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Anti-inflammatory Triterpenoid Saponins from the Stem Bark of *Kalopanax pictus*

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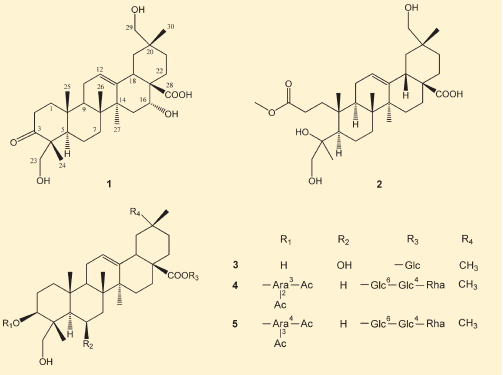
Supporting Information

ABSTRACT: Five new compounds, 16,23,29-trihydroxy-3-oxo-olean-12-en-28-oic acid (**1**), 4,23,29-trihydroxy-3,4-*seco*-olean-12-en-3-oate-28-oic acid (**2**), 3β,6β,23-trihydroxyolean-12-en-28-oic acid 28-*O*-β-D-glucopyranoside (**3**), 3-*O*-[2,3-di-*O*-acetyl-α-L-arabinopyranosyl]hederagenin 28-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (**4**), and 3-*O*-[3,4-di-*O*-acetyl-α-L-arabinopyranosyl]hederagenin 28-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (**5**), as well as 10 known compounds (**6**−**15**), were isolated from the stem bark of *Kalopanax pictus*. Compounds **1**−**5** and **7**−**14** inhibited TNFα-induced NF-κB transcriptional activity in HepG2 cells in a dose-dependent manner, with IC_{50} values ranging from 0.6 to 16.4 μM. Furthermore, the transcriptional inhibitory function of these compounds was confirmed on the basis of decreases in COX-2 and iNOS gene expression in HepG2 cells. The structure−activity relationship of the compounds with respect to anti-inflammatory activity is also discussed.



Kalopanax pictus (Araliaceae) is a deciduous tree growing in East Asian countries. The stem bark of *K. pictus* has been used in traditional medicine to treat rheumatic arthritis, neurotic pain, and diabetes mellitus.[1] Phytochemical studies on the stem bark have demonstrated the presence of hederagenin glycosides, syringin, liriodendrin, and coniferylaldehyde glucosides,[2,3] and the methanol extract of the stem bark of *K. pictus* has been reported to possess cytotoxic,[4] antidiabetic,[5] and anti-inflammatory activities.[6] In this report, we discuss the isolation and structural elucidation of five new (**1**−**5**) and 10 known compounds (**6**−**15**) from the methanol extract of the stem bark of *K. pictus*. The effects of compounds **1**−**15** on TNFα (tumor necrosis factor α)-induced NF-κB transcriptional activity in HepG2 cells were evaluated. To confirm the inhibitory effects of the compounds on NF-κB transcriptional activity, we investigated the effects of the compounds on the upregulation of the pro-inflammatory proteins iNOS (inducible nitric oxide synthase) and COX-2 (cyclooxygenase-2) in TNFα-stimulated HepG2 cells.

RESULTS AND DISCUSSION

A MeOH extract of the dried stem bark of *K. pictus* (290 g) was suspended in H_2O and successively extracted with *n*-hexane, CH_2Cl_2, and EtOAc. The EtOAc- and H_2O-soluble fractions was subjected to multiple chromatographic steps over Diaion HP-20, silica gel, and reversed-phase C_{18}, yielding compounds **1**−**15**. Comparison of the NMR and MS data with reported values led to identification of the structures of the known compounds **6**−**15** as nipponogenin E (**6**),[7] 3β,6β,23-trihydroxyolean-12-en-28-oic acid (**7**),[8] kalopanaxsaponin A (**8**),[3] kalopanaxsaponin B (**9**),[3] kalopanaxsaponin C (**10**),[2] sieboldianoside A (**11**),[9] hederagenin 28-*O*-β-D-glucopyranosyl ester (**12**),[10] dipsacussaponin A (**13**),[11] cauloside D (**14**),[12] and hederagenin (**15**).[13]

Compound **1** was obtained as a white, amorphous powder. The molecular formula was determined to be $C_{30}H_{46}O_6$ from the pseudomolecular ion peak $[M + H]^+$ at m/z 503.3350 (calcd for $C_{30}H_{47}O_6$, 503.3373) in the HR-ESITOFMS. It showed FT-IR absorption bands at 3429, 1729, and 1699 cm^{-1}, suggesting the presence of hydroxy, carboxy, and carbonyl groups, respectively. The ^1H NMR spectrum exhibited five quaternary methyl groups at δ_H 0.88 (Me-24), 1.02 (Me-25), 0.85 (Me-26), 1.39 (Me-27), and 0.97 (Me-30), a broad singlet for an olefinic proton at δ_H 5.33 (H-12) characteristic of the Δ^{12} proton in pentacyclic triterpenes, and a doublet of doublets at δ_H 3.03 (1H, J = 14.4, 4.2 Hz) attributable to H-18 of the Δ^{12} oleanane skeleton.[14] The ^1H NMR spectrum also showed signals for an oxymethine at δ_H 4.42 (1H, br s, H-16) and two oxymethylene groups at δ_H 3.57 (1H, d, J = 11.4 Hz, H-23a), 3.31 (1H, d, J = 11.4 Hz, H-23b), and 3.16 (2H, d, J = 1.8 Hz, H-29). The ^{13}C NMR and DEPT spectra exhibited 30 carbon signals, assignable to five methyls, 11 methylene, five methine, and nine quaternary

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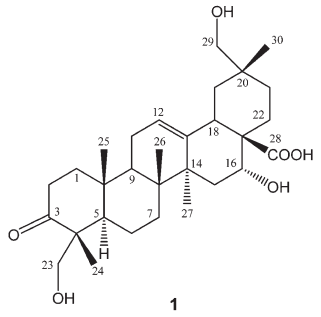
Chart 1



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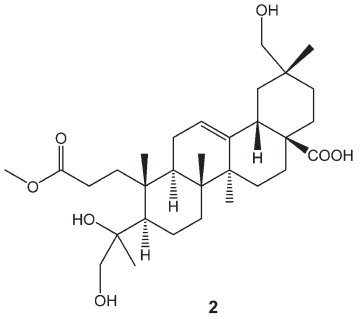

