Food Sci. Biotechnol. Vol. 17, No. 6, pp. 1214 ~ 1220 (2008)

Food Science and Biotechnology

© The Korean Society of Food Science and Technology

Phenolic Compounds from the Fruit Body of *Phellinus linteus* Increase Alkaline Phosphatase (ALP) Activity of Human Osteoblast-like Cells

Ha-Na Lyu, Dae-Young Lee, Dong-Hyun Kim[1], Jong-Su Yoo, Min-Kyung Lee, In-Ho Kim[2], and Nam-In Baek*

Graduate School of Biotechnology and Plant Metabolism Research Center, Kyung Hee University, Yongin, Gyeonggi 446-701, Korea

[1]School of Chemistry, Manchester Interdisciplinary Biocentre, The University of Manchester, 131 Princess Street, Manchester M1 7DN, UK

[2]Korea Food Research Institute, Seongnam, Gyeonggi 463-746, Korea

Abstract Secondary metabolites from the fruit body of *Phellinus linteus* were evaluated for their proliferative effect on human osteoblast-like cells. 3-[4,5-Dimethylthiazole-2-yl]-2,5-diphenyl-tetraxolium bromide (MTT) assay and alkaline phosphatase (ALP) activity assay were used to assess the effect those isolates on the human osteoblast-like cell line (Saos-2). Activity-guided fractionation led to the isolation of ALP-activating phenolic compounds through the extraction of *P. linteus*, solvent partitioning, and repeated silica gel and octadecyl silica gel (ODS) column chromatographic separations. From the result of spectroscopic data including nuclear magnetic resonance (NMR), mass spectrometry (MS), and infrared spectroscopy (IR), the chemical structures of the compounds were determined as 4-(4-hydroxyphenyl)-3-buten-2-one (**1**), 2-(3',4'-dihydroxyphenyl)-1,3-benzodioxole-5-aldehyde (**2**), 4-(3,4-dihydroxyphenyl)-3-buten-2-one (**3**), 3,4-dihydroxybenzaldehyde (**4**), and protocatechuic acid methyl ester (**5**), respectively. This study reports the first isolation of compounds **1-3** and **5** from *P. linteus*. In addition, all phenolic compounds stimulated proliferation of the osteoblast-like cells and increased their ALP activity in a dose-dependent manner (10^{-8} to 10^{-1} mg/mL). The present data demonstrate that phenolic compounds in *P. linteus* stimulated mineralization in bone formation caused by osteoporosis. The bone-formation effect of *P. linteus* seems to be mediated, at least partly, by the stimulating effect of the phenolic compounds on the growth of osteoblasts.

Keywords: *Phellinus linteus*, phenolic compound, alkaline phosphatase (ALP), Saos-2

Introduction

Phellinus linteus, commonly referred to as '*sangwhang*' in Korea, is a fungus belonging to the family Hymenochaetaceae (Aphyllophorales, Basidiomycetes), which is distributed mainly in Asia, tropical America, and North Africa (1). The extracts have long been used as a traditional oriental medicine in Korea and Japan for the treatment of various diseases including arthritis of the knee, oral ulcers, gastroenteric disorders, lymphatic disease, and various cancers (2,3). It has been reported that *P. linteus* has anti-cancer (4,5), anti-tumor (6), anti-mutagenic (7), anti-angiogenic (8), anti-oxidant (9), and immune activity (10). Various polysaccharides and proteoglycans are reported as the principal components of *P. linteus* that manifest pharmacological activities (11). β-Glucan form this mushroom is especially important as it is known to have anti-tumor activity and immuno-stimulating effects (5,9).

Except for polysaccharides, however, there are few reports on other *P. linteus* pharmacologically active compounds. We, therefore, initiated this study to identify principal lower molecular weight compounds of *P. linteus*. Furthermore, only few reports have claimed that extracts of the fruit body of *P. linteus* act to prevent osteoporosis or to treat bone fractures. Screening of extracts from the fruit body of *P. linteus* using 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetraxolium bromide (MTT) assay and alkaline phosphatase (ALP) assay of human osteoblast-like cells (Saos-2) revealed significant osteoblast cell activity (12, 13). Therefore, this study was performed to identify the principal components of the fruit body of *P. linteus* responsible for the proliferation of Saos-2 cells.

Bone is a tissue maintained through continuous osteogenesis and osteolysis regulated by osteoblasts and osteoclasts, respectively (14). Osteoporosis is a common metabolic bone disease usually resulting from an imbalance between osteoblast and osteoclast cell activities which are influenced by multiple physical and physiological factors. Therefore, any remedy for osteoporosis would theoretically involve modifying osteoblast activity to facilitate osteogenesis (bone formation), inhibiting osteoclast activity to suppress osteolysis (bone resorption) (15), or some combination of the two processes.

The current clinical treatments for osteoporosis employ bone resorption inhibitors, estrogen, alendronate, or bisphosphonate (16). However, since these treatments have severe side effects, hormone estrogen replacement therapy is presented as a preventative measure (17). Nevertheless, this therapy is reported to be risky since it may increase the likelihood of uterine cancer and breast cancer (18). The uncertainty of hormone replacement therapy has prompted a search for natural substances that can replace female hormones (19). Accordingly, there is growing interest in

*Corresponding author: Tel: +82-31-201-2661; Fax: +82-31-201-2157
E-mail: nibaek@khu.ac.kr
Received March 28, 2008; Revised May 26, 2008;
Accepted June 9, 2008

finding an appropriate edible phytochemical among existing medicinal resources. In particular, phytoestrogen, a phenolic compound found naturally in certain plants, is structurally similar to estrogen and displays either estrogen effects or anti-estrogen effects according to the target site (20). Therefore, it is necessary to continuously search for indigenous crops rich in phytoestrogen and research phytoestrogen's physiological mechanisms in order to help treat hormone-dependent diseases and chronic aging diseases.

