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Article

Isolation of Flavonoids from the Fruits of *Cornus kousa* Burg.†

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Dried, unripe fruits of *Cornus kousa* Burg. were extracted with 80% aqueous MeOH and the concentrated extracts were partitioned between EtOAc and H_2O. From the EtOAc fraction, four flavonoids were isolated through repeated silica gel, ODS and Sephadex LH-20 column chromatographies followed by a preparative HPLC. Based on the spectroscopic data including NMR, MS and IR, the chemical structures of the compounds were determined as kaempferol (1), astragalin (2), hyperin (3) and isoquercitrin (4). These compounds were isolated for the first time from the fruits of this plant.

Key words: *Astragalin,* Cornus kousa, *hyperin, isoquercitrin, kaempferol, NMR*

Introduction

Cornus kousa Burg. (Cornaceae) is a tree distributed in the mountains of Korea, China and Japan. The fruit of this plant has been used as a hemostatic agent and for the treatment of diarrhea in Korean traditional medicine [Lee, 2003]; also, immuno-regulatory property of fruit-extracts has been reported [Kim *et al.*, 2002]. Some chemical constituents such as isoquercitrin, gallic acid, tannin [Ryu *et al.*, 1971], phenolics and flavonoids [Shaiju *et al.*, 2006] have been reported from the leaves of *C. kousa.* Also, our previous phytochemical researches on the fruits of this plant reported the presence of steroids [Lee *et al.*, 2006] and cytotoxic lignans [Lee *et al.*, 2007]. Our continuing work led to isolation of four flavonoids, which were isolated for the first time from the fruits of this plant. This paper describes the procedures of isolation and the structural elucidation of the flavonoids.

Materials and Methods

Plant materials. The fruits of *Cornus kousa* Burg. (Cornaceae) were collected at the experimental farm in Kyung Hee University in August, 2006. A voucher specimen (KHU060907) was reserved at the Laboratory of Natural Products Chemistry, Kyung Hee University, Suwon, Korea.

General experimental procedures. Optical rotations were measured on a JASCO P-1010 digital polarimeter (Tokyo, Japan). UV spectra were measured on a Shimadzu UV-1601 (Kyoto, Japan). EI-MS was recorded on a JEOL JMSAX 505-WA (Tokyo, Japan) and FAB-MS on a JEOL JMS-700 (Tokyo, Japan). IR spectra were run on a Perkin Elmer Spectrum One FT-IR spectrometer (Buckinghamshire, England). ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were taken on a Varian Unity Inova AS 400 FT-NMR spectrometer (California, USA). HPLC was performed on a Shimadzu LC-10AT (Tokyo, Japan).

Extraction and isolation. The dried and chopped fruits of *C. kousa* (10 kg) were extracted with 80% aqueous MeOH (10 L × 3) three times at room temperature. The extracts were partitioned between EtOAc (2 L × 3) and H_2O (2 L), successively. The EtOAc extract (44 g) was applied to the silica gel (Merck 60A, 70-230 mesh ASTM, Darmstadt, Germany) column (ϕ10 × 60 cm) chromatography (c.c.) and eluted with *n*-hexane-EtOAc (4 : 1 → 2 : 1, 1.5 L) → $CHCl_3$-MeOH (15 : 1 → 13 : 1 → 10 : 1, 1.5 L of each) monitoring by thin layer chromatography (TLC) to provide two fractions. Fraction

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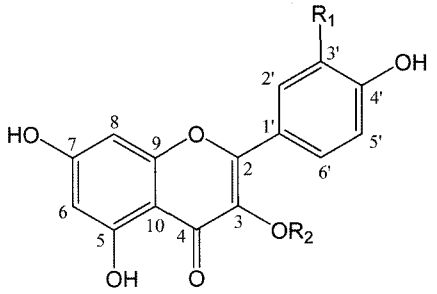
2CKFE1 (2.5 g) was applied to silica gel column chromatography (ϕ4 × 45 cm) and eluted with $CHCl_3$-MeOH-H_2O (13 : 3 : 1, 3 L) to provide one fraction. Subfraction 2CKFE1-2 (88 mg) was subjected to ODS (Merck, octadecyl silica gel, Darmstadt, Germany) column chromatography (ϕ3 × 40 cm) and eluted with MeOH-H_2O (2 : 1, 1 L) to yield compound **1** [35 mg, TLC (RP-18, F_{254}) R_f 0.40, MeOH-H_2O = 2 : 1]. Fraction 2CKFE2 (870 mg) was applied to the ODS column chromatography (ϕ4 × 45 cm) eluted with MeOH-H_2O (2 : 3, 2 L) to provide one fraction. And, subfraction 2CKFE2-1 (310 mg) was applied to silica gel column chromatography (ϕ 4 × 40 cm) and eluted with $CHCl_3$-MeOH-H_2O (12 : 3 : 1, 2.5 L) to provide two fractions. And then, subfraction 2CKFE2-1-1 (35 mg) were applied to Sephadex LH-20 (Amersham Pharmacia Biotech, Uppsala, Sweden) (ϕ2 × 50 cm, 80% MeOH, 500 mL) to give compound **2** [(28 mg, TLC (RP-18, F_{254}) R_f 0.5, MeOH-H_2O = 1 : 1]. Subfraction 2CKFE2-1-2 (95 mg) was purified by HPLC. The analysis were performed on a COSMOSIL $5C_{18}$ Waters column (10 × 250 mm) at column temperature 30°C. The mobile phase composed of CH_3CN-H_2O-TFA (50 : 50 : 0.05, vol. %) was eluted at a flow rate of 1.0 mL/min and the effluent was monitored at 370 nm by UV detector. Two peaks were detected at 2.97 and 3.65 min. The repeated collection of each peak gave two purified flavonoids, compounds **3** (28 mg) and **4** (35 mg).

