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# Lipoxygenase Inhibitory and Antioxidant Activities of Isolated Compounds from Moutan Cortex

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**Abstract –** Phytochemical investigation on the ethyl acetate and n-butanol fractions of Moutan Cortex resulted in the isolation and characterization of a new monoterpene glycoside (**3**) and twenty known monoterpene glycosides (**1**, **2**, **4** - **21**). The structure of **3** was determined by spectroscopic data interpretation and physico-chemical properties. Compounds **1** and **8** presented a remarkable inhibitory activity against lipoxygenase-1 (LOX-1) with $IC_{50}$ values of 45.2 and 37.5 μM, respectively. Compounds **9**, **10**, **13**, **18**, **19**, and **21** showed significant 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging effect with $IC_{50}$ values of 9.8, 25.5, 6.4, 15.2, 18.7, and 23.7 μM, respectively. Benzoylpaeoniflorin (**8**), which exhibited the highest inhibitory effect with an $IC_{50}$ value of 37.5 ± 0.7 μM, was further analyzed the inhibition kinetics by Lineweaver-Burk plots. Results indicated that **8** is a non-competitive inhibitor, and the kinetic parameter values were estimated to be (31.04 μM, *K*i), (0.29 μM/min, $V_m$), and (48.50 μM, $K_m$).
**Keywords –** Moutan Cortex, monoterpenes, LOX-1, DPPH.

## Introduction

Lipoxygenases (LOXs, EC 1.13.11.12), an iron-containing dioxygenase, catalyze the regio- and stereo-specific oxygenation of polyunsaturated fatty acids (PUFA) containing a *cis*,*cis*-1,4-pentadiene hydroperoxidiene unit to produce their corresponding hydroperoxides (Chidananda and Sattur, 2007). In plants, the main substrates are linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) acids. The initial products of lipoxygenases, 9*S* and 13*S* fatty acid hydroperoxides, are proposed to have a regulatory roles in plant and animal metabolism and ability to form free radicals causing membrane damage and accelerate cell death (Gardner, 1995). The animal LOXs-catalyzed oxidation of arachidonic acid yields an unstable epoxy intermediates such as prostaglandins, thrombaxanes, leukotrienes, lipoxins and hepoxilins, which involved in a variety of human disorders such as atherosclerosis, inflammatory bowel disease, psoriasis, asthma, and other immune system disorders (Prigge *et al.*, 1997; Mahesha *et al.*, 2007). 12-lipoxygenase (12-LOX) metabolites have been found to play a central role in the various stages of the metastatic process in tumors and are, therefore, potential targets for anticancer treatment (Dailey and Imming, 1999). The ability of 15-lipoxygenase (15-LOX) (but not other lipoxygenenases) to oxidized low-density lipoproteins (LDL) is suggestive of an important role for this enzyme in the pathogenesis of atherosclerosis (Steinberg *et al.*, 1989; Prigge *et al.*, 1997). The 5-lipoxygenase (5-LOX) pathway of arachidonic acid metabolism forms leukotrienes (LTs) and other eicosanoids with diverse biologic efficiencies that are involved in asthma and other inflammatory diseases (Prigge *et al.*, 1997; Mahesha *et al.*, 2007). Soybean lipoxygenase type 1 (LOX-1) has been widely applied for both mechanistic and inhibitory studies and is commonly accepted as a model for LOXs from other sources because it has similar structure and function with mammalian LOXs (Prigge *et al.*, 1997). Therefore, LOX-1 is used as *in vitro* biochemical model, since it resembles human lipoxygenase in its substrate specificity and inhibitory characteristics (Borgeat *et al.*, 1983).

Free radicals play important role in carcinogenesis through their involvement in breaking of the DNA strand (Pathak and Joshi, 1983). Numerous other pathological

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events are associated with the generation of reactive oxygen species (ROS) constituting a key mechanism for tissue injury. They have significant relevance in the inflammation process, cardiovascular disease (Hertog *et al.*, 1993; Moure *et al.*, 2001), rheumatoid arthritis, and aging process (Halliwel and Gutteridge, 1999).

