JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Coumarins and Lignans from *Zanthoxylum schinifolium* and Their Anticancer Activities

Wei Li,[†] Ya Nan Sun,[†] Xi Tao Yan,[†] Seo Young Yang,[†] Eun-Ji Kim,[‡] Hee Kyoung Kang,[‡] and Young Ho Kim*[,†]

[†]College of Pharmacy, Chungnam National University, Daejeon 305-764, Korea

[‡]School of Medicine and Institute of Medical Science, Jeju National University, Jeju 690-756, Korea

Supporting Information

ABSTRACT: *Zanthoxylum schinifolium* is an aromatic shrub, and its pericarp and leaves are widely used in culinary applications in East Asian countries. It has also long been used in traditional Oriental medicine for treating the common cold, stomach ache, diarrhea, and jaundice. In this study, we identified two new compounds, zanthoxyloside (**1**) and schinifolisatin A (**13**), along with 23 known coumarins (**2**−**12**) and lignans (**14**−**25**), from a methanol extract of the stems of *Z. schinifolium*. The chemical structures of the compounds were determined by mass, 1D-, and 2D NMR spectroscopy. The anticancer effects of the isolated compounds were examined in three human cancer cell lines. Compounds **10**−**12** significantly reduced the proliferation of HL-60 human acute promyelocytic leukemia cells with IC_{50} values of 4.62−5.12 μM. Treatment of PC-3 prostate cancer cells and SNU-C5 colorectal cancer cells with compound **10** resulted in potent antiproliferative activity, with IC_{50} values of 4.39 and 6.26 μM, respectively. Also, compounds **10**−**12** induced the apoptosis of three cancer cells. Furthermore, the induction of apoptosis was accompanied by down-regulation of p-ERK1/2 MAPK, p-AKT, and c-myc levels, in a time-dependent manner. These data suggested that compounds **10**−**12** from *Z. schinifolium* have potential in cancer treatment.

KEYWORDS: *Zanthoxylum schinifolium, Rutaceae, coumarin, lignan, apoptosis, anticancer activity*

INTRODUCTION

Zanthoxylum schinifolium Sieb. and Zucc. is a dioecious shrub with hooked prickly branchlets belonging to the Rutaceae family, found in China, Korea, and Japan.[1] In China, the herb of *Z. schinifolium* has been used to invigorate the circulation of blood, and it was regarded as a drug for various pains.[2] The genus *Zanthoxylum* has more than 200 species. The fruits of these species have a distinctive aroma that is generally described as fresh, floral, spicy, and green. However, many of them also have a distinctive tingling taste. Because of their unique aroma and taste, the fruits of many species of *Zanthoxylum* have been used as a spice in local cuisines in Asia.[3]

Currently, much of the phytochemical research on *Z. schinifolium* has focused on the essential oils, coumarins, flavonoids, and alkaloids of fruits and leaves.[3−6] However, no reported study had described the components of *Z. schinifolium* stems. Previous pharmacological studies on the fruits and leaves of *Z. schinifolium* have determined their medicinal activities, including antiplatelet aggregation,[7] antioxidant,[8] inhibition of the production of monoamine oxidase,[9] anti-inflammatory,[10] and antitumor activities.[11] Essential oils are the major active constituents of *Z. schinifolium*; they have been shown to be effective bacteriostatic/bactericidal, fungicidal, and antiviral agents.[12−15] They are also known to have antioxidant properties, and anti-inflammatory effects result in the suppression of proinflammatory mediator production.[16] Coumarins were shown to possess significant activity with regard to platelet aggregation. In previous work, lignan components of *Z. schinifolium* have not been studied in depth, nor has their bioactivity. Recent reports indicated that coumarins have potent cytotoxic effects against HL-60 and Jukat T cells;[17,18] however, the mechanisms of action of coumarins on anticancer activity are not fully understood.

The Bcl-2 family is separated into antiapoptotic proteins, such as Bcl-2 and Bcl-xL, and pro-apoptotic proteins, such as Bax, Bid, and Bak. During apoptosis, Bax induces apoptosis by the releasing of cytochrome *c* from mitochondria. The released cytochrome c induces the cleavage of caspase-9, followed by the cleavage of caspase-3. The activated caspase-3 induces cleavage of poly (ADP-ribose) polymerase (PARP). In contrast, Bcl-2 inhibits apoptosis through the suppression of cytochrome c release from mitochondria.[19−21]

The mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathways regulate apoptosis, cell survival, and cell growth.[22] Recent studies reported that c-myc, which is an oncoprotein, was stabilized by activation of the ERK 1/2 MAPK pathway.[23] It was also reported that activation of the PI3K/AKT pathway promoted the transcription of c-myc.[24]

In present study, one new coumarin glycoside (**1**) and one new neolignan (**13**), together with 11 coumarins (**2**−**12**) and 12 lignans (**14**−**25**), were isolated from a methanol extract of the stems of *Z. schinifolium*. The anticancer activities of the compounds isolated were investigated in HL-60 (leukemia cells), PC-3 (prostate cancer cells), and SNU-C5 (colorectal

Received: August 6, 2013
Revised: October 14, 2013
Accepted: October 21, 2013
Published: October 21, 2013

cancer cells), using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The effects of compounds **10**−**12** on the induction of apoptosis in three cancer cell lines were also investigated. Because the MAPK and PI3K/AKT pathways are involved in cellular proliferation, differentiation, and apoptosis, ERK 1/2 MAPK and PI3K/AKT activation was investigated.

MATERIALS AND METHODS

General Experimental Procedures. Optical rotations were determined using a Jasco DIP-370 automatic polarimeter. UV spectra were recorded using a Beckman Du-650 UV−vis recording spectrometer. The FT-IR spectra were measured using a Jasco Report-100 infrared spectrometer. The NMR spectra were recorded using a JEOL ECA 600 spectrometer (^{1}H, 600 MHz; ^{13}C, 150 MHz). The LCQ advantage trap mass spectrometer (Thermo Finnigan, San Jose, CA) was equipped with an electrospray ionization (ESI) source, and high-resolution electrospray ionization mass spectra (HR-ESI-MS) were obtained using an Agilent 6530 Accurate-Mass Q-TOF LC/MS system. Column chromatography was performed using a silica gel (Kieselgel 60, 70−230, and 230−400 mesh, Merck, Darmstadt, Germany) and YMC RP-18 resins. Thin-layer chromatography (TLC) was performed using precoated silica gel 60 F_{254} and RP-18 F_{254S} plates (both 0.25 mm, Merck, Darmstadt, Germany).

Plant Material. Dried stems of *Z. schinifolium* were collected from Daejeon, Korea in September 2012 and identified by one of the authors (Y.H.K.). A voucher specimen (CNU 12102) was deposited at the Herbarium of the College of Pharmacy, Chungnam National University.

Extraction and Isolation. Dried stems (2.3 kg) were extracted with MeOH (5 L × 3) under reflux. The MeOH extract (102.0 g) was suspended in water and partitioned with *n*-hexane, EtOAc, and *n*-BuOH. The EtOAc fraction (12.0 g) was subjected to silica gel (5 × 30 cm) column chromatography with a gradient of hexane−EtOAc−MeOH (10:1:0, 6:1:0, 3:1:0, 1.5:1:0.1, 1:1:0.2; 1.5 L for each step) to give six fractions (Fr. 1A−1F). The fraction 1B was separated using a silica gel (1.5 × 80 cm) column chromatography with a gradient of hexane−EtOAc−MeOH (10:1:0.1, 8:1:0.1, 6:1:0.1, 5:1:0.1; 800 mL for each step) elution solvent to give compounds **8** (14.0 mg), **10** (9.0 mg), and **11** (7.5 mg). The fraction 1C was separated using a silica gel (1.5 × 80 cm) column chromatography with a gradient of hexane−acetone−MeOH (7:1:0.1, 6:1:0.1, 5:1:0.1; 1.0 L for each step) elution solvent to give compounds **19** (18.0 mg), **20** (8.0 mg) and **21** (17.0 mg). The fraction 1D was separated using a silica gel (1.0 × 80 cm) column chromatography with a gradient of hexane−EtOAc−MeOH (6:1:0.1, 5.5:1:0.1, 4.8:1:0.1; 1.0 L for each step) elution solvent to give compounds **4** (19.0 mg), **5** (5.0 mg), **6** (8.0 mg), and **7** (59.0 mg). The fraction 1E was separated using a silica gel (1.0 × 80 cm) column chromatography with a gradient of hexane−EtOAc−MeOH (5:1:0.1, 4.5:1:0.1, 4:1:0.1; 2.0 L) elution solvent to give compounds **9** (4.0 mg), **12** (14.0 mg), and **22** (4.5 mg). The fraction 1F was separated using an YMC (1.0 × 80 cm) column chromatography with a MeOH−H_2O (0.75:1; 850 mL) elution solvent to give compounds **13** (9.0 mg), **16** (18.0 mg), and **25** (4.2 mg). The *n*-BuOH fraction (28.0 g) was subjected to silica gel (3.0 × 30 cm) column chromatography with a gradient of CH_2Cl_2−MeOH−H_2O (16:1:0, 10:1:0, 7.5:1:0.1, 3:1:0.15, 1.5:1:0.2; 2.5 L for each step) to give six fractions (Fr. 2A−2F). The fraction 2B was subjected to silica gel (1.0 × 70 cm) column chromatography with a gradient of CH_2Cl_2−MeOH−H_2O (12:1:0, 10:1:0, 8.5:1:0.1, 6.5:1:0.1, 4:1:0.1; 1.5 L for each step) to give 5 subfractions (Fr. 2B-1−2B-6). The fraction 2B-5 was separated using an YMC (1.0 × 80 cm) column chromatography with a gradient of MeOH−H_2O (0.2:1, 0.3:1, 0.6:1, 1:1; 750 mL for each step) elution solvent to give compounds **23** (13.0 mg) and **24** (11.0 mg). The fraction 2B-3 was separated using an YMC (1.0 × 80 cm) column chromatography with a MeOH−H_2O (0.2:1, 0.67:1; 1.0 L for each step) elution solvent to give compounds **14** (18.0 mg) and **15** (7.5 mg). The fraction 2C was separated using an YMC (1.0 × 80 cm) column chromatography with a MeOH−acetone−H_2O (0.2:0.12:1; 750 mL) elution solvent to give compounds **3** (19.0 mg), **17** (11.0 mg), and **18** (14.5 mg). The fraction 2D was separated using an YMC (1.0 × 80 cm) column chromatography with a MeOH−acetone−H_2O (0.15:0.12:1; 750 mL) elution solvent to give compounds **1** (26.0 mg) and **2** (17.8 mg).

