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# The Anti-hyperlipidemic Effect and Constituents of the 19α-Hydroxyursane-type Triterpenoid fraction Obtained from the Leaves of *Rubus crataegifolius*

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**Abstract** – To demonstrate anti-hyperlipidemic activity of the 19α-hydroxyursane-type triterpenoid (19α-HUT)-rich fraction, this fraction was prepared from the extract of *Rubus crataegifolius* leaves. This fraction was found to have anti-hyperlipidemic effect in a high fat diet-induced rat model from the observation of reduction of abdominal fat pad weights, atherogenic index and hypercholesterolemia at 30 and 60 mg/kg (*p.o.*) The 19α-HUT fraction was subjected to $SiO_2$, ODS, and/or Sephadex LH-20 column chromatography to yield a new triterpenoid (**1**) called pomolic acid ester along with nine known triterpenoids which are all 19α-HUTs: euscaphic acid (**2**), tormentic acid (**3**), 23-hydroxytormentic acid (**4**), kaji-ichigoside $F_1$ (**5**), rosamultin (**6**), niga-ichigosides $F_1$ (**7**) and $F_2$ (**8**), suavissimoside $F_1$ (**9**) and coreanoside $F_1$ (**10**). The structure of compound **1** was established as 28-O-formyl-3,19-dihydroxyurs-12-en-28-oic acid on the basis of 2D-NMR spectroscopic data and mass spectrum. Compound **1** was isolated for the first time from natural sources.
**Key words** – *Rubus crataegifolius*, Rosaceae, 19α-hydroxyursane-type triterpenoid, pomolic acid ester, NMR, anti-hyperlipidemic

## Introduction

The unripe fruits of *Rubus coreanus* (Rosaceae), called Rubi Fructus, have been used in traditional Chinese medicine to treat diabetes mellitus, diarrhea, sexual disinclination, amblyopia, and weakness (Moon, 1964). We previously reported that oral administration of this Chinese herb medicine exhibited antinociceptive and antinflammatory (Choi *et al.*, 2003) and anti-rheumatic properties without causing gastric damage in the rat (Nam *et al.*, 2006).

Although the Chinese medicinal drug Rubi Fructus, which is imported from China, is commercially available in Korea, the original plant has been unidentified. *R. craetaegifolius* is widely distributed in the mountainous area of Korea, though *R. coreanus* is being cultivated in Korea. At the ripening stage, the color of *R. coreanus* fruits becomes black whereas that of *R. crataegifolius* becomes red.

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During our investigation, we found that the unripe fruits of Rubus species have a large amount of Rubus-specific triterpenoids, which we assume to be the bioactive constituents; in contrast, the ripe fruits contain a very small amount. On the contrary, since we found that the leaves of Rubus species had a large amount of them, we attempted to isolate 19α-hydroxyursane-type tripterpenoids (19α-HUTs) from the leaves of the Rubus species. We previously revealed that the unripe fruits had a large amount of 19α-HUTs as bioactive substances while the ripe fruits did not. Therefore we attempted to prepare 19α-HUT-rich fraction as the bioactive biomaterial and to isolate the 19α-HUT constituents. We assumed that it is important to prepare 19α-HUT-rich fraction in a high yield, because the substance of 19α-HUTs, euscaphic acid, tormentic acid and 23-hydroxytormentic acid or their analogues had anti-inflammatory and antirheumatic effects, respectively.

Although many triterpenoids belonging to the 19α-HUT has been reported from Rubus species (Wang *et al.*, 2000; Hirai *et al.*, 2000; Jung *et al.*, 2001), the most

popular substances are euscaphic acid, tormentic acid and their analogues. Jung *et al.* (2001) has reported the isolation of 23-hydroxytormentic acid, niga-ichigoside $F_1$ and their related substances from the fruits of *R. crataegifolius* as 19α-HUT constituents. Our isolates obtained from the leaves of the sample plant origin were quite different from the compounds which Jung *et al.* (2001) were able to isolate. Among our isolates, pomolic acid ester (**1**) was isolated for the first time from natural sources. In addition, eight 19α-HUTs were isolated from *R. crataegifolius* for the first time.

We obtained the 19α-HUT fraction from the *R. crataegifolius* leaves to demonstrate that this fraction could be a candidate for a anti-hyperlipidemic biomaterial. High fat diet-induced rat model was used to find anti-hyperlipidemic effect of 19α-HUT fraction. Abdominal fat pad weight changes were estimated in the retroperitoneal, epididymal and total abdominal fat pads to identify the effect on obesity. Serum lipid concentrations were compared to measure total lipids, phospholipids and triglycerides in serum; serum hypercholestrolemia were also estimated to measure total cholesterol, high density lipoprotein (HDL)-cholesterol and low density lipoprotein (LDL)-cholesterol.

## Experimental

**Instruments and reagents** – Melting points were determined using Electrothermal 9100 instrument and uncorrected. Optical rotations were measured on a JASCO DIP-360. IR spectra were recorded in KBr disks on a Hitachi 260-01 spectrometer. EIMS was taken on a JEOL JMS DX-300 spectrometer using 70 eV ionization voltage. The $^1$H-NMR spectra (δ ppm, *J* in Hz) in $C_5D_5N$ or $CD_3OD$ were taken with tetramethylsilane as an internal standard on a Bruker AM-500 spectrometer (500 MHz), while $^{13}$C-NMR spectra were recorded in the same solvent at 125.5 MHz. $^1$H-$^1$H-COSY, HMQC and HMBC spectra were also taken using the same NMR spectrometer. Silica gel (silica gel 60, 0.064 - 0.084 mesh) was purchased for use from Merck Co. (Germany), and Sephadex (Lipophilic Sephadex LH-20) was purchased from Sigma-Aldrich (USA). The column used for medium pressure liquid chromatography (MPLC) was flash column Biotago SF 25 + M0712-2 (Biotago. Inc., USA).

