Antiproliferative Triterpenes from the Leaves and Twigs of Juglans sinensis on HSC-T6 Cells

ARTICLE *in* JOURNAL OF NATURAL PRODUCTS · FEBRUARY 2011

Impact Factor: 3.8 · DOI: 10.1021/np1008202 · Source: PubMed

JOURNAL OF NATURAL PRODUCTS

ARTICLE

pubs.acs.org/jnp

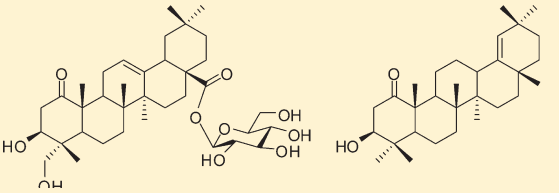
Antiproliferative Triterpenes from the Leaves and Twigs of *Juglans sinensis* on HSC-T6 Cells

Heejung Yang, Eun Ju Jeong, Jinwoong Kim, Sang Hyun Sung, and Young Choong Kim*

College of Pharmacy and Research Institute of Pharmaceutical Science, Seoul National University, Daehak-Dong, Gwanak-Gu, Seoul 151-742, Republic of Korea

Supporting Information

ABSTRACT: Bioassay-guided fractionation of an 80% MeOH extract of leaves and twigs of *Juglan sinensis* has resulted in the isolation of four new triterpenes (**1**−**4**) and 17 known triterpenes (**5**−**21**). The new compounds were determined to be 1-oxo-3β,23-dihydroxyolean-12-en-28-oic acid 28-O-β-D-glucopyranoside (**1**), 1-oxo-3β-hydroxyolean-18-ene (**2**), 3β,23-dihydroxyurs-12-en-28-oic acid 28-O-β-D-glucopyranoside (**3**), and 3β,22α-dihydroxyurs-12-en-28-oic acid 28-*O*-β-D-glucopyranoside (**4**) by spectroscopic analysis. Compounds **2**, **13**, **15**, and **21** showed antiproliferative activities (14.2, 14.8, 15.6, and 11.0% at 100 μM, respectively) in HSC-T6 cells. Flow cytometry assays revealed that these compounds inhibited HSC-T6 proliferation by inducing apoptosis.



Liver fibrosis results from chronic hepatocellular damage in response to viral infection, alcoholic poisoning, drug-induced toxicity, or any other factors that cause damage to hepatocytes.[1−3] The process to liver fibrosis is generally characterized by increased extracellular matrix (ECM) deposition and reduced matrix degradation.[4] In the pathogenesis of liver fibrosis, hepatic stellate cells (HSCs) are the primary cellular source responsible for the excess production of ECM proteins.[5] Once activated, HSCs become proliferative and fibrogenic due to a dramatic increase in the synthesis and deposition of ECM.[6] Therefore, inhibition of HSC proliferation has been considered as an effective treatment for liver fibrosis.[7,8]

During a search for antiproliferative compounds from natural sources employing HSC-T6 cells, it was found that an 80% MeOH extract of leaves and twigs of *Juglans sinensis* Dode. (Juglandaceae) showed significant inhibitory activity on HSC-T6 cell proliferation. HSC-T6 cells, an immortalized rat hepatic stellate cell line, have been widely used as an in vitro assay system to study the action mechanism of the antifibrotic agents.[9−11] *J. sinensis* is a deciduous tree native to some Asian countries such as Korea and China. In Korean traditional medicine, *J. sinensis* has been used for the treatment of airway inflammatory diseases,[12] leucorrhea, scabies, and elephantiasis.[13] A number of flavonoids in the leaves and diarylheptanoids, terpenes, polyphenolics (as mainly tannins), and tetralones in the pericarps have been reported from *Juglans* species.[14−16] In the present study, bioassay-guided fractionation of an 80% MeOH extract of leaves and twigs of *J. sinensis* resulted in the isolation of four new triterpenes (**1**−**4**), 14 known triterpenes (**5**−**9**, **12**−**17**, and **19**−**21**), and three triterpene glycosides (**10**, **11**, and **18**).

RESULTS AND DISCUSSION

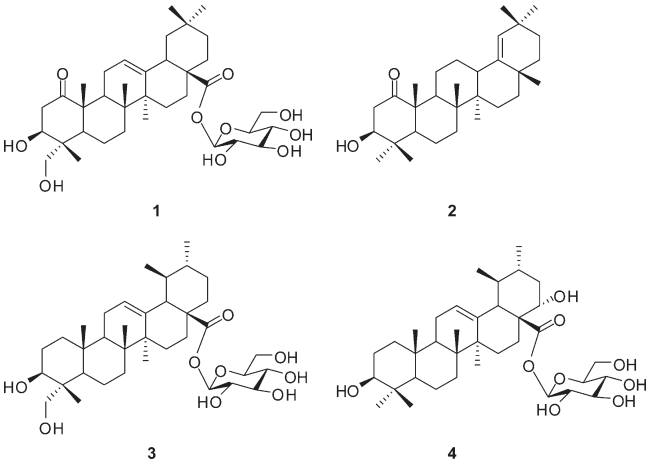
An 80% MeOH extract (2.2 kg) of leaves and twigs of *J. sienensis* (20 kg, dried material) was suspended in distilled water and successively partitioned with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol. The *n*-hexane, $CHCl_3$, and EtOAc fractions showed significant inhibitory activities on the proliferation of HSC-T6 cells (19.6, 16.1, and 53.3% of cell proliferation, respectively, at 100 μg/mL). These fractions were subjected to repeated column chromatography (CC) and HPLC to give the new compounds **1**−**4**, 14 known triterpenes (**5**−**9**, **12**−**17**, and **19**−**21**), and three known triterpene glycosides (**10**, **11**, and **18**) (see Supporting Information, S1). The structures of these compounds were unequivocally determined by 1D, 2D NMR experiments, MS analyses, GC-MS after acid hydrolysis, and comparison with the literature for the known compounds.

