NF-κB Inhibitory Activities of Glycosides and Alkaloids from Zanthoxylum schinifolium Stems

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Zanthoxylum schinifolium is an aromatic shrub, the pericarp and leaves of which are widely used in culinary applications in East Asian countries. In the present study, one new neolignan glycoside, zanthoxyloside A (1) together with 16 known glycosides (2–12) and alkaloids (13–17), were isolated from methanol extract of the stems of Z. schinifolium. The absolute configuration of one known monoterpenoid glycoside (2) was determined. The structures of the isolated compounds were established by one dimensional (1D), 2D NMR and mass spectrometry. The nuclear factor-κB (NF-κB) inhibitory activities of the isolated compounds stimulated with tumor necrosis factor alpha (TNFα) were measured using a luciferase reporter system. Compounds 1, 5, 16, and 17 exhibited significant inhibition of NF-κB activation in a dose-dependent manner. Furthermore, compounds 1, 5, 16, and 17 inhibited TNFα-induced expression of inducible nitric oxide synthase (iNOS) and intercellular adhesion molecule-1 (ICAM-1) mRNA and dose-dependent inhibition of iNOS promoter activity.

Key words Zanthoxylum schinifolium; Rutaceae; glycoside; alkaloid; nuclear factor-κB (NF-κB) inhibitory activity

Zanthoxylum schinifolium Sieb. & Zucc., which belongs to the Rutaceae family, is an aromatic plant, widely used as a pungent condiment and seasoning in Korea, China, Japan and other East Asian countries because of its unique aroma and taste. 1 The leaves and seeds of Z. schinifolium have been used primarily for treatment of various types of pain, the common cold, diarrhea, and jaundice in traditional Chinese medicine. Pharmacological research on Z. schinifolium has focused on its inhibitory effect on monoamine oxidase and its anti-platelet, anti-diabetes, and anti-inflammatory activities. 2–4 Phytochemical studies on Z. schinifolium indicated the main constituents to be coumarins, essential oils, triterpenoids, monoterpenoids, steroids, and flavonoids. 5–7 Coumarins were shown to have significant platelet aggregation activity. Essential oils were found to have antioxidant activity and are used in skin care products. Alkaloids have various bioactivities such as antimicrobial effects. 8 Although there have been many investigations of the active constituents of Z. schinifolium, there have been no detailed reports of the isolation and structural elucidation of glycosides and alkaloids from the stems of this plant or their bioactivities.

Nuclear factor-κB (NF-κB) represents a family of Rel domain-containing proteins, which includes five NF-κB units that can form 15 transcription factors through homo- and heterodimerization. NF-κB plays an important role in the transcriptional regulation of numerous cytokines and adhesion molecules. It is the most extensively studied transcription factor in the immune system. Known inducers of NF-κB activity are highly variable and include reactive oxygen species (ROS), tumor necrosis factor alpha (TNFα), interleukin (IL)-1β, bacterial lipopolysaccharides (LPS), iso-proterenol, cocaine, and ionizing radiation. The activation of NF-κB causes transcription at the eB site, which is involved in many diseases, including inflammatory disorders and cancer. Hence, the inhibition of NF-κB signaling has become a therapeutic target for the treatment of inflammatory diseases and cancer. 9–12 In the present study, the effects of isolated compounds 1–17 from Z. schinifolium on TNFα-induced NF-κB transcriptional activity in human embryonic kidney (293T) cells were evaluated by NF-κB-luciferase assay. To confirm the inhibitory effects of these compounds on NF-κB transcriptional activity, the effects of compounds 1, 5, 16, and 17 on upregulation of the proinflammatory protein inducible nitric oxide synthase (iNOS) and intercellular adhesion molecule-1 (ICAM-1) were evaluated by reverse transcriptase polymerase chain reaction (RT-PCR), as well as their iNOS promoter activities.

