Original paper

Isolation of Aromatic Compounds Produced by *Eurotium herbariorum* NU-2 from Karebushi, a Katsuobushi, and their DPPH-Radical Scavenging Activities

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Aromatic compounds were isolated from an extract of *Eurotium herbariorum* NU-2, which is used in the process of manufacturing karebushi (a katsuobushi). The seventeen compounds were identified as anthraquinone derivatives (A1-A6), eurotinone derivatives (B1, B2), indole-containing diketopiperazine derivatives (C1-C6), and benzaldehyde derivatives (D1-D3). 2-Methyleurotinone (B1), isoechinulin A (C6), tetrahydroauroglaucin (D1), isodihydroauroglaucin (D2), and flavoglaucin (D3) exhibited significantly higher DPPH (1, 1-diphenyl-2-picrylhydrazyl) scavenging activity compared to α -tocopherol, a standard radical scavenger ($P < 0.05$). Asperflavin (A4), isoechinulin B (C1), neoechinulin B (C2), and variecolorin O (C4) had similar activity to α -tocopherol. The extract of *E. herbariorum* NU-2 exhibited high radical scavenging activity compared to other filamentous fungi from katsuobushi, and also contained higher levels of isoechinulin B (C1), echinulin (C5), tetrahydroauroglaucin (D1), and flavoglaucin (D3). The concentration of tetrahydroauroglaucin (D1) in the filamentous fungus extract was highly correlated with the radical scavenging activity.

Keywords: aromatic compound, DPPH radical, *Eurotium herbariorum*, isolation, katsuobushi, scavenging activity

Introduction

Filamentous fungi are used in the manufacture of traditional Japanese fermented foods, including sake, shochu, shoyu, and miso. Fermented foods maintain their favorable qualities and resist oxidative damage during long fermentation periods because they have an antioxidative property, due to the presence of antioxidants (Hu *et al.*, 2004; Taira *et al.*, 2002). Filamentous fungi are thought to be involved in the preservation of fermented foods from oxidative deterioration by the production of secondary metabolites exhibiting antioxidative activity or by the conversion of food components to potent antioxidants (Lin *et al.*, 2006; Esaki *et al.*, 1997).

Fermented foods have been evaluated with respect to their *in vivo* biofunctions and roles in the promotion of health (Parvez *et*

al., 2006). The oxidative stress generated by excess free radicals or reactive oxygen species *in vivo* has been reported to oxidatively modify carbohydrates, lipids, proteins, and nucleic acids and to play a role in the promotion of cancer, arteriosclerosis, and complicating disorders of diabetes mellitus (Murray *et al.*, 2008). The antioxidants of radical scavengers have been shown to have a suppressive effect on oxidative stress *in vivo* and are related to the prevention of these diseases (Kalliora *et al.*, 2006). Functional foods, which contain radical scavengers, have a suppressive effect on oxidative stress and have been promoted for the prevention of life-style diseases and for general good health (Diplock *et al.*, 1998).

Katsuobushi is boiled in water and the resulting extract is used in Japan as a traditional seasoning, katsuo-dashi. There are two

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types of katsuobushi: “arabushi”, which is produced by smoking after boiling, and “karebushi”, which is produced by treating arabushi with mold. The process is a kind of fermentation using *Eurotium* spp. The fermentation imparts a favorable flavor and taste to the commercial product. *E. herbariorum* and *E. repens* are used as mold-starters of karebushi and have been reported to produce functional compounds with antioxidative activity, radical scavenging activity, anti-carcinogenic activity, and a suppressive effect on blood adhesion molecule expression (Miyake *et al.*, 2009; 2010a; 2010b). *E. herbariorum* NE-1, *E. herbariorum* NE-4, and *E. repens* KBN2062 were reported to produce antioxidants, isodihydroauroglauцин, auroglauцин, dihydroauroglauцин, tetrahydroauroglauцин, and flavoglauцин (Miyake *et al.*, 2009; 2010a). *Aspergillus* (*Eurotium*) *repens* MA0197 was reported to produce neoechinulin A, which has antioxidative properties (Yagi and Doi, 1999). *E. herbariorum* NU-2, a mold-starter used in making karebushi, has never been reported to contain radical scavengers; however, a lipase has been purified and characterized from this fungus (Kaminishi *et al.*, 1999). In the present study, we attempted to isolate the aromatic compounds produced by *E. herbariorum* NU-2 and to examine their radical scavenging activities.

Materials and Methods

Materials For the filamentous fungi used as karebushi mold-starters, *E. herbariorum* NU-2, *E. herbariorum* NE-1, and *E. herbariorum* NE-4 were obtained from Ninben Co. (Tokyo, Japan) and *E. repens* KBN2062 was obtained from Bioc Co. (Aichi, Japan). *E. repens* JCM23060, *E. chevalieri* JCM23047 and *E. chevalieri* NBRC4090, filamentous fungi isolated from katsuobushi, were obtained from JCM (Riken Bioresource Center, Ibaragi, Japan) and NBRC (National Institute of Technology and Evaluation, Chiba, Japan). The filamentous fungus YZ was isolated from commercial karebushi produced in Yaizu (Chikiri Shimizu Syoten Co., Ltd., Yaizu City, Shizuoka, Japan), and the filamentous fungus MZ was isolated from that produced in Makurazaki (Makurazaki Katuo Public Co., Makurazaki City, Kagoshima, Japan) by culture using M40Y medium (malt extract 20 g, yeast extract 5 g, sucrose 400 g, agar 20 g, distilled water 1.0 L). The reagents used in the present study were of analytical or HPLC grade (Wako Pure Chemical Industries, Osaka, Japan).