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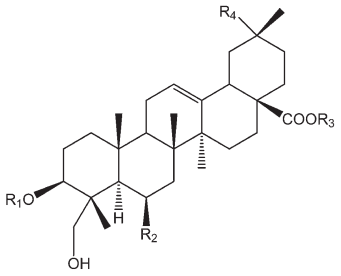

	R_1	R_2	R_3	R_4
3	H	OH	—Glc	CH_3
4	—Ara^{3}(Ac^{2})—Ac	H	—Glc^{6}—Glc^{4}—Rha	CH_3
5	—Ara^{4}(Ac^{3})—Ac	H	—Glc^{6}—Glc^{4}—Rha	CH_3
6	H	H	H	CH_2OH
7	H	OH	H	CH_3
8	—Ara^{2}—Rha	H	H	CH_3
9	—Ara^{2}—Rha	H	—Glc^{6}—Glc^{4}—Rha	CH_3
10	—Ara^{3}(Rha^{2})—Glc	H	—Glc^{6}—Glc^{4}—Rha	CH_3
11	—Ara^{2}—Rha^{3}—Xyl	H	—Glc^{6}—Glc^{4}—Rha	CH_3
12	H	H	—Glc	CH_3
13	H	H	—Glc^{6}—Glc	CH_3
14	—Ara	H	—Glc^{6}—Glc^{4}—Rha	CH_3
15	H	H	H	CH_3

Ara : α-L-arabinopyranosyl
Glc : β-D-glucopyranosyl
Rha : α-L-rhamnopyranosyl
Xyl : β-D-xylopyranosyl

carbons. The ^{13}C NMR spectrum (Table 1) showed signals of a carbonyl (δ_C 218.9, C-3), a carboxy (δ_C 180.2, C-28), two oxymethylenes [δ_C 67.1 (C-23) and 73.8 (C-29)], and an oxymethine carbon (δ_C 74.0, C-16). The ^{13}C NMR spectrum of **1** was similar to that of 23-hydroxy-3-oxolean-12-en-28-oic acid,[15] except for the presence of an oxymethine and oxymethylene group in **1**. The location of a hydroxy group at C-16 was shown by the HMBC correlation between δ_H 4.42 (H-16)/δ_C 48.9 (C-17) and the ^{1}H—^{1}H COSY cross-peak between H_2-15/H-16. The HMBC cross-peaks from δ_H 3.16 (2H, d, J = 1.8 Hz, H-29) to δ_C 35.7 (C-20) and 19.3 (C-30) permitted placement of another hydroxy group at C-29. The downfield chemical shifts of C-16 (δ_C 74.0) and C-29 (δ_C 73.8) and the broad singlet of H-16 (δ_H 4.42) revealed that the C-29 oxymethylene and the C-16 hydroxy group are both α-oriented. On the basis of the above analyses, the structure of compound **1** was established as 16,23,29-trihydroxy-3-oxo-olean-12-en-28-oic acid.

Compound **2** was isolated as a white, amorphous powder, and its molecular formula was established as $C_{31}H_{50}O_7$ by HR-ESITOFMS at m/z 535.3651 $[M + H]^+$ (calcd for $C_{31}H_{51}O_7$, 535.3635). The FT-IR spectrum of **2** revealed hydroxy and carboxy groups on the basis of absorption bands at 3411 and 1701 cm^{-1}, respectively. The ^{1}H NMR spectrum of **2** exhibited the presence of five tertiary methyl singlets at δ_H 1.08 (Me-24), 1.10 (Me-25), 0.82 (Me-26), 1.15 (Me-27), and 0.90 (Me-30) and a broad singlet for an olefinic proton at δ_H 5.26 (H-12) characteristic of the Δ^{12} proton in the pentacyclic triterpenes. The ^{1}H NMR spectrum also showed signals for a methoxy group at δ_H 3.59 (3H, s) and oxymethylene protons at δ_H 3.48 (1H, d, J = 10.8 Hz, H-23a), 3.18 (1H, d, J = 10.8 Hz, H-23b), and 3.16 (2H, s, H-29). The ^{13}C NMR and DEPT spectra displayed the presence of 31 carbons, including six methyls, 11 methylenes, five methines, and nine quaternary carbons. The ^{13}C NMR spectrum (Table 1) of **2** exhibited signals of two carboxy carbons [δ_C 176.5 (C-3) and 180.8 (C-28)], an oxygenated quaternary carbon (δ_C 77.1, C-4), and a methoxy carbon (δ_C 51.1), suggesting that **2** possessed a 4-hydroxy-3,4-*seco*-3-oate ester structural moiety. This was supported by comparison of the NMR spectra of **2** with those of the similar compound 3,4-*seco*-olean-12-en-4-ol-3,28-dioic acid.[16] The difference between these two compounds is the presence of two oxymethylenes and a methoxy group in **2**. The positions of the two oxymethylene groups in **2** at δ_C 70.6 (C-23) and 73.4 (C-29) were determined by the HMBC cross-peaks (Figure 1) between δ_H 1.08 (Me-24)/C-23, δ_H 1.61 (H-5)/C-23, and Me-30/C-29, respectively. On the basis of these data, the planar structure of **2** was established. The relative configuration of **2** was determined by a NOE experiment (Figure 1). In the NOESY spectrum of **2**, the NOE correlations between H-12/H-11a, H-12/H-18, H-18/30, H_3-25/H_3-26, and H_3-25/H-6a revealed the β-orientations of H-11a, H-18, H_3-30, H_3-25, H_3-26, and H-6a. The NOE cross-peaks between H_3-27/H-9, H-9/H-6b, H-6b/H-5, and H-5/H-9 showed the α-orientations of

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H_3-27, H-9, and H-5. On the basis of these data, the structure of compound **2** was established as 4,23,29-trihydroxy-3,4-*seco*-olean-12-en-3-oat-28-oic acid.

