Antifibrotic Activity of Diarylheptanoids from *Betula platyphylla* toward HSC-T6 Cells

Mina Lee,1 Jung Hyun Park,1 Dong Sun Min,2 Hunseung Yoo,2 Jin Ho Park,3 Young Choong Kim,1 and Sang Hyun Sung1,†

1 College of Pharmacy and Research Institute of Pharmaceutical Science, Seoul National University, 2 Natural Product Team, Life Science R&D Center, SK Chemicals, 3 Institute for Life Science, Elcomscience Co., Ltd.

Received November 22, 2011; Accepted June 14, 2012; Online Publication, September 7, 2012

A chemical investigation of the *n*-butanol fraction of the inner bark of *Betula platyphylla* led to the isolation of seven diarylheptanoids, (−)-centrolobol (1), aceroside VII (2), aceroside VIII (3), (3R)-1,7-bis-(4-hydroxyphenyl)-3-heptan-3-ol-O-[2,6-bis-O-(β-D-apiofuranosyl)-β-D-glucopyranoside (4), 1,7-bis-(4-hydroxyphenyl)-5-hepten-3-one (5), platyphyllone (6) and platyphylloside (7). The antifibrotic effects of these isolates were evaluated with HSC-T6 cells by assessing cell proliferation. Among them, compounds 1, 2, 5 and 6 significantly inhibited the proliferation of HSCs in a dose-dependent manner at concentrations from 10 μM to 100 μM. Compound 5 in particular dramatically decreased the collagen content and increased the Caspase-3/7 activity. Taken together, the antifibrotic activity of *B. platyphylla* and its constituents might suggest therapeutic potential against liver fibrosis.

Key words: *Betula platyphylla*; Betulaceae; diarylheptanoid; HSC-T6 cell; antifibrotic activity

Liver fibrosis is a reversible complication of advanced liver disease and represents the consequences of a sustained wound-healing response to chronic liver injury from a variety of causes, including viral, autoimmune, drug-induced, cholestatic and metabolic diseases. Hepatic stellate cells (HSCs; also known as fat-storing cells, Ito cells, or lipocytes) are the primary cell type in the liver responsible for excess collagen synthesis during hepatic fibrosis. HSCs undergo a response known as activation, developing myofibroblast-like phenotype associated with increased proliferation and excessive production of the extracellular matrix (ECM) components, leading to final liver dysfunction and cirrhosis. Following liver injury of any etiology, the activation of HSCs, the crucial aspect in the pathogenesis of hepatic fibrosis, is mediated by various cytokines and reactive oxygen species released from the damaged hepatocytes and activated Kupffer cells. For that reason, suppressing HSC activation may be a useful strategy to prevent or treat hepatic fibrosis. To date, however, therapeutic drugs for liver fibrosis are often insufficient or not available. The HSC-T6 cell line comprises immortalized rat hepatic stellate cells, and has been found to retain all the features of activated stellate cells, including the expression of desmin, α-smooth muscle actin, and glial fibrillary acidic protein, and it can esterify retinol into retinyl esters. We could therefore try to search for antifibrotic compounds from natural products evaluated for their antifibrotic activity by employing HSC-T6 cells.

In the course of searching for natural antifibrotic products, employing HSC-T6 cells as an in vitro assay system, the methanolic extract of the bark of *B. platyphylla* var. japonica (Betulaceae) showed significant inhibition of HSC-T6 cell proliferation. *B. platyphylla*, the birch tree, is widely distributed in Korea, Japan, China, Sahalin and Siberia. The two main products harvested from birch without timber-cutting are the sap and bark. The collected sap can be drunk as a tonic, and syrups, drinks and food additives have also already been commercialized from the birch sap. The bark of *B. platyphylla* has been used in folk medicine for treating various inflammatory diseases, including, arthritis, nephritis, dermatitis and bronchitis. Pharmacological studies of this plant have reported anticancer, anti-arthritis and hepatoprotective activities. Several terpenoids, including betulin and such phenolic compounds as diarylheptanoids and arylbutanoids, have been respectively isolated from the outer bark and inner bark of *B. platyphylla*. However, the antifibrotic activity of this plant has not previously been investigated. We therefore tried to find antifibrotic diarylheptanoids by using an immortalized rat hepatic stellate HSC-T6 cell line as a screening system. Bioassay-guided fractionation of the extract of *B. platyphylla* led to the isolation of seven diarylheptanoids: (−)-centrolobol (1), aceroside VII (2), aceroside VIII (3), (3R)-1,7-bis-(4-hydroxyphenyl)-3-heptan-3-ol-O-[2,6-bis-O-(β-D-apiofuranosyl)-β-D-glucopyranoside (4), 1,7-bis-(4-hydroxyphenyl)-5-hepten-3-one (5), platyphyllone (6) and platyphylloside (7). Among these isolated compounds, 1,7-bis-(4-hydroxyphenyl)-5-hepten-3-one (5) significantly inhibited the proliferation of HSCs at concentrations from 10 μM to 100 μM. Compound 5 in particular showed antifibrotic activity by decreasing the collagen content and increasing the Caspase-3/7 activity.