**Plant material** – The leaves of *R. crataegifolius* were collected in Chiak Mountain, Wonju, Korea and identified by Dr. Sang-Cheol Lim (Prof., Department of Botanical Resources, Sangji University, Korea). The plant collected was dried and crushed for extraction. A voucher specimen (Natchem # 29) was deposited in Laboratory of Natural Product Chemistry, Department of Botanical Resources, Sangji Univeristy, Korea.

**Extraction and preparation of the 19α-HUT fraction** – The dried and crushed leaves of *R. crataegifolius* (2.4 kg) was extracted with MeOH under reflux three times, and the MeOH extract was filtered and evaporated on a rotatory evaporator under reduced pressure and then lyophilized to give a solid mass (156 g) of MeOH extract. This material (150 g) was suspended in $H_2O$ (1 L), and then partitioned with each 0.8 L hexane three times. Thereafter, the aqueous fraction was fractionated with 0.8 L three times. The residual aqueous fraction was extracted with $H_2O$-saturated BuOH in a separating funnel three times; then the BuOH soluble part was concentrated *in vacuo*. The BuOH fraction was suspended in $H_2O$, mixed with 70 g of charcoal to remove phenolic or non-19α-HUTs and then filtered using a filter paper. The filtered solution was poured into Diaion HP-20 column (320 g, 5 × 70 cm) and then eluted with 2.0 L $H_2O$ to elute sugars or ionic substances. This column was successively eluted with 2.0 L MeOH and then the eluate was concentrated to dryness to give a 19α-HUT fraction (18 g). This procedure is shown in Fig. 1.

**Animal** – Sprague-Dawley male rats weighing 190 ± 10 g were purchased from the Hyochang Science,

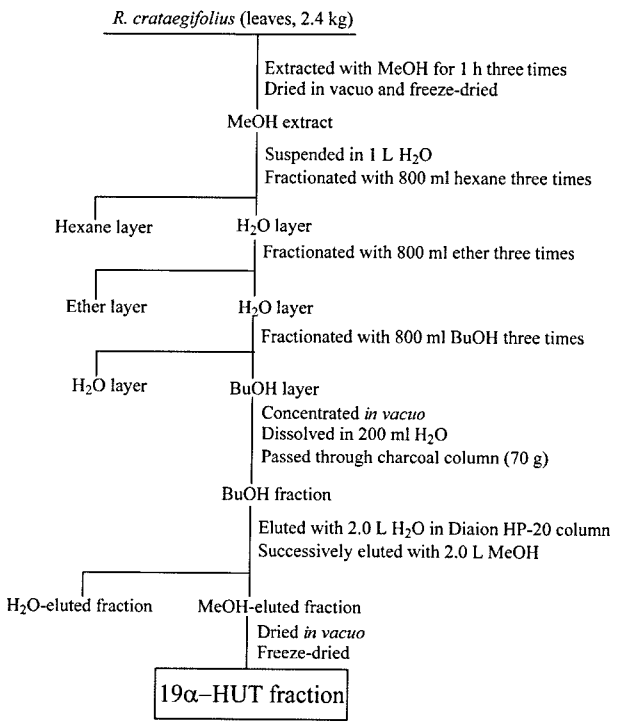



**Fig. 1.** A procedure for extraction and fractionation of the leaves of *R. crataegifolius*.

**Table 1.** Composition of basal and hyperlipidemic diet

| Ingredient | Basal diet (%) | Hyperlipidemic diet |
|---|---|---|
| Casein | 20.0 | 20.0 |
| DL-Methionine | 0.3 | 0.3 |
| Corn starch | 15.0 | 15.0 |
| Sucrose | 50.0 | 34.5 |
| Fiber[1)] | 5.0 | 5.0 |
| Corn oil | 5.0 | – |
| AIN-mineral Mixture[2)] | 3.5 | 3.5 |
| AIN-vitamin Mixture[3)] | 1.0 | 1.0 |
| Choline bitartrate | 0.2 | 0.2 |
| Beef Tallow | – | 20.5 |

[1)] Cellulose: Sigma Co. LTD., USA, [2)] Mineral mixture based on the pattern of Rogers and Haper (1965) contain the following (g/kg diet): calcium phosphate dibasic 500.0, sodium chloride 74.0, potassium citrate monohydrate 220.0, potassium sulfate 52.0, magnesium oxide 24.0, magnesium carbonate 3.5, ferric citrate 6.0, zinc carbonate 1.6, cupuric carbonate 0.3, potassium iodate 0.01, chromium potassium sulfate 0.55, sucrose, finely powered make 1,000 [3)]Vitamin mixture (g/kg diet): thiamine HCl 0.6, biotin 0.02, riboflavin 0.6, cyanocobalamine 0.001, pyridoxine HCl 0.7, retinyl acetate 0.8, nicotinic acid 3.0, DL-tocopherol 3.8, Ca-pantothenate 1.6, 7-dehydrocholesterol 0.0025, folic acid 0.2, methionine 0.005, sucrose, finely powered make 1,000.

maintained under constant conditions (temperature: 20 ± 2 °C, humidity: 40 - 60%, 12 h light/dark cycle) and acclimatized for 2 weeks or more. For 24 h period before the experiment, only water was offered to the animals. To minimize the effect of variations in their diurnal enzyme activity, the animals were sacrificed at 10:00 - 12:00 a.m. All animal experiments were approved by the University of Kyungsung Animal Care and Use Committee, and all procedures were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the Korea National Institute of Health.

