

Saponins and Flavonoid Glycosides from Yellow Sweetclover

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Abstract □ A new saponin, 3-O-[α -L-rhamnopyranosyl(1 \rightarrow 2)- β -D-glucopyranosyl(1 \rightarrow 2)- β -D-glucuronopyranosyl]soyasapogenol B carboxylate (**6**) has been isolated from the medicinal plant yellow sweetclover together with azukisaponin II(**7**), robinin(**8**), and clovin(**9**). **7**, **8**, and **9** are reported for the first time from this plant. The new saponin(**6**) exhibited inhibitory action on leucocyte migration in inflammation.

Keywords □ Yellow sweetclover, Leguminosae, saponin, azukisaponin V carboxylate, azukisaponin II, flavonoid glycoside, robinin, clovin, leucocyte migration inhibitor.

Yellow sweetclover (Herba Meliloti, Leguminosae, DAB 6) has been used as a medicinal herb to relief of inflammation¹. We found that saponin and flavonoid mixture from BuOH soluble fraction of yellow sweetclover showed potent inhibitory activity on leucocyte migration² and reported the isolation of soyasapogenols B(**1**), E(**2**) and a new saponin melilotigenin (**3**) together with kaempferol (**4**) and quercetin(**5**) from this mixture^{3,4}. A further investigation on this mixture has led to the isolation of two saponins, azukisaponin V carboxylate(**6**) and azukisaponin II(**7**) as well as two flavonoid glycosides, robinin (**8**) and clovin(**9**). Of these compounds azukisaponin V carboxylate(**6**) is new and appeared to be one of the constituents responsible for inhibitory activity on leucocyte migration.

RESULTS AND DISCUSSION

The saponin and flavonoid mixture from BuOH fraction of MeOH extract was chromatographic purification yielded the two saponins(**6** and **7**) and two flavonoid glycosides(**8** and **9**). The main compound(**6**), m.p. > 300 °C, was hardly insoluble in alcohol, H₂O, dioxane, and pyridine. Absorptions at 3400 and 1610 cm⁻¹ in the IR spectrum of **6** coupled with the above physical properties, suggested that it was carboxylated form and therefore treated with weak sulfuric acid to yield acidic compound(**10**), m.p. 232-3 °C, which showed strong peak at 1728 cm⁻¹ for carboxyl group in its IR spectrum. Acid hydrolysis of **10** gave soyasapogenol B (**1**), m.p. 259-60 °C, as the genin identified by direct comparison with an authentic sample³ and glucose, glucuronic acid and rhamnose. **10** afforded a methyl

ester(**11**), m.p. 241-3 °C, on CH₂N₂ methylation, which showed seven tertiary methyl singlets at δ 0.79-1.17 and a secondary methyl doublet at δ 1.21(3H, d, J = 6Hz) together with three anomeric proton signals at δ 4.38(1H, d, J = 7Hz), 4.81(1H, d, J = 7Hz), and 5.11(1H, brs), one O-methyl singlet at δ 3.71, and an olefinic proton at δ 5.26(1H, m) in its ¹H-NMR spectrum. Partial hydrolysis of **11** afforded prosapogenins I(**12**) and II(**13**) together with soyasapogenol B(**1**). Prosapogenin I(**12**), m.p. 246-7 °C, gave glucuronic acid and soyasapogenol B(**1**) on acid hydrolysis and identified as 3-O- β -D-glucuronopyranosyl soyasapogenol B methyl ester on the basis of ¹³C-NMR spectral data (Table I). It was confirmed by comparison with the published data⁵. Prosapogenin II(**13**), m.p. 249-53 °C, was acid hydrolyzed to give glucose and glucuronic acid besides soyasapogenol B(**1**). The ¹³C-NMR spectrum of **13** showed signals for glucose, glucuronic acid methyl ester and soyasapogenol B(**1**). The C-2' signal of glucuronic acid moiety was deshielded(6.5 ppm) than prosapogenin I(**12**). Consequently, prosapogenin II(**13**) was suggested to possess a terminal β -D-glucopyranosyl residue attached to the C-2' hydroxyl function in the prosapogenin I(**12**). In the light of these observations, structure of prosapogenin II(**13**) has been assigned as 3-O-[β -D-glucopyranosyl(1 \rightarrow 2)- β -D-glucuronopyranosyl]soyasapogenol B methyl ester, which was identified as azukisaponin II methyl ester by comparison with the published data^{6,7}. The presence of significant glycosidation shift of C-2" signal of the glucopyranoside moiety(2.8ppm) together with a set of signals for terminal rhamnose moiety in the ¹³C-NMR spectrum of **11** indicated that the ter-