Preparation of *Eurotium* spp. extracts Filamentous fungi were precultured in M40Y medium for 7 days at 30°C. The fungal spores were then inoculated onto the slant medium, which had been added to 5 mL of culture medium in a test tube (φ18 x 180 mm), and cultured for 7 days at 30°C. Next, 5 mL of ethyl acetate was added to each test tube, and the tubes were ultrasonicated for 30 min. Individual fungal extracts were obtained from the supernatant after test tubes were centrifuged at 15,000 rpm for 5 min. Extracts were diluted ten-fold with ethanol and then subjected to DPPH radical scavenging activity assay and HPLC determination of aromatic

compounds.

Isolation of aromatic compounds from *E. herbariorum* NU-2 *E. herbariorum* NU-2 was inoculated into M40Y medium in seventy petri dishes (φ90 x 15 mm) and cultured for 9 days at 30°C. The fungi and cultured media were extracted with 2.0 L of ethyl acetate for 24 h at room temperature. The extract was concentrated under reduced pressure. The ethyl acetate extract (3.1 g) was subjected to silica gel column chromatography. The constituents were successively eluted with *n*-hexane-acetone (10:1, 4:1, 3:1, 2:1, 1:1), acetone, and MeOH to give 10 fractions. For each fraction, normal phase column chromatography on silica gel and preparative TLC using appropriate combinations of solvents (*n*-hexane, ethyl acetate, CHCl₃, CH₂Cl₂, diethyl ether, acetone, diisopropyl ether, benzene, and MeOH) resulted in isolation of the following compounds. From fraction 2 (*n*-hexane-acetone 10:1): tetrahydroauroglauцин (**D1**, 18.5 mg), flavoglauцин (**D3**, 4.7 mg), isodihydroauroglauцин (**D2**, 11.8 mg); from fraction 3 (*n*-hexane-acetone 4:1): physcion (**A5**, 4.5 mg); from fraction 4 (*n*-hexane-acetone 3:1): physcion-10,10'-bianthrone (**A1**, 9.0 mg); from fraction 6 (*n*-hexane-acetone 2:1): catenarin (**A2**, 7.0 mg), 2-*O*-methyl-9-dehydroeurotinone (**B2**, 2.5 mg), isoechinulin B (**C1**, 16.5 mg), questin (**A3**, 7.5 mg), neoechinulin B (**C2**, 7.5 mg); from fraction 7 (*n*-hexane-acetone 1:1): 2-methyleurotinone (**B1**, 3.0 mg), asperflavin (**A4**, 29.0 mg), questinol (**A6**, 4.0 mg), isoechinulin A (**C6**, 2.5 mg), neoechinulin A (**C3**, 18.0 mg), echinulin (**C5**, 9.0 mg), and varicolorin O (**C4**, 3.0 mg).

DPPH radical scavenging activity DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity was measured according to the standard method (Miyake *et al.*, 2009). One hundred microliters of 0.13 mg/mL DPPH dissolved in ethanol, 80 μL of 0.1 M Tris-HCl buffer (pH 7.4), and 20 μL of the sample (aromatic compounds and extract of filamentous fungi) were mixed. Each isolated aromatic compound was dissolved in dimethyl sulfoxide and added to the reaction mixture at a final concentration of 50 μM in this assay. The fungal extract, diluted ten-fold with ethanol, was added in this assay. Absorbance at 517 nm was recorded after 1-h incubation at room temperature. The negative control was run with a sample solvent. α-tocopherol was employed in the assay as a standard radical scavenger. Activity was calculated from the percentage decrease with respect to the negative control value. Each value (%) is represented as the mean of quadruplicate measurements. Differences were considered significant at *P* < 0.05 in comparison to α-tocopherol using Dunnett's multiple comparison procedure following one-way analysis of variance.

Determination of aromatic compounds by HPLC The concentration of aromatic compounds in each fungal extract was determined by HPLC (900 series, Jasco Co., Tokyo, Japan), using a YMC-ODS column (YMC-pack φ4.6 x 150 mm, S – 5 mm, YMC Co., Kyoto, Japan), UV detection at 280 nm, mobile solvents of methanol and water containing 5% acetic acid (0 – 10 min; 45% MeOH, 10 – 20 min; 45%→85% MeOH, 20 – 35 min; 85%