Table 1. ^{13}C NMR Data for Compounds 1, 2, and Aglycones of Compounds 3−5[a]

position	1	2	3	4	5
1	37.8	34.7	41.8	39.5	39.5
2	35.7	28.9	27.6	26.6	26.5
3	218.9	176.5	73.8	82.8	83.8
4	47.3	77.1	44.3	43.9	44.0
5	52.5	45.2	49.9	47.8	48.2
6	19.6	22.3	68.7	18.9	18.9
7	32.4	32.1	41.2	33.4	33.3
8	39.6	39.4	39.8	40.7	40.7
9	45.9	39.3	49.5	49.2	49.2
10	36.5	41.2	37.5	37.7	37.7
11	23.6	23.2	24.6	24.1	24.1
12	122.6	123.0	124.1	123.8	123.8
13	144.0	143.9	144.3	145.0	145.0
14	41.9	42.5	43.5	43.1	43.0
15	35.2	27.9	29.0	29.0	29.0
16	74.0	23.1	24.1	24.7	24.6
17	48.9	47.0	48.1	48.2	48.1
18	40.5	41.0	42.5	42.6	42.6
19	41.5	40.3	47.3	47.3	47.3
20	35.7	35.9	31.6	31.6	31.6
21	30.3	28.3	35.0	35.0	35.0
22	30.6	32.1	33.2	33.4	33.4
23	67.1	70.6	66.7	64.2	64.8
24	16.9	21.4	14.2	13.6	13.5
25	14.8	19.8	17.8	16.6	16.7
26	16.7	16.9	19.0	18.0	17.9
27	26.3	25.1	26.5	26.5	26.5
28	180.2	180.8	178.2	178.2	178.2
29	73.8	73.4	33.6	33.6	33.6
30	19.3	18.5	24.1	24.2	24.2
3-OMe		51.1			

[a] Spectra were recorded at 150 MHz in methanol-d_4.

Compounds **3**−**5** were isolated as white, amorphous powders. The monosaccharides obtained after aqueous acid hydrolysis of each compound were identified as glucose, rhamnose, and arabinose by TLC comparison with authentic samples. The absolute configuration of the monosaccharides was determined to be D for glucose and L for rhamnose and arabinose by GC analysis of chiral derivatives of the monosaccharides in the hydrolysate of each compound (see Experimental Section). The relatively large coupling constants (7.2−8.4 Hz) for the anomeric protons in the ^{1}H NMR spectra (Table 2) of these compounds suggested the α-configurations of the arabinopyranosyl moieties and the β-configurations of the glucopyranosyl moieties. The α-configurations of the rhamnopyranosyl moieties were determined from the small coupling constants (1.2 Hz) observed for the anomeric protons (Table 2).

The molecular formula of compound **3** was determined to be $C_{36}H_{58}O_{10}$ from the pseudomolecular ion peak $[M + H]^+$ at m/z 651.4133 (calcd for $C_{36}H_{59}O_{10}$, 651.4108) in the HR-ESI-TOFMS. Its FT-IR spectrum revealed hydroxy and carboxy groups at 3367 and 1731 cm^{-1}, respectively. The ^{1}H NMR spectrum exhibited signals for six tertiary methyls at δ_H 1.02 (Me-24), 1.29 (Me-25), 1.03 (Me-26), 1.10 (Me-27), 0.88 (Me-29), and 0.90 (Me-30) and a broad singlet for an olefinic proton at δ_H 5.26 (H-12) characteristic of the Δ^{12} proton in the pentacyclic triterpenes. In addition, an anomeric proton was also observed at δ_H 5.35 (1H, d, J = 8.4 Hz) in the ^{1}H NMR spectrum. The ^{13}C NMR (Tables 1 and 2) and DEPT spectra exhibited 36 carbon signals, including six methyl, 10 methylene, 12 methine, and eight quaternary carbons. Comparison of NMR data of **3** with reported data led to identification of the aglycone of **3** as 3β,6β,23-trihydroxyolean-12-en-28-oic acid.[8] Acid hydrolysis of **3** with 10% HCl gave 3β,6β,23-trihydroxyolean-12-en-28-oic acid and D-glucose. From the above evidence, the structure of **3** was established to be 3β,6β,23-trihydroxyolean-12-en-28-oic acid 28-*O*-β-D-glucopyranoside.

The molecular formula of **4** was determined to be $C_{57}H_{90}O_{24}$ from the pseudomolecular ion peak $[M + H]^+$ at m/z 1159.5906 (calcd for $C_{57}H_{91}O_{24}$, 1159.5900) in the HR-ESITOFMS. The aglycone of **4** was determined as hederagenin by comparison of its NMR spectroscopic data with reported values.[13] Acid hydrolysis of **4** with 10% HCl produced hederagenin, L-arabinose, D-glucose, and L-rhamnose. The anomeric proton signals at δ_H 4.38 (1H, d, J = 7.8 Hz), 4.47 (1H, d, J = 7.8 Hz), 4.81 (1H, d, J = 1.2 Hz), and 5.31 (1H, d, J = 8.4 Hz) showed HMQC correlations

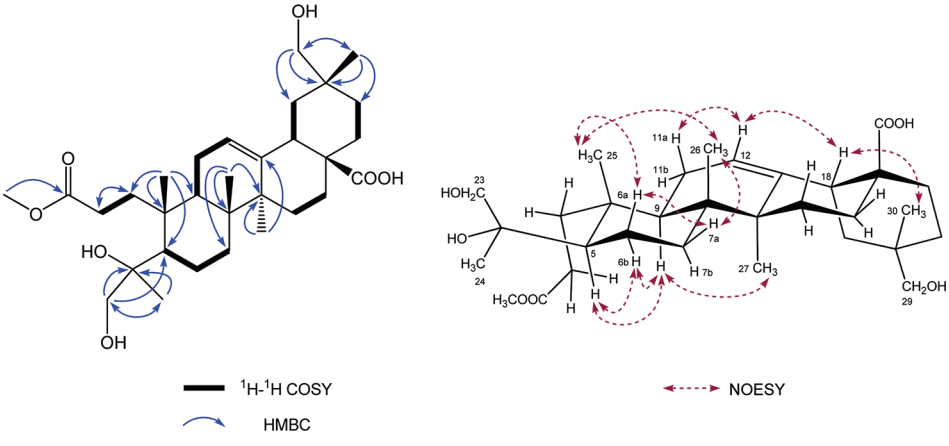

Figure 1. Selective ^{1}H−^{1}H COSY, HMBC, and NOESY correlations of **2**.