Table I. ^{13}C -NMR chemical shifts of saponin(1), saponins(10, 11), prosapogenins(12, 13) and flavonoid glycosides(8, 9)

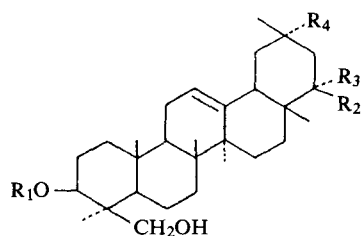
| Carbon | 1* | 10* | 11* | 13* | 12* | Carbon | 8+ | 9++ |
|---------------------|-------|-------|-------|-------|-------|---------|-------|-------|
| C-1 | 39.0 | 38.7 | 38.9 | 39.0 | 39.0 | C-2 | 156.3 | 156.1 |
| C-2 | 28.5 | 26.6 | 26.7 | 26.6 | 27.0 | C-3 | 133.9 | 133.9 |
| C-3 | 80.3 | 91.4 | 91.4 | 91.1 | 89.5 | C-4 | 177.8 | 177.7 |
| C-4 | 43.3 | 43.9 | 44.0 | 44.0 | 44.6 | C-5 | 161.9 | 161.8 |
| C-5 | 56.5 | 56.5 | 56.5 | 56.5 | 56.4 | C-6 | 99.5 | 99.5 |
| C-6 | 19.2 | 18.8 | 18.9 | 18.8 | 18.8 | C-7 | 161.1 | 161.0 |
| C-7 | 33.6 | 33.5 | 33.5 | 33.5 | 33.7 | C-8 | 94.9 | 94.5 |
| C-8 | 40.1 | 40.1 | 40.2 | 40.2 | 40.2 | C-9 | 156.3 | 157.0 |
| C-9 | 48.2 | 48.0 | 48.0 | 48.0 | 48.1 | C-10 | 105.8 | 105.7 |
| C-10 | 37.1 | 36.6 | 36.7 | 36.7 | 36.9 | C-1' | 120.9 | 121.1 |
| C-11 | 24.1 | 24.2 | 24.3 | 24.2 | 24.2 | C-2' | 131.2 | 115.3 |
| C-12 | 122.7 | 123.3 | 122.9 | 123.3 | 123.3 | C-3' | 115.3 | 145.0 |
| C-13 | 145.0 | 144.9 | 145.0 | 145.0 | 145.0 | C-4' | 160.3 | 148.8 |
| C-14 | 42.4 | 42.6 | 42.6 | 42.6 | 42.6 | C-5' | 115.3 | 116.3 |
| C-15 | 26.5 | 26.6 | 26.7 | 26.6 | 27.0 | C-6' | 131.2 | 122.1 |
| C-16 | 28.8 | 28.8 | 28.8 | 28.8 | 28.8 | C-1'' | 102.2 | 102.0 |
| C-17 | 38.1 | 38.1 | 38.2 | 38.1 | 38.2 | C-2'' | 71.4 | 71.2 |
| C-18 | 45.5 | 45.6 | 45.6 | 45.6 | 45.6 | C-3'' | 73.3 | 73.2 |
| C-19 | 46.9 | 46.8 | 47.0 | 47.0 | 47.0 | C-4'' | 68.4 | 68.4 |
| C-20 | 30.9 | 30.9 | 31.0 | 31.0 | 31.0 | C-5'' | 74.0 | 73.8 |
| C-21 | 42.5 | 42.6 | 42.6 | 42.6 | 42.6 | C-6'' | 65.6 | 65.3 |
| C-22 | 75.7 | 75.8 | 75.8 | 75.8 | 75.8 | C-1''' | 100.3 | 100.1 |
| C-23 | 23.6 | 22.9 | 22.9 | 22.8 | 23.4 | C-2''' | 70.6 | 70.2 |
| C-24 | 64.7 | 63.7 | 63.7 | 63.5 | 63.5 | C-3''' | 70.9 | 70.5 |
| C-25 | 16.3 | 15.8 | 15.8 | 15.8 | 15.8 | C-4''' | 72.2 | 72.1 |
| C-26 | 17.1 | 17.2 | 17.2 | 17.2 | 17.2 | C-5''' | 68.4 | 68.4 |
| C-27 | 25.7 | 25.8 | 25.8 | 25.8 | 25.8 | C-6''' | 18.0 | 18.0 |
| C-28 | 28.7 | 28.8 | 28.8 | 28.8 | 28.8 | C-1'''' | 98.8 | 98.7 |
| C-29 | 33.3 | 33.3 | 33.3 | 33.3 | 33.3 | C-2'''' | 70.3 | 70.5 |
| C-30 | 21.2 | 21.1 | 21.2 | 21.2 | 21.2 | C-3'''' | 70.6 | 70.7 |
| C-1' | | 105.3 | 105.3 | 105.2 | 106.6 | C-4'''' | 71.9 | 71.8 |
| C-2' | | 78.3 | 78.3 | 81.9 | 75.4 | C-5'''' | 70.1 | 70.0 |
| C-3' | | 78.4 | 78.3 | 78.2 | 78.0 | C-6'''' | 18.0 | 18.0 |
| C-4' | | 73.6 | 73.5 | 72.6 | 73.3 | | | |
| C-5' | | 77.8 | 76.8 | 77.1 | 77.4 | | | |
| C-6' | | 172.4 | 170.3 | 170.3 | 170.3 | | | |
| (OCH ₃) | | | 52.2 | 52.2 | 52.2 | | | |
| C-1'' | | 102.2 | 102.2 | 104.9 | | | | |
| C-2'' | | 79.0 | 78.6 | 75.8 | | | | |
| C-3'' | | 78.0 | 78.0 | 78.6 | | | | |
| C-4'' | | 72.4 | 72.4 | 70.1 | | | | |
| C-5'' | | 78.5 | 78.4 | 78.3 | | | | |
| C-6'' | | 62.9 | 61.5 | 61.8 | | | | |
| C-1''' | | 102.0 | 102.0 | | | | | |
| C-2''' | | 72.4 | 72.4 | | | | | |
| C-3''' | | 72.7 | 72.8 | | | | | |
| C-4''' | | 74.4 | 74.4 | | | | | |
| C-5''' | | 69.4 | 69.5 | | | | | |
| C-6''' | | 18.9 | 18.9 | | | | | |