ALP is an enzyme present in the liver, bone, and intestines (21). In particular, ALP in bone is specific to the maturation of osteoblasts (22); therefore, bone ALP is a useful marker for osteoblast cell activation (23-25). In this study, in isolate phytoestrogens from the fruit body of *P. linteus* that could potentially be used to treat hormone-dependent diseases such as osteoporosis, the MTT assay, which indicates differentiation and proliferation of Saos-2, and the ALP activity detection assay, were applied to extracts of the fruit body of *P. linteus* after extract fractionation by solvent partitioning and column chromatography. Five phenolic compounds were isolated and characterized as the principal active components facilitating osteogenesis of Saos-2. This study describes the procedures to isolate these compounds and determine their structures. The effect of these active phenolic compounds on the MTT and ALP activities of Saos-2 cells is also presented.

Materials and Methods

Plant materials The fruit body of *Phellinus linteus* was purchased from *sanghwang* mushroom Agricultural Corporation, Gyeongnam, Korea, in May 2005, and was identified by Prof. Dae-Keun Kim, College of Pharmacy, Woosuk University, Jeonju, Korea. A voucher specimen (KHU060314) is reserved at the Laboratory of Natural Products Chemistry, Kyung Hee University, Yongin, Korea.

Chemicals The human osteoblast-like cell line (Saos-2) was obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). MTT, dimethyl sulfoxide (DMSO), penicillin, streptomycin, and RPMI-1640 medium were purchased from Sigma-Aldrich (St. Louis, MO, USA) and fetal bovine serum (FBS) was purchased from JRH Bio Science (Lenexa, KS, USA).

Isolation of active compounds from the fruit body of *P. linteus* The dried fruit body of *P. linteus* (1 kg) was extracted 2 times at room temperature with 80% aqueous methanol (MeOH, 3 L×2). The extracts were partitioned with water (1 L), ethyl acetate (EtOAc, 1 L×2), and normal-butanol (*n*-BuOH, 1 L×2) in succession. The EtOAc extract (10 g) was applied to a silica gel column chromatography (c.c.) (6.5×17 cm) and eluted with a gradient of chloroform ($CHCl_3$):MeOH (15:1→10:1→7:1→5:1→3:1→1:1, 1 L of each) and monitored by thin layer chromatography (TLC) to produce 22 fractions (PLE1 to PLE22). Fraction PLE4 [313 mg, V_e/V_t (elution volume/total volume) 0.10-0.15] was subjected to a silica gel c.c. (4×13 cm) eluted with normal-hexane (*n*-hexane): EtOAc (2:1, 3 L), yielding 18 fractions (PLE4-1 to PLE4-18). Fraction PLE4-6 (27 mg, V_e/V_t 0.25-0.28) was purified by an octadecyl silica gel (ODS) c.c. (2.5×6 cm) and eluted with MeOH:H_2O (1:1, 500 mL) to ultimately produce compound **1** [5.5 mg, V_e/V_t 0.35-0.45, TLC (ODS F_{254}) R_f 0.65, MeOH:H_2O=3:1]. Fraction PLE6 (250 mg, V_e/V_t 0.18-0.20) was applied to an ODS c.c. (3×8 cm) and eluted with MeOH:H_2O (1:3→1:1, 700 mL of each) to ultimately produce compound **2** [22.4 mg, V_e/V_t 0-0.03, TLC (ODS F_{254}) R_f 0.75, MeOH:H_2O=2:1] and compound **3** [17.5 mg, V_e/V_t 0.10-0.13, TLC (ODS F_{254}) R_f 0.66, MeOH:H_2O=2:1]. The concentrated *n*-BuOH fraction (PLB, 31 g) was subjected to a silica gel c.c. (7×23 cm) and eluted with a gradient of $CHCl_3$:MeOH:H_2O (6:4:1 →5:4:1, 4 L of each), resulting in 19 fractions (PLB1 to PLB19). Fraction PLB2 (831 mg, V_e/V_t 0.05-0.12) was subjected to a silica gel c.c. (5×15 cm) and eluted with $CHCl_3$:MeOH (20:1→10:1, 2 L of each) to produce 10 fractions (PLB2-1 to PLB2-10). Fraction PLB2-3 (103 mg, V_e/V_t 0.25-0.40) was applied to an ODS c.c (2.5×5 cm) and eluted with MeOH:H_2O (2:3, 500 mL) yielding compound **4** [7.6 mg, V_e/V_t 0.10-0.15, TLC (ODS F_{254}) R_f 0.72, MeOH:H_2O=2:1] and compound **5** [6.5 mg, V_e/V_t 0.25-0.35, TLC (ODS F_{254}) R_f 0.65, MeOH:H_2O=2:1].

Identification of active compounds from the fruit body of *P. linteus* The melting points (m.p.) of the isolated compounds were determined on a Fisher-John apparatus (Fisher Scientific, Miami, FL, USA) and not corrected. Optical rotation was measured on a polarimeter P-1020 (Jasco, Tokyo, Japan). The infrared (IR) spectrum was obtained with a Perkin model 599B FT-IR spectrometer (Perkin-Elmer, Waltham, MA, USA). Electron impact mass spectrometer (EI-MS) was recorded on a Jeol JMSAX-700 (Tokyo, Japan). Proton nuclear magnetic resonance (^{1}H-NMR, 400 MHz), and carbon NMR (^{13}C-NMR, 100 MHz) spectra were recorded on a Varian Unity Inova AS-400 FT-NMR spectrometer (Varian, Palo Alto, CA, USA). Methanol-d_4 with TMS as internal standard was purchased from Sigma-Aldrich.

