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Short Communication

Cytotoxic Sterols from the Fruits of *Cornus kousa* Burg.

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Cornus kousa Burg. (Cornaceae), a deciduous tree distributed in the mountains of Korea, China, and Japan, is in flower from May to June. Its fruits are red or pink, 2-3 cm in diameter, and very delicious, and the seeds ripen from July to August. In Korea the fruit of this plant has traditionally been used as a hemostatic agent as well as for the treatment of diarrhea [Lee, 2003]. Recently, the immuno-regulatory properties of *C. kousa* fruit extract have been reported [Kim *et al.*, 2002]. Moreover, some chemical constituents such as isoquercitrin, gallic acid, tannin [Ryu *et al.*, 1971], phenolics and flavonoids [Shaiju *et al.*, 2006] have also been reported from the leaves of *C. kousa.* In addition, our previous phytochemical studies on the fruit of this plant demonstrated the presence of steroids [Lee *et al.*, 2006], lignans [Lee *et al.*, 2007a], and flavonoids [Lee *et al.*, 2007b].

Our ongoing work led to the isolation of bioactive sterols from the fruit of *C. kousa* Burg. Through the spectroscopic methods, the chemical structure was revealed to be 6α-hydroxystigmast-4-en-3-one. This steroid and another previously isolated steroid, 6β-hydroxystigmast-4-en-3-one [Lee *et al.*, 2006] were evaluated for their cytotoxicities against the human colon carcinoma (HCT-116), the human breast carcinoma (MCF-7), the human melanoma (SK-MEL-5), and the human ovary carcinoma (SK-OV-3) cell lines.

The fruits of *C. kousa* were collected from the experimental farm in Kyung Hee University, Suwon, Korea and identified by Prof. Dae-Keun Kim, College of Pharmacy, Woosuk University, Jeonju, Korea. A voucher specimen (KHU050914) is reserved at the Laboratory of Natural Products Chemistry, Kyung Hee University, Suwon, Korea.

The optical rotation was recorded on a P-1010 digital polarimeter (JASCO, Tokyo, Japan), and the melting point was measured with a Fisher-John's Melting Point Apparatus (Fisher Scientific, Pittsburg, PA). The UV spectrum was measured on a UV-1601 (Shimadzu, Kyoto, Japan). The IR spectrum was obtained with a Perkin Elmer Spectrum One FT-IR spectrometer (Perkin-Elmer, Norwalk, CT). EI-MS was recorded on a JMSAX 505-WA (JEOL, Tokyo, Japan). ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz), and 2D-NMR spectra were recorded on a AS-400 FT-NMR spectrometer (Varian, Palo Alto, CA). $CDCl_3$ with TMS as an internal standard was purchased from Sigma (St. Louis, MO). RPMI Medium 1640, Dulbecco's Modified Eagle Medium and Penicillin-Streptomycin were purchased from GIBCO BRL (Life Technologies Inc., Grand Island, NY). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and dimethyl sulfoxide (DMSO) were purchased from Sigma.

The dried and chopped fruits of *C. kousa* (1 kg) were extracted with 80% aqueous MeOH (3 L × 3) three times at room temperature. The extracts were successively partitioned with water (1 L), EtOAc (1 L × 3), and *n*-BuOH (0.8 L × 3). The EtOAc extract (4 g) was subjected to the silica gel column chromatography (c.c.) (4.5 x 60 cm) and eluted with *n*-hexane: EtOAc (3 : 1) → $CHCl_3$: MeOH (17 : 1 → 15 : 1 → 13 : 1 → 10 : 1, 1 L each). Monitoring by thin layer chromatography (TLC) produced 21 fractions (CKFE1 to CKFE21). Fraction CKFE5 [160 mg, V_e/V_t (elution volume/total volume) 0.31-0.35] was subjected to the octadecyl silica gel (ODS) c.c. (3 × 25 cm) and eluted with MeOH : H_2O (10 : 1, 300 mL) to give six subfractions (CKFE5-1 to CKFE5-6). Subfraction CKFE5-4 (32 mg, V_e/V_t 0.60-0.70) was applied to ODS c.c. (2.5 × 25 cm) and eluted with MeOH : H_2O (15 : 1, 300 mL) to ultimately produce compound **1** [18 mg, V_e/V_t 0.55-0.75, TLC (RP-18 F_{254}) R_f 0.5, MeOH : H_2O = 6 : 1].