*Recorded at 20MHz in pyridine-d₅+Recorded at 20MHz in DMSO-d₆++Recorded at 50.3MHz in DMSO-d₆

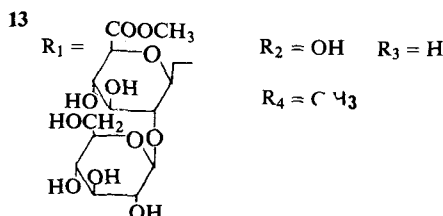
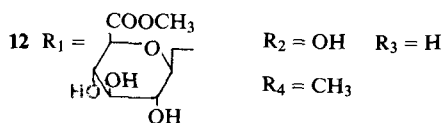
minimal rhamnose moiety in **11** was bound to C-2'' hydroxyl group in the prosapogenin II(**13**). On the basis of these results, the structure of saponin (**10**) can be established as 3-O-[(α -L-rhamnopyranosyl (1 \rightarrow 2)- β -D-glucopyranosyl)] soyasapogenol B, which is identical to azukisaponin V⁸⁾. This saponin (**10**) was previ-

ously isolated from *Vigna angularis* as free form⁸⁾. However, the carboxylate form of azukisaponin V (**6**) is the first isolation from the plant source and the present report confirms the assigned structure from ^{13}C -NMR spectral data.