Results and Discussion

In this study, purification of the EtOAc and n-BuOH fractions of Z. schinifolium stems by silica gel and YMC column chromatography afforded one new neolignan glycoside, zanthoxyloside A (1), and one newly monoterpenoid glycoside, zanthoxyloside B (2), together with 15 known glycosides (3–12) and alkaloids (13–17). Their structures were elucidated as zanthoxyloside A (1), zanthoxyloside B (2), betulalbuside A (3), 2-hydroxy-4-(2-hydroxyethyl)phenyl, 6-(4-hydroxy-3,5-dimethoxybenzoate) O-β-D-glucopyranoside (4), eugenyl-O-β-apiofuranosyl-(1→6)-O-β-D-glucopyranoside (5), isopropyl apio-oligosaccharide (6), benzyl 6-O-β-apoliofuranosyl-O-β-D-glucopyranoside (7), osmanthuside H (8), coniferin (9), syringin (10), homovanillyl alcohol-4’-glycoside (11), rossoside A (12), skimmianine (13), hapterol (14), glycochapelone (15), norcchelerythrine (16), and normitidine (17). Compounds 4, 5, 8, 11, 15, and 17 were isolated from Z. schinifolium for the first time (Fig. 1). Compound 1, a colorless oil, showed a pseudomolecular ion peak [M+H]^+ at m/z 653.2440 (Calcd 653.2445) in high resolution-electrospray ionization-mass spectra (HR-ESI-MS) spectrum, suggesting the molecular formula C_{32}H_{36}O_{15}. The

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IR spectrum of 1 indicated the presence of hydroxy group (3450 cm$^{-1}$), olefinic moiety (1615 cm$^{-1}$), and aromatic moiety (1530 cm$^{-1}$). In the $^1$H-NMR spectrum of 1, three aromatic proton resonances at $\delta_H$ 6.94 (d, $J=1.5$ Hz, H-2'), 7.13 (d, $J=8.0$ Hz, H-5'), and 7.15 (dd, $J=8.0$, 1.5 Hz, H-6') were observed as an ABX system, suggesting that 1 contains a trisubstituted aromatic moiety. Two other proton signals at $\delta_H$ 7.14 (br s, H-5) and 7.23 (br s, H-7) were indicative of the presence of an additional tetrasubstituted aromatic moiety in the structure of 1. Moreover, the $^1$H-NMR spectrum of 1 displayed two methoxy singlets at $\delta_H$ 3.58 and 3.73, two trans double bond protons at $\delta_H$ 6.25 (ddd, $J=16.0$, 6.5, 6.0 Hz, H-11) and 6.68 (d, $J=16.0$ Hz, H-10), and an oxymethine proton at $\delta_H$ 5.99 (d, $J=7.0$ Hz, H-2). Two oxymethylene protons resonated at $\delta_H$ 4.10 (overlap, H-13a), 4.20 (overlap, H-13b), 4.40 (ddd, $J=12.0$, 6.0, 1.5 Hz, H-12a), and 4.72 (overlap, H-12b). The $^{13}$C-NMR spectrum of 1 contained 20 aglycone carbon resonances, which were assigned as two methoxy groups, two methylenes, nine methines and seven quaternary carbon atoms by DEPT-135 experiment (Table 1). An oxymethine proton at $\delta_H$ 5.99 (d, $J=7.0$ Hz, H-2), which coupled to the methine proton $\delta_H$ 3.87 (ddd, $J=7.0$, 6.0, 5.3 Hz, H-3) in the correlation spectroscopy (COSY) spectrum, and these were ascribed to a benzofuran ring. H-3 showed additional coupling with the oxymethine protons at $\delta_H$ 4.10 (overlap, H-13a) and 4.20 (overlap, H-13a), suggested the oxymethylene group attached to a benzofuran ring. Two methoxy groups were placed at C-3/uni2032 and C-8 of aromatic rings on the basis of heteronuclear multiple bond connectivity (HMBC) correlations between $\delta_H$ 3.73/$\delta_C$ 148.7 and $\delta_H$ 3.58/$\delta_C$ 144.6. The correlations between H-2'/C-2, H-6'/C-2, and H-2/C-1' showed the aromatic substitution to take place at C-2. In the COSY spectrum, the proton signal at $\delta_H$ 6.25 (ddd, $J=16.0$, 6.5, 6.0 Hz, H-11) coupled with $\delta_H$ 6.68 (d, $J=16.0$ Hz, H-10), H-10 showed couplings with the oxymethylene protons at $\delta_H$ 4.40 (ddd, $J=12.0$, 6.0, 1.5 Hz, H-12a) and 4.72 (overlap, H-12b), HMBC cross coupling between H-10/C-6 and H-11/C-6 indicated the attachment of a side chain at C-6. From a detailed inspection of these data, associated with the interpretation of COSY, $^1$H-detected heteronuclear multiple quantum coherence (HMQC) and HMBC data, the aglycone of 1 was determined to be dehydrodiconiferyl alcohol.26) Compound 1 had a minus circular dichroism (CD) curve with a minimum at 281 nm, similar to the curve for (−) dehydrodiconiferyl alcohol.26) Enzymatic hydrolysis of 1 produced D-glucose and D-apiose as sugar residues compared with those reported in the literature using a GC experiment after enzymatic hydrolysis. The β-D-configurations of glucose and
apiose residue were attributed by $^{13}$C-NMR chemical shifts ($\delta_C$ 65.3–111.3) and coupling constants (7.0, 2.3 Hz) of the anomeric protons. The HMBC correlations between $\delta_H$ 4.89 (Glc-H1) and $\delta_C$ 69.7 (C-12) indicated 6-O-$\beta$-D-apiofuranosyl-$\beta$-D-glucopyranose was connected at C-12 of aglycone (Fig. 2). Therefore, compound 1 was assigned as (2R,3S)-(−)-dehydrodiconiferyl alcohol 12-(6-O-$\beta$-D-apiofuranosyl-$\beta$-D-glucopyranoside), named zanthoxyloside A.

