays. The fraction 2-c (IC_{50}: 30.2±7.4 μg dry residue/ml) also showed as high activity in DPPH scavenging activity as those of 2-a and 2-b fractions, but FRP activity was lower than those fractions. Thus, 2-a

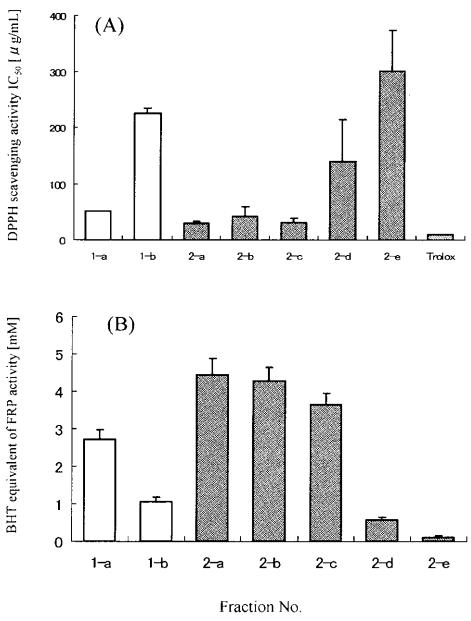

Fig. 2. Antioxidant Activities of FB Part Fractions of Chaga

(A) DPPH scavenging activity (IC_{50} [μg/ml]), (B) FRP activity (BHT equivalent [mM] at 1 mg/ml).

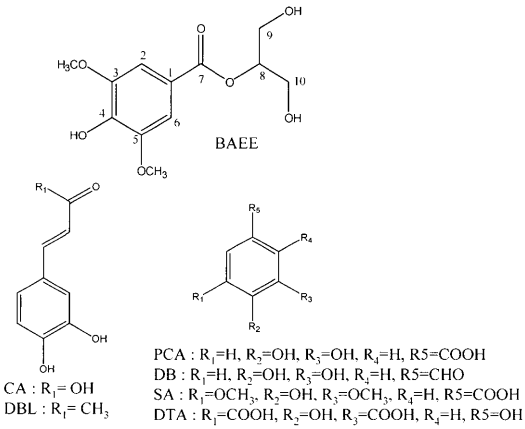

Fig. 3. Structures of Small Phenolic Components Isolated from FB Part of Chaga

and 2-b fractions were further fractionated by RP-18 HPLC and were isolated compounds A (8.8 mg), B (20.4 mg), C (11.4 mg), D (10.0 mg), E (21.0 mg), G (11.0 mg) and F (11.0 mg) from 2-a and compound E (31.6 mg) from 2-b, respectively. From the structural analysis by MS and NMR, compound A was assigned as 4-hydroxy-3,5-dimethoxy benzoic acid 2-hydroxy-1-hydroxymethyl ethyl ester (BAEE) as a new compound firstly isolated from the natural source. Other six were assigned as follows: protocatechuic acid (PCA) as compound B, 3,4-dihydroxybenzaldehyde (DB) as compound C, caffeic acid (CA) as compound D, syringic acid (SA) as compound E, 3,4-dihydroxybenzalacetone (DBL) as compound F and 2,5-dihydroxyterpathalic acid (DTA) as compound G, respectively (Fig. 3).

Antioxidant potentials of those isolated phenolics are shown in Table 2. All of the seven components showed antioxidant activity, especially, DB had the strongest activity in both DPPH scavenging and FRP activities. DB, CA, DBL and DTA showed higher DPPH scavenging activity than

Table 2. Antioxidant Activities of Phenolic Components from Chaga

	DPPH activity IC_{50} [μM]	FRP activity as BHT equivalent [mM]
PCA	53.37±0.34	4.99±0.04
DB	18.06±0.18	5.74±0.30
CA	41.42±0.63	5.46±0.09
SA	50.80±0.26	2.45±0.05
BAEE	345.41±15.07	0.55±0.01
DBL	27.75±0.24	4.60±0.16
DTA	24.84±0.48	1.64±0.09
Trolox	42.00±0.17	

DPPH scavenging activities were shown in IC_{50} [μM], and FRP activities in BHT equivalent [mM] at 1 mM of test samples. Values are expressed as mean±standard deviation.

