CYTOTOXIC TRITERPENOIDS FROM *Cornus kousa* FRUITS

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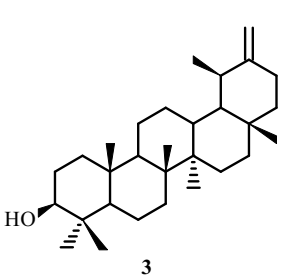
Cornus kousa Burg. (Cornaceae) is a small deciduous tree characterized by brilliant, colorful, and attractive flowers and fruits. It is widely distributed in the northern hemisphere, such as eastern Asia and eastern and northern parts of the United States. *C. kousa* is widely cultivated for ornamental purposes in eastern Asia. The fruits of this plant have been used as a hemostatic agent and for the treatment of diarrhea in Korean traditional medicine [1]. Additionally, immuno-regulatory properties for this fruit extract have been reported [2]. Some chemical constituents such as isoquercitrin, gallic acid, tannin, phenolics, and flavonoids [3] have been reported to be present in the leaves of *C. kousa*. Also, our previous phytochemical research on the fruits of this plant demonstrated the presence of steroids [4], cytotoxic lignans [5], and flavonoids [6].

Bioactivity-guided fractionation led to the isolation of cytotoxic triterpenoids from the fruits of *C. kousa*. When the methanol extracts of *C. kousa* were developed on silica gel TLC, pink and purple spots appeared after spraying with 10% H_2SO_4 solution and heating, indicating the presence of triterpenoids in the extracts. The methanol extract was fractionated into an EtOAc layer, an *n*-BuOH layer, and a H_2O layer through solvent fractionation. The repeated silica gel (SiO_2), octadecyl silica gel (ODS), and Sephadex LH-20 column chromatographies (c.c.) of EtOAc fractions yielded ten triterpenoids, compounds **1**–**10**. The chemical structures of the triterpenoids were determined from spectroscopic analysis, including EI/FAB-MS, IR, PMR, ^{13}C NMR, DEPT, and 2D NMR (COSY, HSQC, HMBC). The compounds were revealed to be ursolic acid (**1**) [7], lupeol (**2**) [8], taraxasterol (**3**) [9], betulinic acid (**4**) [10], betulinic aldehyde (**5**) [11], ursolic aldehyde (**6**) [7], arjunolic acid (**7**) [12], tormentic acid (**8**) [13], asiatic acid (**9**) [14], and 19α-hydroxyasiatic acid (**10**) [15]. Although these compounds were previously isolated from other plants, this is the first report of their isolation from *C. kousa*. All isolated compounds were evaluated for their cytotoxicity against human colon carcinoma (HCT-116), human breast carcinoma (MCF-7), human cervix carcinoma (HeLa), human ovary carcinoma (SK-OV-3), and human melanoma (SK-MEL-5) using the MTT assay. As shown in Table 2, some compounds inhibited the growth of human cancer cell lines. In conclusion, the findings of this study suggest that the methanolic extract of *C. kousa*, as well as its isolated triterpenoids, may prove useful for the treatment of human cancer.

Plant Materials. The fruits of *Cornus kousa* Burg. (Cornaceae) were collected at the experimental farm at Kyung Hee University in August 2006 and identified by Prof. Dae-Keun Kim, College of Pharmacy, Woosuk University, Jeonju, Korea. A voucher specimen (KHU060907) was reserved at the Laboratory of Natural Products Chemistry, Kyung Hee University, Yongin, Korea.



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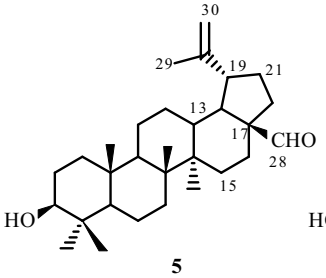

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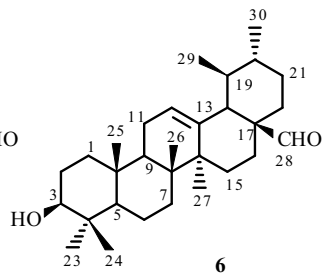