Table 2. ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) Data for Sugar Moieties of Compounds 3−5[a]

	3		4		5	
position	δ_H[b]	δ_C	δ_H	δ_C	δ_H	δ_C
3-*O*-Ara						
1			4.47, d (7.8)	104.4	4.44, d (7.2)	106.3
2			4.94	74.2	3.38	70.6
3			5.01	73.0	4.85	74.4
4			3.82	70.9	5.17	70.0
5			3.88, dd (12.6, 2.4)	64.9	3.87, dd (12.6, 2.4)	64.5
			3.60, d (12.0)		3.69, d (12.0)	
2-Ac			2.08, s	21.3		
				172.0		
3-Ac			2.11, s	21.1	1.99, s	20.9
				172.6		172.3
4-Ac					2.07, s	20.9
						172.2
28-*O*-Glc						
1	5.35, d (8.4)	95.9	5.31, d (8.4)	95.9	5.32, d (8.4)	95.8
2	3.29	74.0	3.30	73.8	3.31	73.8
3	3.39	78.3	3.50	78.2	3.39	78.2
4	3.32	71.2	3.94, dd (9.6, 6.0)	70.7	3.60	70.9
5	3.32	78.7	3.43, t (9.6)	76.8	3.43	76.7
6	3.79	62.5	3.77	69.5	3.76	69.4
	3.65, dd (12.0, 4.2)		4.06, d (11.4)		4.07, d (10.2)	
Glc						
1			4.38, d (7.8)	104.3	4.38, d (7.8)	104.3
2			3.20, t (9.0)	75.4	3.21, t (8.4)	75.3
3			3.26	76.9	3.27	76.8
4			3.52	79.6	3.51	79.5
5			3.38	78.2	3.38	78.1
6			3.62	61.9	3.63	61.9
			3.78		3.77	
Rha						
1			4.81, d (1.2)	103.0	4.82, d (1.2)	102.9
2			3.80	72.5	3.81	72.4
3			3.61	72.3	3.61	72.2
4			3.38	73.9	3.38	73.8
5			3.37	71.0	3.37	70.7
6			1.24, d (6.6)	17.9	1.24, d (6.0)	18.0

[a] Spectra were recorded in methanol-d_4. Coupling constants (J) are in Hz. [b] Protons are overlapped, unless otherwise stated (s, singlet; d, doublet; t, triplet).

with anomeric carbon signals at δ_C 104.3, 104.4, 103.0, and 95.9, respectively, indicating that **4** possessed four sugar units. The downfield chemical shift of C-3 (δ_C 82.8) and the upfield chemical shift of C-28 (δ_C 178.2) in the ^{13}C NMR spectrum of **4** (Table 1) indicated that this compound is a bisdesmosidic saponin. The ^{1}H and ^{13}C NMR data (Table 2) of the monosaccharide residues and the sequence of the sugar residues of **4** were assigned unambiguously on the basis of the HMQC and HMBC spectra. The location of the arabinose at C-3 was identified from the HMBC cross-peak between H-1 of arabinose (δ_H 4.47) and C-3 of the aglycone (δ_C 82.8). The sugar chain at C-28 was also established as α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl from the following HMBC correlations: H-1 of rhamnose (δ_H 4.81) with C-4 of glucose (δ_C 79.6), H-1 of glucose (δ_H 4.38) with C-6 of C-28-glucose (δ_C 69.5), and H-1 of C-28-glucose (δ_H 5.31) with C-28 of the aglycone (δ_C 178.2). In addition to the aglycone and sugar chains, two additional acetyl signals were observed in the NMR spectra. Attachment of the two acetyl groups to C-2 and C-3 of the arabinose was confirmed from HMBC correlations between H-2 (δ_C 4.94) and H-3 (δ_C 5.01) of arabinose with the carbonyl carbons of the two acetyl groups (δ_C 172.0 and 172.6), respectively. On the basis of the data obtained, the structure of **4** was identified as 3-*O*-[2,3-di-*O*-acetyl-α-L-arabinopyranosyl]-hederagenin 28-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside.

The HR-ESITOFMS of **5** exhibited the ion peak $[M + H]^+$ at m/z 1159.5876 (calcd for $C_{57}H_{91}O_{24}$, 1159.5900), consistent with the molecular formula $C_{57}H_{90}O_{24}$. Acid hydrolysis of **5** with 10% HCl gave hederagenin, L-arabinose, D-glucose, and L-rhamnose.

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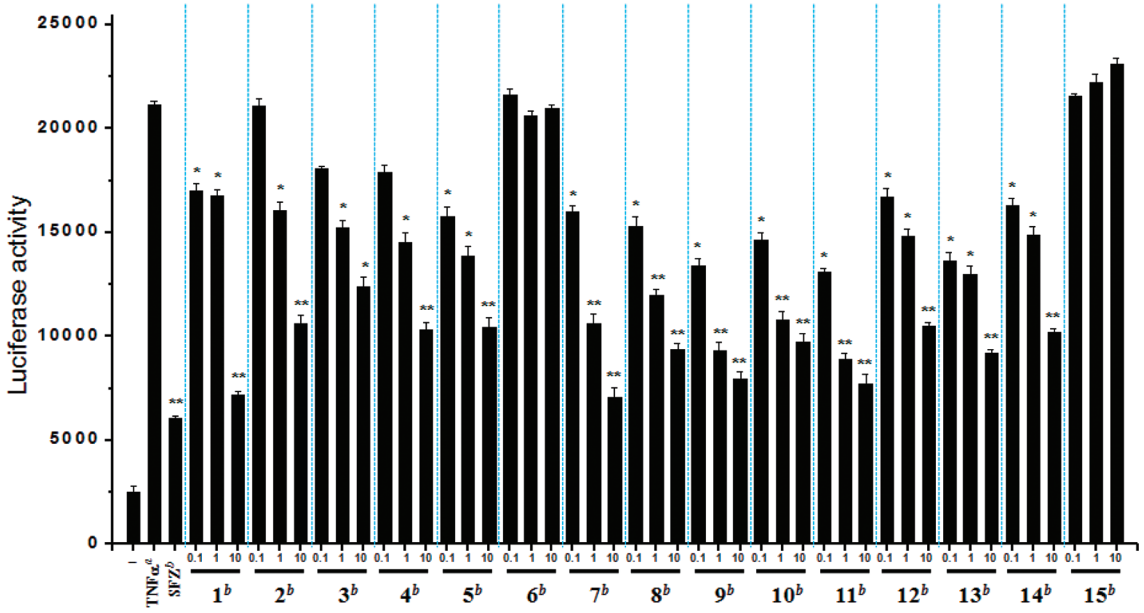

Figure 2. Effects of compounds **1**−**15** on the TNFα-induced NF-κB luciferase reporter activity in HepG2 cells. The values are mean ± SD (n = 6). [a]Stimulated with TNFα. [b]Stimulated with TNFα in the presence of **1**−**15** (0.1, 1, and 10 μM) and sulfasalazine. SFZ: sulfasalazine, positive control (10 μM). Statistical significance is indicated as * ($p < 0.05$) or ** ($p < 0.01$) as determined by Dunnett's multiple comparison test.