Compound 2 was obtained as a white amorphous powder. The molecular formula was determined to be C$_{21}$H$_{36}$O$_{11}$ from a pseudomolecular ion peak [M+H]$^{+}$ at $m/z$ 465.2332 (Calcd 465.2336) in the HR-ESI-MS spectrum. The $^1$H-NMR spectrum of 2 revealed the presence of $\beta$-D-apiose and $\beta$-D-glucose units from the anomeric proton signals at $\delta_H$ 4.15 (d, $J=7.0$ Hz, Glc-H1) and 4.80 (d, $J=2.3$ Hz, Api-H1). The large coupling constants between H-1 and H-2 of sugar units indicated

### Table 1. The $^1$H- and $^{13}$C-NMR Spectroscopic Data of Compounds 1 and 2

|      | $\delta_C^{a,b,c}$ | $\delta_H^{a,d}$ ($J$ in Hz) | $\delta_C^{b,c}$ | $\delta_H^{b,d}$ ($J$ in Hz) |
|------|--------------------|-------------------------------|------------------|-------------------------------|
| 1    |                    |                               | 112.3            | 4.92 (dd, 11.0, 2.0)          |
| 2    | 88.6               | 5.99 (d, 7.0)                 | 113.3            | 144.6 (s)                     |
| 3    | 54.5               | 3.87 (ddd, 7.0, 6.0, 5.3)     | 110.6            | 6.94 (d, 1.5)                 |
| 4    | 130.4              | 7.14 (br s)                   | 114.1            | 4.15 (d, 7.0)                 |
| 5    | 115.9              | 7.23 (brs)                    | 131.0            | 3.87 (m)                      |
| 6    | 111.0              | 5.38 (t, 7.0)                 | 132.9            | 3.87 (m)                      |
| 7    | 144.6              | 7.23 (brs)                    | 132.9            | 3.87 (m)                      |
| 8    | 148.6              | 7.23 (brs)                    | 133.3            | 7.13 (d, 8.0)                 |
| 9    | 132.9              | 6.68 (d, 16.0)                | 132.9            | 6.68 (d, 16.0)                |
| 10   | 123.4              | 6.25 (dd, 16.0, 6.5, 6.0)     | 132.9            | 6.68 (d, 16.0)                |
| 11   | 69.7               | 4.40 (dd, 12.0, 6.0, 1.5)     | 132.9            | 6.68 (d, 16.0)                |
| 12   | 63.8               | 4.10 (m)$^a$                  | 132.9            | 6.68 (d, 16.0)                |
| 13   | 133.3              | 4.20 (m)$^a$                  | 132.9            | 6.68 (d, 16.0)                |
| 1’   | 110.6              | 6.94 (d, 1.5)                 | 132.9            | 6.68 (d, 16.0)                |
| 2’   | 148.7              | 4.15 (m)$^a$                  | 132.9            | 6.68 (d, 16.0)                |
| 3’   | 148.0              | 4.20 (m)$^a$                  | 132.9            | 6.68 (d, 16.0)                |
| 5’   | 116.3              | 7.13 (d, 8.0)                 | 132.9            | 6.68 (d, 16.0)                |
| 6’   | 119.6              | 7.15 (dd, 8.0, 1.5)           | 132.9            | 6.68 (d, 16.0)                |
| 8-OMe| 55.6               | 3.58 (s)                      | 132.9            | 6.68 (d, 16.0)                |