The second saponin (**7**), m.p. 216-8 °C, was hydrolyzed to yield soyasapogenol B(**1**), glucose and

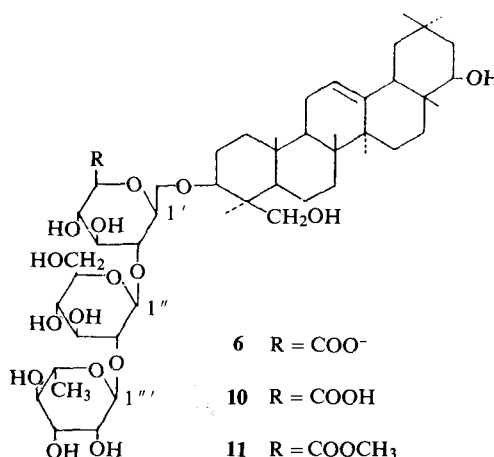


- 1** $R_1 = R_3 = H$ $R_2 = OH$ $R_4 = CH_3$
2 $R_1 = H$ $R_2, R_3 = O$ $R_4 = CH_3$
3 $R_1 = H$ $R_2, R_3 = O$ $R_4 = COOH$



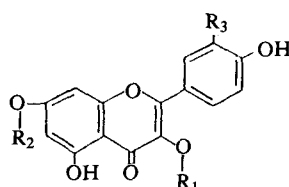
glucuronic acid. It gave a methyl ester(**13**) on CH_2N_2 methylation. The spectral and chemical data of the methyl ester(**13**) corresponded in all respects with those of prosapogenin II. Therefore, the structure of **7** was assigned to be 3-O-[β -D-glucopyranosyl (1 \rightarrow 2)- β -D-glucuronopyranosyl] soya-sapogenol B. This compound was isolated from *V. angularis* as free form⁶) and *Galega officinalis* as carboxylate form⁷).

Compounds **8** and **9** showed positive results in Molisch and Shinoda tests for flavonoid glycosides and gave the same sugars, galactose and rhamnose, on acid hydrolysis together with kaempferol(**4**) from **8** and quercetin(**5**) from **9** as the aglycones. Comparison of the UV spectra of both flavonoids before and after acid hydrolysis indicated that the sites of glycosidation are at the 3 and 7 positions in both compounds. The ¹H-NMR spectra of **8** showed signals corresponding to two moles of rhamnose and one mole of galactose with six aromatic protons for kaempferol(**4**). The ¹H-NMR spectra of **9** showed virtually identical to those of **8** except that signals for aromatic protons indicating that compounds **8** and **9** are the same interglycosidic linkage and the positions of glycosidation which finally determined by ¹³C-NMR spectroscopy. As observed in Table I, the ¹³C-NMR spectra of **8** and **9** are identical to those of robinin and clovin, respectively,



- 6** $R = COO^-$
10 $R = COOH$
11 $R = COOCH_3$

reported in the literatures⁹⁻¹¹). Thus, the structures of **8** and **9** are kaempferol 3-O- α -L-rhamnopyranosyl(1 \rightarrow 6)- β -D-galactopyranoside 7-O- α -L-rhamnopyranoside and the corresponding quercetin analogue, respectively. Both are the first report of the isolation from this plant.