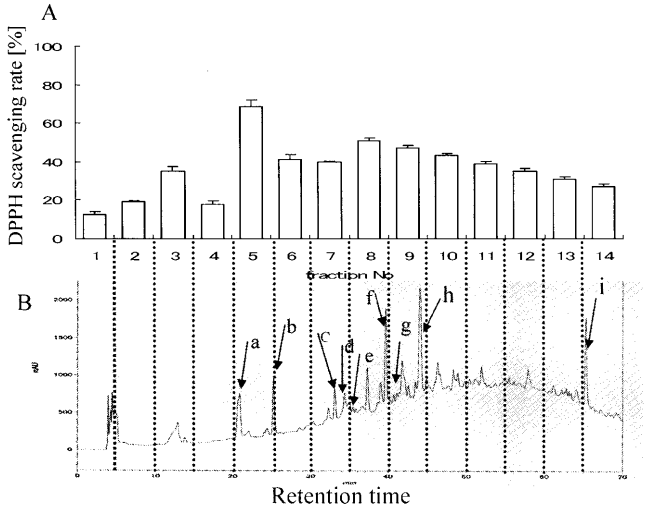

Fig. 4. Antioxidant Activity and Components Distribution Profile in HPLC Chromatogam of 80% MeOH Extracts of FB Part

(A) DPPH scavenging activity of each fraction. (B) Chromatogram of 80% MeOH extract of FB part. a, PAC; b, DB; c, CA; d, SA; e, BAEE; f, DBL; g, DTA; h, unknown peak; i, unknown peak. HPLC conditions were follows: Mightysil RP-18 column (250×4.6 mm i.d., 5 μm), flow rate at 0.7 ml/min. Solvent gradient 1%/min increment from 5 to 80% MeOH containing 0.01% TFA. Monitor wavelength at 245 nm.

Trolox used as reference antioxidant. All the phenolics isolated also showed higher FRP potentials than BHT determined as reference antioxidant except DBL. The activity order for DPPH scavenging and FRP potential were DB>DTA>DBL>CA>Trolox>SA>PCA>BAEE and DB>CA>PCA>DBL>SA>DTA>BHT>BAEE, respectively.

On the other hand, HPLC chromatogram of 2-c fraction (data not shown) revealed that the fraction contains only unknown peak i (see Fig. 4B) and a broad band peaks as seen in the fractions No. 10—12 in Fig. 4B.

Components and Antioxidant Activity Profile of 80% MeOH Extracts of Chaga HPLC chromatogram of 80% MeOH extract of FB part and the DPPH scavenging activity profile in the fractions are given in Fig. 4. It was revealed that PCA, DB, CA, DBL and two unknown peaks were the major components in the 80% MeOH extract. Further, the profile of antioxidant activity distribution in the chromatogram showed rather good consistency with the retention times of presently identified phenolics, such that the major phenolics (PCA, DB and DBL) were distributed in No. 5 and No. 8 fractions which showed relatively high antioxidant activity among the fractions (Fig. 4). Another major peak (unknown peak h) was present in the fraction 9, but was left unidentified.

Discussion

Agaricus blazei Mycelia, *Phellinus linteus*, *Ganoderma lucidum* and *Inonotus obliquus* are the fungi having been used as folk medicines and there are many reports published on anticancer and anti-inflammatory activities.[14,19—21] In this study, the antioxidant activities of these medicinal fungi were comparatively examined first, because oxidative stress plays critical roles in many diseases including cancer and inflammatory disease.[7—9] The results revealed that the antioxidant activity of Chaga decoction was considerably stronger than those of other fungi decoctions, especially in the $O_2^{\cdot-}$ scavenging activity.

Chaga, shapeless overgrowth of *Inonotus obliquus* (persoon) Pilat is comprised of two eye-distinguishable parts, sclerotium (ST) and fruiting body (FB). In this study, the extracts and decoctions were made separately from ST and FB parts, and their antioxidant potentials were precisely examined. Both decoctions of ST and FB parts showed almost same antioxidant potential when evaluated by DPPH scavenging and FRP assays. On the other hand, aqueous MeOH extracts prepared from ST and FB parts showed different antioxidant profile. For example, in the extracts prepared from FB part, both DPPH scavenging and FRP activities increased depending on the MeOH concentration and thus the 80% MeOH extract showed the highest activity. In the ST part preparations, however, the antioxidant recovery profile in these fractions showed less MeOH concentration dependency. Although the 80% MeOH extract showed higher activity than lower MeOH extracts also in the ST preparations, both DPPH scavenging and FRP activities in the 80% MeOH extract were considerably lower than these of FB preparation. In addition, the recovery profiles of DPPH scavenging and FRP activity in 0 to 50% MeOH extract was different between FB and ST preparations such that FRP activity were recovered less than DPPH activity in the 0 to 50% MeOH extract of FB. This indicates the properties of antioxidant principles are different in FB and ST part of chaga.