MeOH), a flow rate of 1 mL/min, and a column temperature of 40°C. Concentration (10 to 1000 µg/mL) of aromatic compounds was correlated with peak area. Individual calibration curves between the HPLC peak area and compound concentrations were calculated as $y = a x$ (where “y” is peak area, “x” is concentration (µg/mL) of compound, and “a” is constant). The individual compound constants are shown as follows: 23,920 (A1), 62,957 (A2), 39,011 (A3), 71,118 (A4), 15,863 (A5), 32,445 (A6), 6,049 (B1), 5,315 (B2), 20,478 (C1), 15,790 (C2), 8,597 (C3), 18,308 (C4), 24,233 (C5), 26,094 (C6), 42,977 (D1), 47,156 (D2), 37,008 (D3). For HPLC determination, each fungal extract was diluted to contain 1000 µg/mL or less of each compound. Each value (µg/mL) for the determination is shown as the mean of duplicate data. The correlation between DPPH radical scavenging activity and the aromatic compound concentration in each fungal extract was examined by calculation of a coefficient of determination (R^2).

Instrumental analysis of isolated compounds ^1H -NMR and ^{13}C -NMR spectra were recorded on a JNM-ECP-500 (JEOL) instrument (500 MHz for ^1H and 125 MHz for ^{13}C , Jeol, Tokyo, Japan). The EI-MS data were obtained with a Jeol JMS-700 spectrometer (Jeol). Anthraquinone derivatives (**A1**–**A6**), eurotinone derivatives (**B1**, **B2**), and indole-containing diketopiperazine derivatives (**C1**–**C6**) were identified by ^1H -NMR and EI-MS analyses.

Physcion-10,10'-bianthrone (A1) ^1H -NMR (CDCl_3 , 500 MHz) δ : 12.18 (1H, s), 12.13 (1H, s), 11.87 (1H, s), 11.82 (1H, s), 6.70 (1H, br s), 6.68 (1H, br s), 6.38 (1H, d, $J = 2.1\text{Hz}$), 6.37 (1H, d, $J = 2.1\text{Hz}$), 6.12 (1H, br s), 6.10 (1H, br s), 6.00 (1H, d, $J = 2.1\text{Hz}$), 5.97 (1H, d, $J = 2.1\text{Hz}$), 4.35 (1H, s), 4.34 (1H, s), 3.84 (3H, s), 3.82 (3H, s), 2.31 (3H, s), 2.29 (3H, s). EI-MS m/z : 538 (M^+ , 2), 270 (100), 269 (90), 255 (24), 241 (41), 227 (42), 198 (14).

Catenarin (A2) ^1H -NMR (acetone- d_6 , 500 MHz) δ : 13.38 (1H, br), 7.29 (1H, br s), 7.19 (1H, s), 6.62 (1H, br s), 2.31 (3H, s). EI-MS m/z : 286 (M^+ , 63), 214 (85), 213 (100), 167 (9).

Questin (A3) ^1H -NMR (acetone- d_6 , 500 MHz) δ : 13.24 (1H, s), 7.37 (1H, br s), 7.22 (1H, d, $J = 2.1\text{Hz}$), 6.97 (1H, br s), 6.79 (1H, d, $J = 2.1\text{Hz}$), 3.86 (3H, s), 2.31 (3H, s). EI-MS m/z : 284 (M^+ , 100), 266 (56), 255 (37), 238 (60), 210 (13), 197 (17).

Asperflavin (A4) ^1H -NMR (acetone- d_6 , 500 MHz) δ : 15.01 (1H, s), 9.20 (1H, br), 6.77 (1H, s), 6.60 (1H, d, $J = 2.1\text{Hz}$), 6.48 (1H, d, $J = 2.1\text{Hz}$), 3.89 (3H, s), 3.05 (1H, d, $J = 15.9\text{Hz}$), 2.98 (1H, br d, $J = 15.9\text{Hz}$), 2.82 (1H, d, $J = 16.8\text{Hz}$), 2.73 (1H, dd, $J = 16.8$, 1.5Hz), 1.36 (3H, s). ^{13}C -NMR (acetone- d_6 , 125 MHz) δ : 203.7 (s), 166.9 (s), 162.8 (s), 161.2 (s), 143.0 (s), 138.6 (s), 116.8 (d), 110.1 (s x 2), 102.9 (d), 98.4 (d), 70.6 (s), 56.1 (q), 52.5 (t), 44.2 (t), 29.2 (q). EI-MS m/z : 288 (M^+ , 100), 270 (13), 230 (49), 215 (13), 212 (16), 202 (10).

Physcion (A5) ^1H -NMR (CDCl_3 , 500 MHz) δ : 12.25 (1H, s), 12.05 (1H, s), 7.56 (1H, d, $J = 1.2\text{Hz}$), 7.30 (1H, d, $J = 2.4\text{Hz}$), 7.01 (1H, d, $J = 1.2\text{Hz}$), 6.62 (1H, d, $J = 2.4\text{Hz}$), 3.87 (3H, s), 2.38 (3H, s). EI-MS m/z : 284 (M^+ , 100), 255 (14), 241 (10), 213 (10).