**High fat diet-induced hyperlipidemia** – Preliminary experimentation was based on blood lipid measurements that hyperlipidemia was induced in rats treated with a high fat diet for totally 6 weeks. A normal diet was given to rats in the untreated control group, while high fat diet was also given to the control and treated groups. Diet composition of normal diet and high fat diet are illustrated in Table 1. After 4 weeks on a high fat diet, sample (19α-HUT fraction) solutions dissolved in saline were administered orally at 30 and 60 mg/kg a day for 2 weeks. The rats were fasted for 8 h after the final sample treatment, anesthetized with $CO_2$, and blood was collected from abdominal aortas. After microcentrifugation of the blood, the obtained sera were preserved at −70 °C.

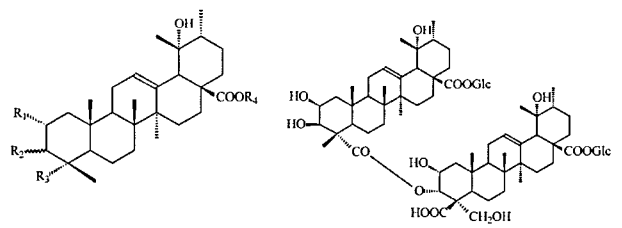
**Measurement of abdominal fat pad weights and serum lipids** – The weights of retroperitoneal-, epididymal- and total abdominal fat pads were measured to find the effect on obesity caused by hyperlipidemia. To find anti-hyperlipidemic effect, the concentrations of total cholesterol, phospholipids and triglycerides in serum were measured. Briefly, serum cholesterol, phospholipids and triglycerides were measured using the kit reagent (AM 202-K, Asan, Korea) prepared based on the enzymatic method of Richmond *et al.* (1976), the kit reagent (Iatron Chem. Co., Japan) on Bagniski *et al.* (1960), and the kit reagent (AM 157S-K, Asan, Korea), (McGown *et al.*, 1983) respectively.

**Measurement of total-, HDL- and LDL cholesterols** – The serum concentration of HDL cholesterol was measured using a kit reagent (AM 203-K, Asan) prepared based on the enzymatic method of Noma *et al.* (1978) LDL cholesterol was calculated according to the equation suggested by Friedewald *et al.* (1972): LDL cholesterol = Total cholesterol – (HDL cholesterol + triglyceride/5).

**Fractionation of 19α-HUT-rich fraction** – The 19α-HUT (15 g) was subjected to silica gel column (280 g, 5 × 57 cm) using $CHCl_3$-MeOH (4 : 1) as a mobile phase and collected with each 50 ml volume. Every collected fraction was checked on TLC by spraying 10% sulfuric acid and combined according to the TLC check to give four fractions. The fraction over 0.50 - 0.75 L volume was dried to afford HUT-F1 fraction (1.1 g). Likewise, each fraction over 0.90 - 1.2 L, 1.4 - 1.75, 1.22 - 1.75 L, and 2.2 - 2.5 L was concentrated to dryness to elicit HUT-F2 (2.9 g), HUT-F3 (2.9 g), and HUT-F4 (4.2 g), respectively, which became targets for further isolation.

**Isolation of compounds 1, 2 and 3** – One g of HUT-F1 was passed into Sephadex LH-20 column with the eluting solvent MeOH to afford a more purified 19α-HUT fraction of HUT-F1. This was subjected to $SiO_2$ column chromatography eluted with $CHCl_3$-MeOH-$H_2O$ (30 : 4 : 1, lower phase) to yield compounds **1** (52 mg), **2** (210 mg), and **3** (280 mg). Physicochemical and spectroscopic data (Mp, $[\alpha]_D$, and $^1$H- and $^{13}$C-NMR spectra) of **2** and **3** were in agreement with the literature data of euscaphic acid and tormentic acid, respectively; compound **2** (euscaphic acid): amorphous powder from MeOH, mp, 266 - 268 °C, $[\alpha]_D^{25}$ +12.0° (c = 0.10, $CHCl_3$); $^1$H- NMR and $^{13}$C-NMR: Literature (Numata *et al.*, 1989); Compound **3** (tormentic acid): amorphous powder from MeOH, mp, 266 - 268 °C, $[\alpha]_D^{25}$ +31.5° (c = 0.80, MeOH); $^1$H- NMR and $^{13}$C-NMR: Literature (Yamagishi *et al.*, 1988). The structures identified are shown in Fig. 2.

**Compound 1** – amorphous powder, mp 250 - 251 °C, $[\alpha]_D^{20}$ +58° (*c* = 0.30, acetone); IR $\nu_{max}$ (KBr, cm$^{-1}$): 3,440 (OH), 2,925 (CH), 1,699, 1,687 (C = O), 1,270,



**10** (Coreanoside $F_1$)

| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | |
|---|---|---|---|---|---|
| **1** | H | β-OH | Me | CHO | Pomolic acid ester |
| **2** | OH | α-OH | Me | H | Euscaphic acid |
| **3** | OH | β-OH | Me | H | Tormentic acid |
| **4** | OH | β-OH | $CH_2OH$ | H | 23-hydroxytormentic acid |
| **5** | OH | α-OH | Me | Glc | Kaji-ichigoside $F_1$ |
| **6** | OH | β-OH | Me | Glc | Rosamultin |
| **7** | OH | β-OH | $CH_2OH$ | Glc | Niga-ichigoside $F_1$ |
| **8** | OH | α-OH | $CH_2OH$ | Glc | Niga-ichigoside $F_2$ |
| **9** | OH | α-OH | COOH | Glc | Suavissimoside $R_1$ |

