

## Phenolic Constituents from *Balanophora laxiflora* with DPPH Radical-Scavenging Activity

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*Balanophora laxiflora* HEMSL. (Balanophoraceae), a dioeciously parasitic plant, has been used as a tonic and for sobering up from drunk by the local people of Yunnan province, China. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay on the acetone extract of the fresh female plant of *B. laxiflora* displayed considerable radical-scavenging activity ( $SC_{50} = 16.4 \mu\text{g/ml}$ ). Further purification of the extract led to the isolation of two new phenolic glycosides, balaxiflorins A and B (**1** and **2**, resp.), together with 17 known phenolic compounds including 3-phenylpropanoids, **3**, **17**–**18**, four lignans, **4**–**7**, nine hydrolyzable tannins, **8**–**16**, and gallic acid (**19**). Their structures were determined by detailed spectroscopic analysis. The free-radical-scavenging activity of the isolated compounds was examined by DPPH assay.

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**Introduction.** – *Balanophora laxiflora* HEMSL. (Balanophoraceae), a dioeciously parasitic plant, is mainly distributed in the subtropic and tropic forests of Southwestern China, Laos, Thailand, and Vietnam [1]. It is normally parasitized on the roots of the evergreen broadleaf trees from Leguminosae, Araliaceae, and Fagaceae families. In Yunnan province of China, it is always found growing on the roots of *Sophora davidii* (FR.) KOMAROV. ex PAVOL. The whole plant of *B. laxiflora* is used as a tonic and for sobering up from drunk by the local people of its growing area of China. As a part of our phytochemical investigation on balanophoraceous plants and to discover potential antioxidative natural products [