*Zanthoxyloside (**1**).* Yellow amorphous powder: $[\alpha]_D^{25}$ −56.8 (*c* 0.1, MeOH); UV (MeOH) 207, 310 nm; IR (KBr) ν_{max} 3540, 1731, 1611, 1581 cm^{-1}; ^{1}H NMR (pyridine-d_5, 600 MHz) and ^{13}C NMR data (pyridine-d_5, 150 MHz), see Table 1; HR-ESI-MS *m*/*z*: $[M + H]^+$ calcd for 487.1452, $C_{21}H_{27}O_{13}$, found 487.1446

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data of Compound 1

	δ_C[a,b]	δ_H[a,c] (*J* in Hz)
2	160.6	
3	115.0	6.32 d (9.5)
4	144.2	7.68 d (9.5)
5	123.4	7.57 d (8.5)
6	113.5	7.36 d (8.5)
7	154.6	
8	137.1	
9	148.6	
10	113.9	
8-OMe	61.4	4.02 s
Glc-1	102.6	5.65 d (7.0)
2	74.6	4.25 m[d]
3	77.7	4.23 m[d]
4	71.5	4.00 t (7.0)
5	78.6	4.25 m[d]
6	69.1	4.07 m[d]
		4.68 m[d]
Api-1	111.2	5.75 d (2.7)
2	77.5	4.70 d (2.7)
3	80.4	
4	75.0	4.28 m[d]
		4.52 d (9.8)
5	65.2	4.11 s

Assignments were done by HMQC and HMBC experiments; *J* values (Hz) are in parentheses. [a]Measured in pyridine-d_5. [b]150 MHz. [c]600 MHz. [d]overlapped.

*Schinifolisatin A (**13**).* Yellowish oil: $C_{30}H_{34}O_8$; $[\alpha]_D^{25}$ +42.6 (*c* 0.1, MeOH); UV (MeOH) 239, 282 nm; IR (KBr) ν_{max} 3420, 1610, 1521, 1072, 1028 cm^{-1}; ^{1}H NMR (methanol-d_4, 600 MHz) and ^{13}C NMR data (methanol-d_4, 150 MHz), see Table 2; HR-ESI-MS *m*/*z*: $[M + H]^+$ calcd for 523.2332, $C_{30}H_{35}O_8$, found 523.2330.

Table 2. The ^{1}H and ^{13}C NMR Spectroscopic Data of Compound 13

	δ_C [a,b]	δ_H [a,c] (J in Hz)
1	132.5	
2	111.9	6.46 br s
3	147.7	
4	146.2	
5	128.9	
6	115.1	6.84 br s
7	130.6	6.41 d (15.8)
8	126.1	6.10 dt (15.8, 5.0)
9	62.5	4.07 d (5.0)
1′	133.2	
2′	109.1	6.79 d (1.8)
3′	144.1	
4′	130.6	
5′	114.8	6.64 d (8.0)
6′	118.4	6.70 dd (8.0, 1.8)
7′	87.9	5.40 d (7.0)
8′	53.8	3.36 m
9′	63.5	3.65 dd (11.0, 5.0)
		3.71 dd (11.0, 7.0)
1″	131.2	
2″	110.7	7.51 d (1.5)
3″	147.9	
4″	144.1	
5″	114.4	6.53 d (8.0)
6″	121.3	6.41 dd (8.0, 1.5)
7″	34.6	2.43 dd (13.6, 6.8)
		2.54 dd (13.6, 6.8)
8″	42.7	1.78 m
9″	60.7	3.46 m
3-OMe	54.8	3.60 s
3′-OMe	55.3	3.74 s
3″-OMe	55.1	3.68 s

Assignments were done by HMQC and HMBC experiments; J values (Hz) are in parentheses. [a]Measured in methanol-d_4. [b]150 MHz. [c]600 MHz.

Enzymatic Hydrolysis. Compound **1** (3.0 mg) was mixed with β-glucosidase (3.0 mg) in water (1.0 mL) and was shaken in a water bath at 37 °C for 12 h. After this, the reaction mixture of **1** was concentrated and then subjected to silica gel (1.0 × 15.0 cm, 40−63 μm) column chromatography with $CHCl_3$−MeOH (15:1, 60 mL) and $CHCl_3$−MeOH−H_2O (7:3:0.5, 60 mL) to afford an aglycone **1a** (1.2 mg) and a sugar fraction. The sugar fraction was concentrated to dryness using N_2 gas. The resulting residue was dissolved in dry pyridine (0.1 mL), and then L-cysteine methyl ester hydrochloride in pyridine (0.06 M, 0.1 mL) was added to the solution. After heating the reaction mixtures at 60 °C for 2 h, 0.1 mL of trimethylsilylimidazole solution was added. Heating at 60 °C was continued for a further 1.5 h. The dried product was partitioned with *n*-hexane and H_2O (0.1 mL each), and the organic layer was analyzed using gas liquid chromatography (GC): DB-5 capillary column (0.32 mm ×30 m); FID detector; column temp, 210 °C; injector temp, 270 °C; detector temp, 300 °C; carrier gas, He (2 mL/min). Under these conditions, standard sugars gave peaks at t_R (min) = 14.12 and 12.24 for L- and D-glucose, t_R (min) = 11.72 and 10.86 for L- and D-apiose, respectively. The peaks of the hydrolysate of **1** were detected at t_R (min) = 12.21 and 10.80, which identified as D-glucose and D-apiose by comparison with the retention time of the authentic samples after treatment with trimethylsilylimidazole in pyridine.

Cell Culture and Reagents. The HL-60 (human acute promyelocytic leukemia), PC-3 (human prostate cancer), and SNU-C5 (human colon cancer) cell lines were obtained from the Korea Cell Line Bank (KCLB) and cultured in RPMI 1640 (Hyclone, UT) medium supplemented with 10% fetal bovine serum (Hyclone, UT), 100 U/mL penicillin, and 100 mg/mL streptomycin (GIBCO, Inc., Grand Island, NY) at 37 °C in a humidified 5% CO_2 atmosphere.

Cell Viability Assay. The effects of isolated compounds on the proliferation of three human cancer cells were evaluated using the MTT assay.[25] Cells were seeded on 96-well microplates in 200 μL (HL-60, 3 × 10^5 cells/mL; PC-3, 5 × 10^4 cells/mL; SNU-C5, 1 × 10^5 cells/mL). After 24 h, the cells were treated with compounds (0.01, 0.1, 1, 10, 50, and 100 μM) for 72 h. At the end of the experimental incubation, the cells were treated with 50 μL (5 mg/mL) of MTT dye and incubated at 37 °C for 4 h. The medium was aspirated and replaced with 150 μL/well dimethyl sulfoxide to dissolve the formazan solution. Cell viabilities were determined by measuring the absorbance at 540 nm using a microplate ELISA reader (Amersham Pharmacia Biotech, NY). Concentration (X-axis)−cell viability (%, Y-axis) curves for compound-treated cancer cells have been obtained. We have determined the IC_{50} values (compound concentration resulting in a 50% inhibition of growth). Results are expressed as means ± standard deviation (SD) from three independent representative experiments. The student's t-test was used to evaluate the data with the following significance levels: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Flow Cytometric Analysis of Apoptosis. The effects of compounds **10**−**12** on cell cycle phase distribution were analyzed by flow cytometry after staining the cells with propidium iodide (PI). HL-60 (3 × 10^5 cells/mL) were treated with compounds **10**−**12** (IC_{50} concentrations), and PC-3 (5 × 10^4 cells/mL) and SNU-C5 (1 × 10^5 cells/mL) were treated with IC_{50} concentrations of compound **10** for 24 and 48 h. The treated cells were washed two times with 0.01 M phosphate-buffered saline (PBS; NaCl 0.138 M; KCl 0.0027 M; pH 7.4) and fixed with 70% ethanol 30 min at 4 °C. The fixed cells were washed twice with cold PBS, incubated with 50 μg/mL RNase A at 37 °C for 30 min, and stained with 50 μg/mL PI solution (Sigma) in the dark for 15 min at 37 °C. The stained cells were analyzed using an EPICS-XL FACScan flow cytometer (Beckman Coulter, Miami, FL). The proportion of cells in G0/G1, S, and G2/M phases was represented as DNA histograms. Apoptotic cells with hypodiploid DNA content were measured by quantifying the sub-G1 peak in the cell cycle pattern. For each experiment, 10 000 events per sample were analyzed, and experiments were repeated three times.