Moutan cortex ("Mok-Dan-Pi" in Korean), the root bark of *Paeonia suffruticosa* Andrew (Paeoniaceae), is an analgesic, sedative, and anti-inflammatory agent that has been used as a remedy for cardiovascular disorders, extravagated blood, and female genital diseases (Ding *et al.*, 1999). Numerous monoterpene glycosides and acetophenones have been isolated from this plant (Ha *et al.*, 2009b). The main compounds found in Moutan Cortex comprise paeonol, benzoylpaeoniflorin, paeoniflorin (Yoshikawa *et al.*, 2000; He *et al.*, 2006). Previous studies have shown that these compounds are largely responsible for pharmacological actions of Moutan Cortex (Wu *et al.*, 2003; Lau *et al*., 2007). However, a detailed study of the antioxidant and lipoxygenase inhibitory activity of isolated compounds from Moutan Cortex has not been conducted. As a part of our ongoing investigations of antioxidant and anti-inflammatory activities from natural sources, the EtOAc fraction significantly exhibited LOX-1 inhibitory activity. In addition, the EtOAc and BuOH fractions showed the potent DPPH radical quenching effect. Thus, extensive chemical and pharmacological studies were made on Moutan Cortex to determine its bioactive compounds. Herein, we report the antioxidant capacity and inhibitory activity of the extract and subsequence fractions, as well as the isolated compounds from Moutan Cortex against DPPH free radical and LOX-1, respectively.

## Results and Discussion

The EtOAc fraction exhibited the most potent LOX-1 inhibitory activity and DPPH radical scavenging effect with $IC_{50}$ value of 45.2 ± 1.1 μg/mL and 10.9 ± 0.5 μg/mL, respectively (Table 1). Previous study reported to the identification of the fifteen known compounds (**1**, **2**, **4** -**8**, **11** - **17**, and **20**) (Ha *et al.*, 2009b). Ongoing to study of this plant, a new compound (**3**) and four known compounds (**9**, **10**, **18**, **21**) were obtained (Fig. 1). The chemical structures of known compounds were identified as quercetin (**9**) (Miyazawa and Hisama, 2003), kaempferol (**10**) (Budzianowski, 1991), methyl gallate (**18**) (Ma *et al.*, 2003), (+)- catechin (**19**) (Schmeda-Hirschmann *et al.*, 2003), and gallic acid (**21**) (Ma *et al.*, 2003).

**Table 1.** DPPH radical scavenging activity and LOX-1 inhibitory effect of MeOH extract and subsequent fractions of Moutan Cortex

| Fractions | LOX-1 | DPPH |
|---|---|---|
| | [a] $IC_{50}$ (μg/mL) | |
| MeOH | 109.9 ± 2.25 | 40.1 ± 0.8 |
| Hexane | 100.1 ± 1.78 | > 150 |
| EtOAc | 45.2 ± 1.17 | 10.9 ± 0.5 |
| BuOH | 129.0 ± 1.56 | 37.5 ± 1.2 |
| [b] Baicalein | 6.3 ± 0.23 | – |
| [c] α-Tocopherol | – | 12.1 ± 0.8 |

[a] Results are represented as percent of control ± SD, n = 3.
[b] Baicalein was a standard inhibitor of LOX-1 (μg)
[c] α-Tocopherol was used as a positive control.

Compound **3** was obtained as colorless crystal needles. The UV absorption exhibited $\lambda_{max}$ 210, 213, and 241 for a benzoylic chromophore. Its IR spectrum exhibited absorption bands for 3529 (OH), 1744 (C = O), 1721 (ester group), 1451 (C = C), 1280 (C-O), 1061 (glycosidic C-O) $cm^{-1}$. Its molecular formula of $C_{23}H_{28}O_{11}$ was determined by HRFABMS and indicated 10 degrees of unsaturation. The positive ion mode HRFABMS of **3** showed a molecular ion peak at *m/z* 481.1972 $[M + H]^+$, corresponding to a molecular formula $C_{23}H_{28}O_{11}$ (481.1991). $^1H$ NMR spectroscopic data of **3** indicated the presence one monosubstituted aromatic ring, one hexosyl moiety, one tertiary methyl, two methines, three methylenes. In addition, $^{13}C$ NMR and DEPT-135 spectra of **3** displayed two quaternary oxygen-bearing carbon ($\delta_C$ 86.9), two carbonyl groups ($\delta_C$ 218.7 and $\delta_C$ 167.8), and two quaternary carbon signals, together with other 17 carbon signals bearing protons. The sugar moiety in **3** was deduced to be a β-D-glucopyranosyl unit because of the anomeric carbon signal at $\delta_C$ 96.7 and proton signal with the large coupling constant value [$\delta_H$ 4.62 (1H, d, $J$ = 7.6 Hz)] and the results of acid hydrolysis. The HMBC displayed the key correlations of H-1' aromeric proton to the C-1 [(H-1'/C-1 ($\delta_H$ 4.62/$\delta_C$ 86.9); H-8/C-1, C-5, C-7 ($\delta_H$ 4.79/$\delta_C$ 86.9, 38.9, 57.2)], showing the connectivities of the β-D-glucopyranoside to the pinan type monoterpene at C-1. The correlations from H-8 to C-7" and H-2",6" to C-7" [H-8/C-7" ($\delta_H$ 4.79/ $\delta_C$ 167.8); H-2", 6"/C-7" ($\delta_H$ 8.02/ $\delta_C$ 167.8)], indicated the linkages of the benzoyl moiety to C-7" (Fig. 2a). Morever, a methyl group was located at C-2, which was confirmed by HMBC correlations between Me-10 /C-1 and C-2 ($\delta_H$ 1.50/ $\delta_C$ 86.9 and 104.3) (Fig. 2a). Structure of **3** was similar to those of paeobrin except for the observation of different signals at C-2 ($\delta_C$ 104.3) and C-4 ($\delta_C$ 218.8) (Okasaka *et*