Compound **1** was a whitish, amorphous powder, $[\alpha]^{25}_{D}$ +39.8 (*c* 0.21, MeOH), and the molecular formula ($C_{36}H_{56}O_{10}$) was established by the positive HRFABMS (*m*/*z* 671.3727 $[M + Na]^+$, calcd for 671.3771). The signals in the ^1H and ^{13}C NMR and HSQC experiments indicated a Δ^{12} oleanane-type triterpene by the chemical shifts of two olefinic carbons at δ_C 143.3 (C-13) and 124.5 (C-12)[17] and included a carbonyl carbon at δ_C 213.1 (C-1), a carboxylic carbon at δ_C 176.4 (C-28), and an oxygenated methylene carbon at δ_C 65.7 (C-23). Furthermore, we deduced the presence of a sugar residue from the signal of an anomeric moiety at δ_H/δ_C 6.29 (1H, d, J = 8.1 Hz, H-1′)/95.6 (C-1′). In the HMBC spectrum of **1**, a carbonyl carbon (δ_C 213.1) correlated with two methylene protons at δ_H 3.44 (1H, t, J = 11.9 Hz, H-2a) and 2.77 (1H, dd, J = 4.8, 11.9 Hz, H-2b) and a tertiary methyl carbon at δ_H 1.35 (3H, s, H-25) (see Supporting Information S2). Thus, the carbonyl carbon was determined to be C-1. From the above information, it was deduced that compound **1** was similar to the known compound

Received: November 11, 2010
Published: February 10, 2011

1-oxo-3β, 23-dihydroxyolean-12-en-28-oic acid (**9**) except for the presence of a sugar residue.[18] A correlation between the anomeric proton at δ_H 6.29 (H-1′) and a carboxylic carbon at δ_C 176.4 (C-28) in the HMBC spectrum placed the sugar residue at C-28, consistent with the alteration of the chemical shift at δ_C 176.4 (−3.6 ppm) in the ^{13}C NMR spectrum.[18] The magnitude of the coupling constant at δ_H 6.29 (J = 8.1 Hz) suggested that a glucose moiety was β-oriented.[19] The sugar was identified as D-glucose by GC analysis. Thus, compound **1** was determined to be 1-oxo-3β,23-dihydroxyolean-12-en-28-oic acid 28-O-β-D-glucopyranoside.

Compound **2** was isolated as whitish, amorphous powder, $[\alpha]^{25}_D$ +50.6 (c 0.62, pyridine). The positive HRFABMS of **2** exhibited m/z 440.3660 $[M]^+$ (calcd for 440.3654), indicating $C_{30}H_{48}O_2$ as its molecular formula. The ^{1}H and ^{13}C NMR spectra and 2D NMR experiments revealed features of a Δ^{18} oleanane-type triterpene with a carbonyl carbon.[20] In the HMBC spectrum, δ_C 212.8 (C-1) showed cross-peaks with δ_H 3.45 (1H, t, J = 10.8 Hz, H-2a), 2.68 (1H, d, J = 10.8 Hz, H-2b), 2.23 (1H, d, J = 10.7 Hz, H-9), and 1.10 (3H, br s, H-25), and δ_C 28.7 (C-23) and 16.6 (C-24) displayed with δ_H 3.71 (1H, d, J = 5.0 Hz, H-3). These data implied that a carbonyl and a carbon attached to an OH were located at C-1 and C-3, respectively (see Supporting Information, S2). The coupling constant of the oxygenated methine doublet at δ_H 3.71 (J = 5.0 Hz) indicated a β-orientation of the C-3 OH group.[18] Thus, compound **2** was identified as 1-oxo-3β-hydroxyolean-18-ene.



Compound **3** was obtained as a whitish, amorphous powder, with $[\alpha]^{25}_D$ +28.8 (c 0.73, MeOH). The positive HRFABMS exhibited a quasi-molecular ion peak at m/z 657.3943 $[M + Na]^+$ (calcd for 657.3979), indicating $C_{36}H_{58}O_9$ as its molecular formula. Compound **3** displayed 36 carbons, of which 30 carbons could be assigned to a triterpene aglycone and 6 carbons to a sugar moiety (see Supporting Information, S35). The chemical shifts of olefinic carbons at δ_C 138.4 (C-13) and 126.2 (C-12) indicated that compound **3** was a Δ^{12} ursane-type triterpene.[17] On the basis of these results, the aglycone of compound **3** was identified as 23-hydroxyursolic acid (see Supporting Information, S2).[21] The anomeric signals at δ_H/δ_C 6.23 (1H, d, J = 8.2 Hz, H-1′)/95.7 (C-1′) showed the presence of a sugar residue in the HSQC analysis. The correlation from δ_H 6.23 (H-1′) to δ_C 176.2 (C-28) placed the sugar residue at C-28. The sugar released on acidic hydrolysis was confirmed to be D-glucose. The coupling constant of a doublet at δ_H 6.23 (J = 8.2 Hz) established the β-configuration of the glucose moiety, similar to that of compound **1**.[19] Thus, compound **3** was determined to be 3β,23-dihydroxyurs-12-en-28-oic acid 28-O-β-D-glucopyranoside.