| 3’-OMe| 55.9              | 3.73 (s)                      | 132.9            | 6.68 (d, 16.0)                |
| Glc-1| 103.4              | 4.89 (d, 7.0)$^a$             | 110.6            | 4.15 (d, 7.0)                 |
| 2    | 74.8               | 4.30 (m)$^a$                  | 75.1             | 3.87 (m)$^a$                  |
| 3    | 77.9               | 4.06 (m)$^a$                  | 78.1             | 3.20 (m)$^a$                  |
| 4    | 71.7               | 4.00 (t, 7.0)                 | 71.8             | 3.16 (t, 7.0)                 |
| 5    | 78.4               | 4.18 (m)$^a$                  | 78.2             | 3.24 (m)$^a$                  |
| 6    | 68.8               | 4.16 (m)$^a$                  | 69.8             | 3.49 (m)$^a$                  |
| Api-1| 111.3              | 5.78 (d, 2.3)                 | 112.2            | 4.80 (d, 2.3)                 |
| 2    | 77.6               | 4.70 (d, 2.3)                 | 77.0             | 3.80 (d, 2.3)                 |
| 3    | 80.3               | 4.70 (d, 2.3)                 | 80.7             | 3.80 (d, 2.3)                 |
| 4    | 75.0               | 4.30 (m)$^a$                  | 75.1             | 3.08 (m)$^a$                  |
| 5    | 65.3               | 4.54 (d, 9.8)                 | 65.6             | 3.47 (s)                      |

Assignments were done by HMQC and HMBC experiments; $J$ values (Hz) are in parentheses. a) Measured in pyridine-$d_5$, b) Measured in methanol-$d_4$, c) 150 MHz, d) 600 MHz, e) Overlapped.
β-linkage. The signals of two methyl groups at δH 1.15 (s, H-9) and 1.57 (s, H-10), three olefinic proton signals at δH 4.92 (dd, J=11.0, 2.0 Hz, H-1a), 5.10 (dd, J=18.0, 2.0 Hz, H-1b), and 5.81 (dd, J=18.0, 11.0 Hz, H-2) indicated a terminal double bond group of the aglycone moiety. The 13C-NMR spectrum of 2 indicated the presence of 10 carbon signals for the aglycone moiety, which were assigned to two methyl signals at δC 23.5 (C-5) and 25.1 (C-8), which was linked to a hydroxyl group. As the optical rotation value of the aglycone moiety, which was determined to be (3R,2S)-3,7-dimethylocta-1,6-dien-3,8-diol (δα = +8.60), the absolute configuration of the 3-position was concluded to be S. 29) The COSY correlation between δH 4.15 (Glc-H1) and δH 5.09 (d, 7.0) supported this structure. Enzymatic hydrolysis of 2 produced D-glucose and α-apiose as sugar residues, the same as 1. As the optical rotation value of the aglycone moiety was [α]D25 25 = +8.50 (c=0.1, MeOH), compared with (3R)-α-d-glucopyranosyl-β-D-apiofuranosyl-β-D-glucopyranoside), named zanthoxylside B. The isolation of 3,7-dimethylocta-1,6-dien-3,8-diol 8-(6-O-β-D-apiofuranosyl-β-D-glucopyranoside) from wine has been reported, but its absolute configuration of the 3-position has not been assigned. This is the first report of its absolute configuration.