Compound **1**: Yellow amorphous oil (MeOH); IR (KBr, ν) 3,356, 2,901, 1,680, 1,598, 1,477, 1,198; EI-MS *m/z*: 162 $[M]^+$, 147 $[M-Me]^+$, 119 $[M-H_2O-Me]^+$; ^{1}H-NMR (400 MHz, CD_3OD, δ) 7.52 (2H, d, *J*=8.4, H-2, 6), 7.43 (1H, d, *J*=16.0, H-7), 6.84 (2H, d, *J*=8.4, H-3, 5), 6.58 (1H, d, *J*=16.0, H-8), 2.34 (3H, s, H-10); ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 199.0 (C-9), 158.1 (C-4), 143.6 (C-7), 130.4 (C-2), 130.4 (C-6), 127.2 (C-1), 125.1 (C-8), 116.2 (C-3), 116.2 (C-5), 27.7 (C-10).

Compound **2**: Pale purple amorphous powder (MeOH); m.p. 131-137°C; $[\alpha]_D$ ±0° (racemic); IR (KBr, ν) 3,329, 3,323, 1,655, 1,647, 1,443, 1,296, 1,167, 878, 754; EI-MS *m/z*: 258 $[M]^+$; ^{1}H-NMR (400 MHz, CD_3OD, δ) 9.67 (3H, s, H-7), 7.30 (1H, dd, *J*=7.2, 2.0, H-6), 7.29 (1H, d, *J*=2.0, H-2), 6.90 (1H, d, *J*=7.2, H-5), 6.84 (1H, d, *J*=1.6, H-2'), 6.74 (1H, d, *J*=6.8, H-5'), 6.74 (1H, d, *J*=6.8, 1.6, H-6'), 5.20 (1H, s, H-7'); ^{13}C-NMR(100 MHz, CD_3OD, δ_C) 192.9 (C-7), 153.5 (C-3), 147.0 (C-4), 146.5 (C-4'), 145.9 (C-3'), 130.9 (C-1'), 130.7 (C-1), 126.3 (C-6), 119.3 (C-6'), 116.1 (C-5), 115.7 (C-5'), 115.2 (C-2), 114.7 (C-2'), 104.7 (C-7').

Compound **3**: Pale purple amorphous powder (MeOH); m.p. 177-178°C; IR (KBr, ν) 3,420, 1,651, 1,603, 1,556, 1,286, 1,115; EI-MS *m/z*: 178 $[M]^+$, 163 $[M-Me]^+$; ^{1}H-NMR (400 MHz, CD_3OD, δ) 7.51 (1H, d, *J*=16.4 Hz, H-7), 7.07 (1H, d, *J*=2.4 Hz, H-2), 6.98 (1H, dd, *J*=8.4, 2.4,

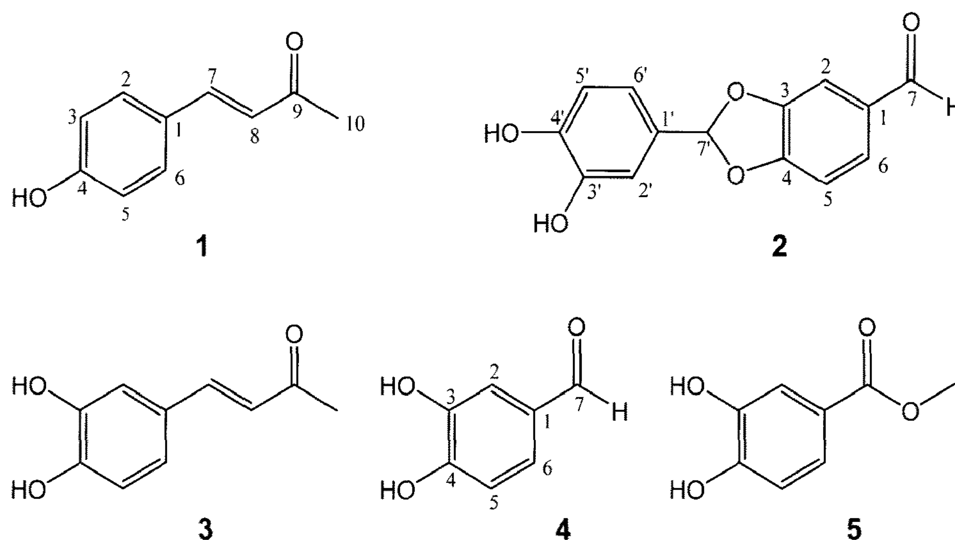

Fig. 1. Chemical structures of compound 1-5 from the fruit body of *Phellinus linteus*. **1**, 4-(4-Hydroxyphenyl)-3-buten-2-one (4-hydroxybenzalacetone; **2**, 2-(3',4'-dihydroxyphenyl)-1,3-benzodioxole-5-aldehyde; **3**, 4-(3,4-dihydroxyphenyl)-3-buten-2-one (3,4-dihydroxybenzalacetone); **4**, 3,4-dihydroxybenzaldehyde (protocatechualdehyde), **5**, 3,4-dihydroxybenzoic acid methyl ester (protocatechuic acid methyl ester).

H-6), 6.78 (1H, d, J=8.4 Hz, H-5), 6.54 (1H, d, J=16.4, H-8), 2.32 (3H, s, H-10); ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 201.39 (C-9), 149.83 (C-4), 146.74 (C-3), 146.74 (C-7), 127.56 (C-1), 124.55 (C-8), 123.44 (C-6), 116.42 (C-5), 115.14 (C-2), 27.02 (C-10).