Compound **1**: Light yellow powder (MeOH); m.p. 178-180; UV (MeOH) λ_{max}: 269, 364 nm; IR (KBr) ν_{max} 3350, 1660, 1610, 1500 cm^{-1}; EI/MS *m/z*: 286 [M^+], 258, 229, 213, 184, 153, 121; ^{1}H-NMR (400 MHz, CD_3OD, δ) 8.06 (2H, d, *J* = 9.2 Hz, H-2'/6'), 6.89 (2H, d, *J* = 9.2 Hz, H-3'/5'), 6.36 (1H, d, *J* = 2.0 Hz, H-8), 6.16 (1H, d, *J* = 2.0 Hz, H-6); ^{13}C-NMR (100 MHz, CD_3OD, δ) see Table 1.

Compound **2**: Yellow amorphous powder (MeOH); m.p. 230-232°C; UV (MeOH) λ_{max}: 218, 268 nm; $[\alpha]^{25}_D$ = +16.0° (*c* = 1.1, MeOH); IR (KBr) ν_{max} 3420, 1680 cm^{-1}; pos. FAB/MS *m/z*: 449, 287; ^{1}H-NMR (400 MHz, CD_3OD, δ) 8.04 (2H, d, *J* = 8.4 Hz, H-2'/6'), 6.87 (2H, d, *J* = 8.4 Hz, H-3'/5'), 6.38 (1H, br s, H-8), 6.19 (1H, br s, H-6), 5.23 (1H, d, *J* = 7.2 Hz, H-1"), 3.18~3.70 (4H, m, H-2", 3", 4", 5"), 3.71 (1H, dd, *J* = 12.0, 2.4 Hz, H-6a"), 3.58(1H, dd, *J* = 12.0, 5.2 Hz, H-6b"); ^{13}C-NMR (100 MHz, CD_3OD, δ) see Table 1.

Compound **3**: Yellow powder (MeOH); m.p. 235-238 °C; UV (MeOH) λ_{max}: 207, 261 nm; $[\alpha]^{25}_D$ = −12.5° (*c* = 0.9, MeOH); neg. FAB/MS *m/z*: 463 [M-H]$^-$, 447, 431, 389, 339, 325, 301; IR (KBr) ν_{max} 3400, 2919, 1656, 1606, 1508 cm^{-1}; ^{1}H-NMR (400 MHz, CD_3OD, δ) 7.84 (1H, d, *J* = 2.4 Hz, H-2'), 7.56 (1H, dd, *J* = 2.4, 8.6 Hz, H-6'), 6.84 (1H, d, *J* = 8.6 Hz, H-5'), 6.34 (1H, d, *J* = 2.0 Hz, H-8), 6.16 (1H, d, *J* = 2.0 Hz, H-6), 5.13 (1H, d, *J* = 8.0 Hz, H-1"), 3.85 (dd, *J* = 3.2, 2.0 Hz, H-4"), 3.82 (dd, *J* = 8.0, 7.8 Hz, H-2"), 3.65 (dd, *J* = 11.0, 3.2, H-6"a), 3.56 (dd, *J* = 11.0, 5.5 Hz, H-6"b), 3.58 (dd, *J* = 7.8, 2.0 Hz, H-3") and 3.48 (m, H-5"); ^{13}C-NMR (100 MHz, CD_3OD, δ) see Table 1.



1 R_1=H; R_2=H
2 R_1=H; R_2=β-D-Glc
3 R_1=OH; R_2=β-D-Gal
4 R_1=OH; R_2=β-D-Glc

Fig. 1. Chemical structures of compounds 1-4 isolated from the fruits of *Cornus kousa*.