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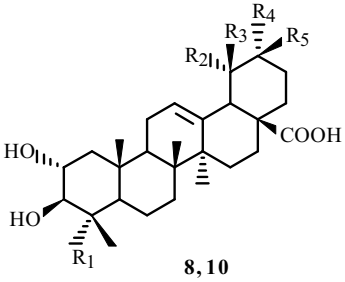

8, 10

8: $R_1 = R_3 = R_4 = CH_3$, $R_2 = OH$, $R_5 = H$
10: $R_1 = CH_2OH$, $R_2 = OH$, $R_3 = R_4 = CH_3$, $R_5 = H$

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TABLE 1. ^{13}C NMR Data of Compounds **1**, **4**, **5**, **6**, **8**, **9** (C_5D_5N), **2**, **3** ($CDCl_3$), **7**, and **10** (CD_3OD) Isolated from the Fruits of *C. kousa*

C atom	1	2	3*	4	5	6*	7	8*	9	10*
1	38.6	38.7	38.0	38.7	39.3	39.0	47.2	47.4	47.9	47.8
2	28.2	27.4	27.5	27.4	27.3	27.2	69.6	68.7	68.9	68.8
3	78.1	78.9	78.9	80.3	78.0	78.1	78.1	83.3	78.1	78.3
4	39.0	38.8	38.7	38.8	39.6	39.4	43.3	39.7	42.7	43.6
5	55.8	55.3	55.3	57.4	55.8	55.4	47.8	55.4	48.0	48.2
6	18.4	18.2	18.3	18.3	18.8	18.4	17.9	18.3	18.6	18.6
7	33.1	34.2	34.3	34.3	34.7	32.8	33.3	32.9	33.3	33.1
8	41.7	40.7	42.1	42.0	41.1	39.3	40.4	40.5	40.1	40.4
9	47.6	50.4	50.4	51.5	50.7	48.0	47.6	47.6	48.1	47.9
10	37.0	37.1	37.2	37.2	37.5	37.0	38.1	38.7	38.9	38.3
11	23.3	20.9	21.1	20.8	21.0	23.3	24.0	24.2	23.9	24.1
12	125.5	25.0	24.8	25.5	25.9	126.4	123.2	126.5	125.5	128.0
13	139.2	38.0	38.8	38.4	39.0	138.2	145.2	139.6	139.2	140.0
14	42.0	42.7	43.0	43.6	42.8	42.2	42.7	42.3	43.8	42.1
15	29.2	27.3	27.4	30.5	28.7	28.2	28.8	31.1	31.2	29.2
16	24.5	35.5	40.0	32.1	29.1	23.4	24.0	26.9	28.8	26.3
17	47.6	42.9	36.3	58.1	59.4	50.2	47.0	48.3	48.2	48.2
18	53.6	48.3	48.3	48.5	48.0	52.6	42.7	54.4	53.6	54.5
19	39.0	47.9	37.7	50.4	48.3	39.2	46.8	72.6	39.5	72.6
20	38.9	150.8	150.8	151.3	150.1	39.0	31.6	41.9	39.9	42.3
21	30.5	29.8	24.0	29.7	29.4	30.2	34.9	26.9	25.0	27.0
22	36.8	39.9	40.8	37.0	33.3	33.2	33.8	38.7	37.6	38.4
23	28.4	27.9	28.0	27.9	28.4	29.8	66.2	29.3	66.4	66.5
24	16.7	15.3	15.4	16.1	16.2	16.7	13.9	17.6	14.5	14.2
25	15.8	16.1	16.0	17.2	16.5	15.8	17.5	16.8	17.5	17.2
26	17.5	15.9	16.1	16.9	16.6	17.6	17.7	17.4	17.6	17.3
27	23.5	14.5	14.6	14.9	14.4	23.3	26.5	24.7	24.5	24.6
28	179.9	18.0	19.3	180.1	206.4	207.1	181.6	179.6	180.5	180.7
29	17.1	19.2	25.1	19.4	19.1	17.0	33.6	27.3	17.6	26.8
30	21.0	109.3	109.1	110.5	110.5	21.2	24.1	16.9	21.5	16.7

*Assignments were carried out based on 2D-NMR (COSY, HSQC, HMBC) experiments.

