# Three New Anti-Oxidative Saponarin Analogs from Young Green Barley Leaves

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**Three new saponarin analogs, 6‴-sinapoylsaponarin, 6‴-feruloylsaponarin and 4′-glucosyl-6‴-sinapoylsaponarin were isolated together with four known compounds from young green barley (*Hordeum vulgare* var. *nudum*) leaves. Their anti-oxidative effects towards superoxide, photo-oxidation of vitamins and the 1, 1-diphenyl-2-picrylhydrazyl radical were tested. The superoxide-scavenging activity depended on the number of free hydroxy groups. In contrast, the inhibition of vitamin oxidation seemed to be due to the flavone skeleton. In addition, the sinapoyl moiety seemed to be important for radical-scavenging activity.**

**Key words** *Hordeum vulgare* var. *nudum*; Gramineae; anti-oxidative; flavonoid; isovitexin; saponarin

Barley (*Hordeum* spp.) is distributed worldwide and one of the most important cultivated plants. Many C-glycosylflavones have been identified in leaf extracts of *Hordeum* spp.,[1] and saponarin (7-glucosylisovitexin, **4**) especially, is the major flavone of barley leaves.[2] Antioxidants are added to food to protect it against damage caused by free radicals. Therefore, effective natural antioxidants are of great interest.

In this paper, we report the structural determination and anti-oxidative effects of five new saponarin analogs, together with two known ones, isolated from young barley leaves.

A methanolic extract of the spray-dried young barley leaves of *Hordeum vulgare* var. *nudum* was partitioned between *n*-hexane and 80% MeOH. After evaporation of the latter phase, the residue was partitioned between ethyl acetate and 40% MeOH. The 40% MeOH extracts were chromatographed on Diaion HP-20, silca-gel and octadecyl silica (ODS) columns to obtain compounds **1**—**7**. Compounds **4**—**7** were identified as saponarin,[3] isovitexin 4′, 7-diglucoside,[3c] isovitexin 7-rhamnosylglucoside[4] and isovitexin,[3] respectively.

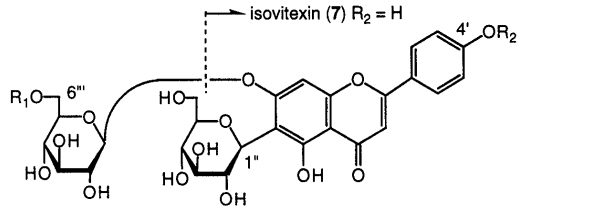
Compound **1** was obtained as a colorless amorphous powder, $[\alpha]_D$ −144° ($H_2O$ : MeOH = 1 : 1). In the positive FAB-MS, **1** showed an $[M+H]^+$ ion at *m/z* 801 which was 206 mass units larger than that of **4**. Alkaline hydrolysis of **1** gave **4**. The $^1$H-NMR spectrum clearly displayed signals due to a sinapoyl group[5] [$\delta$ 3.67 (6H, s), 6.71 (2H, s), 6.37 (1H, d, $J$=16 Hz), 7.48 (1H, d, $J$=16 Hz)] except for those of saponarin moiety. In the $^{13}$C-NMR signals (Table 1) of **1**, acylation shifts[6] [(ppm) : C-5‴ (−3.2), C-6‴ (+2.4)] were observed in the glucosyl moiety at C-7 compared with those of **4**. Therefore, compound **1** was concluded to be 6‴-sinapoylsaponarin.

Compound **2**, a colorless amorphous powder, $[\alpha]_D$ −141° ($H_2O$ : MeOH = 1 : 1) also gave **4** by alkaline hydrolysis. In the $^1$H-NMR spectrum of **2**, although the signals due to the saponarin moiety agreed with those of **1**, the peak-height of the methoxy signal was reduced to one-half that of **1**. The $^{13}$C-NMR spectrum (Table 1) suggested the presence of a feruloyl group,[7] which was also confirmed by observation of a FAB-MS peak due to the $[M+H]^+$ ion at *m/z* 771 being 30 mass units less than that of **1**. Since similar acylation shifts to those of **1** were observed, compound **2** was determined to be 6‴-feruloylsaponarin.

