Suppressive Effects of *Anthriscus sylvestris* Constituents on the Expression and Production of Matrix Metalloproteinase-9 Using Luciferase Transfected Raw 264.7 Cell Based Assay System

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Bioactivity-guided fractionation of *Anthriscus sylvestris* extract, using matrix metalloproteinase-9 (MMP-9) assay, led to the isolation of deoxypodophyllotoxin (1), falcarindiol (2), (-)-hinokinin (3), and (-)-hibalactone (4). All compounds obtained were evaluated for the inhibitory activities against MMP-9 in luciferase and zymographic assays. Of these compounds, compounds 1 (IC₅₀ 0.5 nM) and 2 (IC₅₀ 8.4 µM) were found to inhibit MMP-9 expression in the PGL4.14-MMP-9-Luc plasmid transfected Raw 264.7 cells, and MMP-9 production in a gelatin zymographic assay.

Key words: *Anthriscus sylvestris*, deoxypodophyllotoxin, falcarindiol, luciferase assay, matrix metalloproteinase-9, zymography

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Airway remodeling is a major change responsible for irreversible asthmatic airflow restriction relevant to the progressive subepithelial fibrosis and structural changes in the extracellular matrix (ECM) [Jack *et al.*, 1999]. Matrix metalloproteinases (MMPs) are the key enzymes responsible for ECM degradation [Shapiro and Senior, 1999]. MMPs are a family of zinc-dependent proteolytic enzymes which contain a zinc ion at the active site of catalysis [Nagase and Woessner, 1999]. Among at least 23 kinds of MMPs, MMP-9 (also called gelatinases B, 92kDa) is the most studied and relevant to asthma. MMP-9 can degrade a wide range of ECM except for type I collagen. In particular, MMP-9 possesses potent collagenolytic activity against type IV collagen, the major component of airway basement membrane, suggesting a pivotal role for MMP-9 in starting airway remodeling in asthma [Ohbayashi and Shimokata, 2005].

*Anthriscus sylvestris* Hoffm. (*A. sylvestris*, Umbelíferae) is distributed in Korea, Japanese, Kamchatka, Siberia, and Europe, and its roots have been used as antitussive, antidiuretics, analgesic, antipyretic and cough remedy in Asian countries [Ayres and Loike, 1990]. As a part for MMP-9 inhibitors from plant origin, the methanolic extract of *A. sylvestris* was selected for further investigation due to a significant inhibitory effects on MMP-9 gene expression and its production in cell-based assays. Previous phytochemical investigation revealed that lignans are major secondary metabolites of this plant. It was also found that the extracts or individual constituents from this plant possessed cytotoxic activity, antiinflammatory activities [Lee *et al.*, 2004], and antioxidant activity [Ayres and Loike, 1990].

The present study deals with the activity-guided isolation of lignans and acetylene, and their evaluation for the suppressive effects on MMP-9 production/expression in cell-based assay using the transfection of luciferase and gelatin zymographic assay *in vitro*.

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Abbreviations: ESI, Electrospray ionization; LPS, Lipopolysaccharide; MMP, Matrix metalloproteinase; MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; NMR, Nuclear magnetic resonance; TNF-α, Tumor necrosis alpha

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Material and Methods

**Plant materials.** The roots of *A. sylvestris* Hoffm. (Lot NO. 55687) were purchased in the Dae-Yeon pharmacy (Incheon, Korea). A voucher specimen (PBC-326) has been deposited at the Plant Extract Bank, Korea Research Institute of Bioscience and Biotechnology, Daegu, Korea.

**Reagents and instruments.** Solvents such as methanol, acetone, ethyl acetate, chloroform and hexane were purchased from Duk-san chemical Co. (Daejeon, Korea), and used after re-distillation. HPLC solvents were purchased from Duk-san chemical (Silia gel (Kieselgel 60, 0.040-0.063 mm, Merck, Darmstadt, Germany), and RP-18 resin was carried out on silicagel (Kieselgel 60, 0.040-0.063 mm, Merck, Darmstadt, Germany), and RP-18 resin was purchased from Duk-san chemical Co. (Daejeon, Korea). Column chromatography (TLC) was done on precoated Silica gel plates (70-200 mesh, Merck) and RP-18 F254s plates (70-200 mesh, Merck). Optical rotations were measured with a JASCO DIP-370 digital polarimeter. UV spectra were recorded on a UV-spectrometer Model CM-10 (Spectronics Corp., New York, NY). HR-ESI mass spectrometric analysis was performed using a Waters Q-Tof Premier spectrometer. NMR spectra were recorded on a Bruker DMX 300MHz spectrophotometer. Preparation HPLC analysis was done on a pump 305 (Gilson Co., Middleton, England), detector UV/VIS (Gilson Co.), column Comosil 5C18-MS-II (250×20 mm, 5 μ, Comosil Co.).

**Extraction and isolation of the root of *A. sylvestris*.** The dried roots (10.0 kg) of *A. sylvestris* were extracted with MeOH (1.2 L×3) at room temperature for four days. The methanolic extract (450 g) was suspended in H2O (1 L), and the resulting H2O layer was partitioned with n-hexane (500 mL×3), EtOAc (500 mL×3), n-BuOH (500 mL×3), successively, to give the hexane fraction (ASH, 30.3 g), EtOAc fraction (ASE, 32.4 g), n-BuOH fraction (ASB, 27.4 g), and aqueous fraction (ASW, 370 g), respectively. Compound 1 (1.2 g) was obtained by recrystallization with MeOH from the EtOAc fraction. Because both of ASH and ASE showed significant effects on the assays for what in the preliminary screening, the combined fractions (ASH, 48 g) chromatographed on a silica gel column using a mixture of hexane/acetone (9:1-5:1, v/v, step gradient) to obtain 7 fractions (ASH-1—7). ASHE-6 (389.1 mg) was chromatographed on a RP-18 silica gel column using a mixture of MeOH/H2O (70-100% of MeOH, step gradient) to obtain 6 fractions (ASHE-6 Fr.1–6). ASHE-64 was chromatographed on a preparative TLC using a mixture of CHCl3/MeOH (10/1, v/v), and then purified using a prop. HPLC with a mixture of MeOH/H2O (85/15, v/v), to give compounds 3 and 4 (0.7 mg, and 0.6 mg). ASHE-65 was fractionated by preparation HPLC using a mixture of MeOH/H2O (65/35, v/v) and gave compound 2 (21 mg).

