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Antioxidant Constituents from *Setaria viridis*

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The EtOAc and *n*-BuOH soluble fractions from the aerial part of *Setaria viridis* showed a strong free radical scavenging activity. Six major compounds were isolated from these fractions. They were identified by spectral data as tricin (**1**), *p*-hydroxycinnamic acid (**2**), vitexin 2"-*O*-xyloside (**3**), orientin 2"-*O*-xyloside (**4**), tricin-7-*O*-*β*-*D*-glucoside (**5**) and vitexin 2"-*O*-glucoside (**6**). Among these compounds, **4** and **5** exhibited strong free radical scavenging activities on 1, 1-diphenyl-2-picrylhydrazyl (DPPH). We further studied the effects of these isolated compounds on the lipid peroxidation in rat liver microsomes induced by non-enzymatic method. As expected, **4** and **5** exhibited significant inhibition on ascorbic/Fe^{2+} induced lipid peroxidation in rat liver microsomes.

Key words: *Setaria viridis*, Flavonoids, Antioxidant, Free radical scavenging activity, DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical, Lipid peroxidation

INTRODUCTION

Setaria viridis (Gramineae) is widely distributed in worldwide, and its aerial part is used as anti-inflammatory agent (Kim *et al.*, 1997). There is one report on the chemical constituents of this plant (Shigeo *et al.*, 1986). In the course of a screening to evaluate antioxidant constituents from medicinal plants, we found that EtOAc and *n*-BuOH extracts from the aerial parts of *Setaria viridis* showed a strong free radical scavenging activity. Repeated column chromatography of the EtOAc and *n*-BuOH soluble fractions led to the isolation of six compounds. This paper deals with structure elucidation of these compounds and their antioxidant activities using DPPH free radical scavenging method and lipid peroxidation in rat liver microsomes induced by non-enzymatic methods.

MATERIALS AND METHOD

Instruments and reagents

Melting points were determined on a Fisher-Johns melting point apparatus and were uncorrected. Nuclear magnetic resonances (^{1}H-NMR and ^{13}C-NMR spectra taken at 200 and 50 MHz, respectively) were recorded on a Varian Gemini 200 spectrometer using deuterated solvents as the internal standard. The EI (70 eV) and FAB MS spectra were determined using a Autospec Micromass, UV spectra were obtained using a Hitachi U-2000, and IR spectra in a KBr disk using a Bio-Rad FTS-7. TLC work was carried out using plates coated with silica gel 60 F254 (Merck Co.). All solvents were routinely distilled prior to use. Silica gel column chromatography was performed on Merck silica gel 60 (70-230 mesh and 230-400 mesh). Other reagents were commercial grade without purification.

Plant materials

The aerial part of *Setaria viridis* was collected at Chuncheon, Kangwon Province, Korea in August 2000 and identified taxonomically with respect to morphology. A voucher specimen of the plant was deposited at the College of Pharmacy (KNUP-H-43), Kangwon National University.

Extraction and isolation

The air-dried aerial parts (3 kg) were ground and extracted three times with hot MeOH over a total 4hr period. The resultant extracts were combined and concentrated under reduced pressure to afford 420 g of

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the residue. This MeOH extract was suspended in 10 volumes of water and then partitioned successively with equal volumes of *n*-hexane, EtOAc and *n*-BuOH, leaving a residual water soluble fraction. Each fraction was evaporated in vaccuo to yield the residues of *n*-hexane fraction(fr.) (110 g), EtOAc fr., (3.8 g), and *n*-BuOH fr., (25 g).

The EtOAc soluble fraction (3.8 g) was column chromatographed on a silica gel (250 g, 70-230 mesh, 15 × 50 cm) using stepwise gradient elution with the solvents $CHCl_3$-MeOH (9:1→2:1 v/v) to divide the fraction into four sub-fractions (Fr.1-Fr.4).

Sub-fraction 1 was re-chromatographed on silica gel column (70 g, 70-230 mesh, 2 × 50 cm) by elution with benzene-acetone-MeOH (8:1.5:0.5) to give compound **1** (384 mg). Sub-fraction 2 was re-chromatographed on silica gel column (70 g, 70-230 mesh, 2 × 50 cm) by elution with $CHCl_3$-MeOH-H_2O (4:1:0.1) to give compound **2** (75 mg). On the other hand, the *n*-BuOH soluble fraction (25 g) was column chromatographed on a silica gel (500 g, 70-230 mesh, 15 × 50 cm) using stepwise gradient elution with the solvents EtOAc-MeOH-H_2O (8:2:0.5→7:2:1 v/v) to divide the fraction into four sub-fractions (Fr.1-Fr.4').