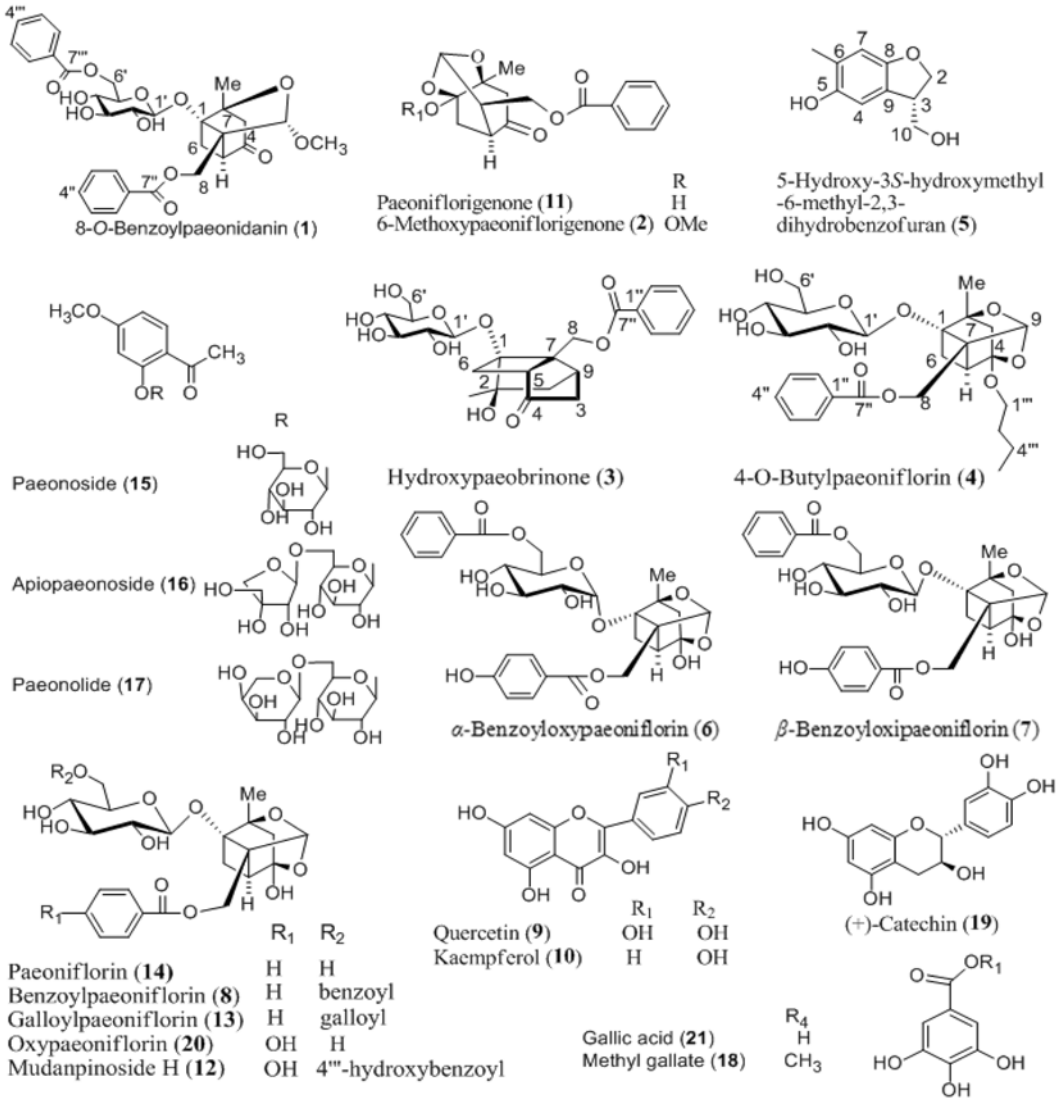


**Fig. 1.** Chemical structures of all isolated compounds from Moutan Cortex (**1** - **21**).

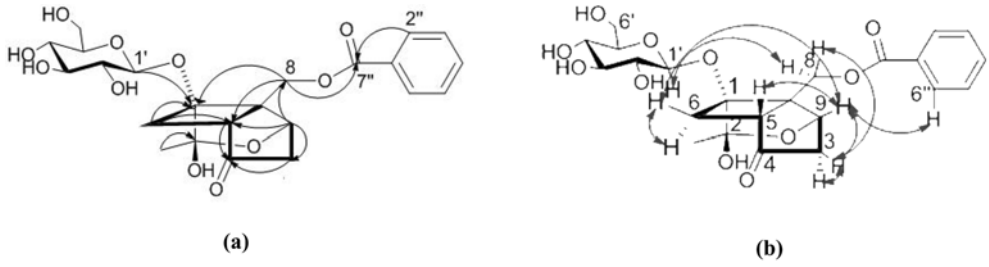


**Fig. 2.** Selected HMBC (a) and ROESY (b) correlations of **3**.

*al.*, 2008). The configurations of the stereogenic centers in the monoterpene unit were initially assigned by comparison to the spectra of related compounds, and were confirmed by the ROESY spectrum. The ROESY spectra also showed cross-correlations between the pairs of protons H-3a/H-3b; H-3b/H-8 and H-9; H-8/H-6, H-1'; H-6a/H-6b; H-9/H-6"; and H-6/H-9 (Fig. 2b). On this basis, the structure of **3** was determined as hydroxypaeobrinone. Compound **3** was considered to be derived from paeoniflorin by the similar process for the production of

**Table 2.** DPPH radical scavenging activity and LOX-1 inhibitory effect of the compounds (**1** - **21**)

| Compounds | LOX-1 | DPPH |
|---|---|---|
| | [a]$IC_{50}$ (μM) | |
| 8-*O*-Benzoylpaeonidanin (**1**) | 45.2 ± 2.1 | > 150 |
| 6-Methoxypaeoniflorigenone (**2**) | 70.4 ± 1.4 | > 150 |
| Hydroxypaeobrinone (**3**) | 75.9 ± 1.7 | > 150 |