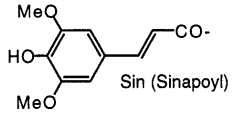
Compound **3**, a colorless amorphous powder, $[\alpha]_D$ −84° ($H_2O$ : MeOH = 1 : 1) gave **5** on alkaline hydrolysis. In the positive FAB-MS, **3** showed an $[M+H]^+$ ion at *m/z* 963 which was 162 mass units larger than that of **1**. In the $^1$H-NMR spectrum of **3**, a sinapoyl group and three anomeric signals were observed. In the $^{13}$C-NMR spectrum (Table 1) of **3**, the signals attributable to the B and C rings together with a glucosyl moiety attached at C-4′ were consistent with those of **5**, whereas the remaining signals were compatible with those of **1**. Consequently, compound **3** was determined to be 4′-glucosyl-6‴-sinapoylsaponarin.

The present study was carried out to investigate the *in vitro* mode of activity of the isolated flavone derivatives in a non-enzymic $O_2^-$ generation system, a 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical as a model of an unsaturated fatty acid radical, and vitamins as a model of a light oxidation system.

Firstly, the anti-oxidative actions of **1**—**7** were determined




| | $R_1$ | $R_2$ |
|---|---|---|
| 6‴-sinapoylsaponarin (**1**) | Sin | H |
| 6‴-feruloylsaponarin (**2**) | Fer | H |
| 4′-glucosyl-6‴-sinapoylsaponarin (**3**) | Sin | Glc |
| saponarin (**4**) | H | H |
| isovitexin 4′, 7-diglucoside (**5**) | H | Glc |
| isovitexin 7-rhamnosylglucoside (**6**) | Rha | H |



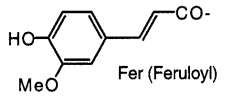




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Table 1. $^{13}$C-NMR Data for Compounds **1**—**7**