Morphological Analysis of Apoptosis by Hoechst 33342 Staining. For detection of apoptosis, HL-60 cells (3 × 10^5 cells/mL) were treated with compounds **10**−**12** (IC_{50} concentrations), and PC-3 (5 × 10^4 cells/mL) and SNU-C5 (1 × 10^5 cells/mL) were treated with IC_{50} concentrations of compound **10** for 24 and 48 h. The cells were incubated in Hoechst 33342 (10 μg/mL medium at final) staining solution at 37 °C for 20 min. The stained cells were observed with an inverted fluorescent microscope equipped with an IX-71 Olympus camera and photographed (magnification ×200).

Western Blot Analysis. HL-60 (3 × 10^5 cells/mL) were treated with compounds **10**−**12** (IC_{50} concentrations), and

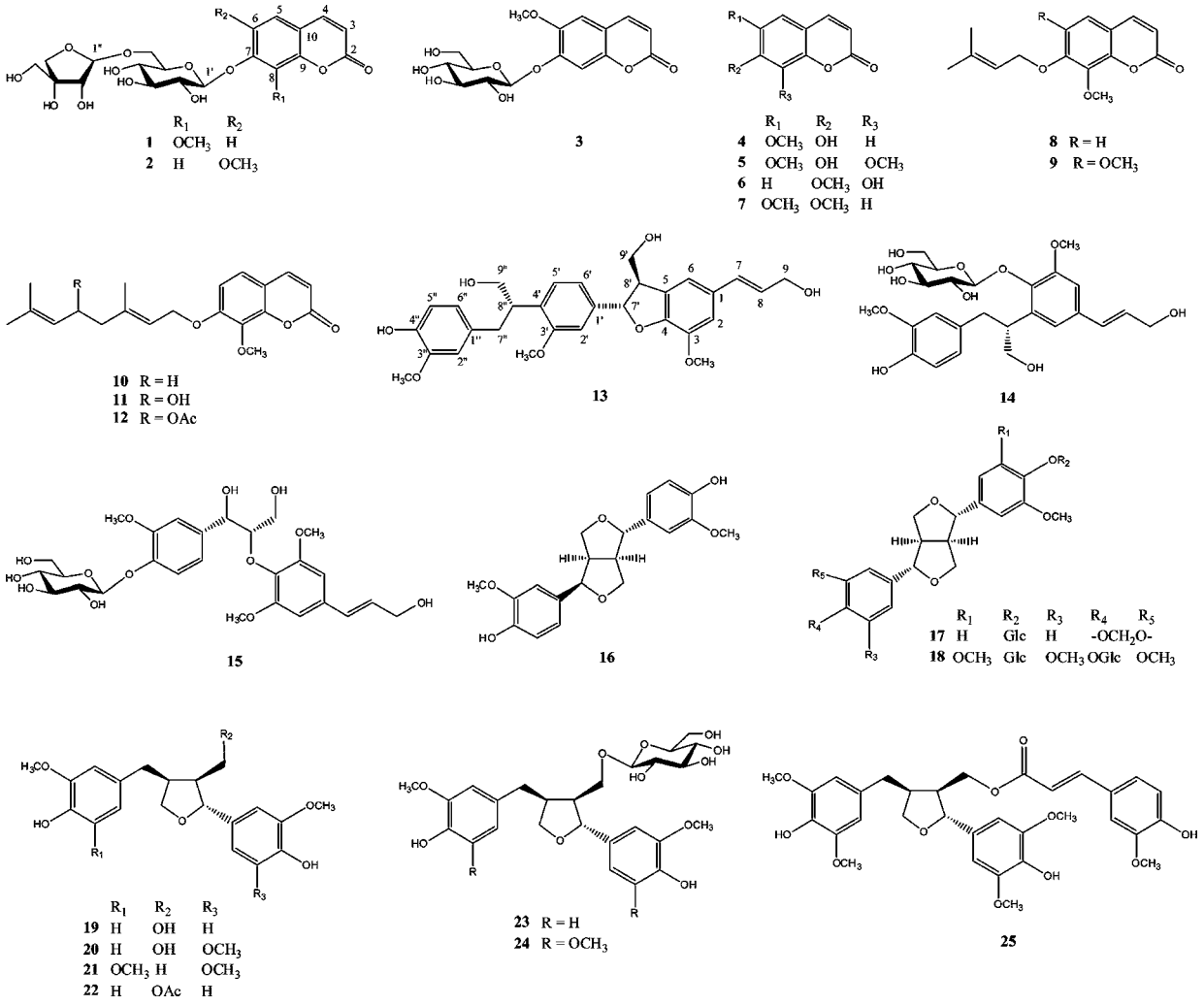

Figure 1. Structures of compounds **1**–**25** from the stems of *Z. schinifolium*.

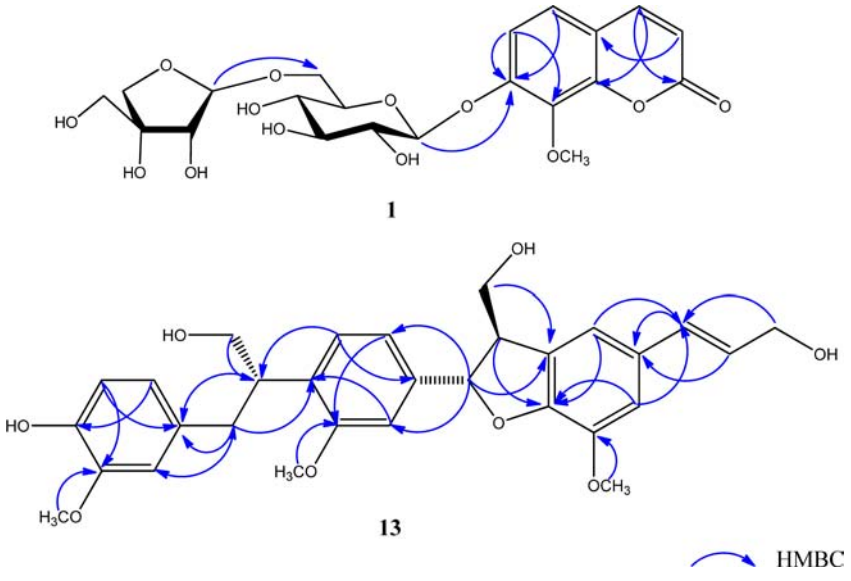

Figure 2. Key HMBC correlations of compounds **1** and **13**.

PC-3 (5×10^4 cells/mL) and SNU-C5 (1×10^5 cells/mL) were treated with IC_{50} concentrations of compound **10** for 12, 24, and 48 h. After treatment, the cells were harvested and washed two times with cold PBS. The cells were lysed with lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1 mM NaVO3, 10 mM NaF, 1 mM

dx.doi.org/10.1021/jf403479c | J. Agric. Food Chem. 2013, 61, 10730–10740

dithiothreitol, 1 mM phenylmethylsulfonylfluoride, 25 μg/mL aprotinin, 25 μg/mL leupeptin, and 1% Nonidet P-40) and kept on ice for 30 min at 4 °C. The lysates were centrifuged at 15 000 rpm at 4 °C for 15 min. The supernatants were stored at −20 °C until use. The protein content was determined by the Bradford assay.[26] The same amounts of lysates were separated on 8−15% SDS-PAGE gels and then transferred onto a polyvinylidene fluoride (PVDF) membrane (BID-RAD, HC, U.S.A.) by glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl [pH 8.8], and 20% MeOH [v/v]) at 100 V for 2 h. After blocking with 5% nonfat dried milk, the membrane was incubated with primary antibody against PARP (1:1000), cleaved caspase-3 (1:1000), cleaved caspase-9 (1:1000), Bcl-2 (1:1000), Bax (1:1000), ERK1/2 (1:1000), p-ERK1/2 (1:1000), c-myc (1:1000), AKT (1:1000), p-AKT (1:1000), and β-actin (1:5000) antibodies and incubated with a secondary HRP antibody (1:5000; Vector Laboratories, Burlingame, VT) at room temperature. The membrane was exposed on X-ray films (AGFA, Belgium), and protein bands were detected using a WEST-ZOL plus Western Blot Detection System (iNtRON, Gyeonggi-do, Korea).

