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Antifibrotic constituents of *Alnus firma* on hepatic stellate cells

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ABSTRACT

Suppression of hepatic stellate cell (HSC) growth and activation have been proposed as therapeutic strategies for the treatment and prevention of liver fibrosis. In the course of screening antifibrotic activity of natural products, the methanolic extract of *Alnus firma* barks (Betulaceae) showed inhibitory activity of cell proliferation on HSC-T6 cells. A new triterpenoid characterized as lup-20(29) en-2,28-diol-3-yl caffeate (**13**) was isolated with 12 known diarylheptanoids (**1**–**12**) from the barks of *A. firma* using bioactivity-guided fractionation. Among these compounds, **2** and **13** significantly inhibited the proliferation of HSCs in dose- and time-dependent manners at concentrations from 10 to 100 μM. Taken together, antifibrotic activities of *A. firma* and its active constituents might suggest the therapeutic potentials against liver fibrosis.

Hepatic fibrosis, a worldwide prevalent health risk, arises from sustained wound healing response to chronic liver injury from a variety of causes including viral, autoimmune, drug induced, cholestatic and metabolic diseases. Hepatic fibrosis is characterized by increased proliferation and excessive deposition of extracellular matrix (ECM) which lead to final liver dysfunction and irreversible cirrhosis.[1,2] Hepatic stellate cells (HSC) (also known as fat-storing cells, Ito cells, or lipocytes) are identified as the primary cell-type in the liver responsible for hepatic fibrosis.[3] Following liver injury, the HSCs undergo 'activation', developing myofibroblasts-like phenotype associated with increased proliferation and excessive production of ECM components, which is the major pathological feature of hepatic fibrosis. Disturbance either the fibrogenic or proliferative responses of HSCs has been proposed as therapeutic targets against hepatic fibrosis.[4] To date, however, the therapeutic drugs of liver fibrosis are still insufficient and/or unavailable. Thus, we tried to search for antifibrotic compounds from natural product.

In the course of searching for antifibrotic natural product employing HSC-T6 cells as an in vitro assay system, the methanolic extract of the stems of *Alnus firma* Sieb. et Zucc. (Betulaceae) showed significant inhibition on HSC-T6 cell proliferation (26.8% of the control at 100 μg/mL. p <0.001). The barks of *A. firma* are widely distributed in Korea and Japan and has been used as a tonic medicine in folk medicine.[5] Chemical constituents such as diarylheptanoids, triterpenoids and flavonoids of *A. firma* have been reported.[6,7] We previously reported the anti-inflammatory effect of diarylheptanoids isolated from *A. firma*.[8] Further investigation on *A. firma* demonstrated the antifibrotic activity of total methanolic extract (26.8% of control, p <0.001). Therefore, in the present study, we have attempted to isolate the antifibrotic constituents from barks of *A. firma*.

The barks of *A. firma* were obtained from SK Forest Co. Ltd, Chungju, Korea in March 2008 and identified by Dr. Jong Hee Park, a professor of the College of Pharmacy, Pusan National University (Busan, Korea). A voucher specimen (SNU-751) has been deposited in the herbarium of the Medicinal Plant Garden, College of Pharmacy, Seoul National University. The air-dried barks of *A. firma* (5.7 kg) were extracted four times for 2 h with 80% MeOH in an ultrasonic apparatus at room temperature. Removal of the solvent under vacuum resulted in total methanolic extract (1.1 kg). This methanolic extract was then suspended in distilled water and successively partitioned with CH_2Cl_2, EtOAc, *n*-BuOH. The CH_2Cl_2 layer was suspended in 90% MeOH and then further partitioned with *n*-hexane. The 90% MeOH fraction, which showed the most potent inhibitory activity (7.7% of control at 100 μg/mL, p < 0.001) was subjected to further isolation.