| | **1** | **2** | **3** | **4** | **5** | **6** | **7** |
|---|---|---|---|---|---|---|---|
| C-2 | 164.0 | 164.1 | 163.4 | 164.1 | 163.3 | 164.3 | 163.6 |
| C-3 | 102.7 | 102.9 | 103.7 | 103.0 | 104.0 | 103.0 | 102.8 |
| C-4 | 181.8 | 181.9 | 181.8 | 181.8 | 181.8 | 181.8 | 181.8 |
| C-5 | 159.5 | 159.5 | 159.6 | 159.9 | 159.6 | 159.2 | 159.9 |
| C-6 | 110.7 | 110.7 | 110.5 | 110.9 | 110.3 | 108.9 | 108.9 |
| C-7 | 161.9 | 161.8 | 162.2 | 162.3 | 162.2 | 162.2 | 163.3 |
| C-8 | 93.7 | 93.7 | 93.6 | 93.7 | 93.7 | 93.5 | 93.7 |
| C-9 | 156.1 | 156.0 | 156.3 | 156.2 | 156.2 | 156.2 | 156.3 |
| C-10 | 105.0 | 104.5 | 104.9 | 105.4 | 104.9 | 105.8 | 103.4 |
| C-1′ | 120.7 | 120.8 | 123.4 | 120.9 | 123.7 | 120.9 | 121.1 |
| C-2′, 6′ | 127.9 | 128.0 | 127.9 | 128.1 | 127.8 | 128.2 | 128.5 |
| C-3′, 5′ | 115.6 | 115.8 | 116.3 | 115.8 | 116.6 | 115.9 | 116.0 |
| C-4′ | 161.0 | 161.0 | 160.2 | 161.0 | 160.2 | 161.0 | 161.2 |
| C-1″ | 73.3 | 73.3 | 73.1 | 73.5 | 73.0 | 73.4 | 73.1 |
| C-2″ | 70.6 | 70.6 | 70.7 | 70.6 | 70.6 | 70.6 | 70.6 |
| C-3″ | 78.8 | 78.8 | 78.8 | 78.8 | 78.8 | 78.8 | 78.9 |
| C-4″ | 70.1 | 70.1 | 69.8 | 69.7 | 69.8 | 69.6 | 70.3 |
| C-5″ | 80.8 | 80.7 | 80.5 | 80.8 | 80.8 | 80.9 | 81.5 |
| C-6″ | 60.7 | 60.5 | 60.2 | 60.7 | 60.7 | 60.7 | 61.5 |
| C-1‴ | 101.4 | 100.9 | 100.6 | 101.3 | 101.3 | 101.4 | |
| C-2‴ | 72.6 | 72.7 | 72.6 | 72.7 | 72.6 | 72.7 | |
| C-3‴ | 75.7 | 75.8 | 75.6 | 75.8 | 75.8 | 75.7 | |
| C-4‴ | 69.7 | 70.1 | 69.6 | 69.7 | 69.7 | 69.6 | |
| C-5‴ | 73.8 | 73.8 | 73.5 | 77.0 | 76.9 | 75.7 | |
| C-6‴ | 63.1 | 63.2 | 63.0 | 60.7 | 60.7 | 68.0 | |
| C-1⁗ | | | 99.6 | | 100.1 | 100.3 | |
| C-2⁗ | | | 73.1 | | 73.4 | 70.2 | |
| C-3⁗ | | | 76.5 | | 76.4 | 70.8 | |
| C-4⁗ | | | 69.2 | | 69.7 | 72.1 | |
| C-5⁗ | | | 77.1 | | 77.0 | 68.0 | |
| C-6⁗ | | | 60.6 | | 60.7 | 17.4 | |
| Sin C-1 | 124.2 | | 124.1 | | | | |
| C-2, 6 | 106.4 | | 105.9 | | | | |
| C-3, 5 | 147.8 | | 147.7 | | | | |
| C-4 | 138.5 | | 138.1 | | | | |
| C-7 | 145.1 | | 145.7 | | | | |
| C-8 | 114.4 | | 114.4 | | | | |
| C-9 | 166.1 | | 166.4 | | | | |
| OMe | 55.9 | | 55.8 | | | | |
| Fer C-1 | | 125.4 | | | | | |
| C-2 | | 111.7 | | | | | |
| C-3 | | 149.2 | | | | | |
| C-4 | | 147.7 | | | | | |
| C-5 | | 115.4 | | | | | |
| C-6 | | 122.3 | | | | | |
| C-7 | | 144.9 | | | | | |
| C-8 | | 114.5 | | | | | |
| C-9 | | 166.0 | | | | | |
| OMe | | 55.6 | | | | | |

Chemical shifts ($\delta$, ppm) were measured in DMSO-$d_6$ at 90 °C.

by superoxide-scavenging activity.[8] Superoxide anions were estimated by spectrophotometic measurement of the product of the reduction of nitro blue tetrazolium (NBT). The superoxide-scavenging activity of **1**—**7** (Fig. 1) might depend upon the number of free phenolic hydroxy groups. In fact, compounds **1**, **2** and **7** having three phenolic hydroxy groups exhibited stronger inhibition than **3**—**6** which have less than two hydroxy groups.