Sub-fraction 2' was re-chromatographed on ODS (70 g, YMC gel, ODS-A, S-150 μm) column by elution with 30% MeOH to give compound **3** (129 mg), compound **4** (39 mg), compound **5** (31 mg) and compound **6** (18.5 mg), respectively.

Compound 1 (Tricin): MP : 291~292°; UVλ_{max}: MeOH 247sh, 269 342 nm; UVλ_{max}: MeOH+NaOH 264, 275.5sh, 417.5 nm; UVλ_{max}: MeOH+NaOAc 275, 321sh, 362 nm; UVλ_{max}: MeOH+NaOAc+H_3BO_3 271, 342.5 nm; IRν_{max} (KBr) cm^{-1}: 3449(OH), 1667(C=O), 1055(C-O); ^{1}H-NMR (DMSO-d_6) δ_H (ppm): 12.96(1H, s, 5-OH), 7.32(2H, s, H-6' and H-2'), 6.97(1H, s, H-3), 6.55(1H, d, *J*=2.0Hz, H-8), 6.20(1H, d, *J*=2.0Hz, H-6) and 3.87(6H, s, OCH_3 × 2); ^{13}C-NMR (DMSO-d_6) δ_C (ppm): see **Table I**; Positive FAB-MS *m/z*: 331 $[M+H]^+$

Compound 2 (*p*-Hydroxycinnamic acid): MP : 213~215°; UVλ_{max}: MeOH 221, 293sh, 313 nm; UVλ_{max}: MeOH +NaOH 221, 334, 341nm; IRν_{max} (KBr) cm^{-1}: 3345(OH), 1648, 1619(C=O), 1502,1432(C=C); ^{1}H-NMR (DMSO-d_6) δ_H (ppm): 7.57(1H, d, *J*=15.8Hz, H-7), 7.58(2H, d, *J*=8.6Hz, H-2 and H-6), 6.86(2H, d, *J*=8.6Hz, H-3 and H-5), 6.36(1H, d, *J*=15.8Hz, H-8), ^{13}C-NMR (DMSO-d_6) δ_C (ppm): 168.05(C-9), 159.68(C-4), 144.20(C-8), 130.14(C-3, and 5), 125.35(C-7), 115.81(C-2 and 6), 115.47 (C-1); EI-MS(70 eV) *m/z*: 164 $[M]^+$

Compound 3 (Vitexin 2"-*O*-xyloside): MP : 209~211°; UVλ_{max}: MeOH 270, 309sh, 341 nm; UVλ_{max}: MeOH+NaOH 280, 330, 388 nm; UVλ_{max}: MeOH+NaOAc 278.5, 300sh, 359.5 nm; UVλ_{max}: MeOH+NaOAc+ H_3BO_3 270, 309sh, 341nm; UVλ_{max}: MeOH+$AlCl_3$ 274.5, 303, 340, 379 nm; UVλ_{max}: MeOH+$AlCl_3$+HCl 273, 302, 339, 378nm; IRν_{max} (KBr)cm^{-1}: 3450(OH), 1660(C=O), 1060(C-O); ^{1}H-NMR (DMSO-d_6) δ_H (ppm): 13.23 (1H, s, 5-OH), 8.11(2H, d, *J*=8.8Hz, H-6'and H-2'), 6.99(2H, d, *J*=8.8Hz, H-3'and H-5'), 6.87(1H, s, H-3), 6.32(1H, s, H-6), 5.322.31(18H, m, sugar protons), 4.76(D_2O exchange 2H, br. d, *J*=10.0Hz, H-1"and H-1'"); ^{13}C-NMR (DMSO-d_6) $_C$ (ppm): see **Table I**; Positive FAB-MS *m/z*: 565 $[M+H]^+$

Compound 4 (Orientin 2"-*O*-xyloside): MP : 203~205°;