Silica gel column chromatography (CC) of 90% MeOH fraction (68.2 g) was carried out using a mixture of CHCl3–MeOH with increasing polarity ($CHCl_3$/MeOH, 100:1→0:100) and yielded nine fractions (MC1–MC9). MC2 was subjected to silica gel CC ($CHCl_3$/MeOH, 25:1→0:100) to give four subfractions (MC2-1–MC2-4). MC2-3 was divided into six fractions (MC2-3-1–MC2-3-6) by MPLC (RediSep silica gel; $CHCl_3$/MeOH, 25:1→0:100; 30 mL/min). Compounds **3** (48 mg) and **13** (202 mg) were isolated from MC2-3-4

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Table 1
^{1}H and ^{13}C NMR data for compound **13** in Pyridine-d_5

Position	Compound **13**	
	δ_C[a]	δ_H[b]
1	48.8	1.34 (1H, m, H-1α), 2.39 (1H, dd, 4.5, 12.0, H-1β)
2	66.6	4.31 (1H, td, 10.8, 4.5)
3	84.9	5.25 (1H, d, 9.6)
4	39.9	
5	55.5	
6	18.6	1.36 (1H, m), 1.46 (1H, m)
7	34.4	1.28 (1H, m), 1.39 (1H, m)
8	41.2	
9	50.6	1.43 (1H, m)
10	38.5	
11	21.2	1.20 (1H, m), 1.47 (1H, m)
12	25.6	1.14 (1H, m), 1.76 (1H, m)
13	37.5	1.80 (1H, m)
14	43.0	
15	27.5	1.04 (1H, overlap.), 1.91 (1H, td, 3.6, 13.2)
16	30.0	1.35 (1H, m), 2.47 (1H, m)
17	48.5	
18	49.1	1.69 (1H, t, 12.0, 11.4)
19	48.4	2.62 (1H, td, 10.9, 6.0)
20	151.2	
21	30.3	1.52 (1H, m), 2.16 (1H, m)
22	34.9	1.22 (1H, m), 2.43 (1H, t, 10.8)
23	28.8	1.03 (3H, s)
24	18.1	1.02 (3H, s)
25	17.6	0.96 (3H, s)
26	16.1	0.99 (3H, s)
27	14.9	1.05 (3H, s)
28	59.4	3.68 (1H, d, 10.8), 4.10 (1H, d, 10.8)
29	110.0	4.76 (1H, br s), 4.89 (1H, br s)
30	19.3	1.78 (3H, m)
1′	127.0	
2′	115.8	7.59 (1H, d, 1.8)
3′	145.4	
4′	147.6	
5′	116.7	7.24 (1H, d, 8.0)
6′	121.9	7.15 (1H, dd, 8.0, 1.8)
7′	145.4	8.03 (1H, d, 16.2)
8′	115.9	6.69 (1H, d, 16.2)
9′	168.0	

[a] Recorded at 150 MHz.
[b] Recorded at 600 MHz. *J* in Hz within parentheses.

by HPLC (YMC hydrosphere H80 C_{18}; 250 × 10 mm; AcCN–H_2O, 45:55; 2 mL/min) and from MC2-3-3 by recrystallization, respectively. MC2-3-2 was chromatographed on MPLC (RediSep silica gel; $CHCl_3$/MeOH, 15:1→0:100; 30 mL/min) to yield eight subfraction (MC2-3-2-1–MC2-3-2-8) and afforded compound **1** (3.5 mg) from MC2-3-2-5. MC4 was subjected to MPLC (RediSep silica gel; $CHCl_3$/MeOH, 10:1→0:100; 30 mL/min) to yield 10 fractions (MC4-1–MC4-10). MC4-3 was fractioned by MPLC (RediSep RP_{18}; MeOH/H_2O, 0:100→100:0; 20 mL/min) (MC4-3-1–MC4-3-5). Compounds **2** (68 mg) and **7** (22 mg) were isolated from MC4-3 on the HPLC (YMC hydrosphere H80 C_{18}; 250 × 10 mm; AcCN–H_2O, 34:66; 2 mL/min; Rt: 10.2 and 8.5 min, respectively). Compounds **4**–**6** and **8**–**12** were previously isolated from EtOAc fraction which also significantly inhibited the proliferation of HSC-T6 cells (22.7% of control at 100 μg/ mL, $p < 0.001$).[8]