The downfield chemical shift of C-3 (δ_C 83.8) and the upfield chemical shift of C-28 (δ_C 178.2) in the ^{13}C NMR spectrum of **5** (Table 1) revealed that **5** is a bisdesmosidic saponin. Linkage of the sugar units was determined by the HMQC and HMBC experiments. Comparison of the NMR spectroscopic data of **5** with those of **4** (Tables 1 and 2) revealed that these compounds possessed the same trisaccharide moiety linked to C-28 of the aglycone. The HMBC correlation of H-1 of the arabinose [δ_H 4.44 (1H, d, J = 7.2 Hz)] with C-3 of the aglycone (δ_C 83.8) indicated that the arabinose was located at C-3. Moreover, two acetyl groups linked to C-3 and C-4 of the arabinose were identified from HMBC correlations between H-3 (δ_H 4.85) and H-4 (δ_H 5.17) of arabinose and the carbonyl carbons of the two acetyl groups (δ_C 172.3 and 172.2), respectively. On the basis of this information, the structure of **5** was established as 3-*O*-[3,4-di-*O*-acetyl-α-L-arabinopyranosyl]hederagenin 28-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside.

The anti-inflammatory activity of compounds **1**−**15** was evaluated through inhibition of a TNFα-induced NF-κB luciferase reporter and by attenuation of TNFα-induced pro-inflammatory gene (iNOS and COX-2) expression in HepG2 cells.

The NF-κB luciferase assay is designed to monitor the activity of NF-κB-regulated signal transduction pathways in cultured cells. The NF-κB-responsive luciferase construct encodes the firefly luciferase reporter gene under the control of a minimal CMV promoter and tandem repeats of the NF-κB transcriptional response element. Using this assay, the inhibitory activity of compounds **1**−**15** on NF-κB activation is readily monitored. Pro-inflammatory agents, such as TNFα, are known to activate the NF-κB pathway.[17] HepG2 cells transfected with the NF-κB luciferase reporter plasmid exhibited approximately a 9-fold increase in the luciferase signal after treatment with 10 ng/mL of TNFα, indicating that transcriptional activity increased compared to untreated cells. The results showed that compounds **1**−**5** and **7**−**14** inhibited TNFα-induced NF-κB transcriptional activity in HepG2 cells in a dose-dependent manner (Figure 2), with IC_{50} values ranging from 0.6 to 16.4 μM (Table 3). Among the compounds tested, compounds **7**, **9**, and **11** showed the most potent effects, with IC_{50} values of 1.4, 0.8, and 0.6 μM, respectively. Remarkably, the inhibitory effects of compounds **9** and **11** were more potent than that of the anti-inflammatory chemotherapy drug sulfasalazine (IC_{50} = 0.9 μM). Other compounds (**1**−**5**, **8**, **10**, and **12**−**14**) exhibited significant activity with IC_{50} values ranging from 3.9 to 16.4 μM.

Table 3. Inhibitory Effects of Compounds 1−15 on the TNFα-Induced NF-κB Transcriptional Activity[a]

compound	IC_{50} (μM)	compound	IC_{50} (μM)
1	6.5 ± 0.35	**9**	0.8 ± 0.05
2	9.4 ± 0.55	**10**	3.9 ± 0.19
3	16.4 ± 0.54	**11**	0.6 ± 0.03
4	8.9 ± 0.65	**12**	9.1 ± 0.63
5	9.3 ± 0.75	**13**	6.2 ± 0.25
6	>20[b]	**14**	9.2 ± 0.54
7	1.4 ± 0.27	**15**	>20
8	5.5 ± 0.32	sulfasalazine	0.9 ± 0.2

[a] The values are mean ± SD (n = 6). [b] A compound is considered inactive with IC_{50} > 20 μM.

Since NF-κB is an important transcription factor involved in regulating the expression of inflammatory NF-κB target genes such as iNOS and COX-2,[18,19] we investigated the effects of compounds **1**−**5** and **7**−**14** on the expression of these genes in TNFα-stimulated HepG2 cells using RT-PCR. HepG2 cells treated with 10 ng/mL TNFα significantly upregulated the mRNA expression of the NF-κB target genes COX-2 and iNOS. Consistent with their inhibitory activity toward NF-κB, compounds **1**−**5** and **7**−**14** significantly inhibited the induction of COX-2 and iNOS mRNA in a dose-dependent manner (Figure 3), indicating that these compounds reduced transcription of these genes. Moreover, the housekeeping protein β-actin was not changed by the presence of compounds **1**−**5** and **7**−**14** at the same concentration.

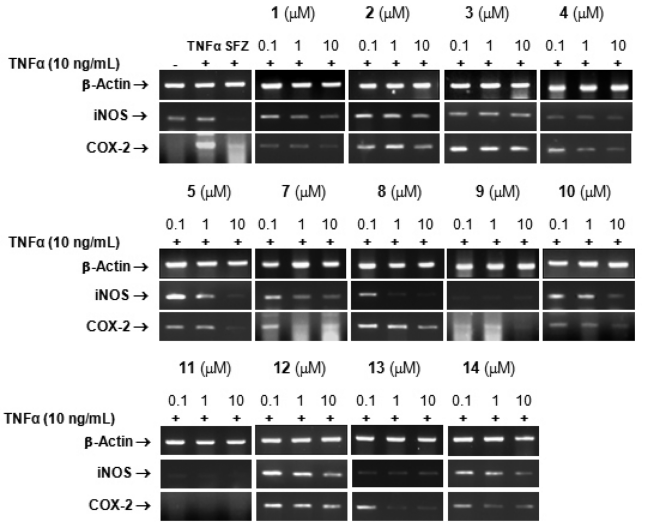

Figure 3. Effects of compounds **1**−**5** and **7**−**14** on iNOS and COX-2 mRNA expression in HepG2 cells.

The anti-inflammatory activity, in combination with the structural properties of compounds **1**−**15**, allowed us to infer certain guidelines regarding the structure−activity relationship. The results showed that compound **15** was not active, whereas all saponins were active, suggesting that the sugar moieties play essential roles in the anti-inflammatory activity. The hydroxy group at C-6 also plays an important role in the anti-inflammatory activity on the basis of a comparison of the structure and activity of compound **15** with those of **3** and **7**. The structures of compounds **4**, **5**, **9**, **10**, **11**, and **14** were found to be similar, except for the sugar moieties located at C-3 of the aglycones. When the arabinose unit at C-3 of the aglycones linked to other saccharides, such as a rhamnose (**9**), a rhamnose-xylose chain (**11**), or a rhamnose and glucose (**10**), the anti-inflammatory activity increased significantly as compared to single arabinose units at C-3 of the aglycones of **4**, **5**, and **14**. Also, the acetyl groups on the arabinose units in compounds **4** and **5** did not affect the activity compared to the activity of compounds **4**, **5**, and **14**. Moreover, the carbonyl (C-3) and hydroxy group at C-16 of compound **1** and the ring A-3,4-*seco* of **2** also played important roles in the anti-inflammatory activity, on the basis of comparison of the structure and activity of compounds **1**, **2**, and **6**. These data may be useful in evaluating the structure−function relationship of other oleanane-type saponins and their aglycons, as well as to develop novel anti-inflammatory agents for medical uses.