Compound **4** had the molecular formula $C_{36}H_{58}O_9$. The ^{1}H and ^{13}C NMR spectra of **4** were similar to those of ursolic acid,[17] except for the signals of an oxygenated proton at δ_H/δ_C 4.33 (1H, m, H-22)/73.6 (C-22) and a sugar residue (see Supporting Information, S35). HMBC correlations between δ_H 1.88 (1H, m, H-21a) and 1.68 (1H, m, H-21b) and δ_C 73.6 (C-22) and between δ_H 6.29 (H-1′) and δ_C 174.9 (C-28) confirmed that the OH and sugar moieties were attached at C-22 and C-28, respectively (see Supporting Information, S2). In the ROESY spectrum, the correlations between δ_H 4.33 (1H, m, H-22) and δ_H 2.56 (1H, d, J = 11.4 Hz, H-18) and 1.01 (1H, m, H-20) indicated an α-configuration of the OH group at C-22. The coupling constant of the doublet at δ_H 6.29 (J = 7.7 Hz) established the β-configuration of the glucose moiety. GC analysis of chiral derivatives of the hydrolysate of compound **4** yielded D-glucose. Taken together, the structure of compound **4** was confirmed as 3β,22α-dihydroxyurs-12-en-28-oic acid 28-O-β-D-glucopyranoside.

The 17 known compounds were identified as oleanolic acid (**5**),[17] maslinic acid (**6**),[22] arjunolic acid (**7**),[23] virgatic acid (**8**),[18] 1-oxo-3β,23-dihydroxyolean-12-en-28-oic acid (**9**),[18] hederagenin 28-*O*-β-D-glucopyranoside (**10**),[24] 2α,3β,23-trihydroxyolean-12-en-28-oic acid 28-*O*-β-D-glucopynoside (**11**),[24] 3β-acetoxyolean-18-en-28-oic acid (**12**),[25] ursolic acid (**13**),[17] 23-hydroxyursolic acid (**14**),[21] corosolic acid (**15**),[26] 2α,3α,23-trihydroxyurs-12-en-28-oic acid (**16**),[27] 3-oxo-23-hydroxyurs-12-en-28-oic acid (**17**),[28] 2α,3β,23α-trihydroxyurs-12-en-28-oic acid 28-*O*-β-D-glucopyranoside (**18**),[29] 3β-hydroxyurs-20-en-28-oic acid (**19**),[30] lupeol (**20**),[31] and 2α,3β-dihydroxylup-20(29)-ene (**21**)[32] (see Supporting Information, S1).

Inhibition of HSC cell proliferation has been considered as an effective treatment for delaying fibrosis in the liver. It is known that HSC-T6 cells, an immortalized hepatic stellate cell line, cultured on plastic plates are spontaneously activated and then lead to myofibroblastic phenotype, which is similar to the process of HSC cell activation in vivo. Thus, HSC-T6 cells were used as the screening tool to determine antifibrotic activity of the isolated compounds.[33,34] The inhibitory activity of compounds **1**−**21** on the proliferation of activated HSC-T6 cells was evaluated (see Supporting Information, S36). These compounds consisted of oleanane type (**1**, **2**, **5**−**12**), ursane type (**3**, **4**, **13**−**19**), and lupane type (**20**, **21**), according to their aglycone moieties (see Supporting Information, S1). Substitutions such as OH (**1**−**21**), carboxyl (**5**−**9**, **12**−**17**, and **19**), oxo (**1**, **2**, **8**, **9**, and **17**), and glucopyranoside (**1**, **3**, **4**, **10**, **11**, and **18**) also divided them into several groups. The ursane-type triterpenoids showed higher inhibitory activities than those of oleanane type on the proliferation of HSC-T6 cells. Ursane-type compounds **13**−**17** (14.8, 50.0, 15.6, 20.6, and 77.9% at 100 μM, respectively) showed more potent inhibitory activities than oleanane-type compounds **5**−**9** (86.6, 83.5, 75.6, 54.9, and 63.6% at 100 μM, respectively). The substitution at C-1 or C-28 also affected the inhibitory activity. Compounds **8** and **9** (54.9 and 63.6% at 100 μM, respectively), having an oxo group at C-1, showed more potent antiproliferative activity than compounds **5**−**7** (86.6, 83.5, and 75.6% at 100 μM, respectively), without an oxo group. The presence of a glucopyranoside moiety at C-28 also affected the inhibitory activity, as can be seen by the differences between

Table 1. Effects (%) of Compounds 2, 13, 15, and 21 on Cell Cycle Progression in HSC-T6 Cells[a]

	24 h				48 h			
compound	sub-G_1	G_0/G_1	S	G_2/M	sub-G_1	G_0/G_1	S	G_2/M
control	1.9 ± 0.6	51.9 ± 7.3	12.1 ± 3.1	20.1 ± 0.7	4.5 ± 0.9	42.3 ± 4.4	8.3 ± 1.2	19.6 ± 2.8
2	1.9 ± 1.3	53.2 ± 3.9	14.0 ± 3.9	17.8 ± 3.2	5.0 ± 0.6	43.6 ± 5.2	10.6 ± 1.7	18.8 ± 4.2
13	2.9 ± 1.9	56.4 ± 8.5	11.9 ± 2.1	18.9 ± 1.9[b]	17.0 ± 8.7[b]	39.7 ± 5.1	9.6 ± 2.2	16.7 ± 1.6
15	12.4 ± 2.8[b]	44.6 ± 7.7[b]	10.6 ± 2.9	20.6 ± 4.9	57.7 ± 4.0[c]	14.7 ± 6.4[b]	4.3 ± 0.0[b]	6.0 ± 3.1[b]
21	1.9 ± 1.1	55.9 ± 1.5	13.7 ± 3.7	19.3 ± 2.2	5.4 ± 1.8	47.2 ± 4.4	11.0 ± 2.4	18.0 ± 5.3