Statistical Analysis. Results are expressed as the mean ± standard deviation (SD). All assays were performed in at least three independent experiments.

RESULTS AND DISCUSSION

Identification of Compounds 1−25. In total, 25 compounds, 12 coumarins (**1**−**12**), and 13 lignans (**13**−**25**) were isolated from a methanol extract of stems of *Z. schinifolium*. Their structures were identified as zanthoxyloside (**1**), hymexelsin (**2**),[27] scopoline (**3**),[28] scopoletin (**4**),[29] phytodolor (**5**),[30] daphnetin 7-methyl ether (**6**),[18] scoparone (**7**),[31] lacinartin (**8**),[32] puberulin (**9**),[33] collinin (**10**),[7] 8-methoxyanisocoumarin H (**11**),[7] acetoxyschinifolin (**12**),[7] schinifolisatin A (**13**), icariside E5 (**14**),[34] citrusin B (**15**),[35] epipinoresinol (**16**),[36] simplexoside (**17**),[37] (+)-(+)-syringaresinol-di-*O*-β-D-glucopyranoside (**18**),[38] (+)-lariciresinol (**19**),[39] 5′-methoxylariciresinol (**20**),[40] (+)-5,5′-dimethoxylariciresinol (**21**),[41] lariciresinol acetate (**22**),[42] (+)-lariciresinol-9-*O*-β-D-glucopyranoside (**23**),[43] alangilignoside C (**24**),[44] and (+)-9′-*O*-*trans*-feruloyl-5,5′-dimethoxylariciresinol (**25**)[45] on the basis of spectral data and chemical evidence, which agreed well with literature reports (Figure 1). Of these, zanthoxyloside (**1**) and schinifolisatin A (**13**) were identified as new compounds. Additionally, puberulin (**9**), icariside E5 (**14**), citrusin B (**15**), simplexoside (**17**), (+)-syringaresinol-di-*O*-β-D-glucopyranoside (**18**), 5′-methoxylariciresinol (**20**), lariciresinol acetate (**22**), alangilignoside C (**24**), and (+)-9′-*O*-*trans*-feruloyl-5,5′-dimethoxylariciresinol (**25**) were isolated from the *Zanthoxylum* genus for the first time.

Table 3. Inhibitory Effect of the Isolated Compounds against Cancer Cells Lines

	cancer cell line IC_{50} ± S.D.[a] (μM)		
compd	HL-60	PC-3	SNU-C5
1	>100	>100	>100
2	>100	>100	>100
3	>100	>100	>100
4	>100	>100	>100
5	19.80 ± 3.05	>100	>100
6	77.66 ± 6.48	>100	>100
7	74.29 ± 1.58	>100	>100
8	28.18 ± 1.68	71.01 ± 2.16	92.93 ± 4.24
9	21.77 ± 1.51	30.11 ± 1.05	67.64 ± 3.75
10	4.62 ± 0.27	4.39 ± 0.58	6.26 ± 0.10
11	5.02 ± 0.14	12.22 ± 0.67	33.50 ± 1.44
12	5.12 ± 0.19	33.81 ± 1.21	35.11 ± 0.41
13	28.70 ± 0.95	42.60 ± 3.30	82.50 ± 3.76
14	>100	>100	>100
15	>100	>100	>100
16	74.43 ± 1.04	58.87 ± 1.20	>100
17	27.81 ± 1.56	>100	>100
18	>100	>100	>100
19	>100	>100	>100
20	>100	>100	>100
21	>100	>100	>100
22	>100	>100	>100
23	>100	>100	>100
24	>100	>100	>100
25	25.20 ± 0.41	72.35 ± 1.85	>100
mitoxantron[b]	0.075 ± 0.005	5.17 ± 0.337	21.60 ± 1.20

[a]Values are means ± SDs (n = 3). [b]Positive control.

Compound **1** was isolated as a yellow amorphous powder. The molecular formula was established as $C_{21}H_{26}O_{13}$ by a quasimolecular-ion peak $[M + H]^+$ at m/z 487.1446 (calcd 487.1452) in the HR-ESI-MS spectrum. A prominent ESI-MS fragment peak occurred at m/z 191 $[M - H - 294]^-$, suggesting the presence of one hexose and one pentose in the structure. The IR spectrum showed absorption bands characteristic of the hydroxy group (3540 cm^{-1}), carbonyl group (1731 cm^{-1}), and an aromatic moiety (1611, 1581 cm^{-1}). Enzymatic hydrolysis of **1** produced D-glucose and D-apiose as sugar residues, by comparison with those reported in the literature using a GC experiment. The ^1H NMR spectrum (Table 1) showed two doublets at δ_H 6.32 (d, J = 9.5 Hz, H-3) and 7.68 (1H, d, J = 9.5 Hz, H-4) and another pair of doublets at δ_H 7.36 (d, J = 8.5 Hz, H-6) and 7.57 (1H, d, J = 8.5 Hz, H-5), in addition to other signals in the nonaromatic range. In the aromatic region, the ^{13}C NMR and DEPT spectra showed a methoxy carbon at δ_C 61.4, nine aromatic region signals in the range of δ_C 113.5−160.6, and 11 sugar moiety signals at δ_C 65.2−111.2. From the above data, compound **1** is likely a coumarin glycoside.[46] The β-D-configurations of the glucose and apiose residues were determined using ^{13}C NMR chemical shifts and coupling constants (7.0, 2.7 Hz) of the anomeric protons. The HMBC correlations between the anomeric proton signal at δ_H 5.65 (Glc-H1) and δ_C 154.6 (C-7) indicated that the glucose residue was connected to C-7 of the aglycone, and the correlation between δ_H 5.75 (Api-H1) and δ_C 69.1 (Glc-C6) demonstrated that the apiose was linked to C-6 of the glucose residue (Figure 2). On the basis of this evidence, the structure of **1** was determined to be 7-hydroxy-8-methoxycoumarin 7-(6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside), named zanthoxyloside.

Compound **13** was isolated as a yellowish oil. Its molecular formula, $C_{30}H_{34}O_8$, was determined on the basis of the positive HR-ESI-MS at m/z 523.2330 $[M + H]^+$ (calcd 523.2332). The ^1H, ^{13}C NMR, and HMQC spectra of **13** revealed three segments (Table 2). The ^1H NMR spectrum of **13** showed the presence of eight aromatic protons: 1,3,4-trisubstituted aromatic ring (ring B and C) signals at δ_H 6.41 (dd, J = 8.0, 1.5 Hz, H-6″), 6.53 (d, J = 8.0 Hz, H-5″), 6.64 (d, J = 8.0 Hz, H-5′), 6.70 (dd, J = 8.0, 1.8 Hz, H-6′), 6.79 (d, J = 1.8 Hz, H-2′), and 7.51 (d, J = 1.5 Hz, H-2″) and 1,3,4,5-tetrasubstituted