Compound **4**: Yellow powder (MeOH); m.p. 230-232 °C; UV (MeOH) λ_{max}: 207, 256 nm; neg. FAB/MS *m/z*: 463 [M-H]$^-$, 447, 423, 389, 297, 204; IR (KBr) ν_{max} 3400, 2919, 1656, 1606, 1508 cm^{-1}; ^{1}H-NMR (400 MHz, CD_3OD, δ) 7.70 (1H, d, *J* = 2.4 Hz, H-2'), 7.55 (1H, dd, *J* = 2.4, 8.6 Hz, H-6'), 6.85 (1H, d, *J* = 8.6 Hz, H-5'), 6.34 (1H, d, *J* = 2.0 Hz, H-8), 6.16 (1H, d, *J* = 2.0 Hz, H-6), 5.22 (1H, d, *J* = 7.2 Hz, H-1"), 3.22~3.51 (4H, m, H-2", 3", 4", 5"), 3.71 (1H, dd, *J* = 12.0, 2.4 Hz, H-6a"), 3.58 (1H, dd, *J* = 12.0, 5.2 Hz, H-6b"); ^{13}C-NMR (100 MHz, CD_3OD, δ) see Table 1.

Results and Discussion

When the methanol extract of *C. kousa* was developed on the silica gel TLC, the spots showed not only the UV absorbance at 254 or 365 nm, but also a yellow colorization by spraying 10% H_2SO_4 solution and then heating the TLC plate, indicating the presence of flavonoids in the extracts. The methanol extract was fractionated into EtOAc layer, *n*-BuOH layer and H_2O layer through solvent fractionation. The repeated silica gel, ODS, Sephadex LH-20 column chromatographies and HPLC of EtOAc fractions supplied four flavonoids, compounds **1-4**. Structural identifications of these compounds were carried out by interpretation of extensive spectroscopic data and comparison with the data described in the literature.

Compound **1** was obtained as light yellow powder, and showed the molecular ion peak (M^+) at *m/z* 286 in the EI/MS spectrum. The IR spectrum showed the absorbance

Table 1. ^{13}C-NMR (100 MHz) of compounds 1, 2, 3 and 4 (in methanol-d_4)

No. of Carbon	Compound 1	Compound 2	Compound 3	Compound 4
2	147.8	158.3	158.5	158.1
3	137.1	135.3	135.4	135.4
4	177.1	178.3	179.1	179.1
5	162.3	162.8	162.7	162.7
6	99.2	99.7	99.7	99.7
7	165.3	165.7	165.7	165.7
8	94.4	94.6	94.6	94.6
9	158.1	158.8	158.2	158.2
10	104.4	104.9	105.5	105.4
1'	123.6	122.6	122.8	122.6
2'	130.5	132.1	115.9	115.8
3'	116.2	116.0	145.6	145.5
4'	160.3	161.4	149.7	149.6
5'	116.2	116.0	117.7	117.4
6'	130.5	132.1	122.8	123.0
1"	-	104.0	105.4	104.3
2"	-	75.6	73.1	75.6
3"	-	78.3	75.0	78.2
4"	-	71.2	69.9	71.1
5"	-	77.9	77.0	78.0
6"	-	62.5	61.9	62.5

bands due to the hydroxyl (3350 cm^{-1}), ketone (1660 cm^{-1}) and aromatic (1610, 1500 cm^{-1}) functions. In the ^{1}H-NMR spectrum, aromatic methine signals at δ 8.06 (2H, d, J = 9.2 Hz) and 6.89 (2H, d, J = 9.2 Hz) of due to a 1',4'-disubstitution of ring B, and at δ 6.36 (1H, d, J = 2.0 Hz) and 6.16 (1H, d, J = 2.0 Hz) of a typical *meta*-coupled pattern due to 1,2,3,5-tetrasubstitution of ring A were observed. The ^{13}C-NMR spectrum showed fifteen carbon signals. The multiplicity of each carbon was determined using DEPT experiment. The signals in the low magnet field region indicated the presence of a conjugated ketone at δ 177.1 (C-4), six oxygenated sp^2 quaternary carbons [δ 165.3 (C-7), 162.3 (C-5), 160.3 (C-4'), 158.1 (C-9), 147.8 (C-2) and 137.1 (C-3)], two sp^2 quaternary carbons [δ 123.6 (C-1') and 104.4 (C-10)] and six sp^2 methine carbons [δ 130.5 (C-2'/6'), 116.2 (C-3'/5'), 99.2 (C-6) and 94.4 (C-8)]. Compound **1** was identified as kaempferol, the most well-known flavonol, by comparison of spectroscopic data with those of literature [Zhang *et al.*, 2006].