**Fig. 2.** Structure of 19α-HUT constituents isolated from *R. crataegifolius*.

1,046 (C-O); EI-MS (70 eV) *m/z* (rel. int.): 509 ($[M-H]^+$, 2.6), 472 ($[M-CO]^+$, 9.8), 454 ([M-CO-$H_2O$], 24.9), 426 ($[M-CO-2\times H_2O]^+$, 58,4), 411 ([M-CO-2×$H_2O$-$CH_3$], 8.7), 354 (19.4), 267 (31.0), 246 (41.2), 146 (100); $^1$H- NMR (500 MHz, $CD_3OD$) δ: 8.57 (1H, s, aldehydic proton), 5.31 (1H, d-like, H-12), 3.18 (1H, dd, $J$ = 3.4, 12 Hz, H-3), 2.59 (1H, dt, $J$ = 3.4, 10.0 Hz, $H_a$-16), 2.53 (1H, s, H-18), 1.36 (3H, s, H-29), 1.21 (3H, s), 1.00[a] (3H, s, H-25), 0.97[a] (3H, s, H-23), 0.95 (3H, d, $J$ = 7.0 Hz, H-30), 0.83[b] (3H, s, H-26), 0.82[b] (3H, s, H-24). [a,b] Values with the same superscript may be interconvertible; $^{13}$C-NMR (125.5 MHz, $CD_3OD$) δ: 40.3 (C-1), 27.1 (C-2), 80.3 (C-3), 38.6 (C-4), 57.2 (C-5), 20.1 (C-6), 34.7 (C-7), 40.3 (C-8), 43.5 (C-9), 43.1 (C-10), 25.1 (C-11), 129.9 (C-12), 140.5 (C-13), 41.5 (C-14), 30.1 (C-15), 27.5 (C-16), 49.0 - 50.2 (overlapped with $CD_3OD$, C-17), 55.6 (C-18), 74.1 (C-19), 43.5 (C-20), 28.1 (C-21), 39.9 (C-22), 29.2 (C-23), 17.0 (C-23), 17.0 (C-24), 16.8 (C-25), 18.0 (C-26), 25.2 (C-27), 172.7 (C-28), 27.8 (C-29), 16,3 (C-30), 172.4 (CHO).

**Alkaline hydrolysis of compound 1** – Twenty mg of compound **1** was dissolved in 5%-NaOH and refluxed for 3 h and cooled. The reaction mixture was acidified with diluted HCl and then partitioned with 50 ml EtOAc three times. The EtOAc-soluble part was washed with 30 ml $H_2O$ twice and dried *in vacuo*. The concentrate was purified on Sephadex LH-20 column using MeOH solvent and then crystallized from MeOH to yield compound **1a**. The physicochemical and spectroscopic data (mp, $[\alpha]_D$, and $^1$H- and $^{13}$C-NMR spectra) of **1a** were in agreement with the literature data for pomolic acid (Kuang *et al.*, 1989); compound **1a**: amorphous powder from MeOH, mp, 284 °C, $[\alpha]_D^{25}$ +39° (c = 0.30, MeOH); $^1$H-NMR (500 MHz, pyridine-$d_5$) δ: 5.56 (1H, br. s, H-12), 3.39 (1H, d, $J$ = 9.7 Hz, H-3), 3.06 (1H, dt, $J$ = 4.2, 11.6 Hz), 2.99 (1H, s, H-18), 1.41 (3H, s, H-23), 1.36 (3H, s, H-29), 1.21 (3H, s, H-27), 1.01 (3H, s, H-24), 0.97 (3H, s, H-26), 0.95 (3H, d, $J$ = 7.0 Hz), 0.86 (3H, s, H-25); $^{13}$C-NMR (125.5 MHz, pyridine-$d_5$) δ: 39.2 (C-1), 26.7 (C-2), 78.0 (C-3), 38.8 (C-4), 55.7 (C-5), 18.7 (C-6), 33.4 (C-7), 40.1 (C-8), 47.6 (C-9), 37.2 , 23.8 (C-11), 127.9 (C-12), 139.7 (C-13), 41.9 (C-14), 28.6 (C-15), 26.9 (C-16), 48.1 (C-17), 54.4 (C-18), 72.5 (C-19), 42.1 (C-20), 26.2 (C-21), 38.3 (C-22), 29.1 (C-23), 16.5 (C-24), 16.3 (C-25), 17.0 (C-26), 24.5 (C-27), 180.4 (C-28), 27.9 (C-29), 15.4 (C-30).

**Isolation of compounds 4, 5 and 6** – The silica gel column (60 g, 3 × 52 cm) chromatography of 2 g of HUT-F2 eluted with $CHCl_3$-MeOH-$H_2O$ (30 : 4 : 1, lower phase) as eluting solvent to afford the three fractions, HUT-F2-1, -2, and -3, which were further purified using MPLC column ($SiO_2$, 3 × 21.5 cm) with the solvent $CHCl_3$-MeOH-$H_2O$ (12 : 2 : 1, lower phase) to yield compounds **4** (230 mg), **5** (35 mg), and **6** (140 mg). The physicochemical and spectroscopic data (Mp, $[\alpha]_D$, and $^1$H- and $^{13}$C-NMR spectra) of **4**, **5**, and **6** were in agreement with the literature data of 23-hydroxytormentic acid, rosamultin and kaji-ichigoside $F_1$, respectively; compound **4** (23-hydroxytormentic acid) – amorphous powder, mp. 288 °C, $[\alpha]_D^{17}$ +22.5° (c = 0.034, MeOH); $^1$H- NMR and $^{13}$C-NMR: Literature (Kim *et al.*, 1993); Compound **5** (rosamultin): amorphous powder, mp. 200 - 4 °C, $[\alpha]_D^{17}$ +10° (c = 0.028, MeOH); $^1$H- NMR and $^{13}$C-NMR: Literature (Kim *et al.*, 1993); Compound **6** (kaji-ichigoside $F_1$): amorphous powder, mp. 214 - 6 °C, $[\alpha]_D^{18}$ +15.2° (c = 0.021, MeOH); $^1$H- NMR and $^{13}$C-NMR: Literature (Kim *et al.*, 1993).