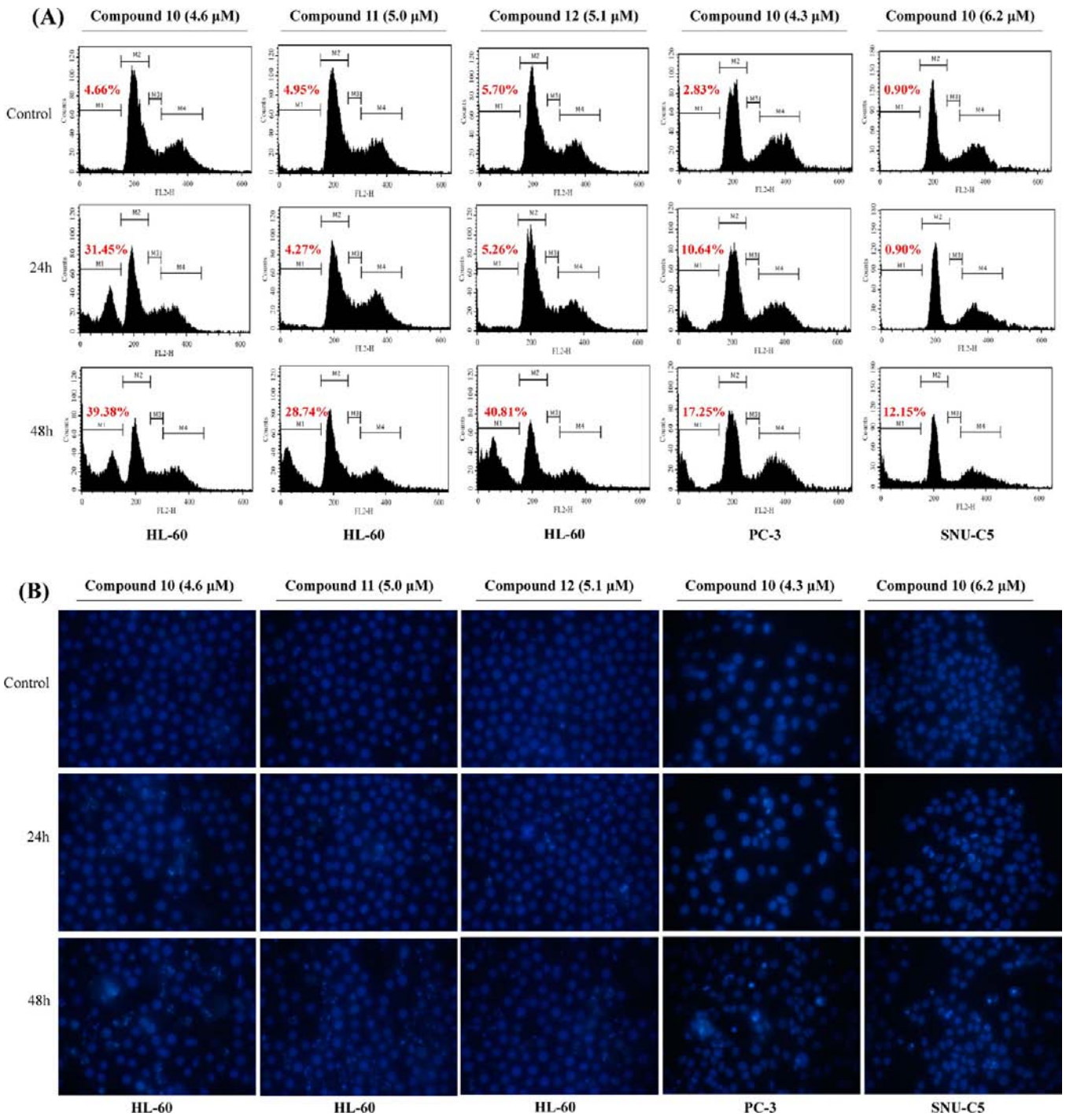

Figure 3. Compounds **10**−**12** induce apoptosis in cancer cells.

aromatic moiety (ring A) signals at δ_H 6.46 (br s, H-2) and 6.84 (br s, H-6). The ^{13}C NMR spectrum of **13** showed the presence of 18 aromatic carbons: 1,3,4-trisubstituted aromatic ring (ring B and C) signals at δ_C 109.1 (C-2′), 110.7 (C-2″), 114.4 (C-5″), 114.8 (C-5′), 118.4 (C-6′), 121.3 (C-6″), 130.6 (C-4′), 131.2 (C-1″), 133.2 (C-1′), 144.1 (C-3′ and 4″), and 147.9 (C-3″) and 1,3,4,5-tetrasubstituted aromatic moiety (ring A) signals at δ_C 111.9 (C-2), 115.1 (C-6), 128.9 (C-5), 132.5 (C-1), 146.2 (C-4), and 147.7 (C-3). The HMBC spectra of **13** revealed spin systems due to three segments: (C-7→C-9), (C-7′→C-9′), and (C-7″→C-9″).[47] *Trans* double bond signals at δ_H 6.10 (dt, J = 15.8, 5.0 Hz, H-8) and 6.41 (d, J = 15.8 Hz, H-7) and an oxymethylene signal at δ_H 4.07 (d, J = 5.0 Hz, H-9) suggested a propenol derivative, attached to C-1 of the aromatic ring A, determined by HMBC correlations at δ_H 6.46 (H-2)/δ_C 130.6 (C-7), δ_H 6.84 (H-6)/δ_C 130.6 (C-7), δ_H 6.10 (H-8)/δ_C 132.5 (C-1), and δ_H 4.07 (H-9)/δ_C 130.6 (C-7). The doublet signal at δ_H 5.40 (d, J = 7.0 Hz, H-7′) could be assigned to a CH adjacent to an aromatic ring and the oxygen atom of the ether linkage in a dihydrobenzofuran unit, while a methine proton signal at δ_H 3.36 (m, H-8′) could be assigned to the other proton of the dihydrobenzofuran ring.[48] An oxymethylene group was linked at the dihydrobenzofuran ring by HMBC correlations at δ_H 3.65 (dd, J = 11.0, 5.0 Hz, H-9′a),

dx.doi.org/10.1021/jf403479c | J. Agric. Food Chem. 2013, 61, 10730−10740

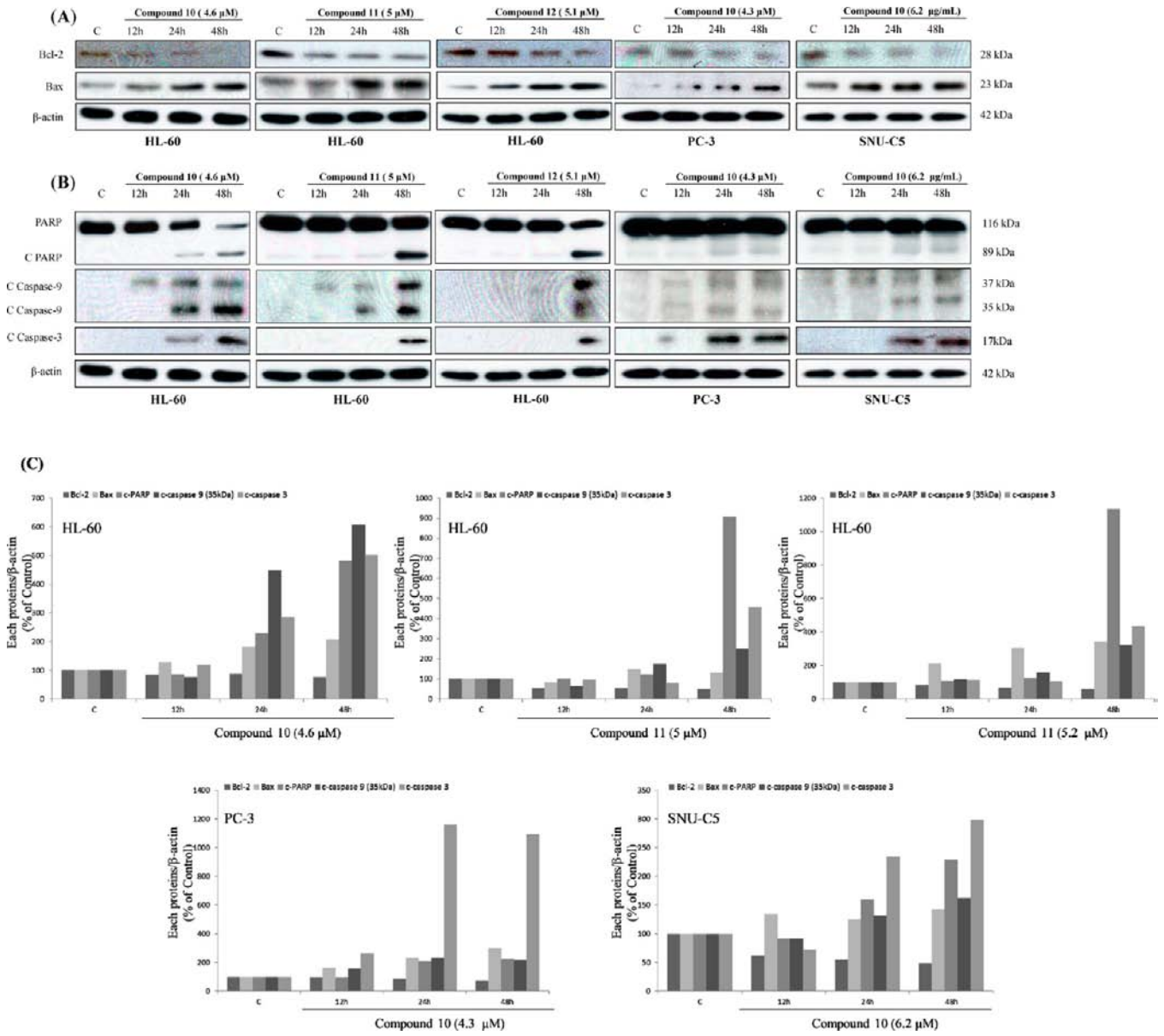

Figure 4. Effects of compounds **10**−**12** on the expression of apoptosis-related proteins.

3.71 (dd, J = 11.0, 7.0 Hz, H-9′b), and δ_C 53.8 (C-8′). This segment was connected to aromatic rings using the HMBC spectrum (Figure 2): the connection with the aromatic ring A was suggested by the correlations between δ_H 5.40 (H-7′)/δ_C 128.9 (C-5), δ_H 3.36 (H-8′)/δ_C 146.2 (C-4), δ_H 3.65 and 3.71 (H-9′)/δ_C 128.9 (C-5), while the connection with the aromatic ring B was suggested by the correlations at δ_H 5.40 (H-7′)/δ_C 109.1 (C-2′) and 5.40 (H-7′)/δ_C 118.4 (C-6′). A propanoid moiety at δ_H 1.78 (m, H-8″), 2.43 (dd, J = 13.6, 6.8 Hz, H-7″a), 2.54 (dd, J = 13.6, 6.8 Hz, H-7″b), and 3.46 (m, H-9″) was determined to be attached to the C-4′ position of ring B and C-1″ position of ring C by HMBC correlations at δ_H 2.43 and 2.54 (H-7″)/δ_C 110.7 (C-2″), 130.6 (C-4′), and 131.2 (C-1″), δ_H 6.64 (H-5′)/ δ_C 42.7 (C-8″), and δ_H 6.41 (H-6″)/ δ_C 34.6 (C-7″). Thus, the three segments of **13** were linked together by two phenyl propanol derivatives, which included the non-aromatic portion of a dihydrobenzofuran ring. Furthermore, three aromatic methoxyl group signals that resonated at δ_H 3.60 (s, 3-OMe), 3.68 (s, 3″-OMe), and 3.74 (s, 3′-OMe) were linked at δ_C 147.7 (C-3), 147.9 (C-3″), and 144.1 (C-3′) by the HMBC spectrum. The relative orientation of the substituents at the 7′- and 8′-position were determined to be *trans* from the observation of cross-peaks between H-7′/H-9′b, and H-8′/H-2′ in the NOESY spectrum, and the stereochemistry of the benzofuran ring was determined as 2*R* and 3*S*, and absolute configuration of H-8″ by HMBC data and comparison of the spectral data with related compounds.[49−51] Based on this evidence, the structure of **13** was concluded to be (2*R*,3*S*)3-hydroxymethyl-5-(3-hydroxypropenyl)-7-methoxy-2-{3-methoxy-4-[1-(3-methoxy-4-hydroxybenzyl)-2-hydroxyethyl] phenyl}-2,3-dihydrobenzofuran, named schinifolisatin A.

