Antioxidative Effects of 6-Methoxysorigenin and Its Derivatives from *Rhamnus nakaharai*

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In the search for potential antioxidants, the naphthalenic compounds, 6-methoxysorigenin (2) and its glycosides [*i.e.* 6-methoxysorigenin-8-*O*-glucoside (3), α-sorinin (4), and 6-methoxysorigenin-8-rutinoside (5)] isolated from *Rhamnus nakaharai* together with two acylates (peracetate and perpropionate) of 2 were evaluated for antioxidant activities using 2,2-diphenyl-1-picrylhydrazyl (DPPH), metal chelating, and electron spin resonance (ESR) assays as well as anti-lipid peroxidation assay. The results showed that 2 possesses the most potent DPPH radical scavenging, metal chelating, and anti-lipid peroxidation activities with IC_{50} values of 3.48, 615.90, and 5.95 μg/ml, respectively. The glycosides 3, 4, and 5 showed decreasing antioxidant activity that was related to an increased substitution at 1,8-dihydroxyl with sugar molecules. This suggests the importance of 1,8-dihydroxyl of 2 in the antioxidative effect. The iron chelation result could further explain the main cause of increasing antioxidant activity in 2. The acylates of 2 (2a peracetate and 2b perpropionate), although lacking a free hydroxyl, also exhibited significant anti-lipid peroxidation effect. ESR results further demonstrated that 2 possesses strong antioxidant activities. Taken together, this study shows that 2 is a potent antioxidant and may also be used for designing new iron chelators for clinical applications.

Key words *Rhamnus nakaharai*; Rhamnaceae; 6-methoxysorigenin; antioxidant activity

Oxidative stress is well known as a major cause of various chronic diseases, namely cardiovascular diseases, rheumatism, diabetes mellitus, and cancer.[1,2] Reactive oxygen species (ROS) are generated *in vivo* during shock, inflammation, and ischemia/reperfusion injury.[3] Antioxidant therapy has been clinically demonstrated to be valuable in the prevention of certain chronic diseases.[3,4] Antioxidants also play an important role in the food industry, because excessive formation of free radicals can accelerate oxidation of lipids in foods and thereby decreases food quality.

Rhamnus nakaharai HAYATA (Rhamnaceae) is traditionally used as a folk medicine in Taiwan for treating constipation, inflammation, malignant tumors, and asthma.[5] 3-*O*-Methyquercetin, the main constituent of the plant, has been reported to inhibit cAMP- and cGMP-phosphodiesterase activity of guinea pig trachealis.[6] It was also demonstrated a selective and competitive inhibitor of phosphosdiesterase subtype 3/subtype 4.[5] Isotorachrysone (**1**), an acetonaphthone isolated from *R. nakaharai*, showed antioxidant[7] and platelet aggregation inhibitory[8] activities. 6-Methoxysorigenin (**2**), isolated from the wood of *R. nakaharai*, possesses a structure closely analogous to **1** (Fig. 1) and is rich in glycosides.[9]

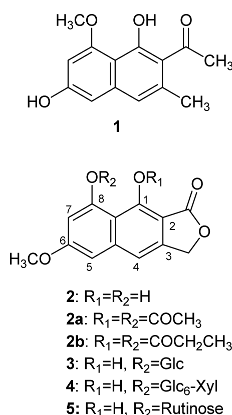

Fig. 1. Chemical Structures of Isotorachrysone (**1**), 6-Methoxysorigenin (**2**) and Its Derivatives

To evaluate the antioxidant effect of **2** and its derivatives, glycosides from the wood of *R. nakaharai* were isolated and characterized. They were 6-methoxysorigenin-8-*O*-glucoside (**3**), α-sorinin (**4**), 6-methoxysorigenin-8-*O*-rutinoside (**5**). In addition, peracylates **2a** and **2b**, and **2** were prepared and evaluated for antioxidant activities using 2,2-diphenyl-1-picrylhydrazyl (DPPH), metal chelating, and electron spin resonance (ESR) assays as well as $FeCl_2$-ascorbic acid-induced lipid peroxidation assay in rat liver homogenates *in vitro*. Their antioxidant activities were compared with those of trolox and with EDTA for results of metal chelating assay.

Results and Discussion

DPPH· is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule.[10] In this assay, all test compounds effectively reduced the stable radical DPPH to the yellow-colored diphenylpicrylhydrazine and their scavenging effect was dose-dependent. It was found that compound **2** possesses the most potent DPPH radical-scavenging activity, with an IC_{50} value of 3.48 μg/ml, which is close to that of trolox (IC_{50}: 3.18 μg/ml) (Table 1).

