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Maslinic Acid, a Triterpenoid from the Root Barks of *Ulmus davidiana* var. *japonica*, Affects the Viability of HSC-T6 Hepatic Stellate Cells†

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Abstract – Activation of hepatic stellate cells (HSCs) characterized by increased proliferation and extracellular matrix deposition is identified as the major pathological feature of hepatic cirrhosis. Therefore, suppression of HSC activation has been proposed as an important antifibrotic therapeutic strategy. In the present study, we investigated the antiproliferative activity of root barks of *Ulmus davidiana* var. *japonica* (Ulmaceae) by employing HSC-T6 hepatic stellate cells as an in vitro assay system. Further investigation of the *n*-hexane and $CHCl_3$ fractions of root barks of *U. davidiana* var *japonica* led to the isolation of six triterpenoids: friedelin (**1**), epifridelanol (**2**), oleanolic acid (**3**), maslinic acid (**4**), β-amyrin (**5**) and α-amyrin (**6**), together with β-sitosterol (**7**) and daucosterol (**8**). Among these compounds, **2**, **3** and **4** significantly inhibited HSC proliferation. In addition, **4** inhibited HSC proliferation in time- and concentration-related manners, via a partially direct toxic effect, as assessed by morphological changes and release of lactate dehydrogenase.
Key words – *Ulmus davidiana* var. *japonica*, Ulmaceae, maslinic acid, antifibrotic, HSC-T6, hepatic stellate cells, triterpenoid

Introduction

Ulmus davidiana Planchon var. *japonica* Nakai (Ulmaceae) is a deciduous tree that is widely distributed in Korea and Japan. In traditional Korean medicine, the barks of stem and root of this plant have been used for treating edema, mastitis and gastric cancer with anti-inflammatory, antibacterial and anticancer properties (Bae, 2000). Previous phytochemical studies of *Ulmus* species have reported the isolation of diverse flavonoids, triterpenoids and lignans (Lee and Kim, 2001; Lee *et al*., 2001, 2008; Kim *et al*., 2007). In addition, beneficial effects of *Ulmus* species on neurodegenerative diseases, osteoporosis, cancer and inflammation have been reported (Lee and Kim, 2001; Kim *et al*., 2008; Choi *et al*., 2009; Zheng *et al*., 2010).

Liver fibrosis and end-stage disease cirrhosis are major health issues arising from chronic liver injury caused by diverse factors including viruses, alcohol and metabolic agents. Liver fibrosis is a common response to most chronic hepatic diseases, characterized by the excess production and deposition of extracelluar matrix (ECM). An early event in the development of hepatic fibrosis is the activation of hepatic stellate cells (HSCs). HSCs have important functions in normal liver, such as retinoid storage, remodeling of ECM and production of growth factors. During liver fibrosis, HSCs undergo a complex activation process characterized by increased proliferation and ECM deposition, which is the major pathological feature of hepatic cirrhosis (Li and Friedman, 1999; Tsukada *et al*., 2006). Therefore, suppression of HSC activation and proliferation, and induction of apoptosis in activated HSCs have been proposed as therapeutic strategies for the treatment and prevention of hepatic fibrosis (Wu and Zern, 2000; Bataller and Brenner, 2005). In the present study, we investigated the antifibrotic activity of root bark of *U. davidiana* var. *japonica*, employing HSC-T6 rat hepatic stellate cells as an *in vitro* assay system. We attempted to isolate antifibrotic compounds and evaluate their antifibrotic activity.

Methods and materials

Plant material – Root barks of *U. davidiana* var.

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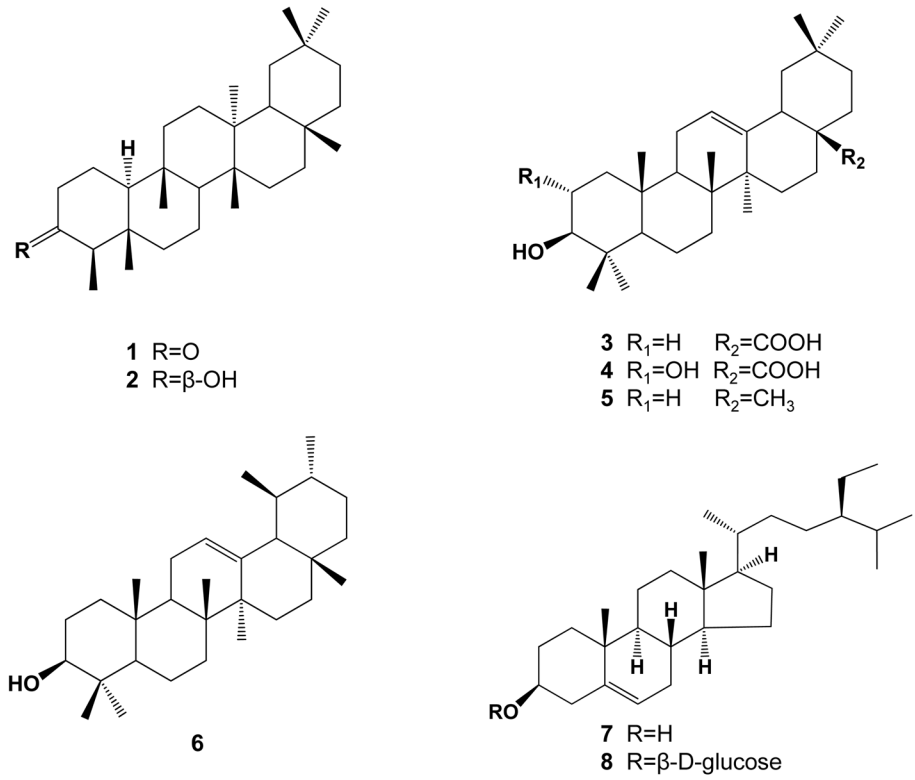