Questinol (A6) ^1H -NMR (acetone- d_6 , 500 MHz) δ : 13.37 (1H,

s), 7.55 (1H, br s), 7.22 (1H, d, $J = 2.3\text{Hz}$), 7.14 (1H, br s), 6.78 (1H, d, $J = 2.3\text{Hz}$), 4.61 (2H, s), 3.85 (3H, s). EI-MS m/z : 300 (M^+ , 73), 282 (33), 271 (19), 254 (24), 241 (19), 225 (18).

2-Methyleurotinone (B1) ^1H -NMR ($\text{DMSO}-\text{d}_6$, 500 MHz) δ : 9.46 (1H, br s), 9.34 (1H, br s), 8.18 (1H, br s), 6.54 (1H, s), 6.30 (1H, d, $J = 2.7\text{Hz}$), 6.25 (1H, d, $J = 2.7\text{Hz}$), 3.80 (2H, br s), 3.70 (3H, s), 2.11 (3H, s). EI-MS m/z : 302 (M^+ , 100), 287 (27), 270 (67), 259 (27), 217 (25).

2-O-Methyl-9-dehydroxyeurotinone (B2) ^1H -NMR ($\text{DMSO}-\text{d}_6$, 500 MHz) δ : 10.02 (1H, br s), 9.50 (1H, br s), 6.62 (2H, s), 6.30 (1H, d, $J = 2.3\text{Hz}$), 6.26 (1H, d, $J = 2.3\text{Hz}$), 3.71 (5H, br s), 2.19 (3H, s). EI-MS m/z : 286 (M^+ , 100), 271 (22), 254 (24), 243 (33), 201 (28).

Isoechinulin B (C1) ^1H -NMR (CDCl_3 , 500 MHz) δ : 8.85 (1H, br s), 8.29 (1H, br s), 7.68 (1H, br s), 7.27 (1H, d, $J = 8.2\text{Hz}$), 7.26 (1H, s), 7.07 (1H, d, $J = 1.6\text{Hz}$), 7.04 (1H, dd, $J = 8.2$, 1.6Hz), 6.06 (1H, dd, $J = 17.4$, 10.4Hz), 5.61 (1H, d, $J = 1.2\text{Hz}$), 5.35 (1H, m), 5.22 (1H, dd, $J = 10.4$, 1.2Hz), 5.18 (1H, dd, $J = 17.4$, 1.2Hz), 4.99 (1H, d, $J = 1.2\text{Hz}$), 3.41 (2H, d, $J = 7.3\text{Hz}$), 1.72 (3H, s), 1.71 (3H, s), 1.52 (6H, s). ^{13}C -NMR (CDCl_3 , 125 MHz) δ : 157.8 (s), 155.7 (s), 144.4 (s), 144.2 (d), 135.1 (s), 133.6 (s), 132.8 (s), 132.2 (s), 126.1 (s), 124.2 (s), 124.0 (d), 123.4 (d), 118.0 (d), 113.4 (d), 113.4 (t), 111.2 (d), 102.8 (s), 102.0 (t), 39.3 (s), 34.5 (t), 27.4 (q x 2), 25.7 (q), 14.0 (q). EI-MS m/z : 389 (M^+ , 100), 346 (7), 321 (15), 320 (56), 236 (31).

Neoechinulin B (C2) ^1H -NMR (acetone- d_6 , 500 MHz) δ : 10.40 (1H, br s), 9.79 (1H, br s), 8.14 (1H, br s), 7.41 (1H, d, $J = 7.8\text{Hz}$), 7.35 (1H, d, $J = 7.8\text{Hz}$), 7.12 (1H, s), 7.12 (1H, dt, $J = 7.8$, 1.3Hz), 7.07 (1H, dt, $J = 7.8$, 1.3Hz), 6.14 (1H, dd, $J = 17.4$, 10.4Hz), 5.35 (1H, s), 5.11 (1H, dd, $J = 17.4$, 1.0Hz), 5.08 (1H, dd, $J = 10.4$, 1.0Hz), 5.02 (1H, s), 1.56 (6H, s). EI-MS m/z : 321 (M^+ , 100), 278 (14), 252 (71), 182 (57).

Neoechinulin A (C3) ^1H -NMR (acetone- d_6 , 500 MHz) δ : 10.27 (1H, br s), 7.88 (1H, br s), 7.39 (1H, br s), 7.35 (1H, d, $J = 8.0\text{Hz}$), 7.26 (1H, d, $J = 8.0\text{Hz}$), 7.06 (1H, dt, $J = 8.0$, 1.0Hz), 7.01 (1H, s), 7.00 (1H, dt, $J = 8.0$, 1.0Hz), 6.10 (1H, dd, $J = 17.4$, 10.7Hz), 5.06 (1H, dd, $J = 17.4$, 1.0Hz), 5.03 (1H, dd, $J = 10.7$, 1.0Hz), 4.24 (1H, q, $J = 7.0\text{Hz}$), 1.51 (6H, s), 1.47 (3H, d, $J = 7.0\text{Hz}$). EI-MS m/z : 323 (M^+ , 89), 280 (11), 254 (100), 182 (55).