† To whom correspondence should be addressed. Tel: +82-2-880-7859; Fax: +82-2-877-2933; E-mail: shsung@snu.ac.kr
Materials and Methods

General experimental procedure. The optical rotation was measured with a Jasco DIP-1000 digital polarimeter. UV spectra were obtained with a Shimadzu UV-201 spectrophotometer, and IR spectra with a Perkin-Elmer 1710 spectrophotometer. NMR spectra were obtained with a Jeol JNM GSX-400 NMR spectrometer (400 and 100 MHz for $^1$H and $^{13}$C, respectively) or a Bruker AMX 500 NMR spectrometer (500 and 125 MHz for $^1$H and $^{13}$C, respectively) in CD$_3$OD, with solvent signals as the internal standards. High-resolution and low-resolution FABMS data were obtained with a Jeol JMS-AXS05SWA instrument. GC was conducted by a $^{9}$NS gas chromatograph (Agilent Technologies Co., USA) with a flame ionization detector (FID). Silica gel (230–400 mesh, Merck), BDS RP (Lichroprep BDS RP-18, 40–63 µm, EM Science), and Sephadex LH-20 (18–110 µm, Pharmacia) were used for column chromatography. TLC was carried out on precoated Kieselgel 60 F254 (0.25 mm, Merck) and RP-18 plates, and spots were visualized by heating after spraying with anisaldehyde–H$_2$SO$_4$. HPLC was performed on an L-7100 pump (Hitachi Co., Japan), an L-7400 UV detector (Hitachi Co., Japan), and a Symmetry C18 column (Phenomenex Co., USA). The other HPLC equipment consisted of a G-321 pump (Gilson Co., USA), a G-151 UV detector (Gilson Co., USA), and an YMC C$_18$ hydrosphere (YMC Co., Japan). All chromatograms were monitored at 210 nm.

Plant material. The bark of *B. platyphylla* was provided by SK &C (Korea). The samples had been collected in afforested land of SK &C which has more than 450,000 *B. platyphylla* trees in 167.6 ha of forest in Chungju. A voucher specimen (SNU-797) has been deposited at the herbarium of the Medicinal Plant Garden, College of Pharmacy, Seoul National University.

Extraction and isolation. The dried plant material (5.7 kg) was pulverized and then extracted with 80% methanol (30L, 3 h by 4) at room temperature. The methanolic extract was concentrated in vacuo to give a crude extract (948.4 g). This crude extract was then suspended in H$_2$O and successively partitioned with CHCl$_3$ (8 L) and n-butanol (8 L), giving a solid residue of 205.3 g and 577.5 g, respectively. Of these fractions, the n-butanol fraction, which showed the significant inhibitory effect (Table 1), was used for isolating the active compounds to yield seven diarylheptanoids (Fig. 1). The n-butanol fraction (577.5 g) was eluted from Diaion HP-20 resin using a water-MeOH gradient as the mobile phase to afford six fractions (B1–B6). B4 (90.7 g) was subjected to silica gel column chromatography to yield fifteen fractions (B4-1–B4-15). Compounds 1, 2, and 3 were isolated by MPLC (RediSep C$_18$) and low-resolution FABMS data were obtained with a Jeol JMS-AXS05SWA instrument.

| Table 1. Inhibitory Activity of the Methanolic Extract and Fractions of *B. Plattphylla* toward HSC-T6 Cell Proliferation |
|-----------------|-----------------|-----------------|
|                 | 50µg/mL         | 150µg/mL        |
| Control         | 100.0 ± 1.1     |                 |
| Total extract   | 71.4 ± 3.2      | 69.9 ± 3.1*     |
| CHCl$_3$ fraction | 31.4 ± 2.3**   | 25.1 ± 5.5**    |
| n-ButOH fraction | 93.1 ± 4.9*    | 42.0 ± 4.0*     |
| Water fraction | 87.4 ± 5.5     | 111.5 ± 7.4     |

HSC-T6 cells were incubated with the methanolic extract and the fractions at a concentration ranging from 50 to 150 µg/mL for 48 h. Cell viability was measured by MTT assay. Each value is expressed as the mean ± SD of three independent experiments, each performed using triplicate wells. The mean value was significantly different (*p < 0.05, **p < 0.01) from the control value.

Fig. 1. Structures of the Diarylheptanoids Isolated from *B. platyphylla* Bark.