**Compound 1.** White crystal (colorless needles); ESI-MS m/z 399.1446 [M+H]+; 1H-NMR (300 MHz, CD3OD, δ) 6.66 (1H, s, H-2), 6.52 (1H, s, H-5), 6.34 (2H, s, H-2,6), 5.95 (-OCH3-O, d, J= 1.2 Hz), 5.93 (-OCH3-O, d, J=1.2 Hz), 4.46 (m, H-9), 3.92 (m, H-9), 3.82 (-OCH3-OH-4), 3.74 (-OCH3-OH-3'), 3.09 (m, H-7), 2.78 (m, H-7); 13C-NMR (75 MHz, CD3OD, δc) 174.9 (C-9'), 152.6 (C-7'), 152.5 (C-3), 147.0 (C-7), 147.6 (C-4'), 137.5 (C-4), 136.2 (C-1'), 130.6 (C-6), 128.2 (C-1), 110.4 (C-5), 108.4 (C-2), 108.3 (C-2'), 108.2 (C-6'), 72.0 (C-9), 47.4 (C-8'), 43.7 (C-7'), 33.0 (C-7), 32.7 (C-8) [David et al., 1995].

**Compound 2.** Colorless oil; ESI-MS m/z 259.1700 [M+H]+; 1H-NMR (300 MHz, CD3OD, δ) 5.90 (1H, dd, J= 16.2, 10.2, 5.1 Hz, H-2), 5.60-5.42 (2H, m, H-9 and 10), 5.39 (1H, dt, J=16.2, 1.5 Hz, H-1a), 5.19 (1H, dt, J=10.2, 1.5 Hz, H-1b), 5.15 (1H, brd, J=8.1 Hz, H-8), 4.84 (1H, s, H-3), 1.39 (2H, m), 1.29 (8H, m), 0.90 (3H, t, J=8.7 Hz, H-7'), 13C-NMR (75 MHz, CD3OD, δc) 138.1 (C-10), 134.1 (C-2), 129.8 (C-9), 116.6 (C-1), 81.1 (C-7), 79.7 (C-4), 70.1 (C-5), 68.7 (C-6), 63.8 (C-3), 58.8 (C-8) [Furumi et al., 1998].

**Compound 3.** Yellow oil; ESI-MS m/z 399.1073 [M+HCOO]+; 1H-NMR (300 MHz, CD3OD, δ) 6.70 (1H, brd, J=8.1 Hz, H-6), 6.67 (1H, s, H-2), 6.60 (2H, m, H-5' and H-5), 5.62 (2H, m, H-5 and 5'), 5.62 (1H, m, H-2 and H-6), 5.90 (OCH3-O, d, J=2.4 Hz), 5.89 (OCH3-O, d, J=3.0 Hz), 4.16 (1H, dd, J=8.1, 7.0 Hz, H-9), 3.93 (1H, dd, J=8.1, 7.0 Hz, H-9), 2.91 (1H, dd, J=9.9, 5.9 Hz, H-7), 2.78 (1H, dd, J=10.5, 7.2 Hz, H-7), 2.61 (1H, m, H-5), 1.86 (2H, m, H-7b and H-7b), 1.87 (4H, m, H-8); 13C-NMR (75 MHz, CD3OD, δc) 178.3 (C-9), 147.9 (C-3), 147.9 (C-3'), 146.5 (C-4'), 146.4 (C-4), 131.6 (C-1), 131.4 (C-1'), 132.4 (C-6'), 127.8 (C-6), 110.4 (OCH3-Oa), 109.9 (OCH3-ob), 109.0 (C-2), 109.1 (C-5'), 109.8 (C-2'), 109.0 (C-5), 47.7 (C-8), 46.5 (C-8'), 42.6 (C-8), 39.0 (C-7), 35.6 (C-7); [Ikeda et al., 1998].

**Compound 4.** Yellow crystals; ESI-MS m/z 379.0922 [M+HCOO]+; 1H-NMR (300 MHz, CD3OD, δ) 7.44 (1H, d, J=1.5 Hz, H-7), 7.12 (1H, d, J=8.1 Hz, H-6), 7.11 (1H, s, H-2), 6.89 (1H, d, J=8.1 Hz, H-5), 6.66 (1H, d, J=8.4 Hz, H-5'), 6.65 (1H, d, J=8.4 Hz, H-6), 6.62 (1H, s, H-2'), 6.03 (-OCH3-O, s), 5.87 (-OCH3-Od, d, J=5.1 Hz), 4.26 (1H, m, H-9), 4.02 (1H, m, H-9), 2.93 (1H, dd, J=12.0, 3.8 Hz, H-7), 2.88 (1H, m, H-8), 2.69 (1H, dd, J=12.0, 8.7 Hz, H-7) [Dodda et al., 1998].

**Cell cultures.** Raw 264.7 (mouse macrophage cell line) and HT-1080 (human fibrosarcoma cell line) were