Variolorin O (C4) ^1H -NMR (acetone- d_6 , 500 MHz) δ : 10.30 (1H, br s), 7.98 (1H, br s), 7.91 (1H, br s), 7.38 (2H, m), 7.11 (1H, s), 7.09 (1H, dt, $J = 8.0$, 1.0Hz), 7.02 (1H, dt, $J = 8.0$, 1.0Hz), 6.13 (1H, dd, $J = 17.4$, 10.4Hz), 5.10 (1H, dd, $J = 17.4$, 1.0Hz), 5.08 (1H, dd, $J = 10.4$, 1.0Hz), 1.66 (3H, s), 1.56 (3H, s), 1.55 (3H, s). EI-MS m/z : 339 (M^+ , 43), 321 (100), 270 (30), 252 (79), 182 (81).

Echinulin (C5) ^1H -NMR (CDCl_3 , 500 MHz) δ : 8.03 (1H, br s), 7.11 (1H, s), 6.78 (1H, s), 6.08 (1H, dd, $J = 17.4$, 10.4Hz), 6.05 (1H, br s), 5.65 (1H, br s), 5.40 (1H, m), 5.33 (1H, m), 5.15 (1H, dd, $J = 10.4$, 1.0Hz), 5.14 (1H, dd, $J = 17.4$, 1.0Hz), 4.38 (1H, dd, $J = 11.7$, 3.6Hz), 4.07 (1H, d, $J = 6.7\text{Hz}$), 3.64 (1H, dd, $J = 14.8$, 3.6Hz), 3.51 (2H, d, $J = 7.3\text{Hz}$), 3.37 (2H, d, $J = 7.3\text{Hz}$), 3.16 (1H, dd, $J = 14.6$, 11.7Hz), 1.85 (3H, s), 1.78 (3H, s), 1.72 (3H, s), 1.71 (3H, s), 1.51

(3H, d, $J = 7.0$ Hz), 1.48 (6H, s). EI-MS m/z : 461 (M^+ , 81), 391 (38), 335 (98), 334 (100), 278 (66), 264 (43).

Isoechinulin A (C6) $^1\text{H-NMR}$ (CDCl_3 , 500 MHz) δ : 8.19 (1H, br s), 7.41 (1H, br s), 7.26 (2H, m), 7.20 (1H, s), 7.04 (1H, br s), 6.06 (1H, dd, $J = 17.4, 10.4$ Hz), 5.35 (1H, m), 5.21 (1H, dd, $J = 10.4, 0.8$ Hz), 5.17 (1H, dd, $J = 17.4, 0.8$ Hz), 4.28 (1H, q, $J = 7.0$ Hz), 3.41 (2H, d, $J = 7.2$ Hz), 1.72 (3H, s), 1.71 (3H, s), 1.60 (3H, d, $J = 7.0$ Hz), 1.52 (6H, s). EI-MS m/z : 391 (M^+ , 100), 323 (26), 322 (80), 251 (18).

Tetrahydroauroglaucin (D1) $^1\text{H-NMR}$ (CDCl_3 , 500 MHz) δ : 11.73 (1H, s), 10.10 (1H, s), 7.02 (1H, s), 6.48 (1H, d, $J = 16.2$ Hz), 5.98 (1H, dt, $J = 16.5, 7.3$ Hz), 5.29 (1H, m), 4.98 (1H, br s), 3.32 (2H, d, $J = 7.3$ Hz), 2.32 (2H, q, $J = 7.3$ Hz), 1.75 (3H, s), 1.70 (3H, s), 1.52 (2H, quintet, $J = 7.3$ Hz), 1.35 (4H, m), 0.92 (3H, t, $J = 7.3$ Hz). $^{13}\text{C-NMR}$ (CDCl_3 , 125 MHz) δ : 196.3, 155.1, 144.8, 142.7, 133.9, 130.4, 125.0, 123.9, 121.0, 120.1, 117.1, 33.4, 31.4, 28.7, 27.2, 25.8, 22.4, 17.8, 14.0. EI-MS m/z : 302 (M^+ , 63), 269 (27), 231 (80), 228 (24), 211 (27), 199 (25), 189 (33), 176 (19), 175 (100), 161 (31), 147 (31).

Isodihydroauroglaucin (D2) $^1\text{H-NMR}$ (CDCl_3 , 500 MHz) δ : 11.94 (1H, s), 10.23 (1H, s), 6.90 (1H, s), 6.02 (1H, m), 6.00 (1H,

m), 5.59 (1H, m), 5.58 (1H, m), 5.28 (1H, m), 4.53 (1H, br s), 3.29 (2H, d, $J = 7.3$ Hz), 2.98 (2H, t, $J = 7.5$ Hz), 2.34 (2H, q, $J = 7.5$ Hz), 1.76 (3H, d, $J = 1.0$ Hz), 1.73 (3H, d, $J = 6.7$ Hz), 1.70 (3H, s). $^{13}\text{C-NMR}$ (CDCl_3 , 125 MHz) δ : 195.4, 155.8, 145.1, 133.9, 132.0, 131.1, 129.4, 128.9, 128.3, 127.4, 125.8, 121.1, 117.3, 34.2, 27.0, 25.8, 24.1, 18.0, 17.8. EI-MS m/z : 300 (M^+ , 51), 282 (16), 267 (10), 239 (10), 227 (14), 226 (17), 219 (48), 211 (15), 163 (100), 149 (21).