Glycohaplopine (15) previously isolated from Haplophyllum perforatum, 24) but the spectroscopic data were not given. Here, we reported the 1H- and 13C-NMR data for glycohaplopine for the first time (Table 2).

The NF-κB inhibitory activity of the isolated compounds was evaluated by inhibition of TNFα-induced NF-κB luciferase reporter. Cell viability was measured using a Cell-Counting Kit (CCK)-8. Compounds 1, 5, 16, and 17 did not exhibit significant cytotoxicity against 293T cells at the concentrations tested, whereas compounds 13-15 showed moderate cytotoxicity (data not shown). 293T cells were treated with 10ng/mL TNFα and showed increased transcriptional activity compared to untreated cells. The compounds were pretreated with transfected 293T cells at various concentrations (0.01, 0.1, 1, 10 µM), followed by stimulation with TNFα. Apigenin was used as a positive control (Fig. 3). The results showed that compounds 1, 5, 16, and 17 exhibited significant inhibition.
of NF-κB activation in a dose-dependent manner, with IC50 values ranging from 0.67 to 37.5 µM, respectively. Compounds 2 and 6–8 inhibited moderate NF-κB transcriptional activity (data not shown). Especially compound 16, which showed significantly activity more potent than positive control. However, other compounds were inactive at the indicated concentrations (IC50 >100 µM, data not shown).

The activation of NF-κB is involved in immunity, inflammation, cell proliferation and negative feedback of the NF-κB signal. NF-κB targets the expression of genes such as iNOS and ICAM-1, which play important roles in the inflammatory response. Compounds 1, 5, 16, and 17 were investigated for their transcriptional inhibitory effects on iNOS and ICAM-1 gene expression. Compound 16 significantly inhibited the induction of iNOS and ICAM-1 mRNA in a concentration-dependent manner; compounds 1, 5, and 17 also showed moderate effects, indicating that these compounds reduced the transcription of these genes (Fig. 4). The housekeeping protein, β-actin was unchanged by the presence of compounds 1, 5, 16, and 17 at the same concentration. Moreover, compounds 1, 5, 16, and 17 also decreased TNFα-induced iNOS promoter activity in a dose-dependent manner, with IC50 values ranging from 0.58 to 82.8 µM, respectively (Table 3). These data suggested that compounds 1, 5, 16, and 17 isolated from Z. schinifolium suppressed TNF-α-induced NF-κB transcriptional activity via inhibition of iNOS gene transcription.

In this study, 12 glycosides (1–12) and 5 alkaloids (13–17) were isolated from the stems of Z. schinifolium. To our knowledge, this is the first report regarding the effects on NF-κB, cell proliferation and negative feedback of the NF-κB. Plant Material Dried stems of Z. schinifolium were collected from Daejeon, Korea in September 2012 and identified by one of the authors (Prof. Young Ho Kim). A voucher specimen (CNU 12102) was deposited at the Herbarium of College of Pharmacy, Chungnam National University.

Extraction and Isolation Dried stems (2.3 kg) of Z. schinifolium were extracted with MeOH (5 L×3) under reflux. The MeOH extract (102.0 g) was suspended in water and partitioned with n-hexane, EtOAc and n-BuOH. The EtOAc fraction (12.0 g) was subjected to silica gel (5×30 cm) column chromatography with a gradient of hexane–EtOAc–MeOH (10:1:0, 6:1:0, 3:1:0, 1.5:1:0.1, 1:1:0.2, 1.5L for each step) to give 6 fractions (Fr. 1A–F). The fraction IC was separated using a silica gel (1.5×80 cm) column chromatography hexane–acetone–MeOH (7:1:0.1, 6:1:0.1, 5:1:0.1; 1.0 L for each step) elution solvent to give compounds 13 (11.0 mg) and 14 (7.0 mg). The fraction 1E was separated using a silica gel (1×80 cm) column chromatography hexane–acetone–MeOH (5:1:0.1, 4:1:0.1; 500 mL for each step) elution solvent to give compounds 16 (25.0 mg) and 17 (8.0 mg). The n-BuOH fraction (28.0 g) was subjected to silica gel (3.0×30 cm) column chromatography with a gradient of CH2Cl2–MeOH–H2O (16:1:0, 10:1:0, 7.5:1:0.1, 3:1:0.15, 1.5:1:0.2; 2.5 L for each step) to give 6 fractions (Fr. 2A–F). The fraction 2A was separated using an YMC (1×80 cm) column chromatography hexane–acetone–MeOH (5:1:0.1, 4:1:0.1; 500 mL for each step) elution solvent to give compounds 15 (16.0 mg), 4 (25.0 mg), 12 (5.0 mg) and 17 (7.0 mg). The fraction 2B was