Next, the anti-oxidative actions of **1**—**7** were measured by inhibition of the photo-oxidation of vitamins (Fig. 2a—c).[9—11] In contrast to the former anti-oxidative experiment, all compounds showed a similar ability to inhibit the oxidation of vitamins $K_1$, $B_6$ and $B_2$. Therefore, the inhibition of vitamin oxidation seems to be due to the flavone skeleton

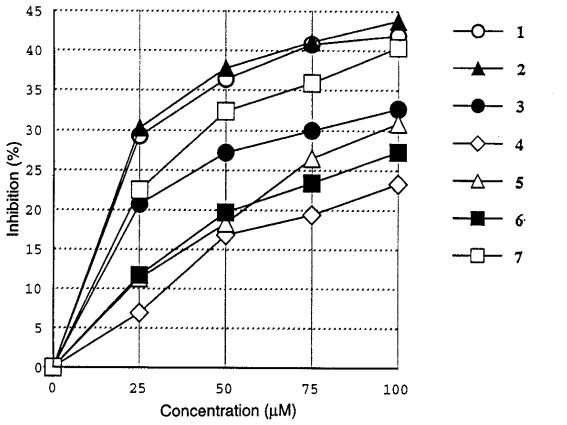


Fig. 1. Effects of Compounds **1**—**7** on Superoxide Anion

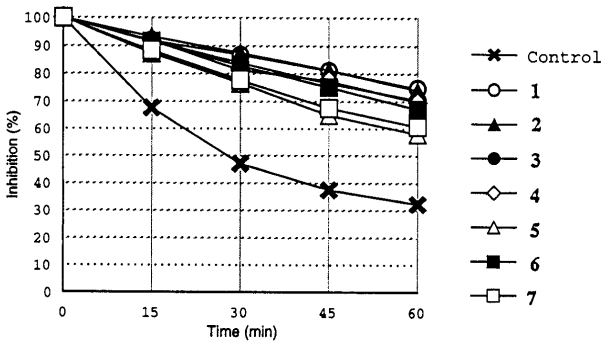


Fig. 2a. Effects of Compounds **1**—**7** on the Photo-Oxidation of Vitamin $B_6$

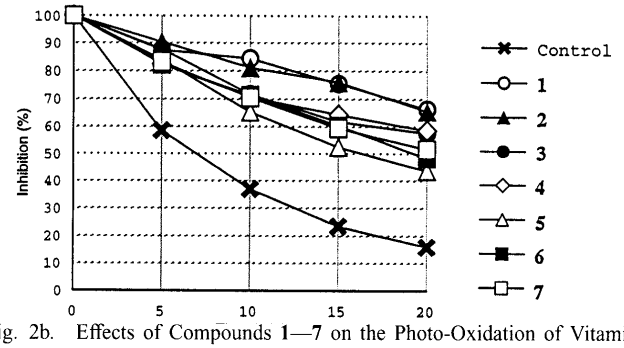


Fig. 2b. Effects of Compounds **1**—**7** on the Photo-Oxidation of Vitamin $K_1$

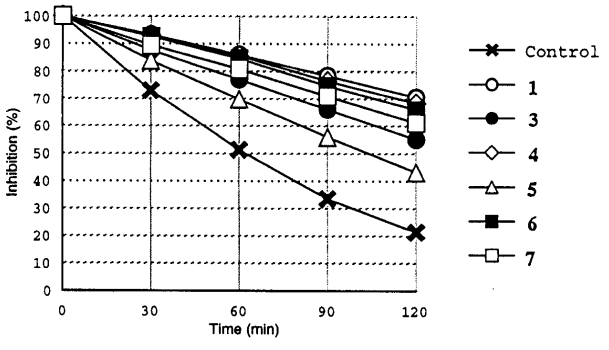


Fig. 2c. Effects of Compounds **1**, **3**—**7** on the Photo-Oxidation of Vitamin $B_2$

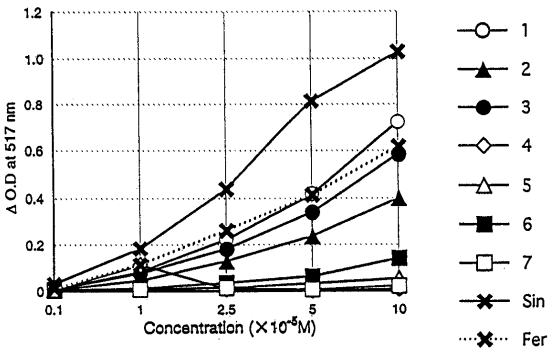


Fig. 3. Effects of Compounds **1**—**7** and Sinapic (Sin), Ferulic (Fer) Acids on Diphenyl-2-picryl-hydrazyl (DPPH) Radical

with the hydroxy group at C-5.