Compound **2** was assumed to be a monoglycoside of compound **1** from the spectroscopic data such as MS, ^{1}H-NMR and ^{13}C-NMR. One anomeric proton signal at δ 5.23 (d, J = 7.2 Hz) and the carbon signal at δ 104.0 (C-1") were observed. The chemical shifts of other glycosidic carbon signals at δ 78.3 (C-3"), 77.9 (C-5"), 75.6 (C-2"), 71.2 (C-4") and 62.5 (C-6") suggested the presence of a β-glucopyranosyl group. The connection between the glucopyranosyl unit (C-1") and the C-3 of the aglycon was verified by the cross-peak observed between δ 5.23 (H-1") and 135.3 (C-3) in the HMBC spectrum. Thus, compound **2** was identified as kaempferol-3-*O*-β-D-glucopyranoside (astragalin) through the comparison of several physical and spectroscopic data with those of literature [Han *et al.*, 2004].

Compound **3** was isolated as yellow amorphous powders. The negative FAB-MS gave a pseudomolecular ion peak at *m/z* 463 [M-H]$^-$. In the ^{1}H-NMR spectrum, the typical proton signals of quercetin moiety, that is, those of an AMX system due to a 1',3',4'-trisubstitution of ring B [δ 7.84 (d, J = 2.4 Hz), 7.56 (dd, J = 2.4, 8.6 Hz) and δ 6.84 (d, J = 8.6 Hz)], and a typical *meta*-coupled pattern for H-8 and H-6 protons [δ 6.34 (d, J = 2.0 Hz) and 6.16 (d, J = 2.0 Hz)], were observed. The ^{13}C-NMR and DEPT spectra of compound **3** showed resonances for twenty one carbons and presence of a quercetin moiety with the exception of monosaccharide moiety (see Table 1.). An anomeric proton signals of compound **3** appeared at δ 5.13 (d, J = 8.0 Hz) and other oxygenated methines and methylene signals of sugar moiety at δ 3.85 (dd, J = 3.2, 2.0 Hz, H-4"), 3.82 (dd, J = 8.0, 7.8 Hz, H-2"), 3.65 (dd, J = 11.0, 3.2, H-6"a), 3.56 (dd, J = 11.0, 5.5 Hz, H-6"b), 3.58 (dd, J = 7.8, 2.0 Hz, H-3") and 3.48 (m, H-5"). Also, the carbon signals of the sugar moiety were observed at δ 105.4 (C-1"), 77.0 (C-5"), 75.0 (C-3"), 73.1 (C-2"), 69.9 (C-4") and 61.9 (C-6") suggesting the presence of β-galactopyranoside units. In the HMBC spectrum, a crosspeak between δ 5.13 (H-1") and 135.4 (C-3) established the connection of quercetin and β-galactose moieties. Thus, the structure of compound **3** was identified as quercetin-3-*O*-β-D-galactopyranoside, hyperin [Lu *et al.*, 1999].

Compound **4** was almost identical with compound **3** with the exception of monosaccharide moiety. An anomeric proton signal of compound **4** was observed at δ 5.22 (d, J = 7.2 Hz) and the ^{13}C-NMR signals of sugar moiety were observed at δ 104.3 (C-1"), 78.2 (C-3"), 78.0 (C-5"), 75.6 (C-2"), 71.1 (C-4") and 62.5 (C-6") suggesting the presence of a β-glucopyranosyl group. The connection between the glucopyranosyl unit (C-1") and the C-3 of the quercetin was verified by the cross-peak of δ 5.22 (H-1") to 135.4 (C-3) in the HMBC spectrum. Therefore, compound **4** was identified as quercetin-3-*O*-β-D-glucopyranoside (isoquercitrin) through the comparison of several physical and spectroscopic data with those of literature [Han *et al.*, 2004]. All the compounds were isolated for the first time from the fruits of this plant.

All the isolated flavonoids have been reported to exhibit antioxidant activity [Zhang *et al.*, 2006; Han *et al.*, 2004; Lu *et al.*, 1999]. Recently, kaempferol (**1**) was examined as an inhibitor of cigarette smoke-induced activation of the aryl hydrocarbon receptor and cell transformation [Guvenalp *et al.*, 2005]. It was reported that astragalin (**2**) had cytotoxicity against four human cancer cell lines [Yan *et al.*, 2005]. Hyperin (**3**) was reported to be a source of antiretroviral for AIDS therapy due to significant anti-HIV-1 activity [Markham *et al.*, 1978]. Isoquercitrin (**4**) has also been found to be a major factor in anti-inflammatory activity [Jeng *et al.*, 1987] and hepatoprotective activity [Puppala *et al.*, 2007]. Therefore, *Cornus kousa* which was used as an edible fruit in Korea, might be very useful for the development of functional food and raw materials of medicine.

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