Compound **1**: White powder ($CHCl_3$); mp 120°C; UV

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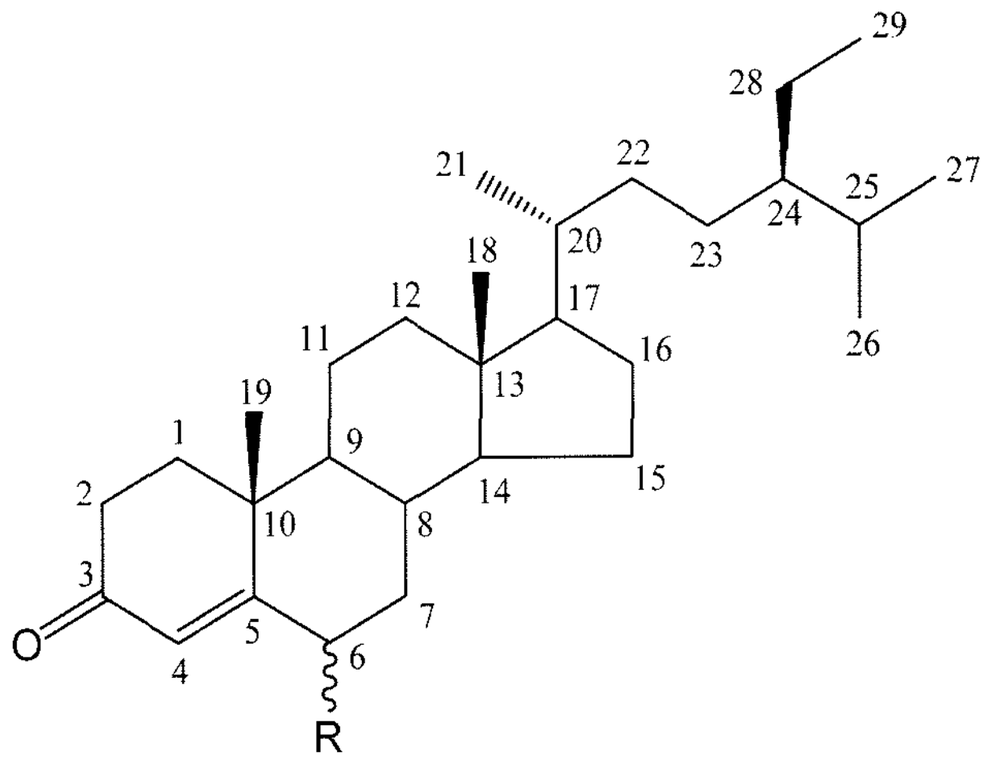
($CHCl_3$) λ_{max} 243 nm; $[\alpha]_D^{21}$ = 100.0° (*c* = 0.50, $CHCl_3$); EI/MS *m/z* (70 eV): 428 $[M]^+$, 413, 410, 287; IR (KBr, cm^{-1}): 3570, 1675, 1640; ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 6.16 (1H, br s, H-4), 4.33 (1H, ddd, *J* = 12.0, 5.6, 1.6 Hz, H-6), 1.17 (3H, s, H-19), 0.92 (3H, d, *J* = 6.8 Hz, H-21), 0.86 (3H, t, *J* = 7.6 Hz, H-29), 0.85 (3H, d, *J* 6.8 Hz, H-26), 0.82 (3H, d, *J* = 6.9 Hz, H-27), 0.71 (3H, s, H-18); ^{13}C-NMR (100 MHz, $CDCl_3$, δ): 199.50 (C-3), 171.71 (C-5), 119.52 (C-4), 68.66 (C-6), 55.94 (C-14), 55.55 (C-17), 53.74 (C-9), 45.81 (C-24), 42.46 (C-13), 41.46 (C-7), 41.46 (C-12), 39.46 (C-10), 36.27 (C-1), 36.12 (C-20), 34.17 (C-22), 33.82 (C-2), 29.75 (C-8), 29.15 (C-25), 28.19 (C-16), 26.06 (C-23), 24.23 (C-15), 23.10 (C-28), 21.07 (C-11), 19.87 (C-26), 19.07 (C-19), 18.75 (C-27), 18.33 (C-21), 12.04 (C-29), 12.00 (C-18).

6β-Hydroxystigmast-4-en-3-one (compound **2**): White powder ($CHCl_3$); mp 186°C; UV ($CHCl_3$) λ_{max} 215 nm; $[\alpha]_D^{21}$ = 11.0° (*c* = 0.70, $CHCl_3$); EI/MS *m/z* (70 eV): 428 $[M]^+$, 413, 410, 287; IR(KBr, cm^{-1}): 3570, 1675, 1640; ^{1}H-NMR and ^{13}C-NMR spectral data were consistent to those of Lee *et al.* [2006].

Cytotoxic activity of each compound was measured in four cell lines, HCT-116, MCF-7, SK-MEL-5, and SK-OV-3, obtained from the Korean Cell Line Bank (KCLB, Korea). A modified microculture tetrazolium (MTT) assay (Mosmann, 1983) was used. The activity of each compound was tested at several concentrations, and the IC_{50} values were calculated.