Table I. ^{13}C-NMR(50MHz) data for compounds **1**, **3**, **4**, **5** and **6**(DMSO-d_6)

comp. / carbon	1	3	4	5	6
C-2	164.22	163.41	163.99	164.21	163.87
C-3	104.48	102.01	102.43	105.43	102.72
C-4	181.90	181.64	182.04	182.18	182.11
C-5	161.51	160.76	160.62	160.19	160.15
C-6	98.89	97.66	98.08	99.57	98.19
C-7	163.76	162.29	162.77	163.10	162.65
C-8	94.25	103.27	103.65	95.38	103.71
C-9	157.43	156.19	156.67	156.96	156.27
C-10	103.67	103.37	103.81	103.85	103.92
C-1'	120.49	121.54	122.00	120.22	121.79
C-2'	103.81	128.46	114.01	104.54	128.94
C-3'	148.29	115.51	145.91	148.30	115.90
C-4'	139.95	160.16	149.70	140.18	160.68
C-5'	148.29	115.51	115.81	148.30	115.90
C-6'	103.81	128.46	119.29	104.54	128.94
OCH_3	56.41			56.41	
D-glucose					
C-1		71.08	71.53	100.21	71.55
C-2		81.33	82.02	73.19	81.82
C-3		77.86	78.41	77.41	78.47
C-4		69.67	70.37	69.69	70.15
C-5		80.39	80.91	76.54	81.20
C-6		60.50	61.37	60.68	60.99
D-xylose					
C-1		105.33	105.87		
C-2		73.20	73.70		
C-3		75.39	75.90		
C-4		68.88	69.33		
C-5		65.02	65.52		
D-glucose					
C-1'					105.20
C-2'					74.39
C-3'					76.03
C-4'					69.49
C-5'					76.26
C-6'					60.42

$UV\lambda_{max}$: MeOH 257, 269, 341 nm; $UV\lambda_{max}$: MeOH+NaOH 269.5, 277.5, 339sh, 400.5 nm; $UV\lambda_{max}$: MeOH+NaOAc 271.5, 356 nm; $UV\lambda_{max}$: MeOH+NaOAc+H_3BO_3 262.5, 379 nm; $UV\lambda_{max}$: MeOH+$AlCl_3$ 269, 379.5 nm; $UV\lambda_{max}$: MeOH+$AlCl_3$+HCl 243, 359 nm; $IR\nu_{max}$ (KBr)cm^{-1}: 3440(OH), 1645(C=O), 1070(C-O); ^{1}H-NMR (DMSO-d_6) δ_H (ppm): 13.23(1H, s, 5-OH), 7.60(2H, m, H-6'and H-2'), 6.96(H, d, *J*=7.8Hz, H-5'), 6.74(1H, s, H-3), 6.31(1H, s, H-6), 5.27~2.44(18H, m, sugar protons), 4.84(D_2O exchange 2H, br. d, *J*=10.0Hz, H-1" and H-1'"); ^{13}C-NMR (DMSO-d_6) δ_C (ppm): see **Table I**; Positive FAB-MS *m/z*: 581 $[M+H]^+$

Compound 5 (Tricin-7-*O*-β-*D*-glucoside): MP : 240~241°; $UV\lambda_{max}$: MeOH 253, 269, 341 nm; $UV\lambda_{max}$: MeOH+NaOH 259.5, 294, 427 nm; $UV\lambda_{max}$: MeOH+NaOAc 253, 268.5, 341 nm; $UV\lambda_{max}$: MeOH+NaOAc+H_3BO_3 253, 269, 341 nm; UV_{lmax}: MeOH+$AlCl_3$ 248, 341, 380 nm; $UV\lambda_{max}$: MeOH+$AlCl_3$+HCl 241, 340, 378 nm; $IR\nu_{max}$ (KBr)cm^{-1}: 3460(OH), 1672(C=O), 1060(C-O); ^{1}H-NMR (DMSO-d_6) $_H$ (ppm): 13.05(1H, s, 5-OH), 7.44(2H, s, H-6'and H-2'), 7.16(1H, s, H-3), 7.02(1H, d, *J*=2.2Hz, H-8), 6.54(1H, d, *J*=2.2Hz, H-6), 3.96($OCH_3$2), 5.512.57(12H, m, sugar protons), 5.09(D_2O exchange 1H, d, *J*=7.8Hz, H-1"); ^{13}C-NMR (DMSO-d_6) δ_C (ppm): see **Table I**; Positive FAB-MS, *m/z*: 493 $[M+H]^+$