General Experimental Procedure. Optical rotations were measured on a JASCO P-1010 digital polarimeter (Tokyo, Japan). Uncorrected melting points were measured with a Fisher-Johns (Fisher Scientific) Melting Point Apparatus (Waltham, USA). EI-MS and FAB-MS data were recorded on a JEOL JMSAX 505-WA (Tokyo, Japan). IR spectra were run on a Perkin–Elmer Spectrum One FT-IR spectrometer (Waltham, USA). PMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were taken on a Varian Unity Inova AS 400 FT-NMR spectrometer (Lake Forest, USA).

Extraction and Isolation of Triterpenoids. The dried and chopped fruits of *C. kousa* (10 kg) were extracted with 80% aqueous MeOH (10 L × 3) at room temperature. The extracts were partitioned successively between EtOAc (2 L × 3) and H_2O (2 L). The EtOAc extracts (44 g) were subjected to SiO_2 c.c. and eluted with *n*-hexane–EtOAc (4:1→2:1)→$CHCl_3$–MeOH (15:1→13:1→10:1), monitoring by thin layer chromatography (TLC) to provide 17 fractions (CKFE1 to CKFE17), which yielded compound **1** [1.58 g, V_e/V_t (elution volume/total volume) 0.66–0.70, TLC (SiO_2 F_{254}) R_f 0.70 in $CHCl_3$–MeOH (7:1)]. Subfraction CKFE4 (190 mg, V_e/V_t 0.21–0.25) was subjected to SiO_2 c.c. and eluted with *n*-hexane–EtOAc (7:1) to give eight fractions (CKFE4-1 to CKFE4-8). Subfraction CKFE4-2 (79 mg, V_e/V_t 0.15–0.40) was further purified by ODS c.c. using MeCN–H_2O (20:1) as eluting solution to give compound **2** [10.3 mg, V_e/V_t 0.70–0.80, TLC (RP-18 F_{254}) R_f 0.40 in MeCN–H_2O (10:1)]. Subfraction CKFE6 (2.40 g, V_e/V_t 0.32–0.40) was subjected to SiO_2 c.c. and eluted with *n*-hexane–EtOAc (7:1) to give 12 fractions (CKFE6-1 to CKFE6-12), which yielded compound **3** [22 mg, TLC (SiO_2 F_{254}) R_f 0.70 in *n*-hexane–EtOAc (1:1)]. Subfraction CKFE6-6 (478 mg, V_e/V_t 0.50–0.65) was subjected to ODS c.c. and eluted with MeOH–H_2O (10:1) to give five fractions (CKFE6-6-1 to CKFE6-6-5) and to ultimately produce compound **4** [35 mg, V_e/V_t 0.30–0.45, TLC (RP-18 F_{254}) R_f 0.55, MeOH–H_2O (5:1)]. Subfraction CKFE6-6-3 (45 mg, V_e/V_t 0.46–0.65) was subjected to Sephadex LH-20 c.c. and eluted with 80% MeOH to produce compound **5** [33 mg, V_e/V_t 0.50–0.90, TLC (RP-18 F_{254}) R_f 0.47, MeOH–H_2O (5:1)].

TABLE 2. Cytotoxicity of Triterpenoids Isolated from the Fruits of *C. kousa* against Human Cancer Cell Lines, IC_{50} values*

Compound	Cancer Cell Lines**				
	HCT-116	MCF-7	HeLa	SK-OV-3	SK-MEL-5
1	19.5	19.8	20.2	21.0	23.4
2	>200	>200	>200	>200	>200
3	100.0	53.3	49.5	100.0	49.6
4	17.4	17.6	15.6	18.0	15.8
5	19.0	17.0	15.0	18.3	32.2
6	200.0	198.2	60.5	>200	200.0
7	25.0	85.4	100.0	92.5	102.8
8	> 200	>200	>200	>200	>200
9	98.2	97.0	75.5	97.6	65.3
10	>200	>200	>200	>200	>200
Doxorubicin***	3.2	10.6	4.8	0.58	1.03
Paclitaxel***	0.05	0.9	1.1	0.46	0.15

*IC_{50} value is the concentration (μM) at which there is 50% inhibition of cell growth. IC_{50} values were calculated from regression lines using five different concentrations on triplicate experiments.