[a] HSC-T6 cells were incubated with compounds **2**, **13**, **15**, and **21** at a concentration of 25 μM for 24 and 48 h. Control is the value for cultures with vehicle for 24 or 48 h. Results are expressed as the mean ± SD of three independent experiments, each performed using triplicate wells. [b] $p < 0.05$ compared with control. [c] $p < 0.005$ compared with control.

the compounds with the same aglycone: compounds **7** (75.6% at 100 μM) and **11** (89.8% at 100 μM), **9** (63.6% at 100 μM) and **1** (90.2% at 100 μM), **14** (50.0% at 100 μM) and **3** (80.9% at 100 μM), and **16** (20.6% at 100 μM) and **18** (83.9% at 100 μM), respectively. Compounds **7**, **9**, **14**, and **16**, with the carboxylic acid at C-28, showed slightly more potent inhibitory activities than compounds **11**, **1**, **3**, and **18**, with a β-glucopyranoside at C-28. These results suggested that a free carboxylic group at C-28 might be important to exert antiproliferative activity in HSC-T6 cells. The inhibitory activity of (−)-EGCG, a positive control, was 83.8% at 10 μM, 41.7% at 50 μM, and 33.4% at 100 μM, respectively. Taken together, it was suggested that the ursane-type skeleton, the presence of an oxo group at C-1, and a free carboxylic group at C-28 were important for inhibitory activity on HSC-T6 cell proliferation.

The morphology of cells treated with the most potent compounds, **2**, **13**, **15**, and **21** (14.2, 14.8, 15.6, and 11.0% at 100 μM, respectively), were changed into apoptotic cells in dose- and time-dependent manners (see Supporting Information, S37). To evaluate the apoptotic effects of these compounds on HSC-T6 cells, we investigated the changes in cell cycle by flow cytometry at concentrations of 25−50 μM. All four compounds showed significant decrease of the number of G_1 phase cells and increase of cells in the sub-G_0/G_1 phase at 50 μM after 48 h incubation (data not shown; the ratio of sub-G_0/G_1 phase is >60%). These results indicated that compounds **2**, **13**, **15**, and **21** inhibited proliferation of HSC-T6 cells by arresting DNA synthesis. Compound **15** demonstrated the most significant inhibitory activity of DNA synthesis at 25 μM (12.4% after 24 h, 57.7% after 48 h) (Table 1; see Supporting Information, S38). At concentrations of 10 and 25 μM of compound **15**, we additionally examined the stage of apoptosis using the Annexin-V/PI double staining assay (Figure 1). The percentages of early apoptosis (Annexin-V^+/PI^-) and late apoptosis (Annexin-V^+/PI^+) were increased dose- and time-dependently. After 24 h incubation with compound **15** at 10 and 25 μM, the percentage of cells in apoptosis stage was about 6.23% (Annexin-V^+/PI^-) and 7.12% (Annexin-V^+/PI^+) at 10 μM and 10.45% (Annexin-V^+/PI^-) and 15.24% (Annexin-V^+/PI^+) at 25 μM, respectively. After 48 h, whereas the percentage of the apoptotic cells was decreased to 4.41% (Annexin-V^+/PI^-) and 9.23% (Annexin-V^+/PI^+) at 25 μM, the percentage of dead cells (Annexin-V^-/PI^+) was dramatically increased to 31.85% at the same concentration. However, the populations of early or late apoptotic cells treated with 10 μM were about 10.14% (Annexin-V^+/PI^-) and 17.80% (Annexin-V^+/PI^+), respectively. These results suggested that the antiproliferative activity of compound **15** in HSC-T6 cells may be exerted by interference of cell proliferation via apoptosis in dose- and time-dependent manners. Further studies are needed to clarify the action mechanism of compound **15** on the inhibition of HSC-T6 cell proliferation.

EXPERIMENTAL SECTION

General Experimental Procedures. Column chromatography (CC) was carried out on Kiesgel 60 silica gel (40−60 μm, 230−400 mesh, Merck, USA), YMC-GEL ODS-A (5−150 μm, YMC), and Sephadex LH-20 (25−100 μM, Pharmacia, NJ, USA), and TLC was carried out on Kiesgel 60 F_{254} coated normal silica gel and RP-18 F_{254} coated reversed-phase silica gel. The ^{1}H and ^{13}C NMR, ^{1}H−^{1}H COSY, HSQC, and HMBC spectra were recorded on a Bruker AMX 400 or 500 spectrometer in pyridine-d_5. Solvent signals were used as internal standards. FT-IR spectra were measured with a JASCO FT/IR-300 spectrophotometer. High-resolution and low-resolution FABMS were obtained on a JEOL JMS-AX505WA. GC was conducted on a 5890 gas chromatograph (Agilent Technologies Co. Ltd., USA) with a flame ionization detector (FID). The HPLC system consisted of a G-321 pump (Gilson Co. Ltd., USA), a G-151 UV detector (Gilson Co. Ltd., USA), and a YMC-Pack Pro C_{18} column (250 mm × 10 mm i.d.; 5 μm), and all chromatograms were monitored at 210 nm. HPLC grade solvents (Fisher Scientific, USA) were used in the MeOH−H_2O system. Detection of DNA cycle and apoptosis on HSC-T6 cells was conducted by flow cytometry (BD Biosciences, FACSCalibur, Franklin Lakes, NJ, USA).

Plant Material. Leaves and twigs of *Juglans sinensis* were collected at the Medicinal Plant Garden, Seoul National University, Goyang, Korea, in September 2008 and air-dried. *J. sinensis* was identified by Dr. Jong Hee Park, a professor of the College of Pharmacy, Pusan National University. A voucher specimen (SNUPH-0329) has been stored in the Herbarium in the Medicinal Plant Garden, Seoul National University.