Compound 6 (Vitexin 2"-*O*-glucoside): MP : 218~220°; $UV\lambda_{max}$: MeOH 269.5, 300sh, 334 nm; $UV\lambda_{max}$: MeOH+NaOH 279.5, 313.5, 380 nm; $UV\lambda_{max}$: MeOH+NaOAc 279, 300sh, 372 nm; $UV\lambda_{max}$: MeOH+NaOAc+ H_3BO_3 271, 341 nm; $UV\lambda_{max}$: MeOH+$AlCl_3$ 305sh, 341, 384 nm; $UV\lambda_{max}$: MeOH+$AlCl_3$+HCl 341, 384 nm; $IR\nu_{max}$ (KBr)cm^{-1}: 3420(OH), 1660(C=O), 1070(C-O); ^{1}H-NMR (DMSO-d_6) δ_H (ppm): 13.24(1H, s, 5-OH), 8.08(2H, d, *J*=8.4Hz, H-6' and H-2'), 6.97(2H, d, *J*=8.4Hz, H-3'and H-5'), 6.82(1H, s, H-3), 6.31 (1H, s, H-6), 5.532.57(21H, m, sugar protons), 4.85(D_2O exchange, 2H, br. d, *J*=10.0Hz, H-1" and H-1'"); ^{13}C-NMR (DMSO-d_6) δ_C (ppm): see **Table I**; Positive FAB-MS, *m/z*: 595 $[M+H]^+$

Acid hydrolysis of compounds 3, 4, 5 and 6

The solution of compounds **3**, **4**, **5** and **6** (each 5 mg) in 10% H_2SO_4 (50 ml) were heated in a water bath for 1 hr then cooled, respectively.

The reaction mixtures were diluted with water, neutralized with $BaCO_3$ and concentrated *in vacuo* to dryness, respectively. The sugar components were determined by TLC.

Sample preparation

The tested samples were dissolved in MeOH-DMSO (1:1) to various concentration.

Preparation of rat liver microsomal suspension

Male Sprague-Dawley rat liver was removed and washed with ice-cold 0.9% saline. The liver was homogenized in 9 vol. of ice-cold 1.15% KCl. To prepare the liver microsomal fraction, the liver was rapidly homogenized in ice-cold 0.25 M sucrose and centrifuged at 9000 g at 4°C for 20 min. The supernatant was centrifuged at 105000 g at 4°C for 60 min. The microsomal pellets were washed 3 times with ice-cold 0.15 M KCl, and then stored at -20°C until the experiments (Nguyen *et al.*, 1998). The protein content of tissue homogenates was measured using Bio-Rad protein assay kit (Maxwell *et al.*, 1999; Na *et al.*, 2001).

DPPH radical scavenging activity

The scanvenging activity corresponded to the intensity quenching DPPH radical (Hatano *et al*, 1989). Five hundred μl of 60 μM DPPH in MeOH-DMSO(1:1) was added to 500 μl of sample solution and allowed to react for 30 min at room temperature, the optical density was measured at 520 nm. For blank, MeOH-DMSO(1:1) was used instead of DPPH solution, and for control, H_2O was used instead of sample solution. The IC_{50} values were calculated from regression lines where the abscissa represented the concentration of test compound and the ordinate the average percent reduction of DPPH radical from three separate tests.

Inhibition effect on lipid peroxidation in rat liver microsomes

The antioxidant activity was determined by quantification of thiobarbituric acid reactive substance (TBA-RS) using a slightly modified method of Buege and Aust (Nguyen *et al*, 1998). The reaction mixture was composed of 0.5 ml tissue homogenate, 0.9 ml phosphate buffer (50 mM, pH 7.4), 0.5 ml of one of the chemical system generating free radicals: 0.01 mM $FeSO_4$ 0.1 mM ascorbic acid (Fe-VC) or 0.01 mM $FeSO_4$ H_2O_2 (the Fenton reagent), and 0.1 of vehicle or a solution of test agents. The reaction mixtures were incubated by adding 1 ml of 10% (w/v) trichloroacetic acid to the mixture. After centrifugation at 8000 g for 10 min, the supernatants were incubated with 1 ml of 0.8% (w/v) TBA at 100°C for 15 min. After a cooling period, TBA-RS generated was spectrophotometrically determined at 532 nm.

