

## Isorhamnetin Glycosides with Free Radical and ONOO<sup>-</sup> Scavenging Activities from the Stamens of *Nelumbo nucifera*

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In this study, we isolated two new isorhamnetin glycosides, designated as nelumboside A (**3**) and nelumboside B (**4**), as well as the previously-characterized isorhamnetin glucoside (**1**) and isorhamnetin rutinoside (**2**), from the *n*-BuOH fraction of *Nelumbo nucifera* stamens. The structures of the two new compounds were then determined, using chemical and spectroscopic techniques. All isolated isorhamnetin glycosides **1-4** showed marked antioxidant activities in the DPPH, and ONOO<sup>-</sup> assays.

**Key words:** *Nelumbo nucifera*, Nymphaeaceae, Isorhamnetin glycoside, Nelumboside A, Nelumboside B, DPPH, ONOO<sup>-</sup>

### INTRODUCTION

*Nelumbo nucifera* GAERTN. (Nymphaeaceae) is a perennial aquatic crop, which is grown and consumed throughout Asia (Van Bergen *et al.*, 1997; Kim, 1996). All components of *N. nucifera* have been used in a variety of medicinal context, in the treatment of diarrhea, gastritis, insomnia, nervous prostration, and as a haemostatic in diabetic patients (La Cour *et al.*, 1995; Mukherjee *et al.*, 1995, 1997; Kim, 1997; Lee *et al.*, 2001). Previously, alkaloids have been isolated from the embryos and leaves of these plants (Furukawa *et al.*, 1965; Tomita *et al.*, 1965; Furukawa, 1966a; 1966b; Koshiyama *et al.*, 1970; Kunitomo *et al.*, 1970; 1971; Wu *et al.*, 2004), procyanidins (Ling *et al.*, 2005) have been extracted from the seed pods, and flavonol glycosides (Jung *et al.*, 2003) have reportedly been obtained from the stamens of *N. nucifera*. In this paper, we report the isolation and structural identification of two new flavonol glycosides (**3** and **4**) from the stamens of *N. nucifera*. We also evaluated the antioxidant effects of all isolated compounds.

### MATERIALS AND METHODS

#### General experimental

<sup>1</sup>H- and <sup>13</sup>C-NMR spectra were measured with a JEOL JNM-ECP 400 (400 MHz for <sup>1</sup>H, 100 MHz for <sup>13</sup>C) spectrometer. The HMQC and HMBC spectra were recorded using pulsed field gradients. Chemical shifts were referenced to the respective residual solvent peaks, and the values were expressed and recorded in  $\delta$ . The FAB-MS data were collected with a JEOL JMS-HX110/110A spectrometer. Optical rotation was assessed using a Perkin-Elmer 341 MC polarimeter. The UV and IR spectra were recorded on a Varian Carry UV-visible spectrophotometer and a Perkin Elmer FT-IR spectrometer, respectively. Column chromatography was conducted using silica (Si) gel 60 (70-230 mesh, Merck, Germany) and RP-18 Lichroprep (Merck, Germany). TLC was performed on precoated Merck Kieselgel 60 F<sub>254s</sub> plates (0.25 mm) and RP-18 F<sub>254s</sub> plates (Merck), and the spots were visualized under UV light, using 50% H<sub>2</sub>SO<sub>4</sub> reagent. All solvents used in column chromatography were of reagent grade, and all had been acquired from commercial sources.

#### Chemicals

The DPPH (1,1-diphenyl-2-picrylhydrazyl), L-ascorbic acid, and DL-2-amino-3-methylbutanoic acid (DL-penicillamine) were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Dihydrorhodamine 123 (DHR 123) and ONOO<sup>-</sup> were purchased from Molecular Probes

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(Eugene, Oregon, U.S.A.) and Cayman Chemical Co. (Ann Arbor, MI, U.S.A.), respectively.

### Plant material

The stamens of *N. nucifera* were purchased in April 2003, from the Herbal Medicine Co-operative Association of Busan Province, Korea, and were authenticated by Hae Young Chung, professor of the College of Pharmacy, Pusan National University. A voucher specimen (no. 20030516) was deposited in the Herbarium of the Medicine Plant Garden, at the College of Pharmacy of Pusan National University.