**Isolation of compounds 7 and 8** – Two g of HUT-F3 were subjected to $SiO_2$ column (60 g, 3 × 52 cm) chromatography eluted with $CHCl_3$-MeOH-$H_2O$ (75 : 25 : 10, lower phase). After checking TLC, HUT-F4-1 and -2 fractions were obtained. These two fractions were further purified using MPLC column ($SiO_2$, 3 × 21.5 cm) with the solvent $CHCl_3$-MeOH-$H_2O$ (10 : 1 : 1, lower phase) to yield compounds **7** (75 mg) and **8** (17 mg). The physicochemical and spectroscopic data (Mp, $[\alpha]_D$, and $^1$H- and $^{13}$C-NMR spectra) of **7** and **8** were in accordance with the literature data of niga-ichigosides $F_1$ and $F_2$, respectively;

Compound **7** (niga-ichigoside $F_1$): amorphous powder, mp, 233 - 6 °C, $[\alpha]_D^{26}$ +11.2° (c = 0.021, MeOH); $^1$H-NMR and $^{13}$C-NMR: Literature (Kim *et al.*, 1993); Compound **8** (niga-ichigoside $F_2$): colorless needles from MeOH-$H_2O$, mp, 214 - 216 °C, $[\alpha]_D^{20}$ +13.2° (c = 0.92, MeOH); $^1$H- NMR and $^{13}$C-NMR: Literature (Seto *et al.*, 1984).

**Isolation of compounds of 9 and 10** – Four g of HUT-F3 were also subjected to $SiO_2$ column (60 g, 3 × 52 cm) chromatography eluted with $CHCl_3$-MeOH-$H_2O$ (75 : 25 : 10, lower phase). After checking TLC, two fractions HUT-F4-1 and -2 were obtained, which were further purified using MPLC column ($SiO_2$, 3 × 21.5 cm) with the solvent $CHCl_3$-MeOH (2 : 1) to yield compounds **9** (62 mg) and **10** (37 mg). Mp, $[\alpha]_D$, and $^1$H- and $^{13}$C-NMR spectral data of **9** and **10** were in agreement with the literature data of suavissimoside $F_1$ and coreanoside $F_1$, respectively; Compound **9** (Suavissimoside $R_1$): colorless needles from MeOH, mp, 285 - 288 °C, $[\alpha]_D^{18}$ +20.6° (c = 0.67, MeOH); $^1$H- NMR and $^{13}$C-NMR: Literature (Gao *et al.*, 1985); Cimpound **10** (Coreanoside $F_1$): colorless needles from MeOH-$H_2O$, mp, 242 - 245 °C (decomp.), $[\alpha]_D^{25}$ +33.2 (c = 0.30, MeOH); $^1$H-NMR and $^{13}$C-NMR: Literature (Ohtani *et al.*, 1990).

***Statistics*** – *Data were expressed as mean* ± S.D. The level of statistical significance was determined by analysis of variance followed by Duncan's new multiple range test.

## Results

To test the anti-hyperlipidemic effects, 19α-HUT fraction was obtained from the leaves of *R. crataegifolius* using solvent fractionation and charcoal and Diaion HP-20 column. The effects of 19α-HUT fraction on a high fat diet-induced rats is shown in Table 2 - 4. The diet-induced hyperlipidemia resulted in a considerable increase of weights in retroperitoneal-, epidydimal- and total abdominal fat pads, as shown in Table 2. Oral administration of 19α-HUT fraction prevented these increase at 30 and 60 mg/g. A significant decrease of hyperlipidemia in total lipid, phospholipid and total triglyceride caused by 19α-HUT fraction the model rat was also observed as shown in Table 3. Hypercholesterolemia in high fat diet-induced rats was also prevented through 19α-HUT fraction treatments. As shown in Table 4, treatments of rats with 19α-HUT fraction increased HDL-cholesterol but rather decreased total cholesterol and LDL-cholesterol. The artherogenic index of the hyperlipidemic rats was 6.29 ± 0.89 but those of 30 and 60 mg/kg-treated groups were 4.88 ± 0.37 and 3.31 ± 0.39, respectively, indicating that atherogenic risks were significantly reduced.

**Table 2.** Abdominal fat fad weight in the normal and diet-induced obesity rats fed 19α-HUT fraction obtained from *R. crataegifolius* for 4 weeks

| Treatment | Dose (mg/kg) | Retroperitoneal | Epididymal | Total-abdomonal |
|---|---|---|---|---|
| | | mg/kg body weight | | |
| Untreated | – | 5.87 ± 0.23$^d$ | 7.48 ± 0.33$^c$ | 13.12 ± 0.42$^d$ |
| Control | – | 12.13 ± 0.77$^a$ | 13.49 ± 0.45$^a$ | 24.97 ± 1.56$^a$ |
| 19α-HUT fr. | 30 | 10.95 ± 0.33$^b$ | 11.90 ± 0.32$^b$ | 21.59 ± 0.36$^b$ |
| | 60 | 9.08 ± 0.30$^c$ | 9.89 ± 0.26$^b$ | 17.58 ± 0.48$^c$ |

Rats were orally treated 19α-HUT fraction daily for consecutive two weeks of orally administration on hyperlipidemic state animal, and sacrificed 24 h after the last treatment of the fraction. Values are mean ± S.D. for nine experiments. Values followed by the same letter are not significantly different ($p < 0.05$).

**Table 3.** Effect of 19α-HUT fraction obtained from *R. crataegifolius* on the serum lipid concentrations in rats fed a hyperlipidemic rats

| Treatment | Dose (mg/kg) | Total lipid | Phospholipid | Triglyceride |
|---|---|---|---|---|
| | | mg/dl | | |
| Untreated | – | 272.4 ± 21.8$^d$ | 123.1 ± 16.8$^c$ | 81.9 ± 8.24$^e$ |
| Control | – | 487.2 ± 40.1$^a$ | 154.9 ± 21.0$^a$ | 198.6 ± 11.3$^a$ |
| 19α-HUT fr. | 30 | 469.7 ± 16.3$^b$ | 132.8 ± 13.1$^{ab}$ | 172.4 ± 6.48$^{ab}$ |
| | 60 | 429.5 ± 16.9$^c$ | 128.2 ± 12.8$^{bc}$ | 151.8 ± 5.69$^b$ |

Values are mean ± S.D. for nine experiments. Values followed by the same letter are not significantly different ($p < 0.05$).