Cell viability, as measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method,[20] showed that compounds **1**−**5** and **7**−**14** had no significant cytotoxicity in HepG2 cells at concentrations that effectively inhibited NF-κB activation and induction of COX-2 and iNOS gene expression (data not shown).

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were determined using a Jasco DIP-370 digital polarimeter. The FT-IR spectra were measured using a Jasco Report-100 infrared spectrometer. ESI mass spectra were obtained using an Agilent 1200 LC-MSD Trap spectrometer. GC was carried out on a Shimadzu-2010 spectrometer: detector, FID; detection temperature, 300 °C; column, SPB-1 (0.25 mm i.d. × 30 m); column temperature, 230 °C; carrier gas, He (2 mL/min); injection temperature, 250 °C; injection volume, 0.5 μL. HR-ESITOF mass spectra were obtained using a JEOL JMS-T100LC spectrometer. The NMR spectra were recorded on a JEOL ECA 600 spectrometer using TMS as an internal standard. TLC was performed on Kieselgel 60 F254 (1.05715; Merck, Darmstadt, Germany) or RP-18 F254s (Merck) plates. Spots were visualized by spraying with 10% aqueous H_2SO_4 solution, followed by heating. CC was performed on silica gel (Kieselgel 60, 70−230 mesh and 230−400 mesh, Merck) and YMC RP-18 resins.

Plant Material. The stem bark of of *K. pictus* was purchased from an herbal market at Kumsan, Chungnam, Korea, in August 2009. The plant material was identified by one of us (Y.H.K.). A voucher specimen (CNU09105) was deposited at the herbarium, College of Pharmacy, Chungnam National University.

Extraction and Isolation. The dried stem bark of *K. pictus* (3 kg) was extracted with hot MeOH. After concentration, the MeOH extract (290 g) was suspended in H_2O and then partitioned successively with *n*-hexane, CH_2Cl_2, and EtOAc to give *n*-hexane (A, 50 g), CH_2Cl_2 (B, 30 g), EtOAc (C, 15 g), and water (D, 195 g) fractions, respectively. Fraction C was chromatographed over silica gel, eluting with MeOH in CH_2Cl_2 (0−100%, stepwise), yielding five fractions (C1−C5). Fraction C1 (2 g) was chromatographed over a silica gel column eluting with CH_2Cl_2−MeOH (10:1) and further purified by YMC RP chromatography using MeOH−H_2O (3:1) as eluent to afford **1** (6 mg) and **2** (10 mg). Fraction C2 (2.2 g) was chromatographed over a silica gel column eluting with CH_2Cl_2−MeOH (17:1) to obtain **6** (10 mg) and **7** (16 mg). Fraction C4 (1.7 g) was separated by YMC RP chromatography using MeOH−H_2O (3:1) as eluent to give **12** (45 mg) and **15** (20 mg). Fraction D was chromatographed on a column of highly porous polymer (Diaion HP-20) and eluted with H_2O and MeOH, successively, to give three fractions (D1−D3). Fraction D2 (26 g) was chromatographed over silica gel, eluting with MeOH in CH_2Cl_2 (0−100%, stepwise), to provide five subfractions (D2A−D2E). Subfraction D2C (2.3 g) was separated by YMC RP chromatography, using MeOH−H_2O (1:1) as eluent, and further purified by CC over silica gel, eluting with CH_2Cl_2−MeOH−H_2O (5:1:0.1), to obtain **9** (55 mg) and **11** (65 mg). Subfraction D2D (3.5 g) was separated by CC over silica gel, using CH_2Cl_2−MeOH−H_2O (4:1:0.1) as eluents, and further purified by YMC RP chromatography, eluting with MeOH−H_2O (1:1), to give **10** (57 mg). Fraction D3 (35 g) was chromatographed over silica gel, eluting with MeOH in CH_2Cl_2 (0−100%, stepwise), to provide four subfractions (D3A−D3D). Subfraction D3B (6.8 g) was then separated by CC over silica gel, using CH_2Cl_2−MeOH−H_2O (5:1:0.1) as eluents, and further purified by YMC RP chromatography, eluting with MeOH−H_2O (2:1), to afford **3** (16 mg), **8** (18 mg), and **13** (15 mg). Subfraction D3D (5.6 g) was separated by YMC RP chromatography, using MeOH−H_2O (15:10) as eluent, and further purified by CC over silica gel, eluting with CH_2Cl_2−MeOH− H_2O (4:1:0.1), to obtain **4** (10 mg), **5** (9 mg), and **14** (23 mg).

16,23,29-Trihydroxy-3-oxo-olean-12-en-28-oic acid (***1***): white, amorphous powder; $[\alpha]^{25}_D$ +4.8 (*c* 0.09, MeOH); FT-IR (CH_3CN) ν_{max} 3429, 2931, 1729, 1699, 1456, 1376, 1260, 1035, 862, 799 cm^{-1}; ^1H NMR data (methanol-d_4, 600 MHz) δ 5.33 (1H, br s, H-12), 4.42 (1H, br s, H-16), 3.57 (1H, d, J = 11.4 Hz, H-23a), 3.31 (1H, d, J = 11.4 Hz, H-23b), 3.16 (2H, d, J = 1.8 Hz, H-29), 3.03 (1H, dd, J = 14.4, 4.2 Hz, H-18), 1.85 (1H, m, H-15a), 1.39 (3H, s, Me-27), 1.37 (1H, m, H-15b), 1.02 (3H, s, Me-25), 0.97 (3H, s, Me-30), 0.88 (3H, s, Me-24), 0.85 (3H, s, Me-26); ^{13}C NMR data (methanol-d_4, 150 MHz), see Table 1; HR-ESITOFMS *m*/*z* 503.3350 $[M + H]^+$ (calcd for $C_{30}H_{47}O_6$, 503.3373).