Finally, the anti-oxidative actions of **1**—**7** together with sinapic acid and ferulic acid were compared monitoring their DPPH reducing activity[12,13] (Fig. 3). Determination of the reducing activity of the DPPH radical was carried out by spectrophotometry. Compounds **1**—**3** showed potent radical-scavenging activity. Furthermore, sinapic acid and ferulic acid themselves also showed strong activity and the former was particularly potent. Despite having three phenolic hydroxy groups, compound **7** did not exhibit any activity. Consequently, the sinapoyl and feruloyl moieties seem to be important for radical-scavenging activity.

Investigation of the free radical-scavenging activity using ESR spin-trapping is now in progress.

## Experimental

**Instruments** $^1$H-NMR (500 MHz) and $^{13}$C-NMR (125 MHz) spectra were recorded at 90 °C in DMSO-$d_6$. Tetramethylsilane was used as an internal standard. FAB-MS (positive ion mode) were measured using a glycerol matrix.

**Reagents** Tris (hydroxymethyl) aminomethane was purchased from Kishida Chemical Co., Ltd. (Osaka, Japan). Hydrochloric acid, dimethyl sulfoxide (DMSO), methanol, ethanol, acetic acid, sodium dihydrogen phosphate and sodium hydroxide were purchased from Hayashi Pure Chemical Industries, Ltd. (Osaka, Japan). Pyridoxine hydrochloride and sodium dodecylsulfate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Phylloquinone, phosphoric acid, sodium 1-pentanesulfonate, NBT, phenazine methosulfate (PMS) and DPPH were bought from Nacalai Tesque, Inc. (Kyoto, Japan). Riboflavin (Japanese Pharmacopoeia Standard) was obtained from the National Institute of Health Science (Tokyo, Japan). $\beta$-Nicotinamide adenine dinucleotide, reduced form, was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

**Plant Material** Young barley leaves were harvested two weeks after germination. The barley leaves were compressed by a sqweezer and then separated from crude fibrous material by filtration to obtain a barley leaf extract. The barley leaf extract so obtained was dried by spray-drying.

**Extraction and Isolation** The spray-dried powders (1.8 kg) were extracted with MeOH, and the extract (161 g) was initially partitioned between *n*-hexane and 80% MeOH. After evaporation of the latter phase, the residue (138 g) was partitioned between ethyl acetate and 40% MeOH. The 40% MeOH extracts (127 g) were chromatographed on Diaion HP-20 to afford fractions 1 to 5, fractions 1, 2, 3 and 4 were separated by silica-gel ($CHCl_3$ : MeOH : $H_2O$ = 7 : 3 : 0.5—6 : 4 : 1) and ODS ($H_2O$–MeOH) column chromatography to provide compounds **1** (0.0022%), **2** (0.0011%), **3** (0.0006%), **4** (0.047%), **5** (0.0007%), **6** (0.0013%) and **7** (0.0006%).

**Compound 1 (6‴-Sinapoylsaponarin)** A colorless amorphous powder, $[\alpha]_D^{25}$ −144° (*c*=0.50, 50% MeOH). HR positive ion FAB-MS *m/z*: 823.2058 ($C_{38}H_{40}NaO_{19}$, Calcd for 823.2061). Positive ion FAB-MS *m/z*: 801 $[M+H]^+$. UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 333 (4.38). $^1$H-NMR (in DMSO-$d_6$): 3.67 (6H, s, sinapoyl OMe×2), 4.74 (1H, d, *J*=9 Hz, 6-*C*-glc-H-1), 5.05 (1H, d, *J*=7 Hz, 7-*O*-glc-H-1), 6.37 (1H, d, *J*=16 Hz, sinapoyl H-8), 6.66 (1H, s, H-8), 6.71 (2H, s, sinapoyl H-2, 6), 6.81 (1H, s, H-3), 6.85 (2H, d, *J*=9 Hz, H-3′, 5′), 7.48 (1H, d, *J*=16 Hz, sinapoyl H-7), 7.78 (2H, d, *J*=9 Hz, H-2′, 6′), 13.41 (1H, s, C-5 OH). $^{13}$C-NMR (in DMSO-$d_6$): Table 1.