Compound **4**: Yellow amorphous powder (MeOH); m.p. 153-155°C; IR (KBr, ν) 3,329, 3,222, 1,652, 1,596, 1,535, 1,445, 1,297, 1,165; EI-MS *m/z*: 138 $[M]^+$, 137 $[M-H]^+$, 109, 81, 63, 53; ^{1}H-NMR (400 MHz, CD_3OD, δ) 9.67 (1H, s, H-7), 7.30 (1H, d, J=8.0, 1.6, H-6), 7.29 (1H, d, J=1.6, H-2), 6.90 (1H, d, J=8.0, H-5); ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 191.9 (C-7), 152.8 (C-4), 146.1 (C-3), 129.5 (C-1), 125.3 (C-6), 115.1 (C-5), 114.1 (C-2).

Compound **5**: Pale purple amorphous powder (MeOH); m.p. 136°C; IR (KBr, ν) 3,338, 1,685, 1,604, 1,521, 1,438, 1,301; EI-MS *m/z*: 168 $[M]^+$, 137, 109, 81, 63, 53; ^{1}H-NMR (400 MHz, CD_3OD, δ) 7.38 (1H, d, J=8.4, 2.0, H-6), 7.39 (1H, d, J=2.0, H-2), 6.77 (1H, d, J=8.4, H-5), 3.80 (3H, s, H-7-OMe); ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 168.9 (C-7), 151.7 (C-4), 146.2 (C-3), 123.6 (C-6), 122.5 (C-1), 117.4 (C-2), 115.8 (C-5), 52.7 (C-7-OMe).

MTT assay Saos-2 cells were grown in RPMI-1640 medium containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained in a humidified incubator at 37°C in a 5% CO_2 atmosphere. Human osteoblast-like cell proliferation was evaluated using an established MTT assay protocol (24). Cells were plated at a density of 2×10^4 cells/96-well plate in 100 μL of medium, and the various isolated compounds were added at a final concentration of 10^{-1} to 10^{-8} mg/mL as solutions containing 1% DMSO. After incubation for 48 hr at 37°C, 0.5 mg/mL MTT was added to each well and incubation continued for 4 hr at 37°C. Formazan crystals were dissolved in DMSO and the absorbance was determined at 550 nm using a microplate reader (SpectraMax 340PC; Molecular Devices, Sunnyvale, CA, USA). The cell proliferation values (%) for the 5 compounds were confirmed using cells cultured without the test compounds as a negative control and cells cultured with NaF (Sigma-Aldrich), 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) (Sigma-Aldrich), and soybean (the seed of *Glycin max*) as a positive control.

ALP activity assay ALP activity was determined using *p*-nitrophenyl phosphate as a substrate following the method of Bowers and McComb (13). The ALP reagent was obtained from Thermo Electron (Louisville, CO, USA). Saos-2 cells were cultured as in the MTT test described previously. After incubation for 48 hr at 37°C, cells were washed with PBS and then lysed with 1% Triton X-100. The lysates were sonicated for 15 sec and centrifuged at 14,000×g for 20 min at 4°C. The clear supernatant was used to measure ALP activity which was determined at 405 nm using a microplate reader (SpectraMax 340PC). The ALP activity values (%) for the 5 compounds were confirmed using cells cultured without the test compounds as a negative control and cells cultured with NaF, $1,25(OH)_2D_3$, and soybean as a positive control.

Results and Discussion

Isolation and identification of active compounds from the fruit body of *P. linteus* The MeOH extracts of the fruit body of *P. linteus* showed an activation effect on bone ALP, a biomarker of osteoblast cell activation. Therefore, activity-guided fractionation of the MeOH extracts was conducted.

The MeOH extracts were partitioned into EtOAc, *n*-BuOH, and H_2O layers through solvent fractionation. Among these 3 layers, the EtOAc and *n*-BuOH fractions showed not only the lowest activity in the MTT assay (Table 1), indicating that they should have little or no cytotoxicity on Saos-2, but also high activity in the ALP assay at most concentration (Table 2). Hence, EtOAc and *n*-BuOH fractions may be effective in stimulating bone

Table 1. The cell proliferation of EtOAc, *n*-BuOH, and H_2O fractions from the fruit body of *P. linteus* on Saos-2 cells

Cell proliferation values for Saos-2 cells (%)[1]			
Concentration (mg/mL)	EtOAc fr.	*n*-BuOH fr.	H_2O fr.
10^{-8}	114.0±10.8[2]	106.3±19.7	106.0±15.4
10^{-7}	134.8±8.9	108.1±13.0	101.6±14.9
10^{-6}	166.9±0.5	108.0±16.1	134.0±13.9
10^{-5}	158.0±8.00	110.0±10.5	105.5±14.7
10^{-4}	151.0±10.6	119.4±12.9	101.0±3.8
10^{-3}	144.9±15.5	123.4±12.5	97.0±7.8
10^{-2}	138.0±5.9	120.0±17.6	95.0±22.7
10^{-1}	132.9±8.6	118.7±13.0	94.3±19.8

[1]Positive control group shows 100% cell proliferation.
[2]Values are means±SD of triplicate determinations.