### Extraction and isolation

The dried *N. nucifera* stamens (5.6 kg) were refluxed with MeOH for three hours. The total filtrate was then concentrated to dryness, *in vacuo*, at 40°C, in order to render the MeOH extract (1.15 Kg). This extract was then suspended in H<sub>2</sub>O, and successively partitioned with CH<sub>2</sub>Cl<sub>2</sub>, EtOAc, and *n*-BuOH, in order to generate CH<sub>2</sub>Cl<sub>2</sub> (285.1 g), EtOAc (40.3 g), and *n*-BuOH fractions (88.2 g), as well as an H<sub>2</sub>O residue (240.9 g). The *n*-BuOH fraction (88.2 g) was then chromatographed with a Si gel column, using CH<sub>2</sub>Cl<sub>2</sub>-MeOH (stepwise), to afford 28 subfractions (Fr. 1 to 28). Fraction 9 (5.7g) was subjected to additional chromatography with CH<sub>2</sub>Cl<sub>2</sub>: MeOH : H<sub>2</sub>O (7:1:0.1, gradient to MeOH) on a silica gel column, resulting in the generation of 14 fractions (Fr. 9-1 to Fr. 9-14). Then, subfractions 5 and 11 were purified using Sephadex LH-20 and 50% MeOH, generating compounds **1** (14 mg) and **2** (11 mg), respectively. Fraction 12 (5.6 g) was subjected to further column chromatography on a silica gel column, with a gradient mixture of CH<sub>2</sub>Cl<sub>2</sub>: MeOH : H<sub>2</sub>O (7:1:0.1, gradient to MeOH). This fraction was then purified *via* Sephadex LH-20, RP-18 gel column chromatography, yielding compound **3** (9 mg). Fraction 14 (8.26 g) was chromatographed on Si gel, using EtOAc-MeOH-H<sub>2</sub>O (24:2:1 to MeOH), resulting in 17 fractions (Fr. 14-1 to 14-17). Fraction 8 (Fr. 14-8, 0.79 g) was subjected to further column chromatography over a Si gel column, using CH<sub>2</sub>Cl<sub>2</sub>-MeOH-H<sub>2</sub>O (65:35:10 to MeOH) to generate 10 fractions (Fr. 14-8-1 to Fr. 14-8-10). Compound **4** (11.3 mg) was then obtained *via* RP-18 column chromatography (40% MeOH) using fraction 5 (Fr. 14-8-5, 0.13 g).

### Acid hydrolysis of compounds **3** and **4**

Compounds **3** and **4** (5 mg each) were dissolved in 5% aqueous HCl (5 mL), and separately refluxed for 3 h each. The reaction mixture was then extracted with EtOAc. The EtOAc fraction (aglycone) and the aqueous fraction (sugar) were concentrated to dryness for the purposes of identification. The success of this step was verified by direct comparison with authentic samples, *via* TLC analyses.

The sugar components were identified as glucose, rhamnose, and lyxose, by direct comparison with authentic samples, *via* TLC analyses on Si gel (developing solvent EtOAc/MeOH/H<sub>2</sub>O/AcOH = 13 : 3 : 3 : 4, *R<sub>f</sub>* 0.30, 0.61, and 0.73, respectively).

### Isorhamnetin 3-O- $\alpha$ -D-lyxopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-glucopyranoside (**3**)

Yellow amorphous powder:  $[\alpha]_D^{20}$  -4.4° (*c* 0.009, MeOH); UV (MeOH)  $\lambda_{max}$  nm (log  $\epsilon$ ): 254 (4.28), 355 (4.20); + NaOMe 271 (4.33), 326 (3.97), 409 (4.37); + NaOAc 269 (4.26), 359 (4.14); + NaOAc + H<sub>3</sub>BO<sub>3</sub> 255 (4.27), 267 (4.23), 356 (4.18); + AlCl<sub>3</sub> 269 (4.28), 355 (4.09), 401 (4.15); + AlCl<sub>3</sub> + HCl 208 (4.57), 269 (4.28), 356 (4.10), 399 (4.14); IR  $\nu_{max}$  3387, 2925, 1655, 1604, 1495, 1355, 1293, 1203, 1127, 1065, 796 cm<sup>-1</sup>; LR-FABMS (negative) *m/z*: 609 [M - H]<sup>-</sup>, 477 [M - H - 132 (lyxose unit)]<sup>-</sup>, 315 [M - 132 (lyxose unit) - 162 (glucose unit) - H]<sup>-</sup>; HR-FAB-MS *m/z*: 633.1415 [M + Na]<sup>+</sup> (Calcd for C<sub>27</sub>H<sub>30</sub>O<sub>16</sub>Na: 633.1432); <sup>1</sup>H- and <sup>13</sup>C-NMR: see Table I.

