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Chemical Components from the Fruit Peels of *Wisteria floribunda* and their Effects on Rat Aortic Vascular Smooth Muscle Cells

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Restenosis after angioplasty is a significant clinical problem.[1] A dominant cellular event is the renarrowing of the vascular lumen after angioplasty, which is due to vascular smooth muscle cell (VSMC) proliferation and migration. After injury, VSMCs proliferate and then migrate into the developing neointima to become the major cellular substrate of restenotic tissue.[2] The pathogenesis of neointimal formation is mediated by VSMCs through phenotype transformation, proliferation, migration, and extracellular matrix rearrangement. Thus, suppressing VSMC proliferation has therapeutic potential for reducing the incidence of occlusive vascular diseases.[2] Vascular injuries release pathological mediators from activated platelets, including platelet-derived growth factor (PDGF), which plays an important role in forming the neointima and, ultimately, in restenosis.[3,4] PDGF expression is high in injured arteries and correlates with neointimal cellular proliferation.[3] PDGF activates intracellular signal transduction pathways that contribute to VSMC proliferation, and the role of PDGF in neointima development has been evaluated in arterial injury models.[5] Thus, inhibiting PDGF-induced VSMC proliferation may provide a therapeutic approach to attenuate the cellular manifestation of vascular disease. Cell proliferation is regulated by many factors during the cell cycle, including cyclin-dependent kinase (cdk) and cyclin family genes, a major group of cell cycle regulatory proteins that regulate cdk activity.[6] Of these, cyclin D1 is a key regulator and marker of cell proliferation, subject to both transcriptional and post-transcriptional regulation.[7]

Wisteria floribunda (Leguminosae) is widely grown in East Asian countries. Its gall extracts are used as an antitumoral preparation in traditional Oriental medicine.[8] Many Oriental medicinists used *W. floribunda* gall extracts for treating patients with breast cancer, stomach cancer, and rheumatoid arthritis. Extracts and isolated compounds from the flowers and seeds of *W. floribunda* also have antioxidant, anticancer, and immunological activities.[8,9]

The methanol extracts of the *W. floribunda* fruit peels showed significant positive activity in a preliminary study in which we screened plant extracts with an inhibitory effect on PDGF-induced VSMC proliferation. To clarify the active components causing this inhibition, a methanol extract of the fruit peels was subjected to various separation procedures. Resulting, 21 compounds were isolated from fruit peels of *W. floribunda* (Fig. 1). Their structures were identified by comparing physicochemical and spectroscopic data to published values for lupeol (**1**),[10] *β*-sitosterol (**2**),[11] daucosterol (**3**),[12] taraxerone (**4**),[13] taraxerol (**5**),[13] 3,4-dimethoxy benzaldehyde (**6**),[14] syringaresinol (**7**),[15] (−)-pinoresinol (**8**),[16] (+)-afzelechin (**9**),[17] (−)-epiafzelechin 7-*O*-*β*-D-glucopyranoside (**10**),[18] (−)-epiafzelechin (**11**),[19] (+)-catechin (**12**),[20] (−)-epicatechin (**13**),[20] dulcisflavan (**14**),[21] kaempferol (**15**),[22] kaempferol 3-*O*-*β*-D-glucopyranoside (**16**),[23] kaempferol 3-*O*-rutinoside (**17**),[24] kaempferol 7-*O*-*β*-D-glucopyranoside (**18**),[25] kaempferol 3,4'-di-*O*-*β*-D-glucopyranoside (**19**),[26] kaempferol 3-*O*-*β*-D-apiofuranosyl-(1→6)-*β*-D-glucopyranoside (**20**),[27] and kaempferol 3,7-di-*O*-*β*-D-glucopyranoside (**21**).[28] Among them, 13 compounds (**9**-**21**) were flavonoids, five compounds (**1**-**5**) were triterpenoids, two compounds (**7**, **8**) were lignans, and one compound (**6**) was phenyl derivatives. This is the first report on a chemical investigation of *W. floribunda* fruit peels.

Next, the isolated compounds were evaluated for their ability to inhibit PDGF-induced VSMC proliferation using a BrdU incorporation assay. Compounds **1**, **4**, **9**, **12**, **13**, and **15** showed the highest inhibitory effects, with IC_{50} values (μM) of 5.4, 7.5, 6.7, 4.3, 4.6, and 3.6, respectively (Table 1). Compounds **3**, **5**-**7**, and **10** were weak active (IC_{50} > 50 μM). The remaining compounds exhibited modest inhibitory effects (IC_{50}s in the range 19.3-46.6 μM). Juglone, IC_{50} 2.4 μM, was used as positive control. The compounds showed no significant cytotoxicity up to 50 μM in the MTT assay suggesting that our results were not due to cytotoxic effects. Cyclin D1 promotes cell growth and its expression is induced by growth factor stimulation.[29] Thus, to examine the underlying mechanism of the *anti*-proliferative effect exert-