**Compound 2 (6‴-Feruloylsaponarin)** A colorless amorphous powder, $[\alpha]_D^{25}$ −141° (*c*=0.50, 50% MeOH). HR positive ion FAB-MS *m/z*: 793.1954 ($C_{38}H_{40}NaO_{19}$, Calcd for 793.1956). Positive ion FAB-MS *m/z*: 771 $[M+H]^+$. UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 329 (4.47). $^1$H-NMR (in DMSO-$d_6$): 3.69 (3H, s, feruloyl OMe), 4.73 (1H, d, *J*=9 Hz, 6-*C*-glc-H-1), 5.05 (1H, d, *J*=7 Hz, 7-*O*-glc-H-1), 6.32 (1H, d, *J*=16 Hz, feruloyl H-8), 6.60 (1H, d, *J*=8 Hz, feruloyl H-6), 6.68 (1H, s, H-8), 6.82 (1H, s, H-3), 6.86 (2H, d, *J*=9 Hz, H-3', 5'), 7.03 (1H, d, *J*=2 Hz, feruloyl H-2), 7.48 (1H, d, *J*=16 Hz, feruloyl H-7), 7.82 (2H, d, *J*=9 Hz, H-2′, 6′), 13.41 (1H, s, C-5 OH). $^{13}$C-NMR (in DMSO-$d_6$): Table 1.

**Compound 3 (4′-Glucosyl-6‴-Sinapoylsaponarin)** A colorless amorphous powder, $[\alpha]_D^{25}$ −84° (*c*=0.50, 50% MeOH). HR positive ion FAB-MS *m/z*: 985.2589 ($C_{38}H_{40}NaO_{19}$, Calcd for 985.2590). Positive ion FAB-MS *m/z*: 963 $[M+H]^+$. UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$): 329 (4.39). $^1$H-NMR (in DMSO-$d_6$): 3.67 (6H, s, sinapoyl OMe×2), 4.74 (1H, d, *J*=9 Hz, 6-*C*-glc-H-1), 4.96 (1H, d, *J*=7 Hz, 4′-*O*-glc-H-1), 5.05 (1H, d, *J*=7 Hz, 7-*O*-glc-H-1), 6.39 (1H, d, *J*=16 Hz, sinapoyl H-8), 6.74 (1H, s, H-8), 6.72 (2H, s, sinapoyl H-2, 6), 6.84 (1H, s, H-3), 7.11 (2H, d, *J*=9 Hz, H-3′, 5′), 7.48 (1H, d, *J*=16 Hz, sinapoyl H-7), 7.88 (2H, d, *J*=9 Hz, H-2′, 6′), 13.35 (1H, s, C-5 OH). $^{13}$C-NMR (in DMSO-$d_6$): Table 1.