| 4-*O*-Butylpaeoniflorin (**4**) | 148.2 ± 2.8 | > 150 |
| 5-Hydroxy-3*S*-hydroxymethyl-6-methyl-2,3-dihydrobenzofuran (**5**) | NT | > 150 |
| α-Benzoyloxypaeoniflorin (**6**) | 90.3 ± 1.8 | > 150 |
| β-Benzoyloxypaeoniflorin (**7**) | 85.2 ± 1.4 | > 150 |
| Benzoylpaeoniflorin (**8**) | 37.5 ± 0.7 | > 150 |
| [c] Quercetin (**9**) | NT | 9.8 0.4 |
| Kaempferol (**10**) | NT | 25.5 2.1 |
| Paeoniflorigenone (**11**) | 81.7 ± 2.1 | > 150 |
| Mudanpinoside H (**12**) | 112.1 ± 1.7 | > 150 |
| Galloyl paeoniflorin (**13**) | 62.8 ± 1.2 | 6.4 0.7 |
| Paeoniflorin (**14**) | 87.1 ± 1.4 | 118.2 ± 2.2 |
| Paeonolide (**15**) | > 150 | > 150 |
| Paeonoside(**16**) | > 150 | > 150 |
| Apiopaeonoside (**17**) | > 150 | > 500 |
| Methyl gallate (**18**) | > 150 | 15.2 1.1 |
| (+)-Catechin (**19**) | 104.2 ± 1.4 | 18.7 1.6 |
| Oxypaeoniflorin (**20**) | > 150 | 87.6 ± 1.7 |
| [b)] Baicalein | 23.4 ± 0.5 | – |
| [c)] Gallic acid (**21**) | – | 23.7 1.2 |

[a] Results are represented as percent of control ± SD, n = 3.
[b] Baicalein was a standard inhibitors of the LOX-1 (μM)
[c] Quercetin and gallic acid were used as positive controls.
NT: Not test

lactiflorin (Okasaka *et al.*, 2008).