Fig. 1. Triterpenoids isolated from the root barks of *U.davidiana* var. *japonica*.

japonica were purchased from a local herbal market in Chungbuk, Korea, in March 2010. They were identified by the herbarium of College of Pharmacy at Chungbuk National University, where a voucher specimen was deposited (CBNU-2010-UDVJ).

Extraction and isolation – *U. davidiana* var. *japonica* stem bark samples (500 g) were extracted two times with 80% methanol (MeOH), which yielded 43.1 g of total methanolic extract. The methanolic extract was then suspended in H_2O and partitioned successively with *n*-hexane, $CHCl_3$, ethyl acetate (EtOAc) and *n*-butanol. The *n*-hexane and $CHCl_3$ fractions, which showed similar thin layer chromoatography patterns, were combined to form one fraction. The combined *n*-hexane and $CHCl_3$ fraction (2.3 g) was subjected to silica gel column chromatography with a mixture of *n*-hexane-EtOAc to give 14 fractions (H1-H14). Compound **1** (24.1 mg), **2** (30.6 mg), **7** (16.8 mg), **3** (3.6 mg) and **8** (9.1 mg) were obtained from H1, H2, H4, H8, and H12, respectively, by recrystallization using MeOH. Compound **5** and **6** (2.9 mg) were obtained as a mixture from H3 by recrystallization using MeOH. Compound **4** (1.5 mg) was obtained from H10 by recrystallization using $CHCl_3$.

Culture of HSC-T6 hepatic stellate cells – An immortalized rat hepatic stellate cell line, HSC-T6, was kindly provided by Prof. SL Freidman (Columbia University, New York). HSC-T6 cells were maintained in 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.

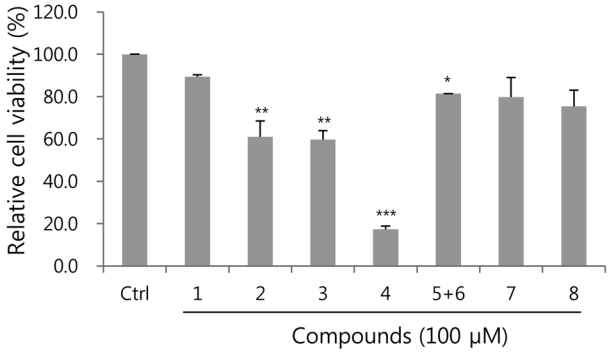

Fig. 2. Effect of compounds isolated from *U.davidiana* var. *japonica* on HSC-T6 cell viability. HSC-T6 cells were incubated with 100 μM of each compound for 48 hr. Cell viability was measured by the MTT assay. The percent of cell viability (%) was calculated as 100 × (absorbance of compound-treated / absorbance of control). Results are expressed as the mean ± S.D. of three independent experiments, each performed using triplicate wells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control.

Measurement of cell viability – Samples to be tested were dissolved in dimethylsulfoxide (DMSO). Our preliminary study showed that DMSO at a final concentration of 0.1% in the medium did not affect cell viability. HSC-T6 cells were treated with vehicle or samples to be tested for 48 hr or as indicated. Cell viability was assessed by an established 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HSC-T6 cells were incubated with 0.5 mg/mL of MTT in the last 2 hr of the culture period tested. Reduction of MTT to formazan was assessed in an ELISA plate reader.

Statistical analysis – The evaluation of statistical significance was determined by the Student's *t*-test with a value of $p \leq 0.05$ were considered to be statistically significant.

Results and discussion

Column chromatographic separation of *n*-hexane- and $CHCl_3$- fractions of root bark of *U. davidiana* var. *japonica* led to the isolation of six triterpenoids (**1** - **6**) and two sterol derivatives (**7**, **8**). The structures of isolated compounds were identified to be friedelin (**1**), epifriedelanol (**2**), oleanolic acid (**3**) maslinic acid (**4**), β-amyrin (**5**), α-amyrin (**6**), β-sitosterol (**7**) and daucosterol (**8**), respectively, by comparing of ^{1}H-, ^{13}C-NMR with previous studies (Mahato and Kundu, 1994; Taniguchi *et*