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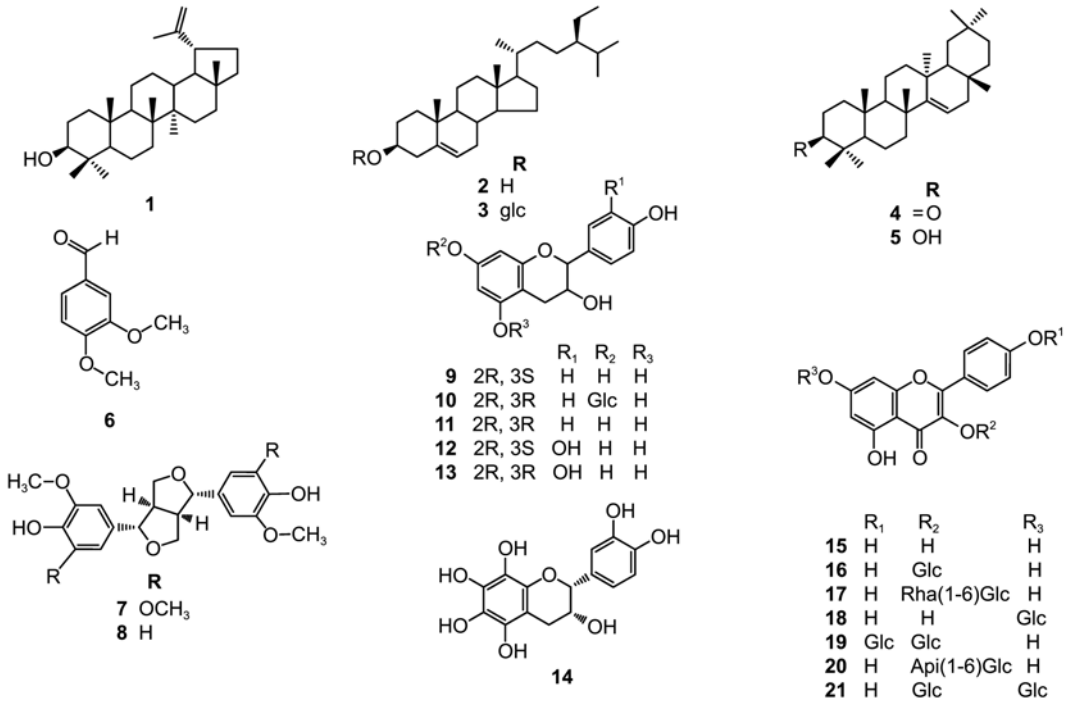

Figure 1. Compounds **1-21** isolated from *Wisteria floribunda* fruit peels.

Table 1. IC_{50} values of compounds (**1-21**) on PDGF-induced VSMCs proliferation

Compounds	IC_{50} values (μM)[a]	Compounds	IC_{50} values (μM)[a]
1	5.4 ± 0.4	**12**	4.3 ± 0.3
2	19.8 ± 1.5	**13**	4.6 ± 0.3
3	> 50	**14**	20.9 ± 1.6
4	7.5 ± 0.5	**15**	3.6 ± 0.3
5	> 50	**16**	32.7 ± 2.4
6	> 50	**17**	21.4 ± 1.6
7	> 50	**18**	21.0 ± 1.6
8	46.6 ± 3.5	**19**	28.6 ± 2.1
9	6.7 ± 0.5	**20**	19.3 ± 1.4
10	> 50	**21**	20.0 ± 1.5
11	39.5 ± 2.9	**Juglone**[b]	2.4 ± 0.3

[a]IC_{50} values were derived from dose-response curves. Data are expressed as means ± SD of three independent experiments (n = 3, $P < 0.05$ *vs.* stimulus control). [b]Juglone was used as positive control.

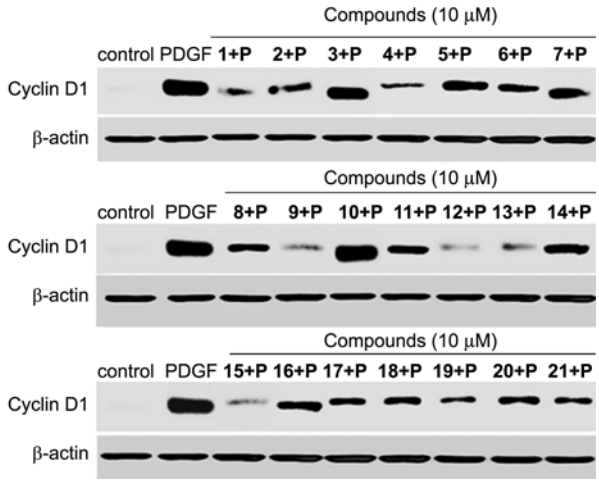

Figure 2. Immunoblot analysis of cyclin D1. VSMCs were treated with or without PDGF (30 ng/mL) in the presence or absence of **1-21** (10 μM) for 24 h, and the total cell lysates were subjected to immunoblotting with antibody against cyclin D1 or β-actin.

ed by the isolated compounds, we tested them on cyclin D1 expression using Western blotting assay. PDGF significantly increased cyclin D1 expression, and pretreatment with the compound suppressed this increase, as observed by lower intensities band. At a concentration of 10 μM, compounds **1**, **4**, **9**, **12**, **13**, and **15** showed inhibitory effects on PDGF-induced cyclin D1 expression in VSMCs (Fig. 2).