Compounds **5**, **15** - **18**, and **20** did not inhibit LOX-1 (Table 2) and compounds **2** - **4**, **6**, **7**, **11** - **14**, and **19** showed moderate inhibitory activity against LOX-1, with $IC_{50}$ values ranging from 62.8 to 148.2 μM. Of these isolated compounds, **1** ($IC_{50}$ 45.2 μM) and **8** ($IC_{50}$ 37.5 μM) exhibited the considerable LOX-1 inhibitory effect as compared with these of positive control (baicalein, $IC_{50}$ value of 23.4 μM) (Table 2). This result suggested that **1** and **8** with higher hydrophobic properties than the others might have highly hydrophobic interaction with active site (hydrophobicity) of LOX-1 (Prigge *et al.*, 1997). In order to detect the nature of inhibition of LOX-1 by monoterpene glycosides, benzoylpaeoniflorin (BPF), which showed the highest inhibitory activity against soybean LOX-1 enzyme, was chosen for performance kinetic experiments. As demonstrated in Fig. 3, BPF showed a dose-dependent inhibitory effect. Herein, the concentration of BPF increased, the enzyme activity was quickly decreased. In addition, the $IC_{50}$ was estimated to

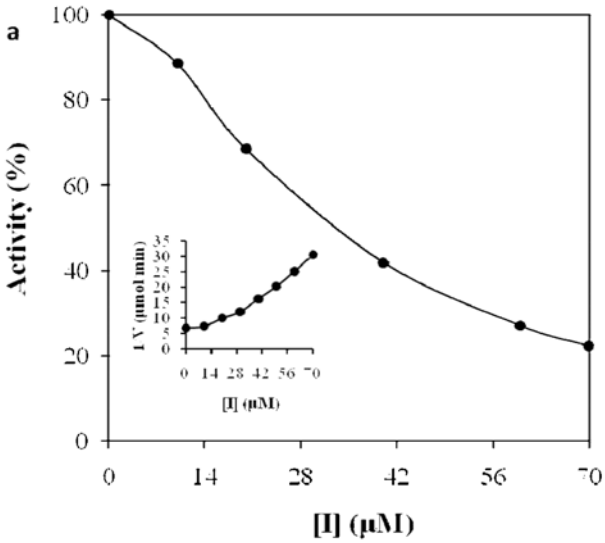


**Fig. 3.** Effects of **8** on the activity of LOX-1 for catalysis of linoleic acid at 25 °C. (Inset) Replots of data as 1/V versus [I].

be 40.61 μM by interpolation from inhibition curve (Fig. 3). A Lineweaver-Burk analysis has demonstrated that

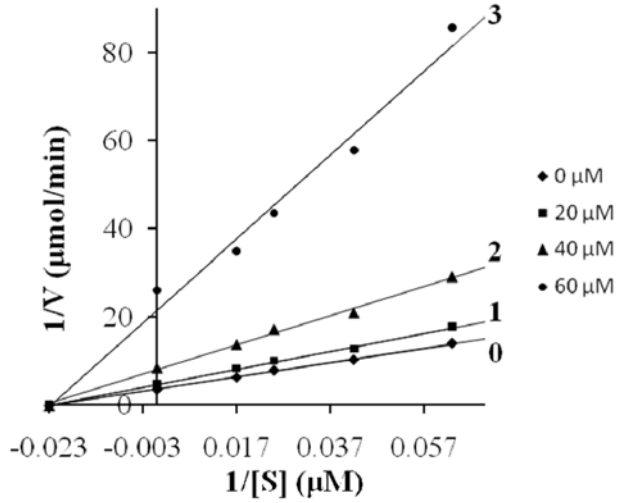


**Fig. 4.** Lineweaver-Burk plots of 13S linoleic acid hydroxyperoxide (13-HPOD) generation by LOX-1 in the presence of **8** at 25 °C, pH 8.8. Concentration of BPF for curves 0 - 3 were 0, 20, 40, 60 μM, respectively.