4 23,29-Trihydroxy-3,4-seco-olean-12-en-3-oat-28-oic acid (***2***): white, amorphous powder; $[\alpha]^{25}_D$ +40 (*c* 0.04, MeOH); FT-IR (CH_3CN) ν_{max} 3411, 2935, 1701, 1456, 1436, 1384, 1265, 1199, 1175, 1042, 933 cm^{-1}; ^1H NMR data (methanol-d_4, 600 MHz) δ 3.59 (3H, s, MeO-3), 1.15 (3H, s, Me-27), 1.10 (3H, s, Me-25), 1.08 (3H, s, Me-24), 0.90 (3H, s, Me-30), 0.82 (3H, s, Me-26), 5.26 (1H, br s, H-12), 3.48 (1H, d, J = 10.8 Hz,

H-23a), 3.18 (1H, d, J = 10.8 Hz, H-23b), 3.16 (2H, s, H-29), 2.87 (1H, dd, J = 13.8, 4.2 Hz, H-18), 2.59 (1H, m, H-2a), 2.39 (1H, m, H-1a), 2.13 (1H, m, H-2b), 1.98 (1H, m, H-16a), 1.87 (1H, m, H-11a), 1.80 (1H, m, H-9), 1.77 (1H, m, H-19a), 1.75 (1H, m, H-7a), 1.71 (1H, m, H-15a), 1.61 (1H, m, H-5), 1.57 (3H, m, H-1b, H-11b, H-16b), 1.56 (1H, m, H-22a), 1.47 (1H, m, H-6a), 1.46 (1H, m, H-7b), 1.45 (1H, m, H-21a), 1.28 (1H, m, H-6b), 1.23 (1H, m, H-22b), 1.10 (1H, m, H-21b), 1.07 (1H, m, H-15b), 1.05 (1H, m, H-19b); ^{13}C NMR data (methanol-d_4, 150 MHz), see Table 1; HR-ESITOFMS m/z 535.3651 $[M + H]^+$ (calcd for $C_{31}H_{51}O_7$, 535.3635).

*3,6β,23-Trihydroxyolean-12-en-28-oic acid 28-O-β-D-glucopyranoside (**3**):* white, amorphous powder; $[\alpha]^{25}_D$ +55.3 (c 0.1, MeOH); FT-IR (CH_3CN) ν_{max} 3367, 2928, 1731, 1456, 1384, 1231, 1063, 1028, 933 cm^{-1}; ^{1}H NMR data (methanol-d_4, 600 MHz) δ 1.29 (3H, s, Me-25), 1.10 (3H, s, Me-27), 1.03 (3H, s, Me-26), 1.02 (3H, s, Me-24), 0.90 (3H, s, Me-30), 0.88 (3H, s, Me-29), 5.26 (1H, br s, H-12), 4.34 (1H, br s, H-6), 3.57 (1H, d, J = 10.8 Hz, H-23a), 3.53 (1H, dd, J = 12.0, 4.2 Hz, H-3), 3.44 (1H, d, J = 10.8 Hz, H-23b); ^{13}C NMR data (methanol-d_4, 150 MHz), see Tables 1 and 2; HR-ESITOFMS m/z 651.4133 $[M + H]^+$ (calcd for $C_{36}H_{59}O_{10}$, 651.4108).

*3-O-[2,3-Di-O-acetyl-α-L-arabinopyranosyl]hederagenin 28-O-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (**4**):* white, amorphous powder; $[\alpha]^{25}_D$ +11 (c 0.1, MeOH); FT-IR (CH_3CN) ν_{max} 3367, 2929, 1732, 1434, 1371, 1237, 1023 cm^{-1}; ^{1}H NMR data (methanol-d_4, 600 MHz) δ 1.13 (3H, s, Me-27), 0.94 (3H, s, Me-25), 0.91 (3H, s, Me-30), 0.88 (3H, s, Me-29), 0.76 (3H, s, Me-26), 0.55 (3H, s, Me-24), 5.22 (1H, br s, H-12), 3.81 (1H, d, J = 11.4 Hz, H-23a), 3.60 (1H, m, H-3), 3.29 (1H, d, J = 11.4 Hz, H-23b); ^{13}C NMR data (methanol-d_4, 150 MHz), see Tables 1 and 2; HR-ESITOFMS m/z 1159.5906 $[M + H]^+$ (cacld for $C_{57}H_{91}O_{24}$, 1159.5900).

*3-O-[3,4-Di-O-acetyl-α-L-arabinopyranosyl]hederagenin 28-O-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (**5**):* white, amorphous powder; $[\alpha]^{25}_D$ +6.3 (c 0.17, MeOH); FT-IR (CH_3CN) ν_{max} 3368, 2929, 1727, 1448, 1373, 1228, 1029 cm^{-1}; ^{1}H NMR data (methanol-d_4, 600 MHz) δ 1.14 (3H, s, Me-27), 0.96 (3H, s, Me-25), 0.91 (3H, s, Me-30), 0.88 (3H, s, Me-29), 0.77 (3H, s, Me-26), 0.69 (3H, s, Me-24), 5.22 (1H, br s, H-12), 3.58 (1H, d, J = 11.4 Hz, H-23a), 3.60 (1H, m, H-3), 3.26 (1H, d, J = 11.4 Hz, H-23b); ^{13}C NMR data (methanol-d_4, 150 MHz), see Tables 1 and 2; HR-ESI-TOFMS m/z 1159.5876 $[M + H]^+$ (cacld for $C_{57}H_{91}O_{24}$, 1159.5900).

Acid Hydrolysis of Saponins. Each saponin (4 mg) was heated in 3 mL of 10% HCl−dioxane (1:1) at 80 °C for 3 h. After the solvent was removed *in vacuo*, and the residue was partitioned between EtOAc and H_2O to give the aglycone and the sugar, respectively. The sugar components in the aqueous layer were analyzed by silica gel TLC by comparison with standard sugars. The solvent system was CH_2Cl_2−MeOH−H_2O (2:1:0.2), and spots were visualized by spraying with 95% EtOH−H_2SO_4−anisaldehyde (9:0.5:0.5, v/v), then heated at 180 °C for 5 min. The R_f values of glucose, arabinose, and rhamnose by TLC was 0.30, 0.5, and 0.75, respectively. The results were confirmed by GC analysis. The aqueous layer was evaporated to dryness to give a residue, dissolved in anhydrous pyridine (100 μL), and then mixed with a pyridine solution of 0.1 M L-cysteine methyl ester hydrochloride (100 μL). After warming at 60 °C for 2 h, trimethylsilylimidazole solution was added, and the reaction solution was warmed at 60 °C for 2 h. The mixture was evaporated *in vacuo* to give a dried product, which was partitioned between *n*-hexane and H_2O. The *n*-hexane layer was filtered and analyzed by GC. The retention times of the persilylated monosaccharide derivatives were as follows: L-arabinose (t_R, 4.72), L-rhamnose (t_R, 5.31 min), and D-glucose (t_R, 14.11 min) were confirmed by comparison with those of authentic standards.