Table 2. The ALP-inducing activity of EtOAc, *n*-BuOH, and H_2O fractions from the fruit body of *P. linteus* on Saos-2 cells

ALP activity values for Saos-2 cells (%)[1]			
Concentration (mg/mL)	EtOAc fr.	*n*-BuOH fr.	H_2O fr.
10^{-8}	130.6±20.9[2]	130.6±12.6	141.7±11.4
10^{-7}	163.3±8.3	140.8±17.8	146.9±9.6
10^{-6}	140.8±16.8	160.2±8.5	151.9±17.1
10^{-5}	136.1±18.5	173.5±24.7	157.0±10.7
10^{-4}	136.7±14.4	167.4±9.7	136.2±5.2
10^{-3}	135.4±15.1	160.7±16.4	134.0±16.5
10^{-2}	136.7±15.5	156.2±16.0	131.9±12.6
10^{-1}	108.8±8.3	90.5±10.7	125.5±10.4

[1]Positive control group shows 100% cell proliferation.
[2]Values are means±SD of triplicate determinations.

formation. Therefore, 5 phenolic compounds (compound **1-5**) were isolated from the principal components of EtOAc and *n*-BuOH fractions.

Compound **1**, a yellow amorphous oil, showed absorbance bands due to hydroxyl (3,356 ν), conjugated carbonyl (1,680 ν), and aromatic (1,598 and 1,477 ν) groups in the IR spectrum and a molecular ion peak at *m/z* 162 $[M]^+$ in the EI-MS spectrum. In the ^{1}H-NMR spectrum, 2 olefin methine signals at δ_H 7.52 (1H, d, *J*=16.0 Hz) and 6.58 (1H, d, *J*=16.0 Hz), which are from a double bond with *trans* configuration, were observed. Also, proton signals at δ_H 7.43 (2H, d, *J*=8.4 Hz) and δ_H 6.84 (2H, d, *J*=8.4 Hz) indicated the presence of a 1,4-disubstituted benzene ring. Additionally, 1 singlet allylic methyl signal at δ_H 2.34 (3H, s) was observed. In the ^{13}C-NMR spectrum, 10 signals were observed; 1 ketone signal at δ_C 199.0, 1 oxygenated olefin quaternary signal at δ_C 158.1, 1 olefin quaternary signal at δ_C 127.2, 6 olefin methine signal at δ_C 130.4 (two), 116.2 (two), 143.6, and 125.1, and 1 methyl signal at δ_C 27.7. This led to the conclusion that compound **1** was phenolic, with a 1,4-disubstituted benzene ring, a *trans* configuration double bond, 1 ketone, and 1 singlet methyl. Compound **1** was finally identified as 4-(4-hydroxyphenyl)-3-buten-2-one (4-hydroxybenzalacetone) by comparison of several physical and spectral data with those in the literature (26).

Compound **2**, a pale purple amorphous powder, showed absorbance bands due to hydroxyl (3,329 ν), conjugated carbonyl (1,655 ν), and aromatic (1,443 ν) groups in the IR spectrum and a molecular ion peak at *m/z* 258 $[M]^+$ in the EI-MS spectrum. In the ^{1}H-NMR spectrum, proton signals indicated the presence of two 1,2,4-trisubstituted benzene rings [{δ_H 7.30 (1H, dd, *J*=7.2, 2.0 Hz), 7.29 (1H, d, *J*=2.0 Hz), 6.90 (1H, d, *J*=7.2 Hz)}, {(δ_H 6.84 (1H, d, *J*=1.6 Hz), 6.74 (1H, d, *J*=6.8 Hz), 6.74 (1H, dd, *J*=6.8, 1.6 Hz)}]. An aldehyde signal at δ_H 9.67 (1H, s) and a dioxymethine signal at δ_H 5.20 (1H, s) were also observed. In the ^{13}C-NMR spectrum, 14 signals, consisting of 1 aldehyde signal at δ_C 192.9 (1H, s), 4 oxygenated olefin quaternary signals at δ_C 153.5, 147.0, 146.5, 145.9, 2 olefin quaternary at δ_C 130.9 and 130.7, 6 olefin methine signals at δ_C 126.3, 119.3, 116.1, 115.7, 115.2, and 114.7, and 1 dioxymethine signal at δ_C 104.7 were observed, leading to the conclusion that compound **2** was phenolic, with two 1,2,4-trisubstituted benzene rings, 1 aldehyde, and 1 dioxymethine. Compound **2** was finally identified as 2-(3',4'-dihydroxyphenyl)-1,3-benzodioxole-5-aldehyde by comparison of several physical and spectral data with those in the literature (27). Compound **3**, a pale purple powder, showed absorbance bands due to hydroxyl (3,420 ν), conjugated carbonyl (1,651 ν), and aromatic (1,603 and 1,556 ν) groups in the IR spectrum and molecular ion peak at *m/z* 178 $[M]^+$ in the EI-MS spectrum. ^{1}H-NMR and ^{13}C-NMR data for compound **3** are very similar to those of compound **1**, except that 1 oxygenated olefin quaternary carbon [δ_C 146.74 (C-3)] replaces instead of 1 olefin methine carbon, indicating the presence of a 1,2,4-trisubstituted benzene ring [δ_H 7.30 (1H, dd, *J*=8.0, 1.6 Hz), 7.29 (1H, d, *J*=1.6 Hz), 6.90 (1H, d, *J*=8.0 Hz)]. Compound **3** was finally identified as 4-(3,4-dihydroxyphenyl)-3-buten-2-one (3,4-dihydroxy-benzalacetone) by comparison of several physical and spectral data with those in the literature (28).