Extraction and Isolation. Dried leaves and twigs (20 kg) of *J. sinensis* were extracted with 80% MeOH (15 L × 3) in an ultrasonic apparatus. After removal of the solvent in vacuo, an 80% MeOH extract (2.2 kg) was suspended in H_2O and successively partitioned into *n*-hexane (48.5 g), $CHCl_3$ (147 g), EtOAc (200 g), and *n*-BuOH (1.4 kg) fractions. The *n*-hexane, $CHCl_3$, and EtOAc fractions, which showed significant inhibitory activity on proliferation of HSC-T6 cells, were each subjected to repeated CC with a gradient elution of solvents. The *n*-hexane fraction was subjected to silica gel CC (45 cm × 10 cm) eluted with mixtures of *n*-hexane−EtOAc (50:1, 10:1, 5:1, 3:1) and $CHCl_3$−MeOH (5:1) and MeOH (v/v) to yield seven fractions (HoH1−HoH7). Compounds **20** (21 mg) from HoH3, and **13** (529 mg) and **21** (10 mg) from HoH5 were isolated using ODS silica gel MPLC (MeOH−H_2O, 8:2), respectively. Fraction HoH4 was further separated into three fractions (HoH4A−4C) on Sephadex LH-20 using a mixture of *n*-hexane−CH_2Cl_2−MeOH (10:10:1). Fraction HoH4B was subjected to an

dx.doi.org/10.1021/np1008202 |J. Nat. Prod. 2011, 74, 751–756

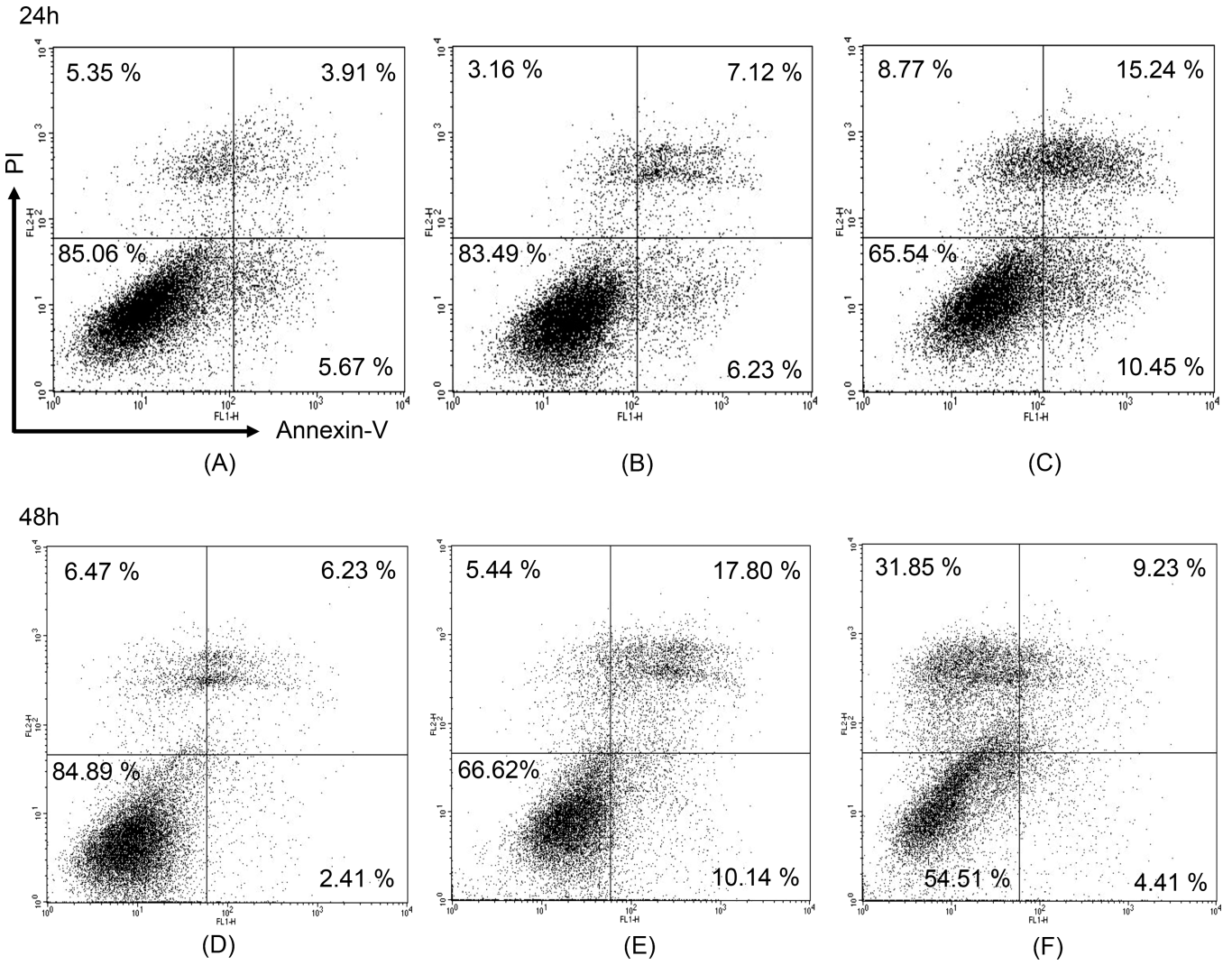


Figure 1. Analysis of apoptosis of compound **15** using flow cytometry. After 24 h incubation with compound **15**, the number of cells during early and late apoptosis (Annexin-V^+/PI^- and Annexin-V^+/PI^+) is increased from 9.58% in the control (A) to 13.35% (B) and 25.69% (C), as the concentration of compound **15** is increased from 10 μM (B) to 25 μM (C). After 48 h treatment with compound **15** at a concentration of 10 μM (E) and 25 μM (F), the number of cells during early and late apoptosis (Annexin-V^+/PI^- and Annexin-V^+/PI^+) is 27.94% (E) and 12.64% (F), respectively. The dead cells (Annexin-V^-/PI^+) treated at 25 μM are increased to 31.85% (F). Values are represented as percentage. Data are representative of three independent experiments.