**Alkaline Hydrolysis of 1—3** A small sample of **1** was hydrolyzed in 2 N NaOH–$H_2O$ at 60 °C for 4 h. After filtration of the mixture, the filtrate was passed through an Amberlite IRA-400 column for desalting. After concentrating the MeOH eluate, the solution was subjected to TLC analysis [TLC, Kiesegel 60 $F_{254}$ (Merck), $CHCl_3$ : MeOH : $H_2O$ (6 : 4 : 1), *Rf*: 0.56 (**4**, saponarin), *n*-BuOH : AcOH : $H_2O$ (4 : 1 : 5, upper), *Rf*: 0.33 (**4**), reagent: 20% aq. $H_2SO_4$] and $^1$H-NMR [4.73 (1H, d, *J*=9 Hz, 6-*C*-glc-H-1), 4.97 (1H, d, *J*=7 Hz, 7-*O*-glc-H-1), 6.88 (1H, s, H-8), 6.75 (1H, s, H-3), 6.94 (2H, d, *J*=9 Hz, H-3′, 5′), 7.90 (2H, d, *J*=9 Hz, H-2′, 6′), 13.48 (1H, s, C-4 OH)]. Similarly, compound **2** gave **4**, while compound **3** gave **5** [[$CHCl_3$ : MeOH : $H_2O$ (6 : 4 : 1), 0.25 (**5**, isovitexin 4′, 7-diglucoside), *n*-BuOH : AcOH: $H_2O$ (4 : 1 : 5, upper), *Rf*: 0.10 (**5**)], [4.98 (1H, d, *J*=7 Hz, 4′-*O*-glc-H-1), 5.00 (1H, d, *J*=7 Hz, 7-*O*-glc-H-1), 6.86 (1H, s, H-3), 6.91 (1H, s, H-8), 7.22 (2H, d, *J*=9 Hz, H-3′, 5′), 8.01 (2H, d, *J*=9 Hz, H-2′, 6′), 13.44 (1H, s, C-5 OH)]].

**Compound 4 (Saponarin)**[3] A colorless needles, mp 229—231 °C, $[\alpha]_D^{25}$ −33° (*c*=0.50, DMSO). Positive ion FAB-MS *m/z*: 595 $[M+H]^+$. $^1$H-NMR (in DMSO-$d_6$): 4.98 (1H, d, *J*=7 Hz, 7-*O*-glc-H-1), 6.90 (1H, s, H-8), 6.76 (1H, s, H-3), 6.96 (2H, d, *J*=9 Hz, H-3′, 5′), 7.91 (2H, d, *J*=9 Hz, H-2′, 6′), 13.43 (1H, s, C-5 OH). $^{13}$C-NMR (in DMSO-$d_6$): Table 1.

**Compound 5 (Isovitexin 4′, 7-Diglucoside)**[3c] A colorless amorphous powder, $[\alpha]_D^{25}$ −51° (*c*=0.50, MeOH). Positive ion FAB-MS *m/z*: 757 $[M+H]^+$. $^1$H-NMR (in DMSO-$d_6$): 4.74 (1H, d, *J*=9 Hz, 6-*C*-glc-H-1), 4.96 (1H, d, *J*=7 Hz, 4′-*O*-glc-H-1), 5.00 (1H, d, *J*=7 Hz, 7-*O*-glc-H-1), 6.86 (1H, s, H-3), 6.91 (1H, s, H-8), 7.22 (2H, d, *J*=9 Hz, H-3′, 5′), 8.01 (2H, d, *J*=9 Hz, H-2′, 6′), 13.36 (1H, s, C-5 OH). $^{13}$C-NMR (in DMSO-$d_6$): Table 1.

**Compound 6 (Isovitexin 7-Rhamnosylglucoside)**[4] A colorless amorphous powder, $[\alpha]_D^{25}$ −100° (*c*=0.50, 50% MeOH). Positive ion FAB-MS *m/z*: 741 $[M+H]^+$. $^1$H-NMR (in DMSO-$d_6$): 1.14 (3H, d, *J*=6 Hz, rha-$H_3$-6), 4.97 (1H, d, *J*=7 Hz, 7-*O*-glc-H-1), 6.78 (1H, s, H-3), 6.89 (1H, s, H-8), 6.98 (2H, d, *J*=9 Hz, H-3′, 5′), 7.90 (2H, d, *J*=9 Hz, H-2′, 6′), 13.59 (1H, s, C-5 OH). $^{13}$C-NMR (in DMSO-$d_6$): Table 1.