- 4** $R_1 = R_2 = R_3 = H$
5 $R_1 = R_2 = H$ $R_3 = OH$
8 $R_1 =$ $R_2 =$ $R_3 = H$
9 $R_1 =$ $R_2 =$ $R_3 = OH$

Table II. The effect of test materials on leucocyte migration and protein exudation in CMC pouch of rats

| Treatment | Dose (mg/pouch) | No. of animals | Leucocyte migration into pouch (cells/mm ³ ± S.E.) | Protein exudation into pouch (mg/ml ± S.E.) |
|-----------------------|--------------------|-------------------|---|---|
| Control | - | 6 | 4313 ± 257.2 | 9.1 ± 0.60 |
| Flavonoid fraction | 6 | 6 | 4640 ± 400.0 | 8.1 ± 0.67 |
| 6 | 6 | 6 | 3030 ± 320.5* | 6.9 ± 0.62* |
| 1 | 2 | 6 | 6236 ± 1529.3 | 8.1 ± 1.0 |
| 7 | 3 | 6 | 4433 ± 109.1 | 9.6 ± 0.72 |
| Aspirin | 20 | 6 | 3015 ± 330.3* | 7.6 ± 0.39 |

*Significantly different from the control group ($p < 0.05$)

The result on the effect of leucocyte migration and protein exudation into carboxymethyl cellulose pouch by test materials was shown in Table II. Aspirin used as reference compound and azukisaponin V carboxylate(6) showed inhibitory actions by 30.1% and 29.7%, respectively, in leucocyte migration. These data were significantly different from the control group ($p < 0.05$) in Student t-test. However, flavonoid fraction, soyasapogenol B(1) and azukisaponin II(7) did not exhibit the action at the doses tested in this experiment. In our previous report², azukisaponin V(10) revealed potent inhibitory action in leucocyte migration at a dose of 6mg/pouch. In the protein exudation into pouch, azukisaponin V carboxylate (6) showed inhibitory action by 24.2% as compared to the control group ($p < 0.05$).

MATERIALS AND METHODS

M.p.'s were determined on a Mitamura-Riken apparatus and are uncorrected. IR spectra were run as KBr discs on a Perkin-Elmer 283B spectrophotometer. Optical rotations were recorded on a Rudolph Autopol III automatic polarimeter. ¹H- and ¹³C-NMR spectra were recorded at 80MHz for ¹H and 20MHz or 50.3MHz for ¹³C, respectively, with a Varian FT-80A or a Bruker AM-200 spectrometer and chemical shifts values are quoted in ppm downfield from TMS as internal standard.

Plant material

The chopped plant material(Herba Meliloti conc., DAB 6) was purchased from Apotheke im Stadthaus, Bonn, West Germany.

Isolation

The plant material(800g) was extracted and frac-

tionated as previous described³. The BuOH fraction was suspended in distilled water to remove water-soluble materials and filtered. The residue (saponin and flavonoid mixture) was chromatographed over a flash column(SiO₂) and eluted with CHCl₃-MeOH-H₂O(520:280:80, lower phase) to give pure saponins(6 and 7) and flavonoid glycosides(8 and 9). A portion of the residue was chromatographed over a polyamide column(polyamide C-200) and eluted with MeOH to give the flavonoid fraction for bioassay.

Azukisaponin V carboxylate(6)

Crystallized from MeOH as amorphous white (380mg); m.p. > 300°C; IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3400(OH), 1610(COO⁻), 1070, 1040(C-O), 805(C=C).

Azukisaponin II(7)

Crystallized from MeOH as needles(50mg); m.p. 216-8°C; $[\alpha]_D^{25}$: +28.4°(c 0.5, MeOH); IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3390(OH), 1730(COOH), 1645(C=C), 1080, 1043, 1030(C-O).

Robinin(8)

Crystallized from MeOH as fine needles(250mg); m.p. 247-8°C; $[\alpha]_D^{25}$: -103.8°(c 0.29, pyridine); IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3400(OH), 1660(α, β -unsaturated C=O), 1600, 1490(C=C), 1005-1090(C=O); UV λ_{max} (MeOH): 247(sh, 4.16), 268(4.29), 320(sh, 4.08), 353(4.23); (CH₃ONa): 244(4.31), 276(4.32), 308(sh, 3.96), 355(sh, 4.20), 396(4.33); (AlCl₃): 277(4.33), 301(3.95), 356(4.14), 403(4.19); (AlCl₃ + HCl): 277(4.29), 301(sh, 3.95), 350(4.15), 400(4.14); (NaOAc): 262(sh, 4.27), 268(4.28), 300(sh, 3.88), 360(sh, 4.10), 403(4.22); (NaOAc + H₃BO₃): 268(4.34), 321(sh, 4.10), 356(4.26); ¹H-NMR(80MHz, DMSO-d₆) δ : 1.07(3H, d, J=5.6Hz, rha-CH₃), 1.13(3H, d, J=5.4Hz, rha-