**HCT-116 (human colon carcinoma), MCF-7 (human breast carcinoma), HeLa (human cervix carcinoma), SK-OV-3 (human ovary carcinoma) and SK-MEL-5 (human melanoma).

*** Doxorubicin and paclitaxel were used as positive controls.

Subfraction CKFE7 (150 mg, V_e/V_t 0.41–0.46) was subjected to ODS c.c. and eluted with MeCN–H_2O (5:1) to give eight fractions (CKFE7-1 to CKFE7-8) which yielded compound **6** [5 mg, V_e/V_t 0.70–0.80, TLC (RP-18 F_{254}) R_f 0.50 in MeCN–H_2O (3:1)]. CKFE8 (10.3 g, V_e/V_t 0.47–0.65) was subjected to SiO_2 c.c. and eluted with $CHCl_3$–MeOH (100:1→50:1→10:1) to give 12 fractions (CKFE8-1 to CKFE8-12). CKFE8-8 (324 mg, V_e/V_t 0.60–0.75) was subjected to ODS c.c. and eluted with MeOH–H_2O (2:1) to give 11 fractions (CKFE8-8-1 to CKFE8-8-11), which produced compound **7** [55 mg, V_e/V_t 0.70–0.80, TLC (RP-18 F_{254}) R_f 0.35, MeOH–H_2O (3:1)]. Subfraction CKFE12 (1.76 g, V_e/V_t 0.76–0.80) was subjected to SiO_2 c.c. and eluted with $CHCl_3$–MeOH (14:1) to give 13 fractions (CKFE12-1 to CKFE12-13). Subfraction CKFE12-9 (66 mg, V_e/V_t 0.75–0.82) was subjected to ODS c.c. and eluted with MeOH–H_2O (3:1) to give three fractions (CKFE12-9-1 to CKFE12-9-3), which yielded compounds **8** [12 mg, V_e/V_t 0.10–0.35, TLC (RP-18 F_{254}) R_f 0.80 in MeOH–H_2O (4:1)] and **9** [20 mg, V_e/V_t 0.70–0.95, TLC (RP-18 F_{254}) R_f 0.50 in MeOH–H_2O (4:1)]. Subfraction CKFE12-7 (19 mg, V_e/V_t 0.75–0.82) was further purified by ODS c.c. using MeOH–H_2O (2:1) as eluting solution to give compound **10** [6.7 mg, V_e/V_t 0.20–0.40, TLC (RP-18 F_{254}) R_f 0.55 in MeOH–H_2O (3:1)].

Cytotoxicity Test. Cytotoxic activity of each triterpenoid was measured against five cell lines: human colon carcinoma (HCT-116), human breast carcinoma (MCF-7), human cervix carcinoma (HeLa), human ovary carcinoma (SK-OV-3), and human melanoma (SK-MEL-5), which were obtained from the Korean Cell Line Bank (KCLB, Korea). A modified microculture tetrazolium (MTT) assay was used [13, 16]. The activity of each compound was tested at several concentrations, and the IC_{50} values were calculated. Doxorubicin and paclitaxel were used as positive controls.

Taraxasterol (3). White powder (in $CHCl_3$), mp 225–227°C, $[\alpha]_D^{25}$ +52.2° (*c* 0.10, $CHCl_3$), EI-MS *m/z*: 426 $[M]^+$, IR spectrum (KBr, cm^{-1}): 3410, 2927, 1659. PMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 4.65 (1H, br.s, H-30a), 4.54 (1H, br.s, H-30b), 3.16 (1H, dd, J = 12.6, 4.8, H-3), 2.35 (1H, d, J = 11.2, H-18), 1.03 (3H, d, J = 6.4, H-29), 1.00 (3H, s, H-26), 0.98 (3H, s, H-23), 0.94 (3H, s, H-27), 0.85 (3H, s, H-25), 0.83 (3H, s, H-28), 0.78 (3H, s, H-24). ^{13}C NMR (100 MHz, $CDCl_3$, δ): see Table 1.