BPF exhibited a non-competitive inhibition against LOX-1. Moreover, the Lineweaver-Burk plot created from enzyme Kinetic Module 1.3 (SPSS Inc.) equipped with Sigma plot 10.0 for non-competitive inhibition manner was applied to determine kinetic parameters ($K_m$, $V_{max}$, and $K_i$) (Fig. 4). Result showed that the mean $K_m$ value 48.50 μM, $V_{max}$ value 0.29 μM/min, and $K_i$ value 31.04 μM, respectively.

Almost monoterpenes, acetophenones, and a dihydrobenzofuran derivative did not scavenge DPPH radical except for **13**, which possessed a three adjacent hydroxy group at 6'-benzoyl moiety, displayed highly potent DPPH radical scavenging effect ($IC_{50}$ value of 6.4 μM) as compared with positive controls **9** ($IC_{50}$ value of 9.8 μM) and **21** ($IC_{50}$ value of 23.7 μM). Besides, **9**, **10**, **18**, **19**, and **20** showed considerable activity (Table 2).

In brief, this study contributes the scientific evidence, at least in part, why Moutan Cortex has traditionally used as an anti-inflammatory agent.

## Experimental Part

**Plant material** – Moutan Cortex, *P. suffruticosa*, was purchased from traditional herbal market in Daejeon, Korea, in January 2007. A specimen of the plant (CNU-1554) has been verified by Prof. K. Bae and deposited at the Herbarium of the College of Pharmacy, Chungnam National University, Korea.

**Chemicals and Reagents** – Soybean lipoxygenase (1.13.11.12) type I-B (LOX-1) was purchased from Sigma-Aldrich Chemical Co., U.S.A.. Linoleic acid (9(*Z*), 12 (*Z*)-octadecadienoic acid, 99%, was from Junsei Chemical Co. Ltds. Baicalein was purchased from Cayman Chemical Company. Unless otherwise indicated, all other chemicals were of analytical grade purity.

**Generals** – Melting points were determined on an Electrothemal apparatus. Optical rotations were measured on a JASCO DIP-370 (Tokyo, Japan) automatic digital polarimeter. UV spectra were taken in MeOH using a JASCO V-550 UV/VIS spectrometer. IR spectra were recorded on a JASCO 100 IR spectrophotometer. The nuclear magnetic resonance (NMR) $^1H$ (300 MHz) and $^{13}C$ (75 MHz) spectra were recorded on a Bruker-DRX-300. Chemical shift were reported in *ppm* downfield from tetramethylsilane (TMS, with *J* in Hz). Mass spectra were obtained with a JEOL MS-700 MStation mass spectrometer. Analytical TLC was performed on Kieselgel 60 $F_{254}$ (Merck) plates (silica gel, 0.25 mm layer thickness) and RP-18 $F_{254}$ (Merck) plates (0.25 mm layer thickness). UV spots were detected by ultraviolet irradiation (254 and 365 nm) and by spraying 10% $H_2SO_4$, followed by heating with a heat gun. Column chromatography was performed with silica gel (70 - 230 and 230 - 400 mesh, Merck), YMC RP-18 resin (30 - 50 μm, Fuji Silysia Chemical Ltd.), Sephadex$^{TM}$ LH-20 (Amersham Biosciences, Uppsala, Sweden).

**Extraction and Isolation** – The Moutan Cortex (20 kg) was extracted three times with MeOH at 60 °C. The combined filtrates were taken to dryness *in vacuo* (40 °C) and residue (3000 g) was stirred with 1000 mL of 95% MeOH and suspended in $H_2O$, then successively partitioned with hexane, EtOAc, BuOH, and $H_2O$.