**Compound 7 (Isovitexin)**[3] Colorless needles, mp 216—219 °C, $[\alpha]_D^{25}$ −1° (*c*=0.50, DMSO). Positive ion FAB-MS *m/z*: 433 $[M+H]^+$. $^1$H-NMR (in DMSO-$d_6$): 4.60 (1H, d, *J*=10 Hz, 6-*C*-glc-H-1), 6.53 (1H, s, H-3), 6.78 (1H, s, H-8), 6.94 (2H, d, *J*=9 Hz, H-3′, 5′), 7.93 (2H, d, *J*=9 Hz, H-2′, 6′), 13.55 (1H, s, C-5 OH). $^{13}$C-NMR (in DMSO-$d_6$): Table 1.

**Measurement of Superoxide Scavenging Activity** The inhibitory effect of samples on the formation of superoxide anions was measured in a reaction system which consisted of 50 $\mu$M PMS, 100 $\mu$M NADH and 60 $\mu$M NBT in 50 mM $KH_2PO_4$–$Na_2HPO_4$ buffer pH 7.4, in a total volume of 1.0 ml with samples dissolved in DMSO. The reaction was started by addition of PMS to the reaction system and, after 3 min of incubation at 25 °C, the absorbance was measured at 560 nm using a Hitachi U-3000 spectrophotometer.

**Measurement of Inhibition of Vitamin Photo-Oxidation** A solution of riboflavin–HCl (200 $\mu$M) or pyridoxine–HCl (200 $\mu$M) was prepared by dissolving it in 200 mM Tris buffer. The phylloquinone solution was prepared by dissolving it in ethanol at a concentration of 200 $\mu$M. The flavones tested were dissolved in 10 ml DMSO and then 2 ml DMSO solution was diluted with distilled water to give a concentration of 200 $\mu$M. The solution of vita-

mins (200 $\mu$l) was mixed with the sample (200 $\mu$l) solution in a 4 ml quartz cell (T-3 UV-10, Nippon Silica Glass Co., Ltd., Tokyo, Japan), covered with aluminium foil during preparation to avoid any effect of light on the vitamin oxidation. The sample cuvettes were then irradiated at 20 °C for 10 to 120 min in a Light-Toron apparatus (Nagano Science Co., Ltd., Osaka, Japan) equipped with six fluorescent chemical lamps, FLR-20S-BL/M (Toshiba Co., Tokyo, Japan). The irradiation intensity was 300 $\mu$W/cm$^2$ and a residual amount of riboflavine–HCl was determined using a Shimadzu HPLC Class-LC10 equipped with a Inertsil ODS-3 column, 5 $\mu$m, 4.6× 150 mm (GL Science Inc., Tokyo, Japan) and a Shimadzu spectrophotometric detector RF-10A (ex. 445 nm, em. 530 nm). The HPLC system was operated at 40 °C and flow rate of the mobile phase, 10 mM $NaH_2PO_4$ (pH 5.5) : methanol (65 : 35), was 0.5 ml/min.

The concentration of pyridoxine–HCl was determined by HPLC-LC using an Inertsil ODS-3 column (5 $\mu$m, 4.6×150 mm) and a Shimadzu UV-VIS Detector SPD-10AV (290 nm) at 50 °C. The mobile phase was 5 mM $NaH_2PO_4$ (pH 2.6) with sodium 1-pentaesulfonate:methanol (9 : 1) and flow rate of the mobile phase was 1.5 ml/min. The determination of phylloquinone was carried out using the HPLC system described above at 30 °C. The mobile phase consisted of MeOH : AcOH (99 : 1) and the flow rate was 1.0 ml/min.

**Measurement of DPPH Radical-Reducing Activity** The samples dissolved in DMSO (0.15 ml) were added to DPPH radical EtOH solution ($1.11\times10^{-4}$ M, 1.35 ml) in a 1.5 ml plastic Dispocell 1938 PS (Kartell, Italy); after a 20 min incubation at 20 °C, the absorbance was measured at 517 nm using a Hitachi U-3000 spectrophotometer.

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