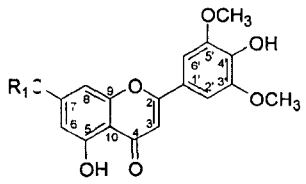
Statistic analysis

Data were expressed as the concentration of MDA nmol/mg protein/ml tissue homogenate and analyzed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test for multiple comparison among groups. Differences of $p<0.05$ were considered

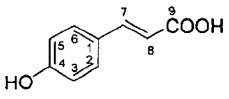
statistically significant.

RESULTS AND DISCUSSION

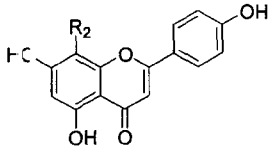
The EtOAc and *n*-BuOH extracts of the aerial parts of *S. viridis* showed a strong free radical scavenging activity. Thus, detailed laboratory investigation was performed on these two active extracts. Bioassay-guided fractionation led to the isolation of compounds **4** and **5** as active principles along with weak active compounds **1** and **6**, and inactive constituents, **2** and **3**. The UV spectrum of compound **1** showed 4', 5, 7-trihydroxy flavone skeleton (Mabry *et al.*, 1970). The ^{1}H-NMR spectrum of **1** exhibited signals due to the C-6 and C-8 positions of the flavone ring at δ6.20 (1H, d, *J*=2.0Hz) and 6.55 (1H, d, *J*=2.0Hz), due to protons of the C-2' and C-6' positions at 7.32(2H, s), due to proton of the C-3 position at 6.97 (1H, s), and due to the two methoxyl groups at 3.86(6H, s). Based on these results and on values previously reported in the literature (Agrawal, 1989; Ulubelen *et al.*, 1984), compound **1** was identified as tricin. Compound **2** was identified as phenyl propanoid, *p*-hydroxycinnamic acid on the basis of spectral analysis as well as comparisons of physical constants with those of reported in the literature (Dey and Harborne, 1989; Pouchert and Behnke, 1993). The UV spectrum of compound **3** showed flavone skeleton and diagnostic shifts as apigenin with free 5, 7, 4'-hydroxyl groups (Mabry *et al.*, 1970). Acid hydrolysis of **3** gave vitexin and xylose (identified by co-TLC with an authentic samples). These data suggested that **3** is vitexin-*O*-xyloside. The attachment cite of xylose was confirmed from its ^{13}C-NMR spectrum. Based on these results and on values previously reported in the literature (Agrawal, 1989; Gluchoff-Fiasson *et al.*, 1989), compound **3** was identified as vitexin 2"-*O*-xyloside.



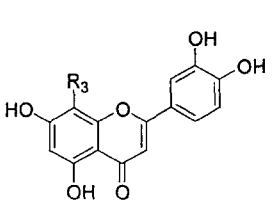
1. R_1 = H
5. R_1 = β-D-glucose



2



3. R_2 = xylose (1'''->2")glucose
5. R_2 = glucose (1'''->2")glucose



4. R_3 = xylose (1'''->2")glucose

Fig. 1. The Structures of Compouds **1-6**

Compound **4** was shown to be luteolin derivative from UV spectrum and it gave evidence for free hydroxyl groups in the 5, 7, 3' and 4' positions (Mabry *et al.*, 1970). Acid hydrolysis of **4** gave orientin and xylose (identified by co-TLC with an authentic samples). These data suggested that **4** is orientin-*O*-xyloside. The attachment cite of xylose was confirmed from its ^{13}C-NMR spectrum. Based on these results and on values previously reported in the literature (Agrawal, 1989; Gluchoff- Fiasson *et al.*, 1989), compound **4** was identified as orientin 2"-*O*-xyloside. The UV spectrum of compound **5** showed 4', 5-dihydroxyflavone skeleton (Mabry *et al.*, 1970). The ^{1}H-NMR features of this compound were similar to those of tricin (**1**) except for the sugar moiety. Acid hydrolysis of **5** gave tricin and glucose (identified by co-TLC with an authentic samples). Based on these results and on values previously reported in the literature (Agrawal, 1989; Ulubelen *et al.*, 1984; Hirai *et al.*, 1986), compound **5** was identified as tricin-7-*O*-β-*D*-glucoside. The ^{1}H, and ^{13}C-NMR spectra of compound **6** similar to those of compound **3** except for the presence of the glucose instead of xylose. Acid hydrolysis of **6** gave vitexin and glucose (identified by co-TLC with an authentic samples). Based on these results and on values previously reported in the literature (Mabry *et al.*, 1970; Agrawal, 1989; Gluchoff- Fiasson *et al.*, 1989), compound **6** was identified as vitexin 2"-*O*-glucoside. Among these compounds, tricin (**1**) and *p*-hydroxycinnamic acid (**2**) were previously reported on the antioxidant activities using 2, 2'-Azobis (2, 4-dimethylvaleronitrile) (AMVN)-initiated oxidation of methyl linolate (Watanabe, 1999), and a competition kinetic test and the in vitro oxidative modification of human low-density lipoprotein (LDL) (Natella *et al.*, 1999), respectively.