The EtOAc fraction (600 g) was separated with silica gel CC using increasing polarity solvents ($CHCl_3$-MeOH 100 : 0, 97 : 3, and 0 : 100) to give six fractions (Fr.1-6). Fraction 5 (9.0 g) was eluted through a Sephadex LH-20 column (MeOH-$H_2O$, 2 : 1) to obtain six fractions (Fr. 5.1 - 5.6). Rechromatography of fraction 5.3 (7.1 g) on a silica gel column with $CHCl_3$-MeOH (20 : 1) generated 6 fractions (Fr. 5.3.1 - 5.3.7). Fraction 5.3.1 (3.5 g) was further separated by an YMC column with MeOH-$H_2O$ (1 : 1) to give 10 subfractions (Fr. 5.3.1.1 - 5.3.1.10). Two compounds **1** (72 mg) and **2** (1,100 mg) was obtained from sub-fraction 5.3.1.2 (2.1 g). Fr. 5.3.2 (0.025 g) was crystallized to obtain **3** (10 mg). Three compounds **5** (8 mg), **6** (20 mg), and **7** (25 mg) were obtained from sub-fraction 5.3.7 (0.2 g) (Ha *et al.*, 2009b).

Fraction 6 (60 g) was combined with the *n*-BuOH fraction (250 g), and then subjected to CC on a silica gel with $CHCl_3$-MeOH eluent ratios of 19 : 1, 9 : 1, 17 : 3,

and pure MeOH, to give fractions (B.1-B.4). Compounds **8** (6.8 g), **9** (20 mg), (**10**, 250 mg), **11** (190 mg), and **12** (5.7 mg) was afforded from sub-fraction B.2 (63 g). B.3 (127 g) was repeatedly subjected to silica gel CC using $CHCl_3$-MeOH (20 : 1) as an eluent to give five subfractions (B.3.1 - 3.5). Fraction B.3.1 (49 g) was subjected to a silica gel CC and eluted using $CHCl_3$ -MeOH mixtures to afford six fractions (B.3.1.1 - 3.1.6). Compounds **13** (200 mg) and **4** (14 mg) were separated from sub-fraction B.3.1.3 (2.6 g) and compounds **14** (6.8 g), **15** (50 mg), **16** (1.2 g), and **17** (100 mg) generated from B.3.1.5 (21.3 g). The third fraction B.3.3 (38 g) was repeatedly applied to a Sephadex LH-20 column, prior to elution with MeOH-$H_2O$ (2 : 1) to give 4 fractions (B.3.3.1-3.3.4) (Ha *et al.*, 2009b). Compounds **18** (6.2 g), **19** (200 mg), and **20** (1.2 g) were yielded from sub-fraction B.3.3.1 (17 g). Compound **21** (2600 mg) was obtained from sub-fraction B.3.4 (25 g). The major constituents of the extract were analyzed and identified. Fraction numbers that were not specifically mentioned contained only minor amounts of the numbered compounds.

**Hydroxypaeobrinone (3):** Colorless needles; mp 195 - 196 ºC; $[\alpha]_D^{25}$ - 7.2 (*c* 0.14, MeOH); UV (MeOH) $\lambda_{max}$ (log ε) 210 (3.99), 213 (3.83), 241 (3.66) nm; IR (KBr) $\nu_{max}$ 3529 (OH), 2939, 1744 (C = O), 1721 (ester), 1600, 1451 (C = C), 1280 (C-O), 1174, 1121, 1061, (glycosidic C-O), 957, 716 $cm^{-1}$; FABMS *m/z* 481.2 $[M + H]^+$; HRFABMS *m/z* 481.1972 $[M + H]^+$, (calcd. for $C_{23}H_{29}O_{11}$ (481.1991); $^1H$ NMR (300 MHz, methanol-$d_4$) δ 1.50 (3H, s, H-10), 2.19 (1H, dd, *J* = 13.6, 4.8 Hz, H-6a), 2.48 (1H, d, *J* = 13.6 Hz, H-6b), 2.54 (1H, dd, *J* = 11.5, 3.4 Hz, H-3a), 2.73 (1H, dd, *J* = 6.3, 4.8 Hz, H-5), 2.79 (1H, d, *J* = 3.4 Hz, H-3b), , 3.34 (1H, m, H-5'), 3.37 (1H, m, H-4'), 3.51 (1H, m, H-3'), 3.67 (1H, dd, *J* = 11.6, 4.8 Hz, H-6'a), 3.84 (1H, d, *J* = 11.6 Hz, H-6'b), 4.62 (1H, d, *J* = 7.6 Hz, H-1'), 4.79 (2H, s, H-8), 4.91 (1H, d, *J* = 3.4 Hz, H-9), 7.50 (2H, t, *J* = 7.8 Hz, H-3'', 5''), 7.64 (1H, tt, *J* = 7.8, 1.2 Hz, H-4''), 8.02 (2H, d, *J* = 7.8 Hz, H-2'', 6''); $^{13}C$ NMR (75 MHz, methanol-$d_4$) δ 16.5 (C-10), 31.8 (C-6), 38.9 (C-5), 48.3 (C-3), 57.1 (C-7), 62.5 (C-6'), 63.9 (C-8), 71.9 (C-4'), 74.9 (C-2'), 76.4 (C-5'), 80.2 (C-3'), 81.8 (C-9), 86.9 (C-1), 96.7 (C-1'), 104.3 (C-2), 130.0 (C-3'', 5''), 130.8 (C-2'', 6''), 130.9 (C-1''), 134.9 (C-4''), 167.8 (C-7''), 218.7 (C-4).