Flavoglaucin (D3) $^1\text{H-NMR}$ (CDCl_3 , 500 MHz) δ : 11.92 (1H, s), 10.25 (1H, s), 6.89 (1H, s), 5.28 (1H, m), 4.41 (1H, br s), 3.29 (2H, d, $J = 7.3$ Hz), 2.88 (2H, t, $J = 7.6$ Hz), 1.75 (3H, s), 1.70 (3H, s), 1.58 (2H, quintet, $J = 7.6$ Hz), 1.40 (2H, quintet), 1.30 (2H, m), 1.28 (4H, m), 0.88 (3H, t, $J = 7.0$ Hz). EI-MS m/z : 304 (M^+ , 100), 249 (49), 215 (26), 199 (20), 189 (26), 187 (26), 177 (26), 175 (29), 173 (26), 163 (51), 161 (34), 149 (29), 147 (26).

Results and Discussion

Isolation of aromatic compounds from *E. herbariorum* NU-

2 Seventeen aromatic compounds were isolated from the extract of *E. herbariorum* NU-2 (Fig. 1). They were identified as six anthraquinone derivatives (**A1-A6**), two eurotinone derivatives (**B1, B2**), six indole-containing diketopiperazine derivatives (**C1-**

Fig. 1. Isolated aromatic compounds from the *Eurotium herbariorum* NU-2 extract.

Compounds **A1-A6** are anthraquinone derivatives, compounds **B1** and **B2** are eurotinone derivatives, compounds **C1-C6** are indole-containing diketopiperazine derivatives, and compounds **D1-D3** are benzaldehyde derivatives.

C6), and three benzaldehyde derivatives (**D1-D3**) (Fig. 1). The anthraquinone derivatives were identified as physcion-10,10'-bianthrone (**A1**), catenarin (**A2**), questin (**A3**), asperflavin (**A4**), physcion (**A5**), and questinol (**A6**). Physcion-10,10'-bianthrone (**A1**) was previously isolated from the seeds of *Cassia torosa* (Kitanaka and Takido, 1982). Catenarin (**A2**) and physcion (**A5**) were previously isolated from the leaves of *Didymocarpus leucocalyx* (Segawa *et al.*, 1999). Questin (**A3**) and asperflavin (**A4**) were previously isolated from the ascomycete, *Microascus tardifaciens* (Fujimoto *et al.*, 1999). Questinol (**A6**) was previously isolated from the roots of *Polygonum cuspidatum* (Kimura *et al.*, 1983). Eurotinone derivatives were identified as 2-methyleurotinone (**B1**) and 2-*O*-methyl-9-dehydroxyeurotinone (**B2**). 2-Methyleurotinone (**B1**) was previously isolated from *Aspergillus variegatus* (Wang *et al.*, 2007b). 2-*O*-Methyl-9-dehydroxyeurotinone (**B2**) was previously isolated from *Eurotium rubrum* (Yan *et al.*, 2012). Indole-containing diketopiperazine derivatives were identified as isoechinulin B (**C1**), neoechinulin B (**C2**), neoechinulin A (**C3**), variegatorin O (**C4**), echinulin (**C5**), and isoechinulin A (**C6**). The compounds (**C1-C6**) were previously isolated from *Aspergillus variegatus* and *Penicillium griseofulvum* (Wang *et al.*, 2007a; Zhou *et al.*, 2010). Benzaldehyde derivatives were identified as tetrahydroauroglaucon (**D1**), isodihydroauroglaucon (**D2**), and flavoglaucon (**D3**). The compounds (**D1-D3**) were previously isolated from *Eurotium* spp. found in katsuobushi (Miyake *et al.*, 2009, 2010a, 2010b). All aromatic compounds isolated from *E.*

herbariorum NU-2 in the present study have been reported to occur in plants and other microorganisms. Neoechinulin A (**C3**), tetrahydroauroglaucon (**D1**), isodihydroauroglaucon (**D2**), and flavoglaucon (**D3**) were previously isolated from mold-starters used in making karebushi (Yagi and Doi, 1999; Miyake *et al.*, 2009; 2010). However, for the first time, the aromatic compounds (**A1-A6**, **B1**, **B2**, **C1**, **C2**, **C4-C6**) were isolated from a mold-starter used in making karebushi.

DPPH radical scavenging activity of aromatic compounds

The isolated aromatic compounds were examined for DPPH radical scavenging activity and compared to α -tocopherol, a standard scavenger (Table 1). 2-Methyleurotinone (**B1**), isoechinulin A (**C6**), tetrahydroauroglaucon (**D1**), isodihydroauroglaucon (**D2**), and flavoglaucon (**D3**) exhibited significantly higher radical scavenging activity than α -tocopherol ($P < 0.05$). Asperflavin (**A4**), isoechinulin B (**C1**), neoechinulin B (**C2**), and variegatorin O (**C4**) had similar activity to α -tocopherol. Of the isolated aromatic compounds, benzaldehyde derivatives (**D1-D3**) were shown to have high activity. We hypothesize that the hydroxyl groups at positions 2 and 5 of the compounds (**D1-D3**) functioned as electron and proton donors and that they expressed the radical scavenging activity. Tetrahydroauroglaucon (**D1**) exhibited higher activity than isodihydroauroglaucon (**D2**) and flavoglaucon (**D3**). The structure of 1'-monoene in the substituent formed by the seven-carbon aliphatic chain on the benzaldehyde derivatives seemed to be related to this high radical scavenging activity. 2-Methyleurotinone (**B1**)

Table1. DPPH radical scavenging activity of aromatic compounds isolated from *Eurotiumher bariorum* NU-2 extracts.