Effects of Compounds 1−25 on the Growth of HL-60, PC-3, and SNU-C5 Cells. To evaluate the effects of the compounds isolated on the growth of HL-60, PC-3, and SNU-C5 cells, cell viability was assessed using the MTT assay. Compounds **10**−**12** reduced HL-60 viable cell percentages significantly, with IC_{50} values of 4.62−5.12 μM, while compounds **5**−**9**, **13**, **15**, **16**, and **25** showed moderate

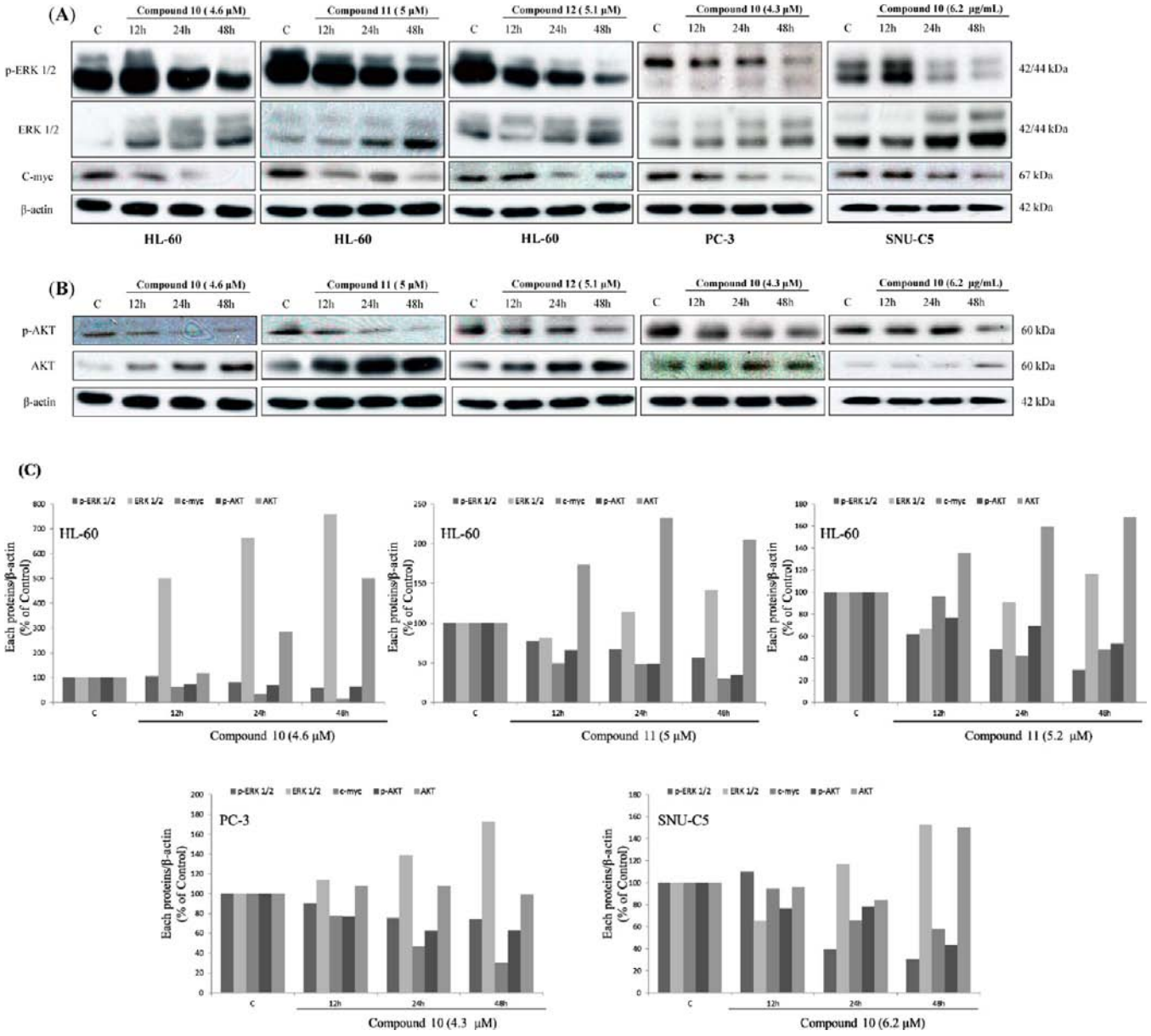

Figure 5. Effects of compounds **10**−**12** on the activation of ERK 1/2 MAPK and PI3K/AKT and c-myc level.

cytotoxic activity, with IC_{50} values ranging from 19.80 to 79.18 μM (Table 3). Furthermore, compound **10** showed the strongest inhibitory effect on the growth of PC-3 and SNU-C5 cells (IC_{50} = 4.39 and 6.26 μM, respectively) and was more potent than the positive control, mitoxantron (IC_{50} = 5.17 and 21.60 μM, respectively). However, compounds **10**−**12** barely inhibited the growth of HEL-299, which is a normal cell line, at concentrations up to 50 μM by under 20% (Figure S7). The other compounds lacked cytotoxic activity on the three cancer cell lines up to 100 μM.

Upon examination of the structure−activity relationship of coumarins (**1**−**12**), compounds **10**−**12** showed the strongest inhibitory effects on the growth of HL-60, PC-3, and SNU-C5 cells, and their structures are similar. When the side-chain unit at C-7 of the aglycone was linked to a *trans*-geraniol derivative (**10**−**12**), the cytotoxic activity increased significantly versus a prenoxy group (**8** and **9**), which showed moderate cytotoxic activity. These results showed that the length of the side-chain unit also plays an important role. With other substituent groups, such as a sugar or sugar chain (**1**−**3**), a hydroxyl group (**4** and **5**), and a methoxy group (**6** and **7**), most did not effectively inhibit the growth of cells. Thus, a *trans*-geraniol derivative or prenoxy group side chain at C-7 seems to be an important functional element. The major active compounds (**10**−**12**) are only found in the *Zanthoxylum* genus, especially in *Z. schinifolium* leaves and stems, and this is a distinguishing characteristic of *Z. schinifolium*.

Effects of Compounds 10−12 on the Induction of Apoptosis. Apoptosis is a complex physiological process that permits the reduction of harmful or unnecessary cells during development, tissue homeostasis, and disease.[52] Apoptotic cell death has typical characteristics, such as chromatin condensation, membrane blebbing, cell shrinkage, and an increased population of sub-G1 hypodiploid cells.[53] To assess whether compounds **10**−**12** could induce apoptosis, cell cycle analysis was performed using PI DNA staining and flow cytometry. Compounds **10**−**12** increased the proportion of the sub-G1 fraction (M1) in a time-dependent manner (Figure 3A). These

dx.doi.org/10.1021/jf403479c | J. Agric. Food Chem. 2013, 61, 10730−10740

results show that compounds **10**−**12** induced apoptosis in cells, supported by the increase in the number of apoptotic bodies, which are readily identifiable by Hoechst 33342 staining in compound-treated cells after 24 and 48 h incubation (Figure 3B).

Effects of Compounds 10−**12 on the Expression of Apoptosis-related Proteins.** When treated with compounds **10**−**12**, with IC_{50} values of 4.62−5.12 μM, we could observe the modulation of apoptosis-related protein levels, such as an increased Bax level, decreased Bcl-2 expression (Figure 4A), cleavage of procaspase-3, cleavage of procaspase-9, and cleavage of PARP in a time-dependent manner (Figure 4B). These data indicated that compounds **10**−**12** induced apoptosis of cells through the modulation of known apoptosis-related proteins.