Compound **13** was isolated as whitish amorphous powder. The molecular formula was determined to be $C_{39}H_{56}O_6$ from the negative HR-FAB-MS at *m/z* = 619.3997 $[M-H]^-$ (calcd for $C_{39}H_{56}O_6$: 619.3999). The ^{1}H NMR spectrum of compound **13** showed evidence for the presence of isopropylene function [δ 4.76 (1H, br s, H-29), 4.89 (1H, br s, H-29), 1.78 (3H, s, H-30)], five *tert*-CH_3 groups [δ 1.05 (3H, s, H-27), 1.03 (3H, s, H-23), 1.02 (3H, s, H-24), 0.99 (3H, s, H-26), 0.96 (3H, s, H-25)], geminal protons at δ 4.10 (1H, d, *J* = 10.8 Hz, H-28) and 3.68 (1H, d, *J* = 10.8 Hz, H-28)], an allylic proton at δ 2.62 (1H, td, *J* = 10.8, 6 Hz, H-28) for the aforesaid triterpenoid skeleton and caffeoyl moiety [δ 8.03 (1H, d, *J* = 16.2 Hz, H-7′), 7.59 (1H, d, *J* = 1.8 Hz, H-2′), 7.24 (1H, d, *J* = 8 Hz, H-5′), 7.15 (1H, dd, *J* = 8, 1.8 Hz H-6′), 6.69 (1H, d, *J* = 16.2 Hz, H-8′)] (Table 1).[9] The coupling constant (9.6 Hz) between proton δ 5.25 (1H, d, *J* = 9.6 Hz, H-3) and δ 4.31 (1H, td, *J* = 10.8, 4.2 Hz, H-2) indicated the hydroxyl groups should have 2α,3β-orientation, which was further supported by the NOESY correlations of H-2 with H-1β and H-25 and of H-3 with H-1α.[10] The connection of caffeoyl group to the C-3 position was also confirmed by the HMBC correlations between the signals at δ_H 5.25 (1H, H-3) and δ_C 168.0 (C-9′). On the basis of the above data, compound **13** was characterized as lup-20(29)en-2,28-diol-3-yl caffeate, which has been newly discovered from nature.

Lup-20(29)en-2,28-diol-3-yl caffeate (**13**); whitish amorphous powder; $[\alpha]_D^{20}$ = +5 (*c* 0.75, $CHCl_3$/MeOH 1:1); IR (KBr) ν_{max}: 3364 (OH), 2941, 1685 (C=O), 1604, 1516, 1454, 1374, 1269, 1177, 1022 cm^{-1}; FABMS: *m/z* 619 $[M-H]^-$; HR FABMS (*m/z*) 619.3997 $[M-H]^-$ (calcd for $C_{22}H_{26}O_{12}$, *m/z* 619.3999).

Compound **1** was identified as 1,7-bis-(4-hydroxyphenyl)-5-hepten-3-one,[12] which has been newly isolated from this plant. Compounds **2**, **3** and **7** were identified as dehydrohirsutanonol, 5-hydroxy-3-platyphyllone and (5*R*)-5-methoxy-1,7-bis-(3,4-dihydroxyphenyl)-3-heptanone by the comparison with literature values (Fig. 1).[11–13] Compounds **4**–**6** and **8**–**12** were identified as diarylheptanoids derivatives as previously reported (Fig. 1).[9]