Aromatic compounds		Radical scavenging activity ¹⁾ (%)
A1	Physcion-10,10'-bianthrone	5.9 ± 1.2
A2	Catenarin	21.9 ± 1.5
A3	Questin	3.2 ± 1.6
A4	Asperflavin	42.1 ± 4.2
A5	Physcion	8.2 ± 1.2
A6	Questinol	3.2 ± 2.2
B1	2-Methyleurotinone	59.4 ± 3.8*
B2	2- <i>O</i> -Methyl-9-dehydroxyeurotinone	10.2 ± 4.5
C1	Isoechinulin	45.5 ± 3.8
C2	Neoechinulin	41.0 ± 2.3
C3	Neoechinulin	21.9 ± 1.1
C4	Variegatorin	44.8 ± 2.9
C5	Echinulin	-3.5 ± 3.3
C6	Isoechinulin	57.2 ± 0.7*
D1	Tetrahydroauroglaucon	67.9 ± 0.8*
D2	Isodihydroauroglaucon	58.6 ± 3.2*
D3	Flavoglaucon	48.2 ± 1.5*
α -Tocopherol		41.4 ± 4.3

Each isolated aromatic compound was added to the reaction mixture to be 50 μ M

¹⁾Data are presented as the mean $\pm$ SD ($N = 4$).

*Value is significantly greater than α -tocopherol at $P < 0.05$.

Table2. The DPPH radical scavenging activity of fungal extracts isolated from katsuobushi and the concentration of aromatic compounds.

Radical scavenging activity (%)											
		<i>E. herbariorum</i> NU-2	<i>E. herbariorum</i> NE-1	<i>E. herbariorum</i> NE-4	<i>E. repens</i> KBN2062	<i>E. repens</i> JCM23060	<i>E. chevalieri</i> JCM23047	<i>E. chevalieri</i> NBRC4090	Filamentous fungi YZ	Filamentous fungi MZ	
		60.3	46.1	49.8	63.5	30.8	35.7	35.4	38.2	35.6	
Aromatic compounds	Retention time for HPLC (min.)	Concentration of aromatic compounds ($\mu\text{g/mL}$)									Coefficient of determination (R^2) ¹⁾
		<i>E. herbariorum</i> NU-2	<i>E. herbariorum</i> NE-1	<i>E. herbariorum</i> NE-4	<i>E. repens</i> KBN2062	<i>E. repens</i> JCM23060	<i>E. chevalieri</i> JCM23047	<i>E. chevalieri</i> NBRC4090	Filamentous fungi YZ	Filamentous fungi MZ	
A1	22.2	1.8	- ²⁾	-	23.4	-	-	-	10.3	5.4	0.045
A2	15.8	3.6	-	0.9	3.6	3.6	0.9	0.8	1.1	-	0.241
A3	10.1	-	-	-	-	-	-	-	-	-	
A4	4.0	-	-	2.1	9.7	-	-	-	-	-	0.445
A5	17.0	3.6	-	-	-	-	-	-	-	-	0.272
A6	5.0	1.0	-	-	7.3	-	-	-	-	-	0.699
B1	4.1	-	-	-	-	-	-	-	-	-	
B2	6.8	-	-	-	-	-	-	-	-	-	
C1	12.3	34.5	3.0	-	4.7	-	-	-	-	-	0.388
C2	7.1	3.0	-	-	36.1	9.9	-	-	-	-	0.310
C3	6.1	-	-	-	-	-	-	-	-	-	
C4	4.9	-	-	-	-	-	-	-	-	-	
C5	17	30.7	8.0	4.7	20.3	19.2	21.6	27.6	17.7	19.3	0.0004
C6	11.6	6.1	-	-	9.1	-	-	-	-	-	0.741
D1	19.5	29.9	17.2	21.0	29.6	4.2	9.3	9.8	9.4	10.1	0.985
D2	16.1	16.2	30.9	21.4	25.1	7.2	7.0	6.1	8.7	8.8	0.517
D3	20.0	30.1	34.3	25.7	24.5	11.9	10.8	8.6	13.3	11.7	0.604

¹⁾ Each data was calculated from correlation between the DPPH radical scavenging activities for extracts of filamentous fungi and the concentration of aromatic compounds in the extract.

²⁾ The peak of the compound was not detected by HPLC.

Fig. 2. HPLC profile of the *Eurotium herbariorum* NU – 2 extract.