Chelating agents, which form σ-bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion.[11] Chelation may inhibit lipid oxidation by stabilizing transition metals. In this study, all test compounds including trolox and EDTA interfered with the formation of ferrous and ferrozine complexes, suggesting that they have chelating activity and are able to capture ferrous ion before ferrozine. Compound **2** (IC_{50}: 615.90 μg/ml) showed a stronger iron-binding capacity than trolox (IC_{50}: 1848.83 μg/

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Table 1. IC_{50} Values of 6-Methoxysorigenin (**2**) and Its Derivatives (**2a**, **2b**, **3**—**5**) by Several Antioxidant Assays

	IC_{50} (μg/ml)[a]							
	2	**2a**	**2b**	**3**	**4**	**5**	Trolox	EDTA
DPPH radical scavenging	3.48±0.02	224.32±4.76	210.43±3.34	170.76±1.49	63.99±1.48	107.14±2.73	3.18±0.05	ND
Metal chelating	615.90±5.79	846.87±29.79	1626.94±13.25	1914.90±27.09	2071.33±5.18	1451.51±25.04	1848.83±49.93	127.92±2.67
Anti-lipid peroxidation	5.95±0.37	21.81±1.09	13.59±0.41	43.94±1.98	127.22±2.25	105.00±1.91	9.66±0.13	ND

a) Values are mean±S.E. ND: not determined.

Table 2. Superoxide Anion Radical Scavenging (SOD-Like) Activity of Compound **2** as Analyzed by Electron Spin Resonance Spectrometry

Compound	Concentration (g/ml)	Signal peak height		SOD activity	SOD-like activity (unit/g)	IC_{50} (mM)
		Mn^{2+}	Radical			
2	0.0493	132.00	95.60	9.374	1.9×10^4	1.04 or 0.256 mg/ml

Calibration curve: $Y=0.235197-0.061957X$; $r=0.997799$; $Y=[I_0/I-1]$; $X=$[SOD (unit)]. I_0: blank; I: the average peak height of the treated compound.

ml) but a weaker activity than EDTA (IC_{50}: 127.92 μg/ml).

Thiobarbituric acid reactive substances (TBARS) method has been widely used in studies of anti-lipid peroxidation activity of natural products/phytochemicals in rat liver homogenate.[12,13] Our results showed that inhibition of malondialdehyde (MDA) formation increases with increasing concentrations of test compounds and trolox. The peracylates **2a** and **2b** also showed notable inhibitory effects with IC_{50} values 21.81 and 13.59 μg/ml, respectively. However, glycosides **3**, **4**, and **5** showed only moderate activity. The potency of anti-lipid peroxidation activity was in the order of **2**>trolox>**2b**>**2a**>**3**>**5**>**4**. Based on the IC_{50} values, compound **2** (IC_{50}: 5.95 μg/ml) demonstrated the best inhibitory effect against lipid peroxidation, which was about two-fold more potent than trolox (IC_{50}: 9.66 μg/ml). The potent anti-lipid peroxidation activity of **2** could be related to its effective iron-binding capacity.

ESR spectral method is considered one of the most effective methods in the study of free radical-scavenging activity of natural products.[14,15] The superoxide anion ($\cdot O_2^-$) is generated by the hypoxanthine–xanthine oxidase system and trapped by DMPO (5,5-dimethyl-1-pyrroline-1-oxide). The DMPO–OOH adduct exhibits an electron spin resonant effect that can be detected by ESR spectrometry. If the DMPO–OOH adduct is reduced, the magnitude of ESR would be decreased. The superoxide anion free radical-scavenging effect of the tested compound can be calculated by a calibration curve that has been established with various concentrations of SOD in the same manner. The ESR spectrum of **2** showed an obvious signal reduction (Fig. 2) and SOD-like scavenging activity (Table 2). This observation further supports the contention that **2** is an excellent antioxidant.

Isotorachrysone (**1**) has been described as a potent antioxidant with iron chelating and potential free radical-scavenging effects on superoxide anion, hydroxyl ($\cdot$OH), hydroperoxyl ($\cdot$OOH), and DPPH radicals, *etc*. It was suggested that the unsubstituted 1,6-dihydroxyl and the 2-acetyl substitution of **1** contribute to these effects.[7] The structure of **2** could even more feasibly behave as a chelator of iron ions because of the 1,8-dihydroxyl and γ-lactonic carbonyl that constitute two chelation sites (Fig. 1). This mechanism could also be supported by the decreased inhibitory effect on lipid peroxidation of the 8-*O*-glycosides of **2** (**3**, **4**, **5**). These glycosides, especially the huge di-glycosides **4** and **5**, can become hindrances of chelation and result in a dramatic decrease of anti-lipid peroxidative activity. On the other hand, the sugar moieties of the glycosides might also act as lipophobic substituents that can prevent contact of the antioxidant with the lipid membrane as well as breaking the chain reaction.