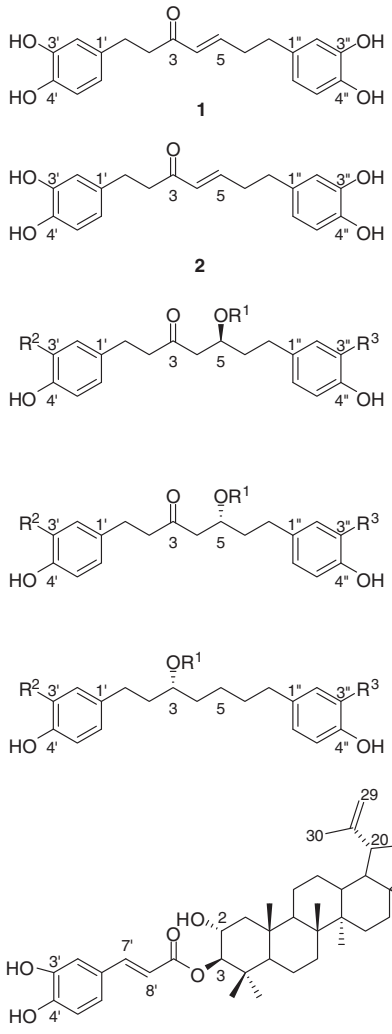

Figure 1. Structures of compounds isolated from *A. firma* barks.

The antifibrotic activity of isolated compounds (**1**–**13**), together with epigallocatechin-3-gallate (EGCG) as a positive control, were examined using HSC-T6 cells.[14] Cell viability and proliferation were assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and BrdU incorporation assay, respectively.[15] Collagen content was measured by Sirius Red-based colorimetric assay.[16] Caspase-3/7 activity was measured with Apo-ONE homogenous caspase-3/7 assay kit using Z-DEVD-rhodamine 110 as a substrate. Statistical significance was determined by 'one-way ANOVA' test using a computerized statistical package with a value of p <0.05 or less considered to be statistically significant.

Antifibrotic activity of compounds **1**–**13** was first evaluated by assessing viability of HSC-T6 cells. As shown in Table 2, compounds **1**–**3**, **5**, **9**–**11** and **13** showed significant inhibitory activity on HSC proliferation with IC50 value around 14.0–54.9 μM.

Because compounds **2** and **13** showed strong inhibitory activity, we further tried to investigate the effect on HSC activation. Inhibition of HSCs viability commonly can be accomplished by various pathways, such as inhibition of cell proliferation and/or induction of cell death. Compounds **2** and **13** decreased HSC-T6 cell viability in dose- and time-dependent manners (Fig. 2). In addition, compounds **2** and **13** significantly inhibited cell proliferation (Fig. 3A). Further investigation on caspase-3/7 activity showed that compounds **2** and **13** increased the caspase-3/7 activity up to 150%

Table 2
Inhibitory activity of compounds **1**–**13** from *A. firma* on HSC-T6 cell proliferation

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
1	34.7	**8**	>100
2	14.0	**9**	39.1
3	54.9	**10**	46.2
4	>100	**11**	54.9
5	34.9	**12**	>100
6	>100	**13**	23.9
7	>100	EGCG	9.9

Values are expressed as the means ± S.D. of three independent experiments, each performed using triplicate wells. EGCG was used as a positive control.

of activated control cells (Fig. 3B). Taken together, we suppose that the antifibrotic activity of compounds **2** and **13** may be achieved by the interference in cell proliferation and by induction of apoptosis but not due to direct toxic effect. Consistent with our hypothesis, treatment of HSCs with compounds **2** and **13** showed did not increase LDH release in the culture medium (data not shown).

Excessive production and deposition of ECM such as collagen are important characteristic of HSC activation. Therefore, the effect on collagen content was evaluated. In our culture system, activated HSC-T6 cells produced high amount of collagen. However, compounds **2** and **13** dramatically decreased collagen content in a dose-dependent manner (Fig. 4).