Measurement of the collagen content. The collagen content was measured by a Sirius Red-based colorimetric assay. Briefly, the cultured HSC-T6 cells were washed with PBS after the treatment, before being fixed with Bouin’s fluid for 1 h. The fixation fluid was removed by suction, and the culture plates were washed by immersing in running tap water for 15 min. The culture plates were air dried and stained by the Sirius Red dye reagent for 1 h. The stained solution was then removed, and the culture was washed with 0.01 N HCl to remove any non-bound dye. The stained material was dissolved in 0.1 N NaOH, and the absorbance was measured at 550 nm against 0.1 N NaOH as a blank. The collagen content (µg/µg of protein) was calibrated from a curve based on the reference standard. The percentage collagen content (%) was calculated as 100 × (absorbance of treated sample/absorbance of control).

Humidified atmosphere of 95% air+5% CO$_2$. The compounds to be tested were dissolved in DMSO to a final culture concentration of 0.1%. A preliminary study had shown that DMSO at a final concentration of 0.1% in the medium did not affect the cell viability. The cells were seeded in 48-well plates at a density of 5 × 10$^4$ cells/mL for the assay and incubated for 24 h. The HSC-T6 cells were treated with the vehicle or the compound to be tested for 48 h. The MTT assay was conducted by incubating the HSC-T6 cells with 0.5 mg/mL of MTT in the last 2 h of the culture period being tested. The reduction of MTT to formazan was assessed by an ELISA plate reader at 540 nm. Cell proliferation (% of the control) of compounds 3–7 was calculated as 100 × (absorbance of treated compound/absorbance of control).
Evaluation of Caspase-3/7 activity. The Caspase-3/7 activity was evaluated with an Apo-ONE Homogenous Caspase-3/7 assay kit, using Z-DEVD-rhodamine 110 as a substrate. After treating with a compound for the indicated time, 100 µL of a homogeneous Caspase-3/7 reagent (buffer + substrate) was added to each well of the cultured HSC-T6 cells in a 96-well plate. The 96-well plate was incubated at room temperature and measured by a Cytofluor II multi-well fluorescence spectrophotometer (excitation at 485 nm and emission at 520 nm).

Statistical analysis. The statistical significance was determined by Student’s t-test, with a value of \( p < 0.05 \) or less being considered as statistically significant.

Results

Isolation of diarylheptanoids from B. platyphylla

The methanolic extract of the bark of B. platyphylla was further fractionated into the CHCl3 and n-butanol fractions which had significantly reduced the cell proliferation at a concentration of 150 \( \mu \)g/mL (Table 1). Betulin, the major component of the CHCl3 fraction, has already been reported to attenuate ethanol-induced liver stellate cell activation.1 Curcumin, one of the diarylheptanoids, is known to inhibit the proliferation of HSC-T6 cells and induce apoptosis.13 The diarylheptanoids reportedly isolated from the bark of B. platyphylla were structurally similar to curcumin.9 We therefore made a further study of the n-butanol fraction containing many diarylheptanoids to search for antifibrotic compounds, although the CHCl3 fraction had shown the most potent antifibrotic activity toward HSC-T6 cells. Activity-guided fractionation of the n-butanol fraction enabled the active constituents to be isolated. Further fractionation and separation by several chromatographic methods yielded seven diarylheptanoids (1–7) from the n-butanol fraction. The structures of these diarylheptanoids were identified as (−)-centrolobol (1), acerside VII (2), aceralde VII (3), (3R)-1,7-bis-(4-hydroxyphenyl)-3-heptan-3-O-[2,6-bis-O-(β-D-apiofuranosyl)-β-D-glucopyranoside (4), 1,7-bis-(4-hydroxyphenyl)-5-hepten-3-one (5), platyphylline (6) and platyphylloside (7) (Fig. 1) by directly comparing their spectroscopic data with those previously reported.14–18

Antiproliferative activity of the diarylheptanoids isolated from B. platyphylla toward HSC-T6 cells

We elucidated the antifibrotic activity of these diarylheptanoids toward HSC-T6 cells by assessing the effect on cell proliferation with an MTT assay. Table 2 shows that compounds 1, 2, 5 and 6 had significant inhibitory activity toward HSC proliferation in a dose-dependent manner with respective IC50 values of 73.7, 75.9, 34.7 and 54.9 \( \mu \)M, while the IC50 values of compound 3, 4 and 7 were more than 100 \( \mu \)M. Compound 5 in particular inhibited cell proliferation by up to 18.5% at the concentration of 100 \( \mu \)M, this being comparable to that of EGCG, a positive control. Since compound 5 had the strongest inhibitory activity among compounds 1–7, we further investigated the antifibrotic activity toward HSC activation.

Inhibitory effect of compound 5 on collagen deposition in HSC-T6 cells

Excessive production and deposition of such ECM as collagen are other important characteristics of HSC activation and are responsible for liver dysfunction. The effect of compound 5 on collagen production in the activated HSC-T6 cells was therefore studied. The activated HSC-T6 cells produced a high amount of collagen in our culture system. Compound 5 dramatically decreased this collagen content in a dose-dependent manner over a concentration ranging from 10 \( \mu \)M to 100 \( \mu \)M (Fig. 2).