### Isorhamnetin 3-O- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 6)-[ $\alpha$ -D-lyxopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-glucopyranoside] (**4**)

Yellow amorphous powder:  $[\alpha]_D^{20}$  -3.89° (*c* 0.011, MeOH); UV (MeOH)  $\lambda_{max}$  nm (log  $\epsilon$ ): 255 (4.25), 268 (4.19), 356 (4.18); + NaOMe 271 (4.31), 326 (3.94), 410 (4.35); + NaOAc 273 (4.26), 321 (4.03), 366 (4.12); + NaOAc + H<sub>3</sub>BO<sub>3</sub> 255 (4.26), 268 (4.21), 358 (4.18); + AlCl<sub>3</sub> 270 (4.27), 364 (4.08), 404 (4.13); + AlCl<sub>3</sub> + HCl 271 (4.25), 360 (4.28), 401 (4.11); IR  $\nu_{max}$  3405, 2930, 1655, 1604, 1561, 1509, 1356, 1293, 1202, 1130, 1082, 813 cm<sup>-1</sup>; LR-FABMS (negative) *m/z*: 755 [M - H]<sup>-</sup>, 623 [M - H - 132 (lyxose unit)]<sup>-</sup>, 609 [M - 146 (rhamnose unit) H]<sup>-</sup>; 315 [M - 132 (lyxose unit) - 146 (rhamnose unit) - 162 (glucose unit) - H]<sup>-</sup>; HR-FAB-MS *m/z*: 779.2031 [M + Na]<sup>+</sup> (Calcd for C<sub>33</sub>H<sub>40</sub>O<sub>20</sub>Na: 779.2011); <sup>1</sup>H- and <sup>13</sup>C-NMR: see Table I.

### DPPH radical scavenging activity assay

According to the modified method, which was first employed by Blois (1958), it was evaluated the DPPH radical scavenging effect. A hundred sixty microliters ( $\mu$ L) of methanolic solution of various sample concentrations (10-320  $\mu$ L/mL) was added to 40  $\mu$ L DPPH methanolic solution ( $1.5 \times 10^{-4}$  M). After vortexing thoroughly and leaving for 30 min at room temperature, the optical density was measured at 520 nm using a microplate reader spectrophotometer VERSAmax (Molecular Devices, CA, U.S.A.). L-Ascorbic acid was employed as a positive control. The antioxidant activity of each sample was expressed in terms of IC<sub>50</sub> ( $\mu$ M required to scavenge DPPH free radicals by 50%) and calculated from the log-dose inhibition curve.