Compound **4**, a yellow amorphous powder, showed absorbance bands due to hydroxyl (3,329 ν), aldehyde (3,222 ν), conjugated carbonyl (1,652 ν), and aromatic (1,596 and 1,535 ν) groups in the IR spectrum and a molecular ion peak at *m/z* 138 $[M]^+$ in the EI-MS spectrum. In the ^{1}H-NMR spectrum, proton signals [δ_H 7.30 (1H, dd, *J*=8.0, 1.6 Hz), 7.29 (1H, d, *J*=1.6 Hz), 6.90 (1H, d, *J*=8.0 Hz)] indicated the presence of a 1,2,4-trisubstituted benzene ring and 1 aldehyde signal at δ_H 9.67 (1H, s) was observed. In the ^{13}C-NMR spectrum, 7 signals, consisting of 1 aldehyde signal (δ_C 191.9), 2 oxygenated olefin quaternary signals at δ_C 152.8 and 146.1, 1 olefin quaternary signal at δ_C 129.5, and 3 olefin methine signals at δ_C 125.3, 115.1, and 114.1 were observed, leading to the conclusion that compound **4** was phenolic, with a 1,2,4-trisubstituted benzene ring, and 1 aldehyde. Compound **4** was finally identified as 3,4-dihydroxy-benzaldehyde (protocatechualdehyde) by comparison of several physical and spectral data with those in the literature (3).

Compound **5**, a pale purple amorphous powder, showed absorbance bands due to hydroxyl (3,338 ν), conjugated carbonyl (1,685 ν), and aromatic (1,604 and 1,521 ν) groups in the IR spectrum and a molecular ion peak at *m/z* 168 $[M]^+$ in the EI-MS spectrum. In the ^{1}H-NMR and ^{13}C-NMR spectra, compound **5** is very similar to compound **4**

Table 3. The cell proliferation of compound 1-5 from the fruit body of *P. linteus* on Saos-2 cells

	Cell proliferation values for Saos-2 cells (%)[1]				
Concentration (mg/mL)	Compound **1**	Compound **2**	Compound **3**	Compound **4**	Compound **5**
10^{-8}	59.4±3.5[2]	51.5±3.3	56.2±3.0	80.8±3.1	86.5±3.7
10^{-7}	108.7±2.8	94.0±3.4	103.9±3.4	102.9±2.3	112.3±4.4
10^{-6}	89.9±3.7	77.6±2.8	123.7±2.8	127.3±3.2	86.1±3.7
10^{-5}	114.9±3.4	124.0±3.0	123.0±3.1	125.2±3.0	127.8±4.0
10^{-4}	85.3±3.3	123.1±2.7	125.3±3.2	127.1±2.8	183.6±3.3
10^{-3}	107.6±2.9	122.2±3.2	117.3±3.2	122.0±3.7	123.9±4.1
10^{-2}	95.2±4.2	113.8±3.2	112.3±3.4	111.9±3.0	108.4±3.9
10^{-1}	54.2±3.1	81.2±3.5	109.5±3.3	108.0±3.0	99.9±3.4

[1]Positive control group shows 100% cell proliferation.
[2]Values are means±SD of triplicate determinations.

Table 4. The ALP-inducing activity of compound 1-5 from the fruit body of *P. linteus* on Saos-2 cells

	ALP activity values for Saos-2 cells (%)[1]				
Concentration (mg/mL)	Compound **1**	Compound **2**	Compound **3**	Compound **4**	Compound **5**
10^{-8}	59.4±3.5[2]	71.4±24.7	157.1±24.7	157.1±65.5	128.6±42.9
10^{-7}	100.0±49.5	100.0±49.5	142.9±65.5	185.7±107.9	157.1±24.7
10^{-6}	128.6±42.9	114.3±24.7	157.1±89.2	257.1±42.9	171.4±85.7
10^{-5}	142.9±49.5	142.9±24.7	200.0±24.7	242.9±24.7	271.4±24.7
10^{-4}	214.3±74.2	185.7±65.5	185.7±49.5	228.6±24.7	242.9±24.7
10^{-3}	185.7±24.7	214.3±74.2	171.4±42.9	157.1±24.7	128.6±42.9
10^{-2}	114.3±65.5	171.4±85.7	85.7±74.2	128.6±42.9	114.3±24.7
10^{-1}	85.7±42.9	142.9±24.7	57.1±24.7	85.7±42.9	71.4±24.7

[1]Positive control group shows 100% cell proliferation.
[2]Values are means±SD of triplicate determinations.

with the exception of 1 methoxy [δ_C 52.7 (C-7-OMe), δ_H 3.80 (H-7-OMe)] and 1 ester group (δ_C 168.9) instead of aldehyde group. Compound **5** was finally identified as 3,4-dihydroxybenzoic acid methyl ester (protocatechuic acid methyl ester) by comparison of several physical and spectral data with those in the literature (29).

From the result of spectroscopic data including NMR, MS, and IR, the chemical structures of isolated compound **1-5** were determined; This study marks the first isolation of compound **1-3** and **5** from *P. linteus*. Compound **4** and **5** are widespread in plants, whereas compound **1-3** have rarely been found.

Compound **1** was reported to exhibit antibacterial activity (30) and cytotoxicity against the human leukemia cell line of K562 (26). Compound **2** was reported to have antioxidative activity (DPPH radical scavenging) (27). Compound **4** was reported to show protective action against peroxidative damage to biomembranes (31), as well as antirheumatic (32), radical scavenging (33), and antiproliferative (34) activities. Compound **5** was reported to exhibit cytotoxicity on human tumor cell lines, inhibitory activity on human lymphocytes proliferation and antioxidative activity (DPPH radical scavenging) (35). There have been few reports on the biological activity of compound **3** to date.