Bioassay showed that triterpenoids containing a taraxer-14-en skeleton with a 3-ketone functional group (**4**), exhibited a stronger effect than did triterpenoids with a 3-alcohol functional group (**5**). This result may be associated with the electrostatic interaction ability of compound **4** at position 3 compared with that of **5**. Kaempferol (**15**), which contains free hydroxyl groups, showed good activity compared with its glycosides. Among the flavan-3-ols, catechin (**12**) and epicatechin (**13**) produced strong effects compared with afzelechin (**11**) and epiafzelechin (**9**), suggesting an increased activity role for the additional hydroxyl group in the B-ring. The results also showed a difference between configurations; the 2*R*,3*S*-configuration (**9**, **12**) may have more efficacy than the 2*R*,3*R*-configuration (**11**, **13**).

In conclusion, the chemical constituents of *W. floribunda* were studied, and flavonoids, triterpenoids and lignans were isolated from fruit peels extracts. The effects of the isolated compounds on PDGF-induced VSMC proliferation were

also investigated. Among them, compounds **1**, **4**, **9**, **12**, **13**, and **15** were the most active components. These results demonstrate that these compounds may inhibit VSMC proliferation by suppressing cyclin D1 expression in VSMCs. Thus, active compounds in *W. floribunda* fruit peels may have therapeutic potential in attenuating vascular occlusive diseases.

Experimental

Plant Material. *W. floribunda* fruit peels were collected in Daejeon, Korea in February 2009 and identified by Prof. KiHwan Bae, College of Pharmacy, Chungnam National University. A voucher specimen (CNU 1389-5) was deposited in the College of Pharmacy, Chungnam National University.

Extraction and Isolation. Dried samples (6 kg) were pulverized and extracted with 95% methanol (18 L) under reflux for 3 × 6 h. The extract was concentrated to dryness under reduced pressure. The residue was suspended in water and partitioned sequentially with hexane, ethyl acetate, and butanol. The crude extracts were subjected to various column chromatography procedures on silica gel, YMC RP-18 gel, and by preparative HPLC (Gilson Trilution system; OptimaPak C_{18} (S-10 μm, 250 × 10 mm i.d.) column; acetonitrile-0.1% HCOOH in water (15:85, v/v); flow rate, 2 mL/min; UV detection, 254 nm] to give compounds **1** (9 mg), **2** (72 mg), **3** (138 mg), **4** (20 mg), **5** (26 mg), **6** (90 mg), **7** (15 mg), **8** (27 mg), **9** (20 mg), **10** (5 mg), **11** (34 mg), **12** (133 mg), **13** (34 mg), and **14** (50 mg), **15** (62 mg), **16** (12 g), **17** (6 mg), **18** (12 mg), **19** (24 mg), **20** (7 mg), and **21** (10 mg).

Cell Culture. VSMCs were isolated from rat thoracic aorta, as described previously.[30] Individual cells were placed in a culture dish and grown in DMEM containing 10% fetal bovine serum (FBS). Cells were passaged by trypsinization, and passages 5-12 were used for experiments.

Cell Viability Assay. Cells were seeded in 96-well plates at a density of 2×10^4 cells/well and cultured for 24 h. After a 48 h incubation of the cells with different concentrations of the test compounds, the cytotoxicity of the compounds was determined by the MTT assay, as described previously.[31] Percent cell viability was calculated based on the absorbance measured relative to the absorbance of control cells exposed to the vehicle alone.

5-Bromo-2'-deoxy-uridine (BrdU) Assay Viable adherent cells were incubated with BrdU labeling solution (10 μM) for 2 h. Cells were fixed in a fixation solution for 30 min at room temperature and incubated with 100 μL anti-BrdU peroxidase-labeled antibody for 90 min. After three washes, substrate solution for colorimetric quantification was added at a final concentration of 100 μL/mL and maintained at room temperature for 5-30 min until color development was sufficient for photometric detection. Absorbance was assayed at 405 nm.

Western Blot Analysis. The cells were collected and washed with phosphate-buffered saline (PBS). The harvested cells were then lysed on ice for 30 min in 100 μL lysis buffer (120 mM NaCl, 40 mM Tris, pH 8, 0.1% Nonidet P-40) and centrifuged (12,000 rpm, 30 min). Supernatants were collected from the lysates and protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Aliquots of the lysates (40 μg of protein) were boiled for 5 min and electrophoresed on 10% SDS-polyacrylamide gels. Proteins in the gels were transferred onto nitrocellulose membranes, which were then incubated with cyclin D1 or mouse monoclonal β-actin antibodies. The membranes were further incubated with secondary anti-mouse or anti-rabbit antibodies. Finally, protein bands were detected using the Enhanced Chemiluminescence Western Blotting Detections Kit (Pierce Biotechnology, Rockford, IL, USA).

Statistical Analyses. Experimental results are expressed as means ± SD. A one-way analysis of variance was used for multiple comparisons followed by Dunnett's test. Differences with a $P < 0.05$ were considered statistically significant.

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