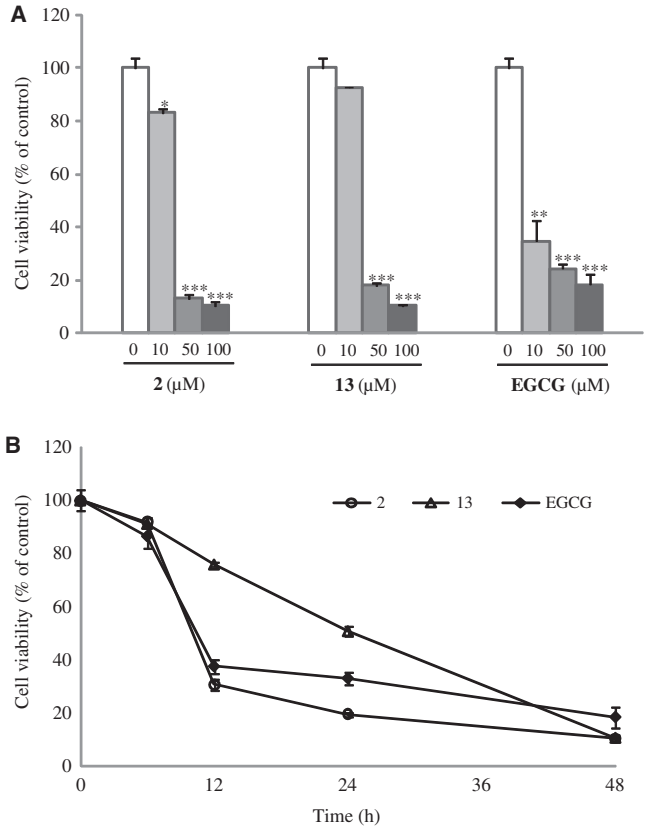

Figure 2. Concentration (A) and time (B) dependent effect of compounds **2** and **13** on cell viability in HSC-T6 cells. HSC-T6 cells were incubated compounds **2** and **13** at the concentration ranging from 10 to 100 μM for 48 h (A), or at 100 μM for indicated time (B). Cell viability was measured by the MTT assay. Values are expressed as the means ± S.D. of three independent experiments, each performed using triplicate wells. Mean value is significantly different (*p <0.05, **p <0.01, ***p <0.001) from the value of control. EGCG was used as a positive control.

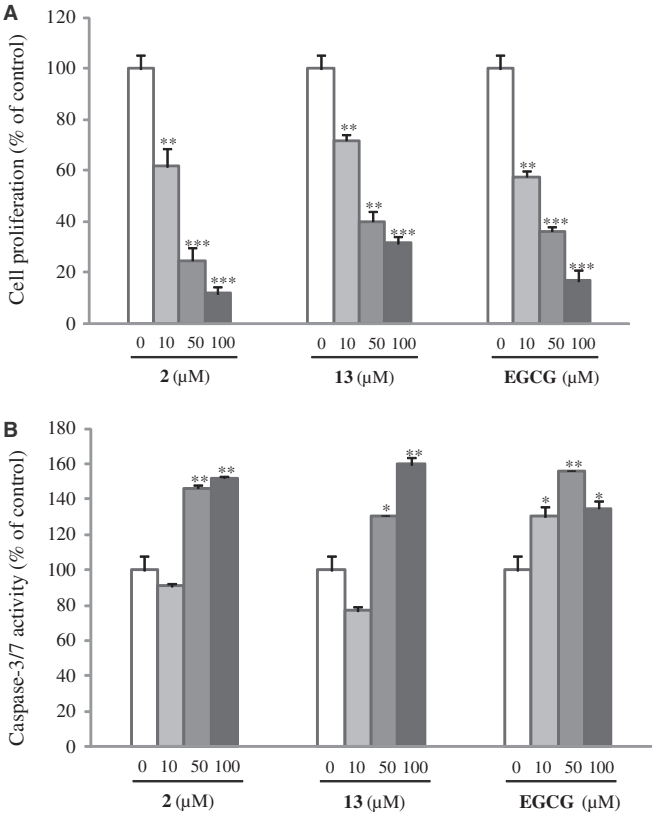

Figure 3. Effect of compounds **2** and **13** on cell proliferation (A) and caspase-3/7 activation (B) in HSC-T6 cells. HSC-T6 cells were treated with compounds **2** and **13** and EGCG at the concentration ranging from 10 to 100 μM for 24 h and for 48 h for proliferation. Cell proliferation and caspase-3/7 activity was measured by BrdU assay and Apo-ONE Homogenous Caspase-3/7 assay kit, respectively. Values are expressed as the means ± S.D. of three independent experiments, each performed using triplicate wells. Significantly different (*p <0.05, **p <0.01, ***p <0.001) from the control. EGCG was used as a positive control.