Orientin 2"-*O*-xyloside (**4**) and tricin-7-*O*-β-*D*-glucoside (**5**) exhibited potent DPPH radical scavenging effects with IC_{50} values of 14 and 12 μg/ml, respectively. Tricin (**1**) and vitexin 2"-*O*-glucoside (**6**) were showed weak DPPH radical scavenging effects with IC_{50} values of 88 and 173 μg/ml, respectively. *p*-hydroxycinnamic acid (**2**) and vitexin 2"-*O*-xyloside (**3**) were considered to be inactive in the antioxidant assay system using DPPH

Table II. DPPH radical scavenging activities of the isolated compounds

Compounds	IC_{50} [a] (μg/ml)
Tricin(**1**)	88
p-Hydroxycinnamic acid(**2**)	592
Vitexin 2"-*O*-xyloside(**3**)	515
Orientin 2"-*O*-xyloside(**4**)	14
Tricin-7-*O*-β-*D*-glucose(**5**)	12
Vitexin 2"-*O*-glucoside(**6**)	173
BHT*	13

*Used as a positive control

[a]Concentration giving a 50% decrease of DPPH radical. The values are the means of triplicate experiments.

Table III. Effects of the isolated compounds on free radical-induced lipid peroxidation in the liver homogenates

Compounds	Concentration (mg/ml)	MDA(nmol/mg protein)	Inhibition(%)	IC_{50}(μg/ml)
	Control	4.83±0.57	-	
	10	1.89±0.32	60.92	
Tricin(**1**)	5	3.02±0.06	37.38	
	1	4.26±0.47	11.83	
	10	1.88±0.72**	61.05	
p-Hydroxycinnamic acid(**2**)	5	2.77±0.83	42.60	6.02±1.43
	1	4.23±0.67	12.37	
	10	3.32±1.11	31.36	
Vitexin 2"-*O*-xyloside(**3**)	5	3.92±0.74	18.92	
	1	4.39±0.19	9.03	
	10	1.83±0.57**	62.12	
Orientin 2"-*O*-xyloside(**4**)	5	3.08±0.66	36.31	6.71±1.09
	1	4.57±0.76	5.28	
	10	2.22±0.65**	54.10	
Tricin-7-*O*-*β*-*D*-glucose(**5**)	5	2.62±0.44	45.81	7.58±0.36
	1	3.50±0.01	27.48	
	10	2.81±0.54	41.79	
Vitexin 2"-*O*-glucose(**6**)	5	3.55±0.75	26.55	
	1	4.59±0.05	4.88	
	10	1.34±0.16**	72.29	
BHT*	5	2.22±0.12	53.96	1.82±0.39
	1	2.48±0.10	48.75	

Data of MDA contents were expressed as the mean ± S.E.M of triplicate tests.
*Used as a positive control
**$p<0.05$

radical scavenging activity as shown in **Table II**.

We further studied the effects of these compounds on the lipid peroxidation in rat liver microsomes induced by nonenzymatic method. As expected, Orientin 2"-*O*-xyloside (**4**) and tricin-7-*O*-*β*-*D*-glucoside (**5**) exhibited significant inhibition on ascorbic acid/Fe^{2+} induced lipid peroxidation in rat liver microsomes (**Table III**). Interestingly, *p*-hydroxycinnamic acid(**2**) showed strong inhibition activity on lipid peroxidation, though it did not showed DPPH free radical scavenging effect.

In conculusion, Orientin 2"-*O*-xyloside (**4**) and tricin-7-*O*-*β*-*D*-glucoside (**5**) exhibited significant free radical scavenging activities and anti-lipid peroxidation activities in the present study. All compounds were isolated for the first time from this plant.

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