CH₃), 4.41(1H, brs, H-1'''), 5.35(1H, d, J=7.1Hz, H-1''), 5.54(1H, brs, H-1'''), 6.44(1H, d, J=2Hz, H-6), 6.80(1H, d, J=2Hz, H-8), 6.87(2H, d, J=8.8Hz, H-3', 5'), 8.09(2H, d, J=8.8Hz, H-2', 6'), 12.55(1H, brs, 5-OH); ¹³C-NMR(20MHz, DMSO-d₆): see Table I.

Clovin(9)

Crystallized from MeOH as stout needles(30 mg); m.p. 200-1 °C; [α]²⁶_D: -90°(c 0.26, MeOH); IR ν^{KBr}_{max} cm⁻¹: 3400(OH), 1660(α, β-unsaturated C=O), 1600, 1495(C=C), 1010-1090(C-O); UV λ_{max}(MeOH): 259(4.42), 269(sh, 4.33), 362(4.31); (CH₃ONa): 271 (4.41), 400(4.34); (AlCl₃): 277(4.45), 300(sh, 3.96), 344(3.75), 442(4.42); (AlCl₃ + HCl): 272(4.41), 301 (sh, 3.93), 365(sh, 4.14), 407(4.27); (NaOAc): 263 (4.37), 298(sh, 3.92), 383(4.16), 418(4.20); (NaOAc + H₃BO₃): 263(4.46), 393(4.32); ¹H-NMR(80MHz, DMSO-d₆) δ : 1.07(3H, d, J=5.6Hz, rha-CH₃), 1.14(3H, d, J=5.2Hz, rha-CH₃), 4.42(1H, brs, H-1'''), 5.35(1H, d, J=6.9Hz, H-1''), 5.54(1H, brs, H-1'''), 6.44(1H, d, J=2Hz, H-6), 6.77(1H, d, J=2Hz, H-8), 6.83(1H, d, J=8.4Hz, H-5'), 7.57(1H, d, J=2Hz, H-2'), 7.68(1H, dd, J=2 and 8.4Hz, H-6'), 12.57(1H, brs, 5-OH); ¹³C-NMR(50.3MHz, DMSO-d₆): see Table I.

Acid treatment of 6

A sample of 6(300mg) was dissolved in 0.02N-H₂SO₄ in 60% dioxane(200 ml) at room temperature and filtered to remove the precipitate. The filtrate was concentrated to the half volume under reduced pressure and added to the crushed ice. The precipitate was filtered, washed and dried. The dried precipitate was dissolved in EtOH followed by recrystallization to yield pure acidic compound(10) as flakes; m.p. 232-3 °C; [α]²⁵_D: +0.7°(c 0.6, MeOH); IR ν^{KBr}_{max} cm⁻¹: 3410(OH), 1728(COOH), 1070, 1045(C-O), 1635, 805(C=C); ¹³C-NMR(20MHz, pyridine-d₃): see Table I.

Acid hydrolysis of 10

A solution of 10(40mg) in 5% H₂SO₄ in dioxane-H₂O(2:1, 25ml) was refluxed for 4hr, concentrated under reduced pressure, and diluted with crushed ice-water to give a precipitate which was collected by filtration and recrystallized from MeOH to give pure soyasapogenol B(1) as needles; m.p. 259-60 °C. Its identity was confirmed by direct comparison with an authentic sample[TLC, m.m.p. and ¹³C-NMR³⁾]. The filtrate was neutralized with BaCO₃ with stirring, filtered, and concentrated in vacuo. L-rhamnose, D-glucose, and D-glucuronic

acid were identified by TLC(precoated cellulose, pyridine-ethyl acetate-acetic acid-water = 36:36:7:21; Rf 0.67 for rhamnose, 0.46 for glucose, and 0.15 and 0.56 for glucuronic acid).