Effects of Compounds 10−**12 on ERK 1/2 MAPK and PI3K/AKT signaling.** The MAPK and PI3K/AKT signaling pathways regulate apoptosis, cell survival, and cell growth.[23] Activation of ERK 1/2 MAPK and AKT also contribute to the cell survival and cell growth via c-myc.[24] Previous studies have demonstrated that taxol induces apoptosis through activation of PI3K/AKT pathways.[54,55] The c-myc is overexpressed in 70% of colorectal tumors.[56] Alterations of chromosome 8, including the c-myc oncogene, have been noted as one of the most common chromosomal abnormalities in prostate cancer progression,[57] and it is well-known that myelogenous leukemia cells, including HL-60, have overexpressed c-myc.[58] To investigate compound-induced intracellular signaling, we analyzed the phosphorylation of ERK1/2 MAPK and AKT and the level of c-myc by Western blotting. Treatment with compounds **10**−**12** decreased p-ERK1/2 MAPK levels and p-AKT levels in conditions that could induce apoptosis in the three human cancer cell lines (Figure 5A). Furthermore, the down-regulation of p-ERK1/2 MAPK and p-AKT was also accompanied by the down-regulation of c-myc in these three human cancer cell lines (Figure 5B). These findings provide evidence demonstrating that the apoptosis-inducing effects of compounds **10**−**12** are mediated by down-regulation of p-ERK 1/2 MAPK, p-AKT, and c-myc.

In this study, 12 coumarins (**1**−**12**) and 13 lignans (**13**−**25**) were isolated from a methanol extract of the stems of *Z. schinifolium*. Zanthoxyloside (**1**) and schinifolisatin A (**13**) were identified as new compounds. To our knowledge, this is the first report of the coumarin and lignan components of stems of *Z. schinifolium* and their cytotoxic effects on HL-60, PC-3, and SNU-C5 cells. These results showed that coumarins and lignans are major components in *Z. schinifolium* stems. Our study demonstrates that coumarins from *Z. schinifolium* inhibited the growth of the three cancer cell lines by inducing apoptosis. Coumarins induced apoptosis through the modulation of known apoptosis-related proteins. Furthermore, the induction of apoptosis was accompanied by the down-regulation of p-ERK 1/2 MAPK, p-AKT, and c-myc in a time-dependent manner. These data suggested that coumarins from the stems of *Z. schinifolium* have potential as a cancer treatment. To prove the therapeutic potential of compounds **10**−**12** for the cancer treatment, we need to investigate in vivo effects of compound **10**−**12** on the cancer prevention and tumor regression in the further study.

■ ASSOCIATED CONTENT

Supporting Information

^{1}H, ^{13}C NMR, HMQC, HMBC, COSY, NOESY, and HR-ESI-MS spectra of compound **1** and **13**. Inhibitory effect of the isolated compounds against cancer cell lines and inhibitory effect of the compounds **10**−**12** against normal human fibroblast cells are available as Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: yhk@cnu.ac.kr. Fax: 82-42-823-6566. Tel.: 82-42-821-5933.

Funding

This study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2012-0006681).

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Paik, S. Y.; Koh, K. H.; Beak, S. M.; Paek, S. H.; Kim, J. A. The essential oils from *Zanthoxylum schinifolium* pericarp induce apoptosis of HepG2 human hepatoma cells through increased production of reactive oxygen species. *Biol. Pharm. Bull.* **2005**, *28*, 802−807.

(2) Cui, H. Z.; Choi, H. R.; Choi, D. H.; Cho, K. W.; Kang, D. G.; Lee, H. S. Aqueous extract of *Zanthoxylum schinifolium* elicits contractile and secretory responses via β1-adrenoceptor activation in beating rabbit atria. *J. Ethnopharmacol.* **2009**, *126*, 300−307.

(3) Yang, X. Aroma constituents and alkylamides of red and green huajiao (*Zanthoxylum bungeanum* and *Zanthoxylum schinifolium*). *J. Agric. Food Chem.* **2008**, *56*, 1689−1696.

(4) Chang, C. T.; Doong, S. L.; Tsai, I. L.; Chen, I. S. Coumarins and anti-HBV constituents from *Zanthoxylum schinifolium*. *Phytochemistry* **1997**, *45*, 1419−1422.

(5) Chen, I. S.; Lin, Y. C.; Tsai, I. L.; Teng, C. M.; Ko, F. N.; Ishikawa, T.; Ishi, H. Coumarins and anti-platelet aggregation constituents from *Zanthoxylum schinifolium*. *Phytochemistry* **1995**, *39*, 1091−1097.

(6) Cheng, M. J.; Yang, C. H.; Lin, W. Y.; Lin, W. Y.; Tsai, I. L.; Chen, I. S. Chemical constituents from the leaves of *Zanthoxylum schinifolium*. *J. Chin. Chem. Soc.* **2002**, *49*, 125−128.

(7) Tsai, I. L.; Lin, W. Y.; Teng, C. M.; Ishikawa, T.; Doong, S. L.; Huang, M. W.; Chen, Y. C.; Chen, I. S. Coumarins and antiplatelet constituents from the root bark of *Zanthoxylum schinifolium*. *Planta Med.* **2000**, *66*, 618−623.

(8) Jang, M. J.; Woo, M. H. Effect of antioxidative, DPPH radical scavenging activity and antithrombogenic by the extract of Sancho (*Zanthoxylum schinifolium*). *Korean J. Nutr.* **2005**, *38*, 386−394.

(9) Jo, Y. S.; Huong, D. T. L.; Bae, K.; Lee, M. K.; Kim, Y. H. Monoamine oxidase inhibitory coumarin from *Zanthoxylum schinifolium*. *Planta Med.* **2002**, *68*, 84−85.

(10) Cao, L. H.; Lee, Y. J.; Kang, D. G.; Kim, J. S.; Lee, H. S. Effect of *Zanthoxylum schinifolium* on TNF-alpha-induced vascular inflammation in human umbilical vein endothelial cells. *Vasc. Pharmacol.* **2009**, *50*, 200−207.

(11) Min, B. K.; Hyun, D. G.; Jeong, S. Y.; Kim, Y. H.; Ma, E. S.; Woo, M. H. A new cytotoxic coumarin, 7-[(*E*)-3′,7′-dimethyl-6′-oxo-2′,7′-octadienyl]oxy coumarin, from the leaves of *Zanthoxylum schinifolium*. *Arch. Pharm. Res.* **2011**, *34*, 723−726.

(12) Cox, S. D.; Mann, C. M.; Markham, J. L.; Bell, H. C.; Gustafson, J. E.; Warmington, J. R.; Wyllie, S. G. The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tea tree oil). *J. Appl. Microbiol.* **2000**, *88*, 170−175.

(13) Cha, J. D.; Jeong, M. R.; Jeong, S. I.; Moon, S. E.; Kil, B. S.; Yun, S. I.; Lee, K. Y.; Song, Y. H. Chemical composition and antimicrobial activity of the essential oil of *Cryptomeria japonica*. *Phytother. Res.* **2007**, *21*, 295−299.

(14) Giordani, R.; Regli, P.; Kaloustian, J.; Mikaïl, C.; Abou, L.; Portugal, H. Antifungal effect of various essential oils against *Candida*