The extract of *Eurotium herbariorum* NU – 2 was analyzed by HPLC, using a ODS column ($\phi 4.6 \times 150$ mm, S – 5 mm), UV detection at 280 nm, mobile solvents of methanol and water containing 5% acetic acid (0 – 10 min; 45% MeOH, 10 – 20 min; 45%→85% MeOH, 20 – 35 min; 85% MeOH), a flow rate of 1 mL/min, and a column temperature of 40°C.

was reported to have higher DPPH radical scavenging activity than 2-*O*-methyl-9-dehydroxyeurotinone (**B2**) (Li *et al.*, 2009), which was also found in the present study. The 9-hydroxyl group of 2-methyleurotinone (**B1**) appeared to be related to this high

scavenging activity. Isoechinulin B (**C1**), neoechinulin B (**C2**), variecolorin O (**C4**), and isoechinulin A (**C6**) exhibited higher activity than neoechinulin A (**C3**), which was reported to exhibit antioxidative and radical scavenging activities (Yagi and Doi,

1999; Li *et al.*, 2004; Kuramochi, 2013). Echinulin (**C5**) exhibited the lowest activity among the indole-containing diketopiperazine derivatives. The double bond between C8 and C9 on the indole-containing diketopiperazine derivatives seemed to be related to the high radical scavenging activity.

DPPH radical scavenging activity of katsuobushi fungal extracts Nine filamentous *Eurotium* spp. fungi were obtained: four filamentous fungi from karebushi mold-starter, three filamentous fungi isolated from katsuobushi, and two filamentous fungi isolated from commercial karebushi. The fungi were cultured in M40Y medium and extracted with ethyl acetate. Each extract was examined for DPPH radical scavenging activity (Table 2). The extracts of *E. herbariorum* NE-1, *E. herbariorum* NE-4, and *E. repens* KBN 2062 (karebushi mold-starters) have been reported to show scavenging activity (Miyake *et al.*, 2009; 2010a). In the present study, the extract of *E. herbariorum* NU-2, a karebushi mold-starter, also exhibited high activity, which was similar to *E. repens* KBN2062. The extracts of three filamentous fungi (*E. repens* JCM23060, *E. chevalieri* JCM23047, and *E. chevalieri* NBRC4090) isolated from katsuobushi and two filamentous fungi (filamentous fungus YZ and filamentous fungus MZ) isolated from commercial karebushi showed lower activity than *E. herbariorum* NU-2.

Determination of aromatic compounds in fungal extracts from katsuobushi The seventeen aromatic compounds isolated from the extract of *E. herbariorum* NU-2 by TLC were analyzed by HPLC using an ODS column. The HPLC profile for the extract of *E. herbariorum* NU-2 is shown in Fig. 2. Questin (**A3**), asperflavin (**A4**), physcion (**A5**), 2-methyleurotinone (**B1**), 2-*O*-methyl-9-dehydroxyeurotinone (**B2**), neoechinulin A (**C3**), and varicolorin O (**C4**) were not detected by HPLC analysis, although the other compounds were detected. The undetected compounds seemed

to be contained at very small amounts in the extract (less than 0.1 µg/mL of extract). The concentration of the compounds in each fungal extract from katsuobushi was determined by HPLC (Table 2). Isoechinulin B (**C1**), echinulin (**C5**), tetrahydroauroglauconin (**D1**), and flavogluconin (**D3**) occurred at higher concentrations (29.9 – 34.5 µg/mL) in the extract of *E. herbariorum* NU-2, and the amounts of isoechinulin B (**C1**) and echinulin (**C5**) were characteristically high in comparison with the other fungal extracts. Asperflavin (**A4**) was not detected in the extract of *E. herbariorum* NU-2 by HPLC, although it was detected in *E. herbariorum* NE-4 and *E. repens* KBN2062. Echinulin (**C5**) and benzaldehyde derivatives (**D1-D3**) were found in all fungal extracts. The extract of *E. repens* KBN2062, which shows a similar activity to that of *E. herbariorum* NU-2, was shown to contain anthraquinone derivatives (**A1**, **A2**, **A4**, and **A6**) and indole-containing diketopiperazine derivatives (**C1**, **C2**, **C5**, and **C6**), and the amounts of compounds **A1** and **C2** were characteristically high. The correlation between the DPPH radical scavenging activity and the concentration of aromatic compounds in the fungal extracts was examined by calculation of the coefficient of determination (R^2) (Table 2). The concentration of tetrahydroauroglauconin (**D1**) in the fungal extracts was highly correlated with radical scavenging activity ($R^2 = 0.985$), as shown in Table 2 and Fig. 3. Notably, the *E. herbariorum* NU-2 extract exhibited both a high tetrahydroauroglauconin (**D1**) concentration (29.9 mg/mL) and high radical scavenging activity.

In the present study, *E. herbariorum* NU-2, a karebushi mold-starter, showed production of aromatic compounds having radical scavenging activities. The compounds with high activity may contribute to antioxidative properties generated during the manufacture of karebushi and the preservation of karebushi quality.

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Fig. 3. The correlation between DPPH radical scavenging activity and tetrahydroauroglauconin (**D1**) concentration in fungal extracts.

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