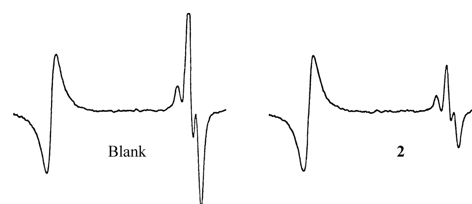

Fig. 2. ESR Spectra of 6-Methoxysorigenin (**2**) and Blank

Acylates **2a** and **2b**, which lack a donable hydrogen atom, retained apparent inhibitory effect on lipid peroxidation. This activity seemed correlated with the length of the fatty acid chain. The manner of lipid peroxidation inhibition of acylates **2a** and **2b** could be different from **2** but similar to the peracetate of isotorachrysone (**1**), which has been shown to possess anti-lipid peroxidative effect.[7] Its activities could be derived from the membrane-stabilizing mechanism that is similar to those of cholesterol and tamoxifen.[16] The antioxidant property of **2** could be similar to isotorachrysone (**1**) since these molecules are structural analogues.

Rhamnaceous-derived crude drugs have long been used as laxatives,[17] which were found to contain 6-methoxysorigenin (**2**) and its derivatives.[8,9,18,19] To date, bioactivities of 6-methoxysorigenin and its derivatives remain largely unknown. This is the first report on their antioxidant activities. Recently, iron chelators have been suggested clinically beneficial in the treatment of iron overload disease and cancer.[20] Structurally, many of these synthetic iron chelators possess a naphthalene nucleus. The present study concludes that 6-methoxysorigenin (**2**) is a potent antioxidant and may also be used for designing new iron chelators for clinical applications.

Experimental

Chemicals L-(+)-Ascorbic acid, thiobarbituric acid (TBA), and ethylenediamine tetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Trolox and ferrous chloride were obtained from Wako Pure Chemical Industries (Osaka, Japan). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from MP Biomedicals Inc. (Eschwege, Germany). All other chemicals used were of analytical grade.

Plant Material The heartwood of *R. nakaharai* was collected from Yang-Ming Shan, Taipei, Taiwan. After confirming the authenticity of the species, a voucher specimen was deposited in the herbarium (T89-Rh-001) of Tajen University, Pingtung, Taiwan.

Analytical Apparatus Melting points were determined by an Electrothermal IA9100 melting point apparatus (ESSLAB, Essex, U.K.). ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded on a Varian Mercury-400 (400 MHz; Varian Inc., CA, U.S.A.) spectrometer whereas the EI-MS was taken on a TurboMass mass spectrometer and FAB-MS on a JMS-HX 110 mass spectrometer (Perkin Elmer Inc., MA, U.S.A.). Optical specific rotation was measured by Jasco model DIP-370 Digital Polarimeter (JASCO Inc, MD, U.S.A.).

Extraction and Isolation The wood was dried and chipped into small pieces followed by weighing about 5 kg for extraction. This was extracted with 10 l of methanol at room temperature for 1 week. The methanolic extract was condensed to obtain a residue of 186 g. This residue was chromatographed on a silica gel (900 g, 70—230#, Merck, Germany) column and eluted with a solvent system of CH_2Cl_2–MeOH. The elution began with CH_2Cl_2 (1500 ml) then with CH_2Cl_2 : MeOH (9 : 1; 2000 ml), followed by CH_2Cl_2 : MeOH (8 : 1; 2000 ml) to obtain 6-methoxysorigenin-8-*O*-glucoside (**3**, 2.1 g). After discarding the solution obtained with CH_2Cl_2 : MeOH (8 : 1—6 : 1; each 1500 ml), elution with CH_2Cl_2 : MeOH (4 : 1; 2500 ml) was performed to obtain 6-methoxysorinin (**4**, 4.2 g). The same solution of **4** was further analyzed with RP-18 TLC to obtain another blue-fluorescing spot with *Rf* 0.56, which was close to that of **4**. Hence it was further chromatographed on a 25×600-mm RP-18 column (60 μm, Merck, Germany). Elution with 60% MeOH–H_2O solvent system 250 ml led to the isolation of 6-methoxysorigenin 8-*O*-rutinoside (**5,** 87 mg). Structure determination of **3**, **4**, and **5** was characterized by spectrometric analyses and further confirmed with the reported data.[18,19] Compound **2** was obtained from acid hydrolysis of the glycoside **3**. The preparation of acylates (**2a**, **2b**) was achieved by treating **2** with pyridine and related acid anhydrides.