ODS silica gel MPLC gradient eluting with mixtures of MeOH−H_2O (6.5:3.5, 7:3, 8:2, 9.5:0.5, and 10:0, respectively) to give compounds **12** (8.5 mg) and **2** (4.5 mg). Compound **19** (45 mg) was isolated from HoH4B by recrystallization (MeOH). Compound **14** (1.2 g) was obtained from HoH6 using Sephadex LH-20 with a mixture of *n*-hexane−CH_2Cl_2−MeOH (10:10:1). Fractions HoCS and HoCSB were separated from the $CHCl_3$ fraction by silica gel CC (10 cm ×20 cm). The column was washed with a mixture of *n*-hexane−EtOAc (5:1) to give fraction HoCS (soluble in the mixture of *n*-hexane−EtOAc, 5:1, 25 g) and fraction HoCSB (the residue was washed with MeOH, 116 g). Fraction HoCS was subjected to silica gel CC ($CHCl_3$−MeOH = 50:1, 30:1, 15:1, 10:1) to afford three fractions (HoCS1−3). Compounds **9** (300 mg), **15** (500 mg), and **16** (650 mg) were isolated from HoCS2 by ODS silica gel MPLC (MeOH−H_2O = 6:4, 8:2). Fraction HoCSB was separated into five fractions (HoCSB1−5) using HP-20 resin eluted with a gradient of aqueous MeOH (MeOH−H_2O = 0:1, 1:4, 5:5, 4:1, 1:0). Compound **5** (30 mg) was isolated from HoCSB1 by recrystallization (MeOH). Fraction HoCSB2 was divided into six fractions (HoCSB2A−F) by ODS silica gel MPLC (MeOH−H_2O = 6:4). Compounds **6** (22 mg), **8** (11 mg), and **17** (9 mg) were purified from HoCS2E by ODS silica gel HPLC using MeOH−H_2O (8:2, 2 mL/min). The EtOAc fraction was subjected to silica gel CC eluted with $CHCl_3$−MeOH of increasing polarity (50:1, 30:1, 10:1, 5:1, 3:1, 0:1) to give 10 fractions (HoE1−10). By ODS silica gel CC eluting with a mixture of MeOH−H_2O, compound **7** (500 mg) was obtained from HoE5 (MeOH−H_2O = 7:3, 9:1). Fraction HoE7 was subjected to ODS silica gel CC (10 cm ×20 cm) to afford five fractions (HoE7A−E). Compounds **1** (5 mg) and **18** (125 mg) were obtained from HoE7D by ODS silica gel HPLC with MeOH−H_2O (8:2, 2 mL/min). Fraction HoE7E was separated into three fractions (HoE7E1−3) by Sephadex LH-20 with MeOH, and HoE7E1 was chromatographed by ODS silica gel HPLC (MeOH−H_2O = 8:2) to give compounds **10** (5.6 mg), **3** (5 mg), and **4** (4.5 mg). Fraction HoE10 was separated into six fractions (HoE10A−F) by ODS silica gel CC (MeOH−H_2O, 8:2), and compound **11** (500 mg) was obtained from HoE10F by ODS silica gel MPLC (MeOH−H_2O, 8:2).

1-Oxo-3*β*, 23-dihydroxyolean-12-en-28-oic acid 28-*O*-*β*-D-glucopyranoside (1): whitish, amorphous powder; $[\alpha]^{25}_D$ +39.8

dx.doi.org/10.1021/np1008202 |*J. Nat. Prod.* 2011, 74, 751–756

(*c* 0.21, MeOH); IR ν_{max} 3388, 2926, 1699, 1650, 1540, 1457, 1072 cm^{-1}; ^1H NMR (400 MHz, pyridine-d_5) and ^{13}C NMR (100 MHz, pyridine-d_5) data, see Supporting Information S35; FABMS *m/z* 671 $[M + Na]^+$; HRFABMS *m/z* 671.3727 $[M + Na]^+$ (calcd for $C_{36}H_{56}O_{10}Na$, 671.3771).

1-Oxo-3β-hydroxyolean-18-ene (2): whitish, amorphous powder; $[\alpha]^{25}_D$ +50.6 (*c* 0.62, pyridine); IR ν_{max} 3388, 2950, 1702, 1539, 1453, 1376, 1303, 1252, 1137, 1041 cm^{-1}; ^1H NMR (500 MHz, pyridine-d_5) and ^{13}C NMR (125 MHz, pyridine-d_5) data, see Supporting Information S35; FABMS *m/z* 463 $[M + Na]^+$; HRFABMS *m/z* 440.3660 $[M]^+$ (calcd for $C_{30}H_{49}O_2Na$, 440.3654).

3β,23-Dihydroxyurs-12-en-28-oic acid 28-*O*-β-D-glucopyranoside (3): whitish, amorphous powder; $[\alpha]^{25}_D$ +28.8 (*c* 0.73, MeOH); IR ν_{max} 3388, 2925, 1732, 1617, 1540, 1456, 1386, 1074 cm^{-1}; ^1H NMR (500 MHz, pyridine-d_5) and ^{13}C NMR (125 MHz, pyridine-d_5) data, see Supporting Information S35; FABMS *m/z* 657 $[M + Na]^+$; HRFABMS *m/z* 657.3943 $[M + Na]^+$ (calcd for $C_{36}H_{58}O_9Na$, 657.3979).