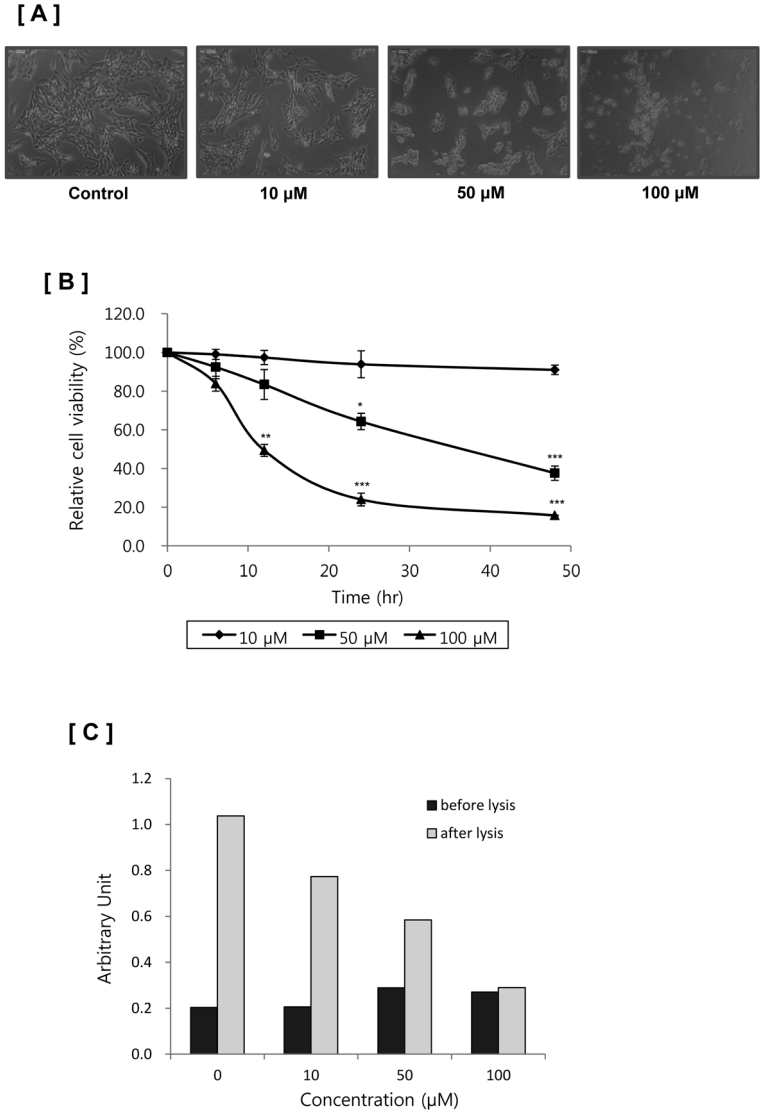

Fig. 3. Concentration- and time-dependent effects of compound 4 on HSC-T6 cells. HSC-T6 cells were incubated with compound **4** at the concentrations ranging from 10 - 100 μM for the indicated times. Cells were observed with a phase contrast microscope [A]. Cell viability was measured by the MTT assay [B] or by LDH release [C]. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control.

al., 2002; Lee *et al*., 2005; Saimaru *et al*,. 2007; de Melo *et al*,. 2010; Li *et al*., 2010).

Next, we investigated the antiproliferative activity of these constituents in HSC-T6 cells by assessing the cell viability using a MTT assay. Among the compounds isolated, **4** showed potent inhibitory activity, followed by **2** and **3**. Compounds **2**, **3** and **4** inhibited cell viability up to 61.0%, 59.6% and 17.3% of control, respectively, at a concentration of 100 μM for 48 hr incubation (Fig. 2). Compound **4** decreased HSC proliferation in dose- and time-dependent manners (Fig. 3).

Decreased cell viability generally can be achieved by two pathways: inhibition of cell proliferation or induction of cell death. Induction of cell death usually can be divided in necrosis and apoptosis. Treatment of compound **4** showed differential effects depending on concentration treated. At 100 μM, morphological changes similar to necrosis were observed (Fig. 3A). In addition, lactate dehydrogenase (LDH) release into medium increased compared to control and lysis of cells did not induce any further LDH release. At the concentration of 10 μM, little morphological change indicative of necrosis was observed (Fig. 3A) and LDH release into medium was comparable to that of control. Total cell numbers decreased at the concentration of 10 μM as assessed by LDH release after cell lysis. Therefore, we supposed that maslinic acid (**4**) exerted anti-proliferative activity on HSCs by differential mechanism depending on its dose. Recent studies suggest the anti-tumor activity of maslinic acid in several cell lines, in part by induction of apoptosis (Reyes-Zurita et al., 2009; Hsum et al., 2011). Based on previous reports together with our present study, the antiproliferative and/or apoptotic activity of maslinic acid might play an important role in the prevention of fibrosis and cancer.

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-talk of the various cell types. Liver fibrosis can be induced by hepatocellular damage, which causes an inflammatory response leading to HSC activation. Several studies have reported anti-inflammatory activity of the stem and root barks of *U. davidiana* var. *japonica* and its constituents (Kim *et al*., 2007; Choi *et al*, 2009). Therefore, we expect that the antifibrotic activity of these compounds might be potentiated *in vivo*, by the collaboration of different mechanism including anti-inflammatory activity. This possibility needs to be investigated.

Acknowledgement

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