Characterization of Compounds 2, 2a, 2b 6-Methoxysorigenin **2**: Brownish powders ($CHCl_3$–MeOH); mp >300 °C; ^{1}H-NMR (400 MHz, pyridine-d_5): δ 3.71 (3H, s, OCH_3), 5.07 (2H, s, γ-lactonic CH_2), 6.58 (1H, s, H-4), 6.63 (1H, d, J=2.0 Hz, H-7), 6.68 (1H, d, J=2.0 Hz, H-5), 14.50 (1H, br, OH), 15.10 (1H, br, OH); ^{13}C-NMR (100 MHz, pyridine-d_5): δ 172.2 (C-1), 113.7 (C-2), 144.0 (C-3), 103.1 (C-4), 98.1 (C-5), 164.5 (C-6), 98.3 (C-7), 162.5 (C-8), 102.8 (C-9), 143.4 (C-10), 176.6 (γ-lactonic C=O), 70.2 (γ-lactonic CH_2), 55.0 (OCH_3); EI-MS *m/z*: 246 $[M]^+$, 217 (base peak).

6-Methoxysorigenin Peracetate **2a**: Colorless needles ($CHCl_3$–MeOH); mp 244 °C; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 2.41, 2.45 (each 3H, s, $COCH_3$), 3.95 (3H, s, OCH_3), 5.52 (2H, s, γ-lactonic CH_2), 7.13 (1H, d, J=2.4 Hz, H-7), 7.50 (1H, d, J=2.4 Hz, H-5), 7.97 (1H, s, H-4); EI-MS *m/z*: 330 $[M]^+$, 288 $[M-42]^+$, 246 $[M-84]^+$ (base peak).

6-Methoxysorigenin Perpropionate **2b**: Colorless masses ($CHCl_3$–MeOH); mp 189 °C; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.33, 1.35 (each 3H, t, J=7.6 Hz, propionate CH_3), 2.69 (2H, m, propionate CH_2), 2.82 (2H, br, propionate CH_2), 3.89 (3H, s, OMe), 5.18 (2H, s, γ-lactonic CH_2), 6.86 (1H, d, J=2.4 Hz, H-7), 6.97 (1H, d, J=2.4 Hz, H-5), 7.46 (1H, s, H-4); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 9.0, 9.2 (propionate CH_3), 27.7, 27.8 (propionate CH_2), 56.0 (OMe), 68.7 (γ-lactonic CH_2), 104.9 (C-7), 113.3 (C-9), 114.8 (C-5), 117.1 (C-2), 117.7 (C-4), 141.6 (C-3), 142.1 (C-10), 146.1 (C-8), 148.4 (C-1), 159.9 (C-6), 168.3 (γ-lactonic C=O), 172.7, 173.1 (propionate C=O); EI-MS *m/z*: 358 $[M]^+$, 302 $[M-C_2H_5CO+1]^+$, 246 $[\text{aglycone}]^+$.

6-Methoxysorigenin-8-*O*-glucopyranoside **3**: Brownish granules (CH_2Cl_2–MeOH); mp 223 °C.

6-Methoxysorigenin-8-*O*-premeveroside (α-Sorinin) **4**: Brownish granules (MeOH); $[\alpha]_D^{22}$ $-58°$ (c=0.1, MeOH); mp 186—187 °C.

6-Methoxysorigenin-8-*O*-rutinoside **5**: Brownish needles (pyridine–MeOH); mp 257 °C.

DPPH Radical-Scavenging Activity DPPH radical-scavenging activity was determined according to the methods of Blois[21] and Chang *et al.*[22]

Metal Chelating Activity The chelation of ferrous ions by various compounds was estimated by the method of Dinis *et al.*[23] with slight modifications.

Anti-lipid Peroxidation Assay The effect of 6-methoxysorigenin and its derivatives on $FeCl_2$-ascorbic acid-induced lipid peroxidation in rat liver homogenate was determined by the methods of Janero[12] and Lu *et al.*[13]

Electron Spin Resonance (ESR) Spectrometry ESR assay was used further to confirm the antioxidant activity of compound **2**. The experimental procedure was conducted according to the method as described by Kohno *et al.*[24]

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