For the determination of active ingredients of Chaga, the 80% MeOH extract of FB part was examined as the source for isolation because the antioxidant activity of the 80% MeOH extract from FB part was the highest among the aqueous MeOH extracts and was also the same level as those of decoctions from both FB and ST parts.

Solvent partition of the 80% MeOH extracts of FB part revealed that the antioxidant activity was concentrated in EtOAc fraction (1-a) indicating the active compounds were rather hydrophobic. Finally, seven phenolic compounds were isolated from the further separated fractions 2-a and 2-b, and their structures were chemically assigned as PCA, CA, SA, DB, DTA, BAEE and DBL (Fig. 3). Although 2-c fraction also showed strong antioxidant activity as high as these of 2-a and 2-b (Fig. 4), the HPLC chromatogram (data not shown) showed the presence of large noncharacteristic broad band peak as appeared in the fractions 10—12 in Fig. 4B together with many small peaks including unknown peak i. It was thus considered that polymeric compounds such as melanin[22] played major role in the antioxidant activity in this fraction. Although the content of unknown peak h and i looked rather

high in the chromatogram of 80% MeOH extract of FB part as shown in Fig. 4B, their contribution to the antioxidant property of Chaga might not be large. The reason in that the fraction No. 14 in Fig. 4 containing unknown peak i showed only weak DPPH scavenging activity. Similarly, the antioxidant activity of fraction 9 in Fig. 4B was not higher than that of fraction 8, even though the fraction 9 contains larger amount of unknown peak h than DBL which located nearly between fraction 8 and 9. However, further study will be needed to clarify the contribution of unknown peak h.

Among the seven phenolics isolated here from the FB of Chaga, BAEE and DTA were unique because no report has been published on these compounds isolated from natural source so far, although DTA is commercially available as a reagent. Other phenolics (PCA, SA, DB and CA) have been isolated from many plants, and DBL from other Fungi.[23—26] Moreover, all these small phenolic compounds have not been discussed to data as the antioxidant components in Chaga. Antioxidant properties of PCA, CA, SA, DB and DBL have been studied elsewhere both *in vitro* and *in vivo*,[24,27—31] but not for BAEE, thus the antioxidant activities of seven phenolics were determined under the same condition and the result was summarized in Table 2. It was revealed that methylation of adjacent OH groups of free OH at 4 position caused significant decrease of FRP activities such as BAEE and DTA. Among these seven components, PCA, DB and DBL were suggested to be the major contributor for the antioxidant potential of 80% MeOH extract of FB part of Chaga. Especially, DBL was considered to be the principal antioxidant component in the extracts of FB part thus in Chaga, since the content of DBL was approximately 2 times larger than other components such as DB and PCA that were also major in the 80% MeOH extracts of both FB and ST part (data not shown). When HPLC chromatogram of ST part was compared with FB, the contents of these seven components were less than those in 80% MeOH extract of FB (date not shown). Indeed, the antioxidant activity of the 80% MeOH extract of ST part was significantly lower than that of FB part (Fig. 1).

The chemical structure of unknown peak h was left unassigned in the present study, although certain antioxidant activity was expected. It may be, however, concluded that 80% MeOH extract of FB part contained no more meaningful ingredients contributing to the antioxidant activity other than seven components identified here.

Further, it was notified that HPLC chromatograms of decoctions of both FB and ST parts were greatly different from that of 80% MeOH extract (data not shown). Presently identified seven phenolic compounds were not significantly determined in the decoctions of both ST and FB parts, although the antioxidant potential of decoctions were as high as those of 80% MeOH extracts (see Fig. 1). It was thus suggested that the antioxidant principles in the decoctions might not be the small phenolics but other substances such as Melanin that was reported elsewhere in the decoction of Chaga as the antioxidant ingredients[12] or other phenolic polymers. Further study is now underway to figure out the significant contribution of these small molecular phenolics to the physiological function of Chaga.

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