3β,22α-Dihydroxyurs-12-en-28-oic acid 28-*O*-β-D-glucopyranoside (4): whitish, amorphous powder; $[\alpha]^{25}_D$ +28.9 (*c* 0.3, MeOH); IR ν_{max} 3388, 2925, 1732, 1617, 1457, 1387, 1070 cm^{-1}; ^1H NMR (500 MHz, pyridine-d_5) and ^{13}C NMR (125 MHz, pyridine-d_5) data, see Supporting Information S35; FABMS *m/z* 657 $[M + Na]^+$; HRFABMS *m/z* 657.3954 $[M + Na]^+$ (calcd for $C_{36}H_{58}O_9Na$, 657.3979).

General Acid Hydrolysis. Each compound (2 mg) was dissolved in 1 N HCl (dioxane−H_2O, 1:1, 1 mL), and each solution was heated to 90 °C for 2 h. After neutralizing each acidic solution with silver carbonate, the solvent was evaporated to dryness under N_2. Each reaction mixture was extracted with $CHCl_3$ and H_2O, successively, and the aqueous layer was concentrated to dryness. Each residue was dissolved in 0.1 mL of dry pyridine, and L-cysteine methyl ester hydrochloride dissolved in dry pyridine (0.06 M, 0.1 mL) was added to the solution. Each mixture was reacted at 60 °C for 2 h, and 0.1 mL of (trimethylsilyl)imidazole dissolved in H_2O was added, followed by heating to dryness at 60 °C for 2 h. Each dried reactant was partitioned between *n*-hexane and H_2O (0.1 mL), and the *n*-hexane fraction was subjected to gas chromatography (GC) (column: DB-5, i.d. 0.25 mm, length 30 m). The conditions for GC were FID detector; column temperature 210 °C; injector temperature 270 °C; detector temperate 300 °C; helium carrier gas (2.0 kg/cm^3). Under these conditions, the sugars of each reactants were identified by comparison with authentic standards: t_R (min) 10.45 (D-glucose), 11.04 (L-glucose), 8.47 (D-arabinose), 8.31 (L-arabinose), 8.92 (D-rhamnose), and 8.66 (L-rhamnose).

Culture of HSC-T6 Cell Line. An immortalized rat hepatic stellate cell line, HSC-T6, was provided by Prof. S. L. Friedman (Columbia University, New York). HSC-T6 cells were maintained in DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin (Sigma, St. Louis, MO, USA), and 100 μg/mL streptomycin (Sigma, St. Louis, MO, USA) at 37 °C in a humidified incubator containing 5% CO_2 gas.

Assessment of Cell Proliferation. Compounds to be tested (**1**−**21**) were dissolved in DMSO (final concentration, 0.1%). 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 **1**−**21** at concentrations from 10 to 100 μM for 48 h. Inhibitory activity of each compound on cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[35] HSC-T6 cells were incubated with 2 mg/mL of MTT for 2 h. Reduction of MTT to formazan was assessed in an ELISA plate reader at 540 nm. Inhibitory activity of compounds **1**−**21** on cell proliferation was calculated as 100 × (abs. of well treated with compounds/abs. of control treated with vehicle). Data were expressed as the mean of three independent experiments. (−)-EGCG was used as a positive control.

Flow Cytometry for Analyzing the DNA Cycle and Apoptosis. For measuring the DNA cycle and apoptosis, HSC-T6 cells were seeded in 12-well plates at a density of 5×10^5 cells/mL for 24 h and treated with compounds **2**, **13**, **15**, and **21** at 10 and 25 μM. After 24 or 48 h, cells were collected, washed twice with PBS ($NaCl_2$ 137 mM, KCl 2 mM, Na_2HPO_4 100 mM, KH_2PO_4 2 mM; pH 7.4), and centrifuged at room temperature. For evaluating the DNA cycle, cell pellets were suspended in ice-cold 70% ethanol for fixation at 4 °C overnight. After centrifuging, cell pellets were resuspended in 600 μL of PI/RNASE staining buffer (BD Pharmingen, Franklin Lakes, NJ, USA) and incubated at room temperature for 30 min before analysis. For measuring apotosis, cell pellets were prepared by an FITC-Annexin V apoptosis detection kit (BD Pharmigen, Franklin Lakes, NJ, USA) according to the manufacturer's manual. DNA cycle and apoptosis of HSC-T6 were evaluated by flow cytometry (BD Biosciences, FACSCalibur, Franklin Lakes, NJ, USA).

Statistical Analysis. The evaluation of statistical significance was determined by the one-way ANOVA test, with $^b p < 0.05$, and $^c p < 0.001$ considered to be statistically significant.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. The structures of compounds **1**−**21**, 1D and 2D NMR spectra of compounds **1**−**4**, and details about the effects of bioactive compounds on cell morphology, DNA synthesis, and apoptosis in HSC-T6 cells are available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: 82-2-880-7842. Fax: 82-2-888-2933. E-mail: youngkim@snu.ac.kr.

■ ACKNOWLEDGMENT

This research was supported by a grant (2010K000832) from the Brain Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology, Korea. We thank Professor S. L. Friedman (Columbia University, New York) for kindly providing the HSC-T6 cells.

■ REFERENCES

(1) Schuppan, D.; Krebs, A.; Bauer, M.; Hahn, E. G. *Cell Death Differ.* **2003**, *10*, S59–67.

(2) Cubero, F. J.; Urtasun, R.; Nieto, N. *Semin. Liver Dis.* **2009**, *29*, 211–221.

(3) Kasper, H. U.; Drebber, U.; Hirsch, I.; Dienes, H. P. *Pathologe* **2006**, *27*, 175–181.

(4) Wu, J.; Zern, M. A. *J. Gastroenterol.* **2000**, *35*, 665–672.