**Acid Hydrolysis of 3** – Compounds **3** (2 mg) were dissolved in 1.0 N HCl (dioxane$H_2O$, 1 : 1, v/v, 1.0 mL) and then heated to 80 ºC in water bath for 3 h. The acidic solution was neutralized with silver carbonate and the solvent thoroughly driven out under $N_2$ gas overnight. After extraction with $CHCl_3$, the aqueous layer was concentrated to dryness using $N_2$ gas. The residue was dissolved in 0.1 mL of dried pyridine, and then L-cysteine methyl ester hydrochloride in pyridine (0.06 M, 0.1 mL) was added to the solution. The reaction mixture was heated at 60 ºC for 2 h, and 0.1 mL of trimethylsilylimidazole solution was added, followed by heating at 60 ºC for 1.5 h. The dried product was partitioned with hexane and $H_2O$ (each 0.1 mL), and the organic layer was analyzed by gas liquid chromatography: Column: column SPB-1 (0.25 mm × 30 m); detector FID, column temp 210 ºC, injector temp 270 ºC, detector temp 300 ºC, carrier gas He (30 mL/min) (Ahn *et al.*, 2006). The retention times of persilylated glucose was identified to be 14.11, when compared with the authentic solutions prepared by the same reaction from the standard monosaccharides (the retention times of persilylated D-glucose and L-glucose were 14.11 and 14.26, respectively).

**Lipoxygenase assay** – The enzyme assay was performed as previously reported with slight modifications (Ha *et al.*, 2009a). Soybean lipoxygenase (131.000 U/mL) was preincubated in 100 mM borate buffer (pH 8.8) for 5 min in a 10 mm path length cuvette at room temperature. The reaction was started by the addition of a linoleic acid (40 μM final concentration) and the change of absorbance at 234 nm was monitored to determine the formation of conjugated dienes. The samples receiving the same volumes of DMSO served as controls. All compounds were dissolved in DMSO unless otherwise indicated.

**Inhibitory effect of test compounds on LOX activity** – Soybean lipoxygenase (131.000 U/mL) was preincubated in 0.5 ml of 100 mM borate buffer (pH 8.8) in the presence of test compound of various concentrations for 5 min in a 10 mm path length cuvette at room temperature. The reaction was started by the addition of a linoleic acid (40 μM final concentration) and the change of absorbance at 234 nm was monitored as describe above

The % inhibition of LOX activity by isolates was calculated from the ΔOD values at 234 nm at the end of 3 min.

Inhibition (%) = [(Ac – As) / Ac]*100

Where Ac and As are the absorbance of the control (without test sample) and the sample, respectively. Results are expressed as a Mean ± SD of at least triplicate measurements. The $K_i$, $K_m$, and $V_m$ values were determined by varying the inhibitor (0, 10, 20, 40, and 60 μM) and substrate (0, 16, 24, 40, and 60 μM) concentrations with the constant enzyme concentration (2.75 μg/mL) using Linewearver-Burk plot.

**DPPH radical scavenging assay** – DPPH radical scavenging activity was determined by the described

method (Ha *et al.*, 2009a). Briefly, each sample in DMSO (5 μL) was added to 150 μM DPPH (195 μL) solution in 96 well plates. The resulting solution was incubated at room temperature for 30 min and the absorbance of the reaction mixture was measured at 520 nm on a micro plate reader.

**Data analysis** – The data analysis was performed by using Sigma plot 10.0 (SPSS Inc., Chicago, IL). Inhibition mode was analyzed by enzyme Kinetic Module 1.3 (SPSS Inc.) equipped with Sigma plot 10.0.

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