In Vitro Anti-inflammatory Assay. *NF-κB-Luciferase Assay.* The NF-κB-luciferase plasmid was first transfected into HepG2 cells. After a limited amount of time, the cells were lysed and luciferin, the substrate of luciferase, was introduced into the cellular extract along with Mg^{2+} and an excess of ATP. Under these conditions, luciferase enzymes expressed by the reporter vector could catalyze the oxidative carboxylation of luciferin. Cells were seeded at 1.5 × 10^5 cells per well in 12-well plates and grown for 24 h. All cells were transfected using WelFect M Gold (WelGENE Inc., Daegu, South Korea), as guided by the manufacturer. Luciferase activity was assayed using an LB 953 Autolumat (EG&G Berthold, Nashua, NH).[21] The transfected HepG2 cells were pretreated for 1 h with either vehicle (DMSO) or compounds, followed by 1 h of treatment with 10 ng/mL TNFα. Unstimulated HepG2 cells were used as a negative control (−). Cells were then harvested, and luciferase activity was assayed. The NF-κB-luciferase plasmid was kindly provided by Dr. Kyoon E. Kim (Chungnam National University, Daejeon, Korea).

RNA Preparation and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Total RNA was extracted using Easy-blue reagent (Intron Biotechnology, Seoul, Korea). Approximately 2 μg of total RNA was subjected to reverse transcription using Moloney murine leukemia virus reverse transcriptase and oligo-dT primers (Promega, Madison, WI) for 1 h at 42 °C. PCR for synthetic cDNA was performed using a Taq polymerase premixture (TaKaRa, Japan). The PCR products were separated by electrophoresis on 1% agarose gels and stained with EtBr. PCR was conducted with the following primer pairs: iNOS sense 5′-TCATCCGCTATGCTGGCTAC-3′, iNOS antisense 5′-CTCAGGGTCACGGCCATTG-3′, COX-2 sense 5′-GCCCAG-CACTTCACGCATCAG-3′, COX-2 antisense 5′-GACCAGGCAC-CAGACCAAAGACC-3′, β-actin sense 5′-TCACCCACACTGTGC-CCATCTACG-3′, and β-actin antisense 5′-CAGCGGAACCGCT-CATTGCCAATG-3′. The specificity of products generated by each set of primers was examined using gel electrophoresis and further confirmed by a melting curve analysis.

HepG2 cells were pretreated in the absence and presence of compounds for 1 h, then exposed to 10 ng/mL TNFα for 6 h. Total mRNA was prepared from the cell pellets using Easy-blue. The levels of mRNA were assessed by RT-PCR.

■ ASSOCIATED CONTENT

Supporting Information. FT-IR, 1D and 2D NMR, and HR-ESI-TOF mass spectra for the new compounds **1**−**5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Kim, T. J. *Korean Resources Plants*; Seoul National University Press: Seoul, 1996; Vol. 2, pp 169−200.

(2) Shao, C.-J.; Kasai, R.; Xu, J.-D.; Tanaka, O. *Chem. Pharm. Bull.* **1989**, *37*, 311–314.

(3) Sano, K.; Sanada, S.; Ida, Y.; Shoji, J. *Chem. Pharm. Bull.* **1991**, *39*, 865–870.

(4) Lee, K. T.; Sohn, I. C.; Park, H. J.; Kim, D. W.; Jung, K. O.; Park, K. Y. *Planta Med.* **2000**, *66*, 329–332.
(5) Park, H. J.; Kim, D. H.; Choi, J. W.; Park, J. H.; Han, Y. N. *Arch. Pharm. Res.* **1998**, *21*, 24–29.
(6) Lee, E. B.; Li, D. W.; Hyun, J. E.; Kim, I. H.; Whang, W. K. *J. Ethnopharmacol.* **2001**, *77*, 197–201.
(7) Miyakoshi, M.; Shirasuna, K.; Hirai, Y.; Shingu, K.; Isoda, S.; Shoji, J.; Ida, Y.; Shimizu, T. *J. Nat. Prod.* **1999**, *62*, 445–448.
(8) Khan, I. A.; Sticher, O.; Rali, T. *J. Nat. Prod.* **1993**, *56*, 2163–2165.
(9) Sawada, H.; Miyakoshi, M.; Isoda, S.; Ida, Y.; Shoji, J. *Phytochemistry* **1993**, *34*, 1117–1121.
(10) Panov, D. A.; Grishkovets, V. I.; Kachala, V. V.; Shashkov, A. S. *Chem. Nat. Compd.* **2006**, *42*, 61–66.
(11) Kizu, H.; Hirabayashi, S.; Suzuki, M.; Tomimori, T. *Chem. Pharm. Bull.* **1985**, *33*, 3473–3478.
(12) Strigina, L. I.; Chetyrina, N. S.; Isakov, V. V.; Elkin, Y. N.; Dzizenko, A. K.; Elyakov, G. B. *Phytochemistry* **1975**, *14*, 1583–1586.
(13) Tori, K.; Sera, S.; Shimaoka, A.; Tomita, Y. *Tetrahedron Lett.* **1974**, *15*, 4227–4230.
(14) Shamma, M.; Glick, R. E.; Mumma, R. O. *J. Org. Chem.* **1962**, *27*, 4512–4517.
(15) Verma, D. K.; Singh, S. K.; Nath, G. *Indian Drugs* **1997**, *34*, 390–392.
(16) Shirane, N.; Hashimoto, Y.; Ueda, K.; Takenaka, H.; Katoh, K. *Phytochemistry* **1996**, *43*, 99–104.
(17) Paul, A. G. *Eukaryon* **2005**, *1*, 4–5.
(18) Wong, H. R.; Menendez, I. Y. *Biochem. Biophys. Res. Commun.* **1999**, *262*, 375–380.
(19) Pahl, H. L. *Oncogene* **1999**, *18*, 6853–6866.
(20) Scudiero, D. A.; Shoemaker, R. H.; Paull, K. D.; Monks, A.; Tierney, S.; Nofziger, T. H.; Currens, M. J.; Seniff, D.; Boyd, M. R. *Cancer Res.* **1988**, *48*, 4827–4833.
(21) Kim, K. K.; Park, K. S.; Song, S. B.; Kim, K. E. *Mol. Carcinog.* **2010**, *49*, 259–270.