(5) Chor, J. S.; Yu, J.; Chan, K. K.; Go, Y. Y.; Sung, J. J. *J. Gastroenterol. Hepatol.* **2009**, *24*, 853–859.

(6) Tsukada, S.; Parsons, C. J.; Rippe, R. A. *Clin. Chim. Acta* **2006**, *364*, 33–60.

(7) Zardi, E. M.; Dobrina, A.; Ambrosino, G.; Margiotta, D.; Polistina, F.; Afeltra, A. *Curr. Med. Chem.* **2008**, *15*, 1628–1644.

(8) Friedman, S. L. *Gastroenterology* **2008**, *134*, 1655–1669.

(9) Wu, J. H.; Wang, Y. R.; Huang, W. Y.; Tan, R. X. *World J. Gastroenterol.* **2010**, *16*, 3911–3918.

(10) Lim, C. S.; Kim, E. Y.; Lee, H. S.; Soh, Y.; Sohn, Y.; Kim, S. Y.; Sohn, N. W.; Jung, H. S.; Kim, Y. B. *Biosci. Biotechnol. Biochem.* **2010**, *74*, 477–483.

(11) Dai, L.; Ji, H.; Kong, X. W.; Zhang, Y. H. *Acta Pharmacol. Sin.* **2010**, *31*, 27–34.

(12) Lee, Y. C.; Kim, S.-H. *Phytochem. Lett.* **2008**, *1*, 6–10.
(13) Bae, K. H. *Medicinal Plants of Korea*; Kyo-Hak Publishing Co.: Seoul, 2000; p 49.
(14) An, R. B.; Kim, H. C.; Tian, Y. H.; Kim, Y. C. *Arch. Pharm. Res.* **2005**, *28*, 529–533.
(15) Liu, J. X.; Di, D. L.; Wei, X. N.; Han, Y. *Planta Med.* **2008**, *74*, 754–759.
(16) Fukuda, T.; Ito, H.; Yoshida, T. *Phytochemistry* **2003**, *63*, 795–801.
(17) Seebacher, W.; Simic, N.; Weis, R.; Saf, R.; Kunert, O. *Magn. Reson. Chem.* **2003**, *41*, 636–638.
(18) Okada, Y.; Omae, A.; Okuyama, T. *Chem. Pharm. Bull.* **2003**, *51*, 452–454.
(19) Agrawal, P. K. *Phytochemistry* **1992**, *31*, 3307–3330.
(20) Kagawa, M.; Minami, H.; Nakahara, M.; Takahashi, H.; Takaoka, S.; Fukuyama, Y. *Phytochemistry* **1998**, *47*, 1101–1105.
(21) Tapondjou, L. A.; Lontsi, D.; Sondengam, B. L.; Shaheen, F.; Choudhary, M. I.; Atta ur, R.; van Heerden, F. R.; Park, H.-J.; Lee, K.-T. *J. Nat. Prod.* **2003**, *66*, 1266–1269.
(22) Taniguchi, S.; Imayoshi, Y.; Kobayashi, E.; Takamatsu, Y.; Ito, H.; Hatano, T.; Sakagami, H.; Tokuda, H.; Nishino, H.; Sugita, D.; Shimura, S.; Yoshida, T. *Phytochemistry* **2002**, *59*, 315–323.
(23) Kundu, A. P.; Mahato, S. B. *Phytochemistry* **1993**, *32*, 999–1002.
(24) Panov, D. A.; Grishkovets, V. I.; Kachala, V. V.; Shashkov, A. S. *Chem. Nat. Compd.* **2006**, *42*, 61–66.
(25) González, A. G.; Fraga, B. M.; González, P.; Hernandez, M. G.; Ravelo, A. G. *Phytochemistry* **1981**, *20*, 1919–1921.
(26) Wen, X.; Sun, H.; Liu, J.; Cheng, K.; Zhang, P.; Zhang, L.; Hao, J.; Zhang, L.; Ni, P.; Zographos, S. E.; Leonidas, D. D.; Alexacou, K.-M.; Gimisis, T.; Hayes, J. M.; Oikonomakos, N. G. *J. Med. Chem.* **2008**, *51*, 3540–3554.
(27) Lee, I. K.; Kim do, H.; Lee, S. Y.; Kim, K. R.; Choi, S. U.; Hong, J. K.; Lee, J. H.; Park, Y. H.; Lee, K. R. *Arch. Pharm. Res.* **2008**, *31*, 1578–1583.
(28) Fourie, T. G.; Matthee, E.; Snyckers, F. O. *Phytochemistry* **1989**, *28*, 2851–2852.
(29) Monti, D.; Candido, A.; Silva, M. M. C.; Kren, V.; Riva, S.; Danieli, B. *Adv. Synth. Catal.* **2005**, *347*, 1168–1174.
(30) Lobo-Echeverri, T.; Rivero-Cruz, J. F.; Su, B. N.; Chai, H. B.; Cordell, G. A.; Pezzuto, J. M.; Swanson, S. M.; Soejarto, D. D.; Kinghorn, A. D. *J. Nat. Prod.* **2005**, *68*, 577–80.
(31) Razdan, T. K.; Harkar, S.; Qadri, B.; Qurishi, M. A.; Khuroo, M. A. *Phytochemistry* **1988**, *27*, 1890–1892.
(32) Hasan, M.; Burdi, D. K.; Ahmad, V. U. *Phytochemistry* **1991**, *30*, 4181–4183.
(33) Li, D.; Friedman, S. L. *J. Gastroenterol. Hepatol.* **1999**, *14*, 618–633.
(34) Chen, A.; Zhang, L. *J. Biol. Chem.* **2003**, *278*, 23381–23389.
(35) Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55–63.