Experimental General Experimental Procedures Optical rotations were determined using a Jasco DIP-370 automatic polarimeter. The FT-IR spectra were measured using a Nicolet 380 FTIR spectrometer. GC was carried out on a Shimadzu-2010 spectrometer: detector, FID; detection temperature, 300°C; column, SPB-1 (0.25 mm i.d.×30 m); column temperature, 230°C; carrier gas, He (2 mL/min) injection temperature, 250°C; injection volume, 0.5 µL. The NMR spectra were recorded using a JEOL ECA 600 spectrometer (1H, 600 MHz; 13C, 150 MHz), and HR-ESI-MS were obtained using an Agilent 6530 Accurate-Mass Q-TOF LC/MS system. Column chromatography was performed using a silica gel (Kieselgel 60, 70–230, and 230–400 mesh, Merck, Darmstadt, Germany), YMC RP-18 resins, and thin layer chromatography (TLC) was performed using pre-coated silica-gel 60F254 and RP-18F254S plates (both 0.25 mm, Merck, Darmstadt, Germany).

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Table 3. Inhibitory Effects of Compounds 1, 5, 16, and 17 on the TNF-α-Induced NF-κB Transcriptional and iNOS Promoter Activity in 293T Cells

| Compound | IC50 (µM) |
|----------|-----------|
|          | NF-κB     | iNOS     |
| 1        | 14.0±2.14 | 82.8±8.24|
| 5        | 17.4±3.33 | 82.0±9.96|
| 16       | 0.67±0.04 | 0.58±0.03|
| 17       | 37.5±5.01 | 46.2±8.05|
| Apigenin  | 1.6±0.28  | 1.6±0.21  |

a) The values are means±S.D.s (n=3). b) Positive control.

Fig. 4. Effects of Compounds 1, 5, 16, and 17 on iNOS and ICAM-1 mRNA Expression in 293T Cells

+: Cells were treated without TNFα and compounds; +: cells were treated with 10 µg/mL TNFα only; +0.1, 1, 10: cells were treated with 20 ng/mL TNFα and compounds.
subjected to silica gel (1.0×70 cm) column chromatography with a gradient of CH₂Cl₂–MeOH–H₂O (12:1:0, 10:1:0, 8.5:1:0.1, 6.5:1:0.1, 4:1:0.1, 1:5 L for each step) to give 6 sub-fractions (Fr. 2B-1–2B-6). The fraction 2B-2 was separated using an YMC (1×80 cm) column chromatography with a MeOH–H₂O (0.15:1, 0.22:1, 0.27:1, 750 mL for each step) elution solvent to give compounds 9 (8.4 mg), 10 (11.0 mg), and 11 (5.0 mg). The fraction 2B-3 was separated using an YMC (1×80 cm) column chromatography with a MeOH–H₂O (0.2:1, 0.67:1, 1.0 L for each step) elution solvent to give compounds 1 (9.0 mg) and 2 (12.0 mg). The fraction 2C was separated using an YMC (1×80 cm) column chromatography with a MeOH–acetone–H₂O (0.2:0.12:1, 750 mL) elution solvent to give compounds 5 (12.0 mg) and 7 (17.0 mg). The fraction 2E was separated using a silica gel (0.8×80 cm) column chromatography with CHCl₃–MeOH–H₂O (4.5:1:1.0, 2.5 L) to give compounds 6 (11.0 mg) and 8 (9.0 mg).

Zanthoxylsides A (1): Colorless oil; [α]D²⁵ = −19.2 (c = 0.3, MeOH); IR (KBr) νmax: 3450, 1615, 1530 cm⁻¹; ¹H-NMR (pyridine-d₅, 600 MHz) and ¹³C-NMR data (pyridine-d₅, 150 MHz), see Table 1; HR-ESI-MS: m/z 653.2445 [M+H]⁺ (Caled for 653.2445).