Compound **1**, was isolated as a white powder from $CHCl_3$, exhibiting a UV absorption maximum at 243 nm and a molecular ion peak (M^+) at *m/z* 428 in the EI/MS spectrum. The compound was freely soluble in chloroform. IR absorption bands at 3570, 1675, and 1640 cm^{-1} were characteristic of the hydroxyl, the ketone, and the aromatic groups, respectively. The NMR spectra of compound **1** were almost identical with those of the previously isolated steroid, 6β-hydroxystigmast-4-en-3-one (compound **2**), with the exception of the coupling pattern of H-6 (compound **1**, ddd, *J* = 12.0, 5.6, 1.6 Hz; compound **2**, br. s) and chemical shift of C-6 (compound **1**, δ 68.66; compound **2**, δ 73.10). The ^{1}H-NMR spectrum showed two tertiary singlet methyl signals at δ 0.71 (H-18), 1.17 (H-19) and three doublet methyl signals at δ 0.82 (*J* = 6.9 Hz, H-27), 0.85 (*J* = 6.8 Hz, H-26), and 0.92 (*J* = 6.8 Hz, H-21), and a triplet methyl signal at 0.86 (*J* = 7.6 Hz, H-29). Furthermore, the presence of one olefine proton at δ 6.16 (br s, H-4) indicated the presence of a stigmast-4-en type structure as found in compound **2**. An oxygenated methine proton exhibited a signal at δ 4.33 (ddd, *J* = 12.0, 5.6, 1.6, H-6), assignable to 6-β proton, thus confirming the presence of α-OH at the H-6 position, because H-6α of 6β-hydroxystigmast-4-en-3-one (compound **2**) was



6α-hydroxystigmast-4-en-3-one (compound 1) : R = α–OH

6β-hydroxystigmast-4-en-3-one (compound 2) : R = β–OH

Fig. 1. Chemical structures of compounds 1 and 2 isolated from the fruit of *Cornus kousa*.

observed at δ 4.32 as broad singlet. The ^{13}C-NMR and DEPT spectra of compound **1** also showed resonances for 29 carbons and confirmed the presence of a pair of olefine carbons at δ 171.71 (C-5) and 119.52 (C-4), and one ketone carbon at δ 199.50 (C-3). An oxygenated methine carbon signal of C-6 was observed at δ 68.66, indicating the stereostructure of 6-hydroxy to be α-configuration, because an oxygenated methine carbon with β-OH such as that of 6β-hydroxystigmast-4-en-3-one (compound **2**) was obsereved at δ 73.1. In the high magnetic field, six methyl signals at δ 19.87 (C-26), 19.07 (C-19), 18.75 (C-27), 18.33 (C-21), 12.04 (C-29), and 12.00 (C-18) were observed. Therefore, the structure of compound **1** was identified as 6α-hydroxystigmast-4-en-3-one. Compound **1** was isolated from this plant for the first time. Steroids containing the conjugated ketone together with the allylic alcohol rarely occur in nature; such steroids have been reported to exhibit several biological activities [Carcache *et al.*, 2006; Chen *et al.*, 2006].

The cytotoxicity of 6α-hydroxystigmast-4-en-3-one (compound **1**) along with the stereoisomer, 6β-hydroxystigmast-4-en-3-one (compound **2**), was evaluated using the MTT assay on four cancer cell lines. Both compounds **1** and **2** showed potent cytotoxicity in a dose-dependent manner against three tested cancer cell lines except MCF-7; the IC_{50} values of compounds **1** and **2** were respectively 20.5 ± 0.5 and 18.2 ± 0.1 mM against HCT-116, 47.3 ± 0.7 and 50.3 ± 0.3 mM against SK-MEL-5, and 37.4 ± 0.4 and 31.4 ± 0.5 mM against SK-OV-3 (Table I). The ketone group in this skeleton structure might be a key factor in the cytotoxic activity.

Table 1. The cytotoxicity of compounds 1 and 2 isolated from the fruit of *C. kousa* against human cancer cell lines

Compound	IC_{50} values (μM) [a]			
	Cancer Cell Lines			
	HCT-116	MCF-7	SK-MEL-5	SK-OV-3
1	20.5 ± 0.5	>50	47.3 ± 0.7	37.4 ± 0.4
2	18.2 ± 0.1	>50	50.3 ± 0.3	31.4 ± 0.5

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

Chang *et al.* [2003] reported that some stigmastane-sterols show no or very low cytotoxicity against some tumor cell lines; in particular, β-sitosterol, the best well known stigmastane-type sterol, showed a much lower cytotoxicity (ED_{50} > 50 mM) against the human colon cancer cell than those of compounds **1** or **2**. Based on these results, it can thus be concluded that compounds **1** and **2** may be potential candidates for use in the treatment of some human cancer cells.

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