**Table I.** <sup>1</sup>H- and <sup>13</sup>C-NMR spectral data of compounds **3** and **4** in DMSO-*d*<sub>6</sub>

No	<b>3</b>		<b>4</b>	
	<sup>13</sup> C	<sup>1</sup> H	<sup>13</sup> C	<sup>1</sup> H
2	155.9		155.5	
3	132.8		132.4	
4	177.1		176.3	
5	161.2		161.0	
6	98.8	6.20 (1H, d, <i>J</i> = 1.9)	99.8	6.01 (1H, d, <i>J</i> = 2.2)
7	164.6		164.8	
8	93.7	6.45 (1H, d, <i>J</i> = 1.9)	94.3	6.22 (1H, d, <i>J</i> = 2.2)
9	156.3		156.8	
10	103.8		102.3	
1'	121.0		121.0	
2'	113.3	7.93 (1H, d, <i>J</i> = 1.9)	113.1	7.79 (1H, d, <i>J</i> = 2.2)
3'	146.9		146.9	
4'	149.5		149.6	
5'	115.3	6.93 (1H, d, <i>J</i> = 8.6)	115.3	6.89 (1H, d, <i>J</i> = 8.6)
6'	122.3	7.58 (1H, dd, <i>J</i> = 1.8, 8.6)	122.3	7.52 (1H, dd, <i>J</i> = 2.2, 8.6)
1''	98.6	5.68 (1H, d, <i>J</i> = 7.3)	99.0	5.50 (1H, d, <i>J</i> = 7.5)
2''	79.6	3.48 (1H, t, <i>J</i> = 5.1 Hz)	78.9	3.46 (1H, m)
3''	77.1	3.48 (1H, t, <i>J</i> = 5.1 Hz)	76.9	3.46 (1H, m)
4''	69.7	3.13 (1H, m)	70.1	3.60 (1H, t, <i>J</i> = 5.9)
5''	77.4	3.13 (1H, m)	75.7	3.27 (1H, d, <i>J</i> = 5.9)
6''	60.5	3.57 (1H, m), 3.33 (1H, m)	66.9	3.70 (2H, m)
1'''	102.3	4.79 (1H, d, <i>J</i> = 4.8)	101.9	4.83 (1H, d, <i>J</i> = 4.3)
2'''	70.6	3.55 (1H, m)	70.4	3.23 (1H, d, <i>J</i> = 5.9)
3'''	72.0	3.41 (1H, m)	71.9	3.46 (1H, m)
4'''	65.8	3.63 (1H, m)	65.4	3.66 (1H, d, <i>J</i> = 8.9)
5'''	62.7	3.68 (1H, dd, <i>J</i> = 6.2, 11.3), 3.21 (1H, dd, <i>J</i> = 2.7, 11.3)	62.1	3.74 (1H, d, <i>J</i> = 11.3), 3.22 (1H, dd, <i>J</i> = 5.9, 12.4)
1''''			100.9	4.35 (1H, s)
2''''			70.3	3.40 (1H, d, <i>J</i> = 2.0)
3''''			70.5	3.06 (1H, t, <i>J</i> = 9.1)
4''''			71.7	3.46 (1H, m)
5''''			68.3	3.23 (1H, d, <i>J</i> = 5.9)
6''''			17.7	0.97 (3H, d, <i>J</i> = 6.2)
OH		12.57 (1H, s)		12.51 (1H, s)
OCH <sub>3</sub>	55.6	3.85 (3H, s)	55.6	3.83 (3H, s)

**Peroxyinitrite scavenging activity assay**

ONOO<sup>-</sup> scavenging activity was assessed by the modified Kooy's method, which involved the monitoring of highly fluorescent rhodamine 123, which was rapidly produced from non-fluorescent DHR 123 in the presence of ONOO<sup>-</sup> (Kooy *et al.*, 1994). In Brief, the rhodamine buffer (pH 7.4) consisted of 50 mM sodium phosphate dibasic, 50 mM sodium phosphate monobasic, 90 mM sodium chloride, 5 mM potassium chloride, and 100 μM DTPA. The final DHR 123 concentration was 5 μM. The

buffer in this assay was prepared prior to use and placed on ice. The samples were dissolved in 10% DMSO at concentrations of 5 - 100 μM for the isolated compounds. The background and final fluorescent intensities were measured 5 minutes after treatment with and without the addition of authentic ONOO<sup>-</sup> (10 μM), dissolved in 0.3 N sodium hydroxide. The fluorescence intensity of the oxidized DHR 123 was evaluated using a microplate fluorescence reader FLx 800 (Bio-Tek Instruments Inc.) at excitation and emission wavelengths of 480 and 530 nm,

respectively. ONOO<sup>-</sup> scavenging activity values were expressed as the means  $\pm$  SD ( $n = 3$ ) of the final fluorescence intensity minus the background fluorescence, *via* the detection of DHR 123 oxidation. DL-Penicillamine was employed as a positive control.

### Statistical analysis

All values were expressed as the mean  $\pm$  SD of three identical experiments.

## RESULTS AND DISCUSSION

The combination of silica gel and RP-18 column chromatography, conducted with the *n*-BuOH soluble portions of methanolic extracts of *N. nucifera* stamens, resulted in the isolation of compounds 1-4. Compounds 1-4 were observed as yellow amorphous powders, which yielded characteristic flavonoid glycoside color reactions, i.e., pink on the Mg-HCl test, and positive on the Molisch test. Compounds 1 and 2 were identified as isorhamnetin-3-*O*- $\beta$ -D-glucopyranoside and isorhamnetin 3-*O*-rutinoside, according to the results of comparisons with published spectral data (Hörhammer *et al.*, 1966; Markham *et al.*, 1978).