Zanthoxylside B (2): White amorphous powder; [α]D²⁵ = −45.7 (c = 0.2, MeOH); IR (KBr) νmax: 3428, 2950, 1661, 1071 cm⁻¹; ¹H-NMR (methanol-d₄, 600 MHz) and ¹³C-NMR data (methanol-d₄, 150 MHz), see Table 1; HR-ESI-MS: m/z 465.2323 [M+H]⁺ (Caled for 465.2336).

Enzymatic Hydrolysis Compounds 1 and 2 (3.0 mg each) were mixed with β-glucosidase (3.0 mg) in water (1.0 mL) respectively and were shaken in a water bath at 37°C for 12 h. After this, the reaction mixture of 1 was concentrated and then subjected to silica gel (1.0×15 cm, 40–63 μm) column chromatography with CHCl₃–MeOH–H₂O (15:1:70 mL) and CHCl₃–MeOH–H₂O (7:3:0.5, 60 mL) to afford an aglycone 1a (1.2 mg) and a sugar fraction. The reaction mixture of 2 was concentrated and then subjected to silica gel (1.0×15 cm, 40–63 μm) column chromatography with CHCl₃–MeOH–H₂O (20:1, 50 mL) and CHCl₃–MeOH–H₂O (7:3:0.5, 60 mL) to afford an aglycone 2a (1.1 mg) and a sugar fraction. The reaction mixture of 2 was concentrated and then subjected to silica gel (1.0×15 cm, 40–63 μm) column chromatography with CHCl₃–MeOH (20:1, 50 mL) and CHCl₃–MeOH–H₂O (7:3:0.5, 60 mL) to afford an aglycone 2a (1.1 mg) and a sugar fraction. The sugar fractions were concentrated to dryness using N₂ gas. The resulting residues were dissolved in dry pyridine (0.1 mL), and then L-cysteine methyl ester hydrochloride in pyridine (0.064 mol, 0.1 mL) was added to the solution. After heating the reaction mixtures at 60°C for 2 h, 0.1 mL of trimethylsilylimidazole solution was added. Heating at 60°C was continued for a further 1.5 h. The dried products were partitioned with n-hexane and H₂O (0.1 mL each), and the organic layers were analyzed using gas liquid chromatography (GC): DB-5 capillary column (0.32 mm×30 m); hydrogen flame ionization detector (FID) detector; column temp., 210°C; injector temp., 270°C; detector temp., 300°C; carrier gas He (3 mL/min). Under these conditions, standard sugars gave peaks at tR (min) = 12.24 and 14.12 for L- and D-glucose, tR (min) = 11.72 and 10.86 for L and D-apiose, respectively. The peaks of the hydrolysate of 1 were detected at tR (min) = 14.17 and 10.81; 2 were detected at tR (min) = 14.19 and 10.83, which identified as D-glucose and D-apiose by comparison with the retention time of the authentic samples after treatment with trimethylsilylimidazole in pyridine.

(5)-(+)-3,7-Dimethylocta-1,6-diene-3,8-diol (2a): Amorphous powder; [α]D²⁵ +8.50 (c = 0.3, MeOH); ¹H-NMR (methanol-d₄, 600 MHz): δH 1.21 (3H, s, H-9), 1.60 (2H, m, H-4), 1.71 (1H, brs, H-10), 2.20 (2H, m, H-5), 3.96 (2H, s, H-8), 5.12 (1H, dd, J = 11.0, 2.0 Hz, H-1a), 5.22 (1H, dd, J = 18.0, 2.0 Hz, H-1b), 5.41 (1H, t, J = 7.0 Hz, H-6), 5.85 (1H, dd, J = 18.0, 11.0 Hz, H-2). ¹³C-NMR (methanol-d₄, 150 MHz): δC 13.0 (C-10), 23.5 (C-5), 27.6 (C-9), 42.8 (C-4), 69.0 (C-3), 73.6 (C-8), 112.0 (C-1), 128.1 (C-6), 136.1 (C-7), 146.5 (C-2).