Effect of compound 5 on Caspase-3/7 activity in HSC-T6 cells

The induction of growth inhibition or cell death of HSCs is a potential strategy to treat hepatofibrosis. Compound 5 decreased the HSC-T6 cell viability in a dose-dependent manner (Table 2). The activation of Caspases, especially Caspases 3 and 7, is a biochemical hallmark of apoptosis.19 We therefore measured the Caspase-3/7 activity with the Apo-ONE Homogenous Caspase-3/7 assay kit, using Z-DEVD-rhodamine 110 as a substrate.11 Further investigation of this activity showed that compound 5 increased the Caspase-3/7 activity by up to 215% of activated control cells (Fig. 3).
Hepatic stellate cells (HSCs) are considered to play a key role in the pathogenesis of liver fibrosis. Inhibiting HSC cell proliferation has been considered as an effective treatment for delaying fibrosis in the liver. It is known that HSC cells, an immortalized hepatic stellate cell line, cultured on plastic plates were spontaneously activated and then led to a myofibroblastic state in vivo.\(^1\) HSC-T6 cells were therefore used as a screening method to evaluate the antifibrotic activity of the isolated compounds. The antiproliferative activity of the seven diarylheptanoids from \(B.\) platyphylla was first evaluated by assessing the viability of HSC-T6 cells which enabled the structure-activity relationship of these compounds to be analyzed. Curcumin, a well-known diarylheptanoid, has been reported to have a beneficial effect on hepatic fibrosis.\(^2\) The seven diarylheptanoids tested in our present study had the same skeleton and mainly differed in the numbers and positions of the hydroxyl groups and glycosyl moieties. Among these seven diarylheptanoids, compound 5, which was very similar to curcumin with an \(\alpha,\beta\)-unsaturated ketone showed much more potent activity than the other diarylheptanoids. It has been reported that the \(\alpha,\beta\)-unsaturated ketone moiety was an important structural determinant for the activity.\(^3\) The addition of a hydroxyl group in the heptane chain and substitution of the glucose moiety tended to decrease the biological activity as shown in diarylheptanoids 1–7. We therefore presume that substitution at C-3 and C-5 might be important for the inhibitory activity of diarylheptanoids toward HSC proliferation; this needs to be clarified with more diverse derivatives. Inhibiting the viability of HSCs can be commonly accomplished by such methods as inhibiting cell proliferation and/or inducing cell death. It has recently been shown that recovery from established experimental fibrosis occurred through the apoptosis of HSCs.\(^4\) It is suggested that the apoptosis of HSCs resolves fibrosis by facilitating net matrix degradation through elimination of the source of both matrix and metalloproteinase inhibitors, providing proof of the concept that HSC apoptosis would be a promising therapeutic strategy for established hepatic fibrosis.\(^5\)–\(^8\) Caspases (cysteine aspartate-specific proteases) are a family of intracellular proteins involved in the initiation and execution of apoptosis. Activation of the execution Caspases is often referred to as the apoptotic commitment point;\(^9\) therefore, Caspase-3/7 activity was evaluated in present study to investigate the cell death of HSCs. It has been reported that the activation of PPAR\(\gamma\) was required for curcumin to suppress ECM gene expression and to contribute to the induction of HSC apoptosis by stimulating the Caspase 3 activity, increasing the abundance of pro-apoptotic Bax and reducing the level of anti-apoptotic Bcl-2 in activated HSCs in vitro.\(^10\) Our present study has shown that the antifibrotic activity of compound 5 resulted, in part, by the increase of Caspase-3/7 activity. Collagen production was also dramatically decreased by the treatment with compound 5. Taken together, we presume that the antifibrotic activity of compound 5 could be achieved by interference with cell proliferation and by induction of apoptosis, and not be due to a direct toxic effect. Consistent with this hypothesis, treating HSCs with compound 5 did not increase LDH release in the culture medium (data not shown). Liver fibrosis can be induced by hepatocellular damage which causes an inflammatory response leading to HSC activation. Diarylheptanoids isolated from species of Betulaceae are known to have anti-inflammatory activity.\(^11\) We therefore expect that the antifibrotic activity of these compounds might be potentiated with an \textit{in vivo} model by the collaboration of different mechanisms including anti-inflammatory activity. This needs to be elucidated by further investigation and will provide further insight into new approaches to liver fibrosis.

**Acknowledgments**

This work was supported by the Global Leading Technology Program (GLST-OSP project no. 10039320) of the Office of Strategic R&D Planning (OSP) funded by the Ministry of Knowledge Economy, Republic of Korea.

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