albicans. Potentiation of antifungal action of amphotericin B by essential oil from *Thymus vulgaris*. *Phytother. Res.* **2004**, *18*, 990−995.
(15) Tragoolpua, Y.; Jatisatienr, A. Anti-herpes simplex virus activities of *Eugenia caryophyllus* (Spreng.) Bullock & S.G. Harrison and essential oil, eugenol. *Phytother. Res.* **2007**, *21*, 1153−1158.
(16) Brand, C.; Ferrante, A.; Prager, R. H.; Riley, T. V.; Carson, C. F.; Finlay-Jones, J. J.; Hart, P. H. The water-soluble components of the essential oil of *Melaleuca alternifolia* (tea tree oil) suppress the production of superoxide by human monocytes, but not neutrophils, activated in vitro. *Inflammation Res.* **2001**, *50*, 213−219.
(17) Ju, Y.; Still, C. C.; Sacalis, J. N.; Li, J.; Ho, C. T. Cytotoxic coumarins and lignans from extracts of the northern prickly ash (*Zanthoxylum americanum*). *Phytother. Res.* **2001**, *15*, 441−443.
(18) Fang, Z.; Jun, D. Y.; Kim, Y. H.; Min, B. S.; Kim, A. K.; Woo, M. H. Cytotoxic constituents from the leaves of *Zanthoxylum schinifolium*. *Bull. Korean Chem. Soc.* **2010**, *31*, 1081−1084.
(19) Hanahan, D.; Weinberg, R. A. The hallmarks of cancer. *Cell* **2000**, *100*, 57−70.
(20) Zimmermann, K. C.; Green, D. R. How cells die: apoptosis pathways. *J. Allergy Clin. Immunol.* **2001**, *108*, S99−103.
(21) Huang, T. C.; Lai, C. S.; Pan, M. H. Induction of apoptosis by luteolin through cleavage of Bcl-2 family in human leukemia HL-60 cells. *Eur. J. Pharmacol.* **2005**, *509*, 1−10.
(22) Rasola, A.; Sciacovelli, M.; Chiara, F.; Pantic, B.; Brusilow, W. S.; Bernardi, P. Activation of mitochondrial ERK protects cancer cells from death through inhibition of the permeability transition. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 726−731.
(23) Yeh, E.; Cunningham, M.; Arnold, H.; Chasse, D.; Monteith, T.; Ivaldi, G.; Hahn, W. C.; Stukenberg, P. T.; Shenolikar, S.; Uchida, T.; Counter, C. M.; Nevins, J. R.; Means, A. R.; Sears, R. A signalling pathway controlling c-myc degradation that impacts oncogenic transformation of human cells. *Nat. Cell Biol.* **2004**, *6*, 308−318.
(24) Kim, D.; Chung, J. Akt: versatile mediator of cell survival and beyond. *J. Biochem. Mol. Biol.* **2002**, *35*, 106−115.
(25) Scudiero, D. A.; Shoemaker, R. H.; Paull, K. D.; Monks, A.; Tierney, S.; Nofziger, T. H.; Currens, M. J.; Seniff, D.; Boyd, M. R. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Res.* **1988**, *48*, 4827−4833.
(26) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(27) Nagaiah, K.; Krupadanam, G. L. D.; Srimannarayana, G. Coumarins from the bark of *Xeromphis uliginosa*. *Fitoterapia* **1992**, *63*, 378−379.
(28) Sun, J.; Yue, Y. D.; Tang, F.; Guo, X. F. Coumarins from the leaves of *Bambusa pervariabilis* McClure. *J. Asian Nat. Prod. Res.* **2010**, *12*, 248−251.
(29) Zakaria, I.; Ahmat, N.; Jaafar, F. M.; Widyawaruyanti, A. Flavonoids with antiplasmodial and cytotoxic activities of *Macaranga triloba*. *Fitoterapia* **2012**, *83*, 968−972.
(30) Deng, Y.; Tang, T. J.; Li, X.; Wu, F. E. A new aporphine alkaloid from *Sabia yunnanensis*. *Nat. Prod. Res.* **2007**, *21*, 28−32.
(31) Kuo, P. C.; Hwang, T. L.; Lin, Y. T.; Kuo, Y. C.; Leu, Y. L. Chemical constituents from *Lobelia chinensis* and their anti-virus and anti-inflammatory bioactivities. *Arch. Pharm. Res.* **2011**, *34*, 715−722.
(32) Harayama, T.; Katsuno, K.; Nishita, Y.; Fujii, M. Revision of structure of a new coumarin isolated from *Artemisia carvifolia* wall. *Chem. Pharm. Bull.* **1994**, *42*, 1550−1552.
(33) Kanlayavattanakul, M.; Ruangrungsi, N.; Watanabe, T.; Ishikawa, T. Chemical constituents of *Pterocaulon redolens*. *Heterocycles* **2003**, *61*, 183−187.
(34) Lee, D. Y.; Lee, D. G.; Cho, J. G.; Bang, M. H.; Lyu, H. N.; Lee, Y. H.; Kim, S. Y.; Baek, N. I. Lignans from the fruits of the red pepper (*Capsicum annuum* L.) and their antioxidant effects. *Arch. Pharm. Res.* **2009**, *32*, 1345−1349.
(35) Yuan, C. S.; Sun, X. B.; Zhao, P. H.; Cao, M. A. Antibacterial constituents from *Pedicularis armata*. *J. Asian Nat. Prod. Res.* **2007**, *9*, 673−677.
(36) Yang, G. Z.; Hu, Y.; Yang, B.; Chen, Y. Lignans from the bark of *Zanthoxylum planispinum*. *Helv. Chim. Acta* **2009**, *92*, 1657−1664.
(37) Yoo, S. W.; Kim, J. S.; Kang, S. S.; Son, K. H.; Chang, H. W.; Kim, H. P.; Bae, K.; Lee, C. O. Constituents of the fruits and leaves of *Euodia daniellii*. *Arch. Pharm. Res.* **2002**, *25*, 824−830.
(38) Houghton, P. J.; Lian, L. M. Iridoids, iridoid-triterpenoid congeners and lignans from *Desfontainia spinosa*. *Phytochemistry* **1986**, *25*, 1907−1912.
(39) Della Greca, M.; Mancino, A.; Previtera, L.; Zarrelli, A.; Zuppolini, S. Lignans from *Phillyrea angustifolia* L. *Phytochem. Lett.* **2010**, *4*, 118−121.
(40) Fiorentino, A.; Della Greca, M.; D' Abrosca, B.; Oriano, P.; Golino, A.; Izzo, A.; Zarelli, A.; Monaco, P. Lignans, neolignans and sesquilignans from *Cestrum parqui l'Her*. *Biochem. Syst. Ecol.* **2007**, *35*, 392−396.
(41) Yang, M. C.; Lee, K. H.; Kim, K. H.; Choi, S. U.; Lee, K. R. Lignan and terpene constituents from the aerial parts of *Saussurea pulchella*. *Arch. Pharm. Res.* **2007**, *30*, 1067−1074.
(42) Pullela, S. V.; Takamatsu, S.; Khan, S. I.; Khan, I. A. Isolation of lignans and biological activity studies of *Ephedra viridis*. *Planta Med.* **2005**, *71*, 789−791.
(43) Gan, L. S.; Zhan, Z. J.; Yang, S. P.; Yue, J. M. Two new terpenoid glucosides from *Aster flaccidus*. *J. Asian Nat. Prod. Res.* **2006**, *8*, 589−594.
(44) Achenbach, H.; Benirschke, M.; Torrenegra, R. Alkaloids and other compounds from seeds of *Tabernaemontana cymosa*. *Phytochemistry* **1997**, *45*, 325−335.
(45) Kwon, H. C.; Choi, S. U.; Lee, J. O.; Bae, K. H.; Zee, O. P.; Lee, K. R. Two new lignans from *Lindera obtusiloba* blume. *Arch. Pharm. Res.* **1999**, *22*, 417−422.
(46) Piao, S. J.; Qiu, F.; Chen, L. X.; Pan, Y.; Dou, D. Q. New stilbene, benzofuran, and coumarin glycosides from *Morus alba*. *Helv. Chim. Acta* **2009**, *92*, 579−587.
(47) Braca, A.; De Tommasi, N.; Morelli, I. I.; Pizza, C. New metabolites from *Onopordum illyricum*. *J. Nat. Prod.* **1999**, *62*, 1371−1375.
(48) Yoshikawa, K.; Kinoshita, H.; Kan, Y.; Arihara, S. Neolignans and phenylpropanoids from the rhizomes of *Coptis japonica* var. *dissecta*. *Chem. Pharm. Bull.* **1995**, *43*, 578−581.
(49) Otsuka, H.; Kashima, N.; Nakamoto, K. A neolignan glycoside and acylated iridoid glucosides from stem bark of *Alangium platanifolium*. *Phytochemistry* **1996**, *42*, 1435−1438.
(50) Shen, Y.; Hsieh, P.; Kuo, Y. Neolignans glucosides from *Jasminum urophyllum*. *Phytochemistry* **1998**, *48*, 719−723.
(51) Yuasa, K.; Ide, T.; Otsuka, H.; Ogimi, C.; Hirata, E.; Takushi, A.; Takeda, Y. Lignan and neolignan glycosides from stems of *Alangium premnifolium*. *Phytochemistry* **1997**, *45*, 611−614.
(52) Porter, A. G.; Janicke, R. U. Emerging roles of caspase-3 in apoptosis. *Cell Death Differ.* **1999**, *6*, 99−104.
(53) Yang, Y.; Zhu, X.; Chen, Y.; Wang, X.; Chen, R. p38 and JNK MAPK, but not ERK1/2 MAPK, play important role in colchicines-induced cortical neurons apoptosis. *Eur. J. Pharmacol.* **2007**, *576*, 26−33.
(54) Frenette, P. S.; Weiss, L. Sulfated glycans induce rapid hematopoietic progenitor cell mobilization: evidence for selectin-dependent and independent mechanisms. *Blood* **2000**, *96*, 2460−2468.
(55) Sweeney, E. A.; Lortat-Jacob, H.; Priestley, G. V.; Nakamoto, B.; Papayannopoulou, T. Sulfated polysaccharides increase plasma levels of SDF-1 in monkeys and mice: involvement in mobilization of stem/progenitor cells. *Blood* **2002**, *99*, 44−51.
(56) Arango, D.; Mariadason, J. M.; Wilson, A. J.; Yang, W.; Corner, G. A.; Nicholas, C.; Aranes, M. J.; Augenlicht, L. H. C-Myc overexpression sensitises colon cancer cells to camptothecin-induced apoptosis. *Br. J. Cancer* **2003**, *89*, 1757−1765.
(57) Hawksworth, D.; Ravindranath, L.; Chen, Y.; Furusato, B.; Sesterhenn, I. A.; McLeod, D. G.; Srivastava, S.; Petrovics, G. Overexpression of c-myc oncogene in prostate cancer predicts biochemical recurrence. *Prostate Cancer Prostatic Dis.* **2010**, *13*, 311−315.

(58) Dalla- Favera, R.; Westin, E.; Gelmann, E. P.; Martinotti, S.; Bregni, M.; Wong-Staal, F.; Gallo, R. C. The human onc gene c-myc: structure, expression, and amplification in the human promyelocytic leukemia cell line HL-60. *Haematol. Blood Transfus.* **1983**, *28*, 247−254.