Betulinic Aldehyde (5). Colorless amorphous powder (in MeOH), mp 193°C, $[\alpha]_D^{20}$ +19.5° (*c* 0.10, $CHCl_3$), EI-MS *m/z*: 440 $[M]^+$, IR spectrum (KBr, cm^{-1}): 3413, 2850, 2760, 1720, 1651. PMR (400 MHz, C_5D_5N, δ, ppm, J/Hz): 9.80 (1H, s, H-28), 4.89 (1H, br.s, H-30a), 4.74 (1H, br.s, H-30b), 3.45 (1H, dd, J = 11.0, 4.8, H-3), 3.06 (1H, ddd, J = 11.0, 10.8, 4.9, H-19), 1.71 (3H, s, H-29), 1.21 (3H, s, H-27), 1.01 (3H, s, H-23), 0.95 (3H, s, H-25), 0.91 (3H, s, H-26), 0.80 (3H, s, H-24). ^{13}C NMR (100 MHz, C_5D_5N, δ): see Table 1.

Ursolic Aldehyde (6). White powder (in $CHCl_3$), mp 217–219°C, $[\alpha]_D^{25}$ +62.4° (*c* 0.90, $CHCl_3$), EI-MS *m/z*: 440 $[M]^+$, IR spectrum (CaF_2 window, $CHCl_3$, cm^{-1}): 3322, 2911, 2890, 1715, 1645. PMR (400 MHz, C_5D_5N, δ, ppm, J/Hz): 9.47 (1H, s, H-28), 5.36 (1H, br.s, H-12), 3.46 (1H, dd, J = 10.8, 4.8, H-3), 1.09 (3H, s, H-27), 0.98 (3H, s, H-23), 0.96 (3H, d, J = 6.4, H-30), 0.92 (3H, s, H-24), 0.84 (3H, d, J = 6.4, H-29), 0.76 (3H, s, H-25), 0.74 (3H, s, H-26). ^{13}C NMR (100 MHz, C_5D_5N, δ): see Table 1.

Tormentic Acid (8). White powder (in MeOH), mp 272–275°C, $[\alpha]_D^{20}$ +29.1° (*c* 0.10, MeOH), EI-MS *m/z*: 488 $[M]^+$, IR spectrum (KBr, cm^{-1}): 3447, 1754, 1696. PMR (400 MHz, C_5D_5N, δ, ppm, J/Hz): 5.58 (1H, br.s, H-12), 4.09 (1H, ddd, J = 10.0, 9.4, 3.6, H-2), 3.37 (1H, d, J = 9.4, H-3), 3.04 (1H, s, H-18), 1.70 (3H, s, H-27), 1.42 (3H, s, H-29), 1.26 (3H, s, H-23), 1.11 (3H, d, J = 5.7, H-30), 1.07 (3H, s, H-24), 1.00 (3H, s, H-25), 0.98 (3H, s, H-26). ^{13}C NMR (100 MHz, C_5D_5N, δ): see Table 1.

19α-Hydroxyasiatic Acid (10). Amorphous powder (in MeOH), mp 290–292°C, $[\alpha]_D^{25}$ +25.8° (*c* 0.50, MeOH), FAB-MS *m/z*: 527 $[M+Na]^+$, IR spectrum (CaF_2 window, $CHCl_3$, cm^{-1}) 3425, 2931, 1735, 1689, 1047. PMR (400 MHz, CD_3OD, δ, ppm, J/Hz): 5.25 (1H, br.s, H-12), 3.66 (1H, m, H-2), 3.44 (1H, d, J = 10.4, H-23a), 3.35 (1H, d, J = 9.6, H-3), 3.21 (1H, d, J = 10.4, H-23b), 2.45 (1H, s, H-18), 1.18 (3H, s, H-27), 1.12 (3H, s, H-29), 1.05 (3H, s, H-24), 0.97 (3H, J = 6.5, H-30), 0.85 (3H, s, H-25), 0.74 (3H, s, H-26). ^{13}C NMR (100 MHz, CD_3OD, δ): see Table 1.

Cytotoxic Activity of Isolated Triterpenoids against Human Cancer Cell Lines. Betulinic acid (**4**) and betulinic aldehyde (**5**), lupane triterpenoids having a carbonyl group at C-28, and ursolic acid (**1**) exhibited significant inhibitory activity against human cancer cell lines (Table 2).

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