Effect of active compounds from *P. linteus* on the cell proliferation and ALP activity of Saos-2 cell The ALP activity responses of Saos-2 to compounds **1-4** are shown in Table 3 and 4. These compounds have comparatively high ALP activity with little or no associated cytotoxicity to human osteoblast-like cells. Also, these 5 phenolic compounds increased the ALP activity of Saos-2 at most concentration. The MTT assay results indicate that compound **1-5** show no cytotoxicity in cells at concentrations of 10^{-7} to 10^{-2} mg/mL (Table 3). In the ALP activity assay, however, these compounds increased ALP activity in Saos-2 compared to the control. Compound **1** showed ALP activity of 100.0±49.5 to 214.3±74.2% at concentration of 10^{-7} to 10^{-2} mg/mL, compound **2** showed ALP activity of 100.0±49.5 to 214.3±74.2% at concentration of 10^{-7} to 10^{-1} mg/mL, compound **3** showed ALP activity of 142.9±65.5 to 200.0±24.7% at concentration of 10^{-8} to 10^{-3} mg/mL, compound **4** showed ALP activity of 128.6±42.9 to 257.1±42.9% at concentration of 10^{-8} to 10^{-2} mg/mL, and compound **5** showed ALP activity of 114.3±24.7 to 271.4±24.7% at concentration of 10^{-8} to 10^{-2} mg/mL. Therefore the 5 phenolic compounds isolated from the fruit body of *P. linteus* are the main components responsible for osteogenesis activity in *P. linteus*.

Although these phenolic compounds showed almost equivalent effects on ALP activity to a well-known ALP activating hormone (estradiol, E2), and a well-known phytoestrogen (genistein), which increased the ALP activity by 54% at 2.72×10^{-6} mg/mL (36) and by 95% at

2.70×10^{-3} mg/mL (37), respectively, comparing to negative control, the idea that the compounds are occurred in edible natural source could prove the significance of the compounds to treat physiological disorders caused by osteoporosis. The compounds have comparatively high ALP activity with little or no cytotoxicity. Therefore, it could be suggested that these compounds could improve mineralization in bone formation thereby counteracting osteoporosis (38, 39). Compound **1** has a phenyl moiety with oxygen atom at *para*-position and sp^2 carbon at benzyl-position, and compound **2-5** with 2 oxygen atoms at *meta*- and *para*-position and sp^2 carbon at benzyl-position, which are similar as a partial structure of isoflavones. We, therefore, suggest such moiety could be key-structure to increase ALP activity.

P. linteus is well known in traditional oriental medicine as an effective natural resource for treating various cancers and immune disease. However, identification of *P. linteus* compounds that might demonstrate relevant bioactivity for the recovery of bone mass or treatment of osteoporosis has not previously been reported. In summary, active compounds likely related to bone formation derived from the fruit body of *P. linteus* were isolated and characterized. Out of the 5 compounds, 4 were precisely identified by NMR, MS, and IR for the first time.

Acknowledgment

This work was supported by the Technology Development Program from the Agricultural Research & Development Promotion Center, and by a scholarship (the second excellent research paper scholarship in 2007) from the Graduate School of Kyung Hee University.