The results of high-resolution FAB mass spectrometry (HR-FAB-MS, positive ion mode) of compound 3 revealed a *quasi*-molecular ion peak at  $m/z$  633.1415 [ $M + Na$ ]<sup>+</sup> (calcd 633.1432), which was determined to be consistent with a molecular formula of C<sub>27</sub>H<sub>30</sub>O<sub>16</sub>. The NMR spectral data of compound 3 indicated that it is fundamentally a cognate of compound 1, except for the presence of an additional sugar unit, as evidenced by an extra anomeric proton occurring at  $\delta$  4.79 ( $J = 4.8$  Hz, H-1''), and a corresponding anomeric carbon occurring at  $\delta$  102.3 (C-1''). Acid hydrolysis yielded isorhamnetin, lyxose, and glucose, all of which were identified by direct comparison with authentic samples, *via* TLC analyses. We assumed that the sugars were in the common *D*-configuration, as

this is normally the configuration observed in association with plant glycosides. The coupling constant, 7.3 Hz ( $\delta$  5.68), was consistent with the diaxial coupling between the protons on C-1 and C-2 in a  $\beta$ -linked glucose, whereas the 4.8 Hz ( $\delta$  4.79) coupling constant represented diequatorial coupling occurring between the protons on C-1 and C-2 in an  $\alpha$ -linked lyxose ( $J = 5.3$  Hz) (Sung *et al.*, 2002). The UV spectrum of compound 3 revealed absorption maxima typical of a number of 3-hydroxy substituted flavonols, at 254 nm and 354 nm (Mabry *et al.*, 1970). The bathochromic shift of band I in the presence of AlCl<sub>3</sub> and AlCl<sub>3</sub>/HCl suggested the presence of a free 4'-hydroxyl group (Mabry *et al.*, 1970). This notion was further bolstered by the observation of the NaOMe absorption spectrum, which displayed a bathochromic shift with an increase in intensity relative to the spectrum obtained in methanol alone. Similarly, the compound exhibited a bathochromic shift of band II in NaOAc for flavonols containing the 7-hydroxyl group (Mabry *et al.*, 1970; Markham, 1982). Therefore, the glycosylation site was suggested to be located at C-3, which was further confirmed by the observed HMBC correlation peak between the anomeric proton at  $\delta_H$  5.68 and  $\delta_C$  132.8 (C-3). This, coupled with the other prominent peaks observed in the negative FAB mass spectrum at  $m/z$  477 [ $M - 132 - H$ ]<sup>-</sup> (loss of lyxose) and  $m/z$  315 [ $M - 132 - 162 - H$ ]<sup>-</sup> (loss of lyxose and glucose), was consistent with the successive loss of two sugar moieties, thereby suggesting that lyxose was a terminal sugar, in the form of lyxosylglucoside. The attachment position of the lyxose to the residual sugar was deduced from the <sup>13</sup>C-NMR spectral data, and was verified by the results of the <sup>1</sup>H-<sup>13</sup>C-COSY studies. In the <sup>13</sup>C-NMR spectrum, the C-2'' of glucose was observed to have shifted downfield at  $\delta$  79.6, whereas the anomeric carbon of lyxose had simultaneously undergone an upfield shift at  $\delta$  102.3. Similarly, in the <sup>1</sup>H-<sup>13</sup>C-COSY spectrum, the glucosylated carbon peak at  $\delta$  79.6 was correlated with its proton, at  $\delta$  3.49 (H-2''). Support for this notion came from HMBC correlations observed between the lyxose anomeric proton at  $\delta$  4.79 with the carbon located at  $\delta$  79.6 (C-2''), and between the glucose anomeric proton at  $\delta$  5.68 with the carbon located at  $\delta$  132.8 (C-3). Therefore, based on the spectroscopic data, the structure of compound 3 was identified as isorhamnetin 3-*O*- $\alpha$ -D-lyxopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-glucopyranoside, and was designated with the trivial name, nelumboside A.