Methylation of 10

A sample of 10(230mg) was methylated in MeOH solution with ethereal CH₂N₂. After usual workup, the reaction product was crystallized from MeOH to give 11 as shining plates; m.p. 241-3 °C; [α]²⁴_D: +0.8°(c 0.8, MeOH); IR ν^{KBr}_{max} cm⁻¹: 3400(OH), 1745(COOCH₃), 1640, 810(C=C), 1072, 1040(C-O); ¹H-NMR(80MHz, CDCl₃-CD₃OD-D₂O-TFA): see text; ¹³C-NMR(20MHz, pyridine-d₃): see Table I.

Partial hydrolysis of 11

A solution of 11(210mg) in 0.1N-H₂SO₄ in 75% dioxane(15ml) was refluxed for 1.5hr, to which crushed ice was added and filtered. The precipitate was washed with distilled water and chromatographed over SiO₂ column. Elution with CHCl₃-MeOH(6:1) gave 10 subfractions. Subfraction No. 7 was crystallized from MeOH to afford prosapogenin II(13, 50mg) as plates; m.p. 249-53 °C; [α]²⁴_D: +20.3°(c 0.4, MeOH); IR ν^{KBr}_{max} cm⁻¹: 3460, 3400(OH), 1735(COOCH₃), 1650 (C=C), 1090, 1080, 1053, 1031(C-O); ¹³C-NMR(20MHz, pyridine-d₃): see Table I. Subfraction No. 5 was rechromatographed over SiO₂ column, followed by elution with CHCl₃-MeOH(9:1) and crystallized from MeOH to prosapogenin I(12, 20 mg) as needles; m.p. 246-7 °C; [α]²⁴_D: +33.7°(c 0.25, MeOH); IR ν^{KBr}_{max} cm⁻¹: 3420(OH), 1745(COOCH₃), 1640(C=C), 1092, 1052, 1030(C-O); ¹³C-NMR(20MHz, pyridine-d₃): see Table I.

Acid hydrolysis of 12 and 13

Acid hydrolysis of 12 and 13 was separately performed by refluxing each prosapogenin(4mg) with 5% H₂SO₄ in 60% dioxane for 4hr. Soyasapogenol B(1) was identified as the genin in each case. Glucuronic acid from 12 and glucuronic acid and glucose from 13 were detected by TLC as described above.

Acid hydrolysis of 7

A solution of 7(10mg) was worked up as for the acid hydrolysis of 6 to give 1. The aqueous layer furnished D-glucose and D-glucuronic acid.

Acid hydrolysis of 8 and 9

Acid hydrolysis of 8(30mg) and 9(10mg) was separately performed as described above. Kaemp-

ferol(4) from 8 and quercetin(5) from 9 were identified as the genin by direct comparison with authentic samples. D-galactose and L-rhamnose were identified in each case.

Inhibitory action on leucocyte migration and protein exudation

The effect of test materials on leucocyte migration and protein exudation into carboxymethyl cellulose pouch in rats was tested with modification of the method of Ishikawa *et al.*¹². Two percent CMC solution in physiological saline was sterilized. Five ml of CMC solution was injected into the air sac which was made before 24hr by injecting subcutaneously on the dorsum of Sprague-Dawley rats (180-200g) with 7ml of air. In the treatment group, the material suspended in sterilized 2% CMC solution was injected likewise into the air sac. Six hr after the treatment, the animals were sacrificed and the whole fluid in the sac was collected. For staining the leucocytes 0.5ml of pouch fluid was mixed with 4.5ml of Turk solution for 10min and the number of leucocytes was counted with hemocytometer. Another 0.1ml aliquot of the pouch fluid was diluted with 5ml of physiological saline and its protein concentration was measured by Lowry's method¹³.

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