References

1. Dai YC, Xu MQ. Studies on the medicinal polypore, *Phellinus baumii* and its kin, *P. linteus*. Mycotaxon 67: 191-200 (1998)
2. Heo J. *Dongeuibogam* (Publication on Oriental Medicine in Chosun Dynasty). Namsandang Press, Seoul, Korea. p. 719 (1993)
3. Kang H-S, Choi J-H, Cho W-K, Park J-C, Choi J-S. A sphingolipid and tyrosinase inhibitors from the fruiting body of *Phellinus linteus*. Arch. Pharm. Res. 27: 742-750 (2004)
4. Choi J-H, Ha T-M, Kim Y-H, Rho Y-D. Studies on the main factors affecting the mycelial growth of *Phellinus linteus*. Korean J. Mycol. 24: 214-222 (1996)
5. Han S-B, Lee C-W, Jeon Y-J, Hong N-D, Yoo I-D, Yang K-H, Kim H-M. The inhibitory effect of polysaccharides isolated from *Phellinus linteus* on tumor growth and metastasis. Immuopharmacology 41: 157-164 (1999)
6. Ikekawa T, Nakanishi M, Uehara N, Chihara G, Fukuoka F. Antitumor action of some basidiomycetes, especially *Phellinus linteus*. Gann 69: 155-157 (1968)
7. Ji J-H, Kim M-N, Chung C-K, Ham S-S. Antimutagenic and cytotoxicity effects of *Phellinus linteus* extracts. J. Korean Soc. Food. Sci. Nutr. 29: 322-328 (2000)
8. Song K-S, Cho S-M, Lee J-H. B-lymphocyte stimulating polysaccharide from mushroom *Phellinus linteus*. Chem. Pharm. Bull. 43: 2105-2108 (1995)
9. Lee J-W, Baek S-J, Bang K-W, Kang S-W, Kang S-M, Kim B-Y, Ha I-S. Biological activity of polysaccharide extracted from the fruit body and cultured mycelia of *Phellinus linteus* IY001. Korean J. Food Sci. Technol. 32: 726-735 (2000)
10. Kim H-M, Han S-B, Oh G-T, Kim Y-H, Hong D-H, Hong N-D, Yoo I-D. Stimulation of humoral and cell mediated immunity by polysaccharide from mushroom *Phellinus linteus*. Int. J. Immunopharmacol. 18: 295-303 (1996)
11. Lee J-W, Bang K-W. Biological activity of *Phellinus* spp. Korean J. Food Ind. Nutr. 6: 25-33 (2001)
12. Carmichael J, De Graff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of tetrazolium-based semiautomated colorimetric assay assessment of chemosensitivity testing. Cancer Res. 47: 936-942 (1987)
13. Bowers GN, McComb RB, Upretti A. 4-Nitrophenyl phosphate-characterization of high-purity materials for measuring alkaline phosphatase activity in human serum. Clin. Chem. 27: 135-143 (1981)
14. Dempster DW, Lindsay R. Pathogenesis of osteoporosis. Lancet 34: 797-801 (1993)
15. Lindsay R. Managing osteoporosis: Current trends, future possibilities. Geriatrics 42: 35-39 (1987)
16. Pole HAP, Felsenberg D, Hanley DA, Stenpan J, Munoz-Torres M, Wolkins TJ, Qui-sheng G, Galich M, Vandormael K, Yates AJ. Multinational placebo-controlled, randomised trial of the effects of alendronate on bone density and fracture risk in post-menopausal women with law bone mass: Results of the FOSIT. Osteoporosis Int. 9: 461-468 (1999)
17. Ettinger B. Overview of estrogen replacement therapy: A historical perspective. P. Soc. Exp. Biol. Med. 217: 2-5 (1998)
18. Schafer JM, Lee ES, O'Regan RM, Yao K, Jordan VC. Rapid development of tamoxifen-stimulated mutant p53 breast tumors (T47D) in athymic mice. Clin. Cancer Res. 6: 4373-4380 (2000)
19. Kronenberg F, Fugh-Berman A. Complementary and alternative medicine for menopausal symptoms: A review of randomized, controlled trials. Ann. Intern. Med. 137: 805-813 (2002)
20. Farnsworth NR, Bingel AS, Cordell GA, Crane FA, Fong HS. Potential value of plant as sources of new antifertility agents. J. Parmcol. Sci. 64: 717-754 (1975)
21. Hofmann WE, Everds N, Pignatello M, Solter PF. Automated and semiautomated analysis of rat alkaline phosphate isoenzyme. Toxicol. Pathol. 22: 633-638 (1994)
22. Kress BC, Mizrahi IA, Armour KW, Marcus R, Emkey RD, Santora AC. Use of bone alkaline phosphatase to monitor alendronate therapy in individual postmenopausal osteoporotic women. Clin. Chem. 45: 1009-1017 (1999)
23. Ha H-K, Ho J-N, Shin S-M, Kim H-J, Koo S-J, Kim I-H, Kim C-S. Effects of Eucommiae Cortex on osteoblast-like cell proliferation and osteoclast inhibition. Arch. Pharm. Res. 26: 929-936 (2003)
24. Delmas PD. Biochemical markers of bone turnover. J. Bone Miner. Res. 8: S549-S555 (1993)
25. Eastell R, Robins SP, Colwell T, Assiri AMA, Riggs BL, Russell RG. Evaluation of bone turnover in type I osteoporosis using biochemical markers specific for both bone formation and bone resorption. Osteoporosis Int. 3: 255-260 (1993)
26. Ducki S, Hadfield JA, Lawrence NJ, Liu CY, McGown AT, Zhang W. Isolation of E-1-(4'-hydroxyphenyl)-but-1-en-3-one from *Scutellaria barbata*. Planta Med. 62: 185-186 (1996)
27. Tagashira M, Ohtake Y. A new antioxidative 1,3-benzodioxole from *Melissa officinalis*. Planta Med. 64: 555-558 (1998)
28. Baranovsky A, Schmitt B, Fowler DJ, Schneider B. Synthesis of new biosynthetically important diarylheptanoids and their oxa- and fluoro-analogues by three different strategies. Synthetic Commun. 33: 1019-1045 (2003)
29. Tsuda T, Watanabe M, Ohshima K, Yamamoto A, Kawakishi S, Osawa T. Antioxidative components isolated from the seed of tamarind (*Tamarindus indica* L.). J. Agr. Food Chem. 42: 2671-2674 (1994)
30. Reddy PS, Jamil K, Madhusudhan P, Anjani G, Das B. Antibacterial activity of isolates from *Piper longum* and *Taxus baccata*. Pharm. Biol. 39: 236-238 (2001)
31. Liu GT, Ahang TM, Wang BE, Wang YW. Protective action of seven natural phenolic compounds against peroxidative damage to biomembranes. Biochem. Pharmacol. 43: 147-152 (1992)
32. Watanabe K, Hayashi H, Mori Y. Effect of a benzylidene derivative, a novel antirheumatic agent, on IL-1 production. Pharm. Res. 28: 57-72 (1993)
33. Kikuzaki H, Kawai Y, Nakatani N. 1,1-Diphenyl-2-picrylhydrazyl radical scavenging active compounds from greater cardamom

(*Amomum subulatum* Roxb.). J. Nutr. Sci. Vitaminol. 47: 167-171 (2001)

34. Wang Y, Hasuma T, Yano Y, Morishima Y, Matsui-Yuasa I, Otani S. Induction of apoptosis in CTLL-2 cells by protocatechualdehyde. Anticancer Res. 21: 1095-1101 (2001)
35. Wilairat R, Kijjoa A, Pinto M, Nascimento MSJ, Silva AMS, Eaton G, Herz W. Constituents of *Schisandra verruculosa* and their cytotoxic effect on human cancer cell lines. Pharm. Biol. 44: 411-415 (2006)
36. Gray TK, Flynn TC, Gray KM, Nabell LM. 17β-Estradiol acts directly on the clonal osteoblastic cell line UMR106. P. Natl. Acad. Sci. USA 84: 6267-6271 (1987)
37. Yamaguchi M, Gao YH. Anabolic effect of genistein and genistin on bone metabolism in the femarol-metaphyseal tissues of elderly rats: The genistein effect is enhanced by zinc. Mol. Cell. Biochem. 178: 377-382 (1998)
38. Nishibe S, Hisada S, Inagaki I. Lignans of *Trachelospermum liukiuense* and *T. foetidum*. Chem. Pharm. Bull. 21: 674-675 (1973)
39. Sakamura S, Terayama Y, Kawakatsu S, Ichihara A, Saito H. Conjugated serotonins and phenolic constituents in safflower seed. Agr. Biol. Chem. Tokyo 44: 2951-2954 (1980)