The 19α-HUT fraction prepared from the leaves of *R. crataegifolius* to obtain biomaterial for anti-hyperlipidemic therapeutics was separated using $SiO_2$- and/or Sephadex LH column chromatography to yield ten 19α-HUT constituents. Their structures were determined on the basis of physicochemical and spectroscopic evidences.

The melting point of compound **1** was 250 - 251 °C and its molecular formula was deduced to be $C_{31}H_{48}O_4$ at m/z 509 of $[M-H]^+$, 472 ($[M-CO]^+$, 9.8), 454 ($[M-CO-H_2O]^+$, 24.9), and 426 ($[M-CO-2\times H_2O]^+$ in the EI mass spectrum. In the $^1$H-NMR spectrum of **1**, the signal of an olefinic proton was observed at δ 5.31 and attributable to H-12 in the $\Delta^{12}$-ursene-type triterpenoid, while a typical doublet peak due to H-30 in 19α-HUT was shown at δ 0.95 (3H, d, $J$ = 7.0 Hz). A peak of δ 1.36 (19-$CH_3$), which was shown at a lower region based on 19-OH linkage, together with another five singlet methyl peaks indicate that compound **1** is a 19α-hydroxy-$\Delta^{12}$-ursene-type triterpenoid. The peak of H-3 and formyl (CHO) proton were prominently observed at δ 3.18 (1H, dd) and δ 8.57 (1H, s), respectively. In particular, the formyl proton

**Table 4.** Effect of 19α-HUT fraction obtained from *R. crataegifolius* on serum cholesterol and artherogenic index in rats fed a hyperlipidemic diet

| Treatment | Dose (mg/kg) | Cholesterol (mg/dl) | | | A.I. |
|---|---|---|---|---|---|
| | | Total | HDL | LDL | |
| Untreated | – | 79.8 ± 6.45[d] | 38.8 ± 1.49[a] | 24.6 ± 4.21[d] | 1.05 ± 0.13[d] |
| Control | – | 179.3 ± 10.2[a] | 24.6 ± 1.13[d] | 115.0 ± 8.92[a] | 6.29 ± 0.89[a] |
| 19α-HUT fr. | 30 | 157.2 ± 6.32[b] | 26.7 ± 2.08[c] | 96.0 ± 4.33[b] | 4.88 ± 0.37[b] |
| | 60 | 138.2 ± 5.40[c] | 32.0 ± 1.86[b] | 75.8 ± 3.36[c] | 3.31 ± 0.39[c] |

Values are mean ± S.D. for nine experiments. Values followed by the same letter are not significantly different ($p < 0.05$).

shown at δ 8.57 was comparable with the peak δ 8.08 of formyl proton in α-amyrin formate isolated from *Marsdenia formosana* (Ito *et al.*, 1978).

In the $^{13}$C-NMR spectrum of **1**, total thirty-one carbon peaks were observed. Among them, the peaks of oxycarbons were distinguishably observed at δ 74.1 (C-19) and δ 80.3 (C-3); the chemical shifts were similar to those of pomolic acid but not quite identical. In particular, no carboxyl peak at C-28 was found in the $^{13}$C-NMR of **1** though it is present in pomolic acid (**1a**), while two carbonyl peaks of **1** were found at δ 172.4 and 172.7. Therefore, this compound cannot be pomolic acid. Compound **1** with two such carbonyl peaks in the $^{13}$C-NMR spectrum was different from common pomolic acid derivatives reported in the literature by Liang et al. (Cheng *et al.*, 1992), Nakanish *et al.* (1982), and Houghton *et al.* (1986). As shown in Table 1, pomolic acid has a carboxyl peak at $\delta_C$ 180.6, while compound **1** did not have it. Generally, acid anhydrides have such characteristic peaks in the $^{13}$C-NMR spectra due to two carbonyls near the chemical shift of compound **1**. In the HMQC spectrum, a formyl proton shown at $\delta_H$ 8.57 was correlated with an anhydride carbonyl carbon at $\delta_C$ 172.7; these results indicate the presence of anhydride. In the HMBC spectrum, as shown in Fig. 3, a peak at $\delta_H$ 2.59 due to $H_a$-16 was crossed to $\delta_C$ 55.6 (C-17) and 172.7 (formyl carbon) indicating that acid anhydridic group is attached at C-17. As shown in the Experimental section, the assignment of $^{13}$C-NMR spectroscopic data of **1** named pomolic acid ester was completed aided by 2D-NMR spectroscopy. Furthermore, alkaline hydrolysis of **1** produced pomolic acid confirming **1** is 28-*O*-formyl-3,19-dihydroyurs-12-en-28-oate (pomolic acid ester). Compound **1** was for the first time isolated from natural sources.

Compounds **2 - 10** were identified as euscaphic acid, tormentic acid, 23-hydroxytormentic acid, kaji-ichigoside $F_1$, rosamultin, niga-ichigoside $F_1$, niga-ichigoside $F_2$, suavissimoside $R_1$, coreanoside $F_1$, respectively, by comparing physicochemical data (mp, $[\alpha]_D$) and $^1$H- and

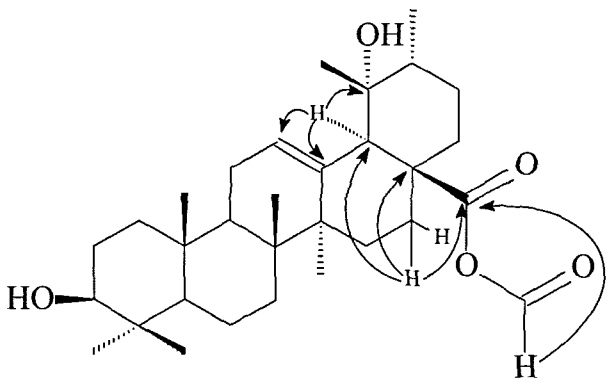



**Fig. 3.** Selected HMBC correlation of compound **1** isolated from *R. crataegifolius*.

$^{13}$C-NMR spectroscopic data of **2-10** with their data in the literature.

## Discussion

We previously reported the anti-hyperlipidemic effect of tormentic acid and euscaphic acid belonging to the 19α-HUT group (Park *et al.*, 2005). Therefore, we attempted to obtain a pure 19α-HUT fraction from Rubus species for the preparation of anti-hyperlipidemic biomaterial. During the investigation of the extraction of 19α-HUT fraction, we found that the leaves of *R. crataegifolius* is a better source for the preparation than the fruits because of their high yield and more available plant material. The fruits of *R. crataegifolius* are edible whereas the leaves are inedible, though a very high yield of 19α-HUT fraction could be obtained from the leaves.

As described in the Results section, 19α-HUT fraction exhibited a significant anti-hyperlipidemic effect in high fat diet-induced rats suggesting that this fraction could be used for biomaterial. Some 19α-HUT constituents such as euscaphic acid, tormentic acid, 23-hydroxytoemtic acid, rosamultin, and niga-ichigoside $F_1$ have been reported to have biological activities such as anti-nociceptive, anti-inflammatory (Choi *et al.*, 2003), and anti-tumor (Mura-

kami *et al.*, 2002). In this study, 19α-HUT fraction was prepared as the anti-hyperlipidemic biomaterial since it could be obtained in a high yield. The ursane-type triterpenoids without 19α-OH, e.g., asiatic acid, 23-hydroxyursolic acid (data not shown) and 2a-hydroxyurolic acid (Kim *et al.*, 2000) were generally more toxic against the cancer cells than the 19α-HUTs, suggesting 19α-OH' s role in 19α-HUTs.

From some Rubus species, the constituents of diterpenes (Chou *et al.*, 1987) tannins (Tanaka *et al.*, 1993) and flavonoids (Gudej, 2003) have been reported as well as triterpenoids. After the removal of nonpolar diterpenes, any salts or sugars using solvent fractionation and charcoal and Diaion HP-20 column as shown in Fig. 1, the 19α-HUT fraction were prepared and only triterpennoid spots were observed on TLC. To find the composition of 19α-HUTs, chromatographic isolation procedures were undertaken and finally led to the isolation of euscaphic acid, tormentic acid, 23-hydroxytormentic acid, rosamultin, kaji-ichigoside $F_1$, niga-ichigoside $F_1$ and suavissimoside $F_1$ as known compounds and pomolic acid ester (**1**) as a new compound, which all belonged to the 19α-HUT. It is our suggestion that 19α-HUTs could be obtained from the Rubus species rather than the triterpenoids lacking of 19α-OH.

It is of our interest that euscaphic acid, kaji-ichigoside $F_1$, niga-ichigoside $F_1$ are the epimers of tormentic acid, rosamultin and niga-ichigoside $F_2$ with respect to $C_3$-OH configuration, respectively. However, Ilex species contains only the 19α-HUTs with 3β-OH but without its epimeric OH implying a different biosynthetic pathway of the 19α-HUT from that of Rubus species. In particular, coreanoside $F_1$ belongs to dimeric triterpenoid (Arimoto *et al.*, 1993a; Arimoto *et al.*, 1993b). Compounds **2**, **3**, **5**, **6**, **8**, **9**, and **10** in addition to compound **1** have not been isolated from *R. crataegifolius*. All the isolates were conclusively 19α-HUTs, demonstrating the pure composition of 19α-HUTs in the fraction responsible for anti-hyperlipidemic biomaterial.

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## References

Arimoto, K., Yoshikawa, K., and Arihara, S., Triterpenoid saponins of Aquifoliaceous plants. XI. Ilexosides XVI-XIV from the leaves of *Ilex rotunda* Thunb. *Chem. Pharm. Bull.* **41**, 39-42 (1993a).

Arimoto, K., Yoshikawa, K., and Arihara, S., Triterpenoid saponins of Aquifoliaceous plants. XII. Ilexosides XLVI-LI from the leaves of *Ilex rotunda* Thunb. *Chem. Pharm. Bull.* **41**, 77-80 (1993b).

Bagniski, E. and Zak., B., Micro-determination of serum phosphate and phospholipids. Clinica. Chimica. Acta. **5**, 834-838 (1960).

Cheng, D.-L. and Cao, X.-P., Pomolic acid derivatives from the root of *Sanguisorba officinalis*. *Phytochemistry* **31**, 1317-1320 (1992).

Choi, J., Lee, K.T., Ha, J., Yun, S.Y., Ko, C.D., Jung, H.J., and Park, H.J., Antinociceptive and anti-inflammatory effects of niga-ichigoside F1 and 23-hydroxytormentic acid obtained from *Rubus coreanus*. *Biol. Pharm. Bull.* **26**, 1436-1441 (2003).

Chou, W.H., Oinaka, T., Kanamaru, F., Mizutani, K., Chen, F.H., and Osamu, T., Diterpene glycosides from leaves of Chinese *Rubus chingii* and fruits of *R. suavissimus* and identification of the source plant of the Chinese folk medicine "Fu-pen-zi". *Chem. Pharm. Bull.* **35**, 3021-3024 (1987).

Friedewald, W.T., Levy, R.I., and Fredrickson, D.S., Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin. Chem.* **24**, 499-502 (1972).

Gao, F.G., Chen, F.H., Tanaka, T., Kasai, R., Seto, T., and Tanaka, O., 19α-Hydroxyursane-type triterpene glucosyl esters from the roots of *Rubus suavissimus* S. Lee. *Chem. Pharm. Bull.* **33**, 37-40 (1985).

Gudej, J., Kaempferol and quercetin glycosides from *Rubus idaeus* L. leaves. *Acta Pol. Pharm.* **60**, 313-315 (2003).

Hirai, N., Suige, M., Wada, M., Lahlou, E.H., Kamo, T., Yoshida, R., Tsuda, M., and Ohigashi, H., Triterpene phytoalexins from strawberry fruit. *Biosci. Biotechnol. Biochem.* **64**, 1707-1712 (2000).

Houghton, P.J. and Lian, L.M., Ttritrpenoids from *Sesfontania spinosa*. *Phytochemistry* **25**, 1939-1944 (1986).

Ito, K. and Lai, J., Study on the constituents of *Marsdenia tinctoria* var. *tomentosa Masamune*. *Yakugaku Zasshi* **98**, 249 (1978).

Jung, S.W., Shin, M.H., Jung, J.H., Kim, N.D., and Im, K.S., A triterpene glucosyl from the roots of *Rubus crataegifolius*. *Arch. Pharm. Res.*, **24**, 412-415 (2001).

Kim, Y.H. and Kang, S.S., Triterpenoids from Rubi Fructus (Bogbunja). *Arch. Pharm. Res.* **16**, 109-113 (1993).

Kim, Y.K., Yoon, S.K., and Ryu, S.Y., Cytotoxic triterpenes from stem bark of *physocarpus intermedius*. *Planta Med.* **66**, 485-486 (2000).

Kuang, H.-X., Kasai, R., Ohtani, K., Liu, Z.-S., Yuan, C.-S., and Tanaka, O., Chemical constituents of pericarps of Rosa davurica Pall., a traditional Chinese medicine. *Chem. Pharm. Bull.* **37**, 2232-2233 (1989).

McGowan, M.W. and Artiss, J.D., Strandbergh, D. R, Zak, B., A peroxidase-coupled method for the colorimetric determination of serum triglycerides. *Clin. Chem.* **29**, 538-542 (1983).

Moon, K.S., Constituents and Uses of Medicinal Herbs, Ilweolseogak, Seoul, pp. 310-311, 1964.

Murakami, C., Ishijima, K., Hirota, M., Sakaguchi, K., Yoshida, H., and Mizushina, Y., Novel anti-inflammatory compounds from *Rubus sieboldii*, triterpenoids, are inhibitors of mammalian DNA polymerases. *Biochim.Biophys. Acta* **1596**, 193-200 (2002).

Nakanishi, T., Terai, H., Nasu, M., Miura, I., and Yoneda, K., Two triterpenoid glycosides from leaves of *Ilex cornuta*. *Phytochemistry* **21**, 1373-1377 (1982).

Nam, J.H., Jung, H.J., Choi, J., Lee, K.T., and Park, H.J., The antigastropathic and anti-rheumatic effect of niga-ichigoside $F_1$ and 23-hydroxytormentic acid isolated from the unripe fruits of *Rubus coreanus* in a rat model. *Biol. Pharm. Bull.* **29**, 967-970 (2006).

Noma, A., Nezu-Nakayama, K., Kita, M., and Okabe, H., Simultaneous determination of serum cholesterol in high- and low-density

lipoproteins with use of heparin, $Ca^{2+}$, and an anion-exchange resin. *Clin. Chem.* **24**, 1504-1508 (1978).

Numata, A., Yang, P., Takahashi, C., Fujiki, R., Nabae, M., and Fujita, E., Cytotoxic triterpenes from a Chinese medicine, Goreishi.*Chem. Pharm. Bull.* **37**, 648-651 (1989).

Ohtani, K., Miyajima, C., Takahashi, T., Kasai, R., Tanaka, R., and Hahn, D.R., Naruhashi N, A dimeric triterpene-glycoside from Rubus coreanus. *Phytochemistry* **29**, 3275-3280 (1990).

Park, H.J., Nam, J.H., Jung, H.J., Lee, M.S., Lee, K.T., Jung, M.H., and Choi, J.W., Inhibitory Effect of Euscaphic Acid and Tormentic acid from the Roots of *Rosa rugosa* on High Fat Diet-Induced Obesity in the Rat. *Kor. J. Pharmacogn.* **36**, 324-331 (2005).

Richmond, W., Use of cholesterol oxidase for assay of total and free cholesterol in serum by continuous flow analysis. *Clin. Chem.* **22**, 1579-1588 (1976).

Seto, T., Tanaka, T., Tanaka, O., and Naruhashi, N., β-Glucosyl esters of 19α-hydroxyursolic acid derivatives in leaves of Rubus species. *Phytochemistry* **23**, 2829-2834 (1984).

Tanaka, T., Tachibana, H., Nonaka, G., Nishioka, I., Hsu, F.L., Kohda, H., and Tanaka, O., Tannins and related compounds. CXXII. New dimeric, trimeric and terameric ellagitanins, labertianins A-D, from *Rubus lambertianus* Seringe. *Chem. Pharm. Bull.* **41**, 1214-1220 (1993).

Wang, S.Y. and Lin, H.S., Antioxidant activity and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. *J. agric. Food Chem.* **48**, 140-146 (2000).

Wang, J.S., Qiu, Z.Y., Xia, Y.P., Li, H.J., Ren, L.Y., and Zhang, L., The protective effects of total glycosides *Rubus parviflolius* on cerebral ischemic in rat. *Zhongguo Zhong Yao Za Zhi* **31**, 138-141 (2006).

Yamagishi, T., Zhang, D.-C., Chang, J.-J., McPhail, D.R., McPhail, A.T., and Lee, K.-H., The cytotoxic principles of *Hyptis capitata* and the structures of the new triterpenes hyptatic acid-A and -B. *Phytochemistry* **27**, 3213-3216 (1988).

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