Oxidation of (R)-(−)-Linalool: (R)-(−)-Linalool (0.5 g) was oxidized with SeO₂ (0.45 g) in 20 mL of EtOH at 50°C for 3 h. The reaction mixture was diluted with H₂O and then extracted three times with EtOAc. The residue obtained on evaporation of the EtOAc layer was subjected to silica gel (1.0×80 cm) column chromatography with CHCl₃–MeOH (20:1, 650 mL) to give (R)-(−)-3,7-dimethylocta-1,6-diene-3,8-diol (69.0 mg).

(R)-(−)-3,7-Dimethylocta-1,6-diene-3,8-diol: The ¹H- and ¹³C-NMR spectra were essentially the same as those of the natural compound (2a).

Cell Toxicity Assay CCK-8 (Dojindo, Kumamoto, Japan) was used to analyze the effect of compounds on cell toxicity according to the manufacturer’s instructions. Cells were cultured overnight in 96-well plate (ca. 1×10⁴ cells/well). Cell toxicity was assessed after the addition of compounds on dose-dependent manner. After 24 h of treatment, 10 μL of the CCK-8 solution was added to triplicate wells, and incubated for 1 h. Absorbance was measured at 450 nm to determine viable cell numbers in wells.

NF-κB and iNOS-Luciferase Assay 293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA, U.S.A.) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 10 μg/mL streptomycin at 37°C and 5% CO₂. The luciferase vector was first transfected into 293T cells. After a limited amount of time, the cells were lysed, and luciferin, the substrate of luciferase, was introduced into the cellular extract along with Mg²⁺ and an excess of ATP. Under these conditions, luciferase enzymes expressed by the reporter vector could catalyze the oxidative carboxylation of luciferin. Cells were seeded at 2×10⁵ cells per well in a 12-well plate and grown. After 24 h, cells were transfected with inducible NF-κB or iNOS firefly luciferase reporter and constitutively expressing Renilla reporter. After 24 h of transfection, medium was changed to assay medium (Opti-MEM+0.5% FBS+0.1 mM NEAA+1 mM sodium pyruvate +100 units/mL penicillin+10 μg/mL streptomycin) and cells were pretreated for 1 h with either vehicle (1% DMSO in water) and compounds, followed by 1 h of treatment with 20 ng/mL TNFα for 23 h. Unstimulated 293T cells were used as a negative control (−). apigenin was used as a positive control. Dual luciferase assay was performed 48 h after transfection, and promoter activity values are expressed as arbitrary units using a Renilla reporter for internal normalization.

RNA Preparation and RT-PCR Total RNA was extracted using Easy-blue reagent (Intron Biotechnology, Seoul, Korea). Approximately 2 μg total RNA was subjected to reverse transcription using Moloney murine leukemia virus (MMLV) reverse transcriptase and oligo-dT primers (Promega, Madison, WI, U.S.A.) for 1 h at 42°C. PCR for synthetic cDNA was performed using a Taq polymerase premixture (TaKaRa, Japan). The PCR products were separated by electrophoresis on 1% agarose gels and stained with EtBr.
PCR was conducted with the following primer pairs: iNOS sense 5′-TCATCCGCTATGGCTGAC-3′, iNOS antisense 5′-CTCGAGTGTGCAGCCATTG-3′, ICAM-1 sense 5′-CTG CAGACAGTGACCACC-3′, ICAM-1 antisense 5′-GTCAG TTTCCCAGGAA-3′, β-actin sense 5′-TCCCTCCACAAG TCCATGTG-3′, and β-actin antisense 5′-CAGCGG ACCCGTCTATGGCAA-3′. The specificity of products generated by each set of primers was examined using gel electrophoresis and further confirmed by a melting curve analysis. HepG2 cells were pretreated in the absence and presence of compounds for 1 h, then exposed to 20 ng/mL TNFα for 6 h. Total mRNA was prepared from the cell pellets using EasyBlue. The levels of mRNA were assessed by RT-PCR.

Statistical Analysis All data represent the mean±standard deviation (S.D.) of at least three independent experiments performed in triplicate. Statistical significance is indicated as *p<0.05 and **p<0.01 as determined by Dunnett’s multiple comparison test.

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