Compound 4 displayed a positive HR-FAB mass ion at  $m/z$  779.2031 [ $M + Na$ ]<sup>+</sup> (calcd 779.2011), which was consistent with the molecular formula C<sub>33</sub>H<sub>40</sub>O<sub>20</sub>. The IR and UV spectra of this compound were almost completely identical to those observed with compound 3, indicating their similar natures. The negative ion FAB mass spectrum of compound 4 revealed a *quasi*-molecular ion peak [ $M -$

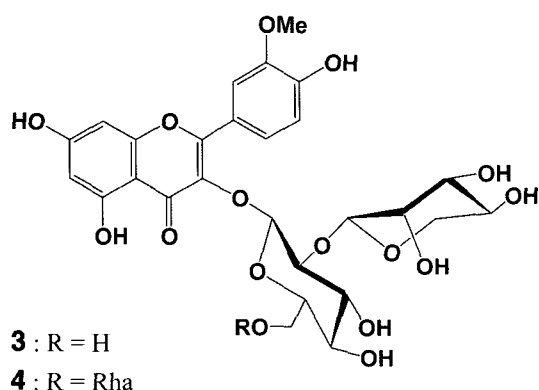


Fig. 1. Structures of 3 and 4

H]<sup>-</sup> at *m/z* 755, and fragment peaks at *m/z* 315 [M - 132 - 146 - 162 - H]<sup>-</sup> (loss of lyxose, rhamnose, and glucose), *m/z* 609 [M - 146 - H]<sup>-</sup> (loss of rhamnose), and *m/z* 623 [M - 132 - H]<sup>-</sup> (loss of lyxose), corresponding to the successive loss of three sugar moieties, thereby indicating that compound **4** possesses one more deoxyhexose than does compound **3**. The NMR spectral data of compound **4** indicated that it is fundamentally a cognate of **3**, except for the presence of one additional sugar unit, as was evidenced by an extra anomeric proton at  $\delta$  4.35 (s, H-1<sup>'''</sup>), one methyl signal at  $\delta$  0.97 (*J* = 6.2 Hz, H-6<sup>'''</sup>), and a corresponding anomeric carbon at  $\delta$  100.9 (C-1<sup>'''</sup>), and one methyl carbon at  $\delta$  17.7 (C-6<sup>'''</sup>). Acid hydrolysis resulted in the formation of isorhamnetin, lyxose, glucose, and rhamnose, all of which were identified by direct comparison with authentic samples, *via* TLC analyses. We assumed that the glucose and lyxose existed in the D-configuration, and that the rhamnose existed in the L-configuration, as these configurations are the most frequently encountered among plant glycosides. The coupling constant, 7.5 Hz ( $\delta$  5.50), was consistent with diaxial coupling between the protons on C-1 and C-2 in a  $\beta$ -linked glucose, whereas the 4.3 Hz ( $\delta$  4.83) and 0 Hz ( $\delta$  4.35) represented diequatorial coupling between the protons on C-1 and C-2, in an  $\alpha$ -linked lyxose and rhamnose. In our comparison of the <sup>13</sup>C-NMR and HMBC spectra of compound **4** with those of compound **3**, signals due to the C-5 and C-6 of the glucosyl moiety were determined to have shifted by -1.7 and +6.4 ppm, thereby indicating the presence of one more terminal rhamnose, linked to the C-6-OH group in the glucosyl moiety. This was supported further by the results of HMBC experiments, which revealed long-range coupling occurring between the anomeric proton of the rhamnosyl moiety at  $\delta$  4.35 and the methylene carbon ( $\delta$  66.9) assignable to the C-6 of the glucosyl moiety. Therefore, compound **4** was structurally identified as isorhamnetin 3-O- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 6)-[ $\alpha$ -D-lyxopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-glucopyranoside], and was designated with the trivial name, nelumboside B.

We also evaluated the antioxidant effects of isolated compounds **1-4** in the DPPH and ONOO<sup>-</sup> assays. All isolated isorhamnetin glycosides **1-4** showed potent antioxidant activities. The IC<sub>50</sub> values of **1-4** were as follows: 11.76, 9.01, 9.21, and 7.43  $\mu$ M in the  $\cdot$ DPPH assay (positive control, L-ascorbic acid, IC<sub>50</sub> 11.93  $\mu$ M), and 3.34  $\pm$  0.62, 2.56  $\pm$  0.37, 2.62  $\pm$  0.40 and 2.12  $\pm$  0.43  $\mu$ M in the ONOO<sup>-</sup> assay (positive control, DL-penicillamine, IC<sub>50</sub> 5.1  $\pm$  0.23  $\mu$ M).

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