Antifibrotic compounds isolated from *A. firma* are twelve diarylhepatonoids and triterpene derivatives. Curcumin, a well known diarylhepatnoid, has been reported for beneficial effect on hepatic fibrosis.[17,18] Twelve diarylheptanoids tested in our present study are same skeleton and mainly differ in the numbers and positions of hydroxyl groups and glycosyl moieties. Among twelve diarylheptonoids, compounds **1** and **2**, which have carbonyl at C-3 and double bond at C-4,5 showed the most potent activity. Generally, addition of hydroxyl group to C-5 decreased antifibrotic activity as shown in compounds **4** and **6**–**8**. However, substitution of carbonyl group at C-3 to hydroxyl group still possesses antifibrotic activity as shown in compounds **9**–**11**. Therefore, we carefully suppose that substitution at C-3 and C-5 might be important for the inhibitory activity of diarylheptanoids on HSC proliferation, which needs to be clarified with more diverse derivatives.

Liver is composed of several different cell types including hepatocytes, HSCs and Kupffer cells and exerts physiological roles as well as pathological condition by cross-talking of various cell types. Liver fibrosis can be induced by hepatocellular damage, which causes inflammatory response leading to HSC activation. In our previous study, diarylheptanoids showed anti-inflammatory activity.[9] Therefore, we expect that antifibrotic activity of these compounds might be potentiated at in vivo model, by the collaboration of different mechanism including anti-inflammatory activity, which needs to be elucidated by further investigation. This will provide further insight into the design of new approaches to liver fibrosis.

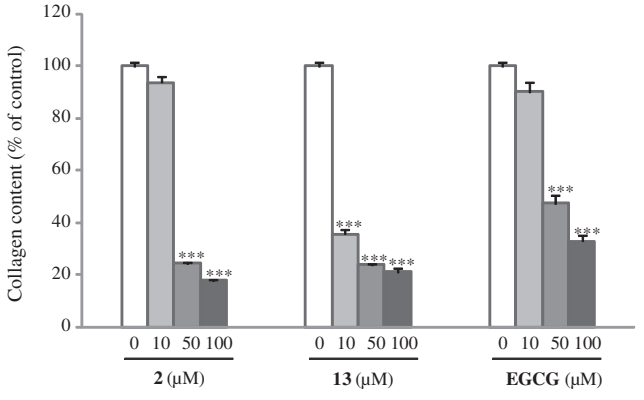

Figure 4. Effect of compounds **2** and 1**3** on collagen deposition in HSC-T6 cells. HSC-T6 cells were treated with compounds **2** and **13** at the concentration ranging from 10 to 100 μM for 48 h. Collagen content was measured by Sirius Red-based colorimetric assay. Values are expressed as the means ± S.D. of three independent experiments, each performed using triplicate wells. Significantly different (***p <0.001) from the control. EGCG was used as a positive control.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.03.074.

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14. An immortalized rat hepatic stellate cell line, HSC-T6, was kindly provided by Professor S. L. Friedman (Columbia University, New York, NY). HSC-T6 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 95% air-5%CO_2. Compounds to be tested were dissolved in DMSO (final culture concentration, 0.1%). A preliminary study showed that DMSO at a final concentration of 0.1% in media did not affect the cell viability. For the assay, the cells were seeded in 48-well plates at a density of [5×10^4 cells/mL] and incubated for 24 h. HSC-T6 cells were treated with vehicle or compounds to be tested for 48 h.
15. MTT assay was conducted by the incubation of HSC-T6 cells with 0.5 mg/mL of MTT in the last 2 h of the culture period tested. Reduction of MTT to formazan was assessed in an ELISA plate reader at 540 nm. The effect on cell proliferation was measured by BrdU incorporation assay according to manufacturer's protocol. Cell viability and cell proliferation (% of control) of compounds **1**–**13** on was calculated as 100 × (absorbance of treated compound/absorbance of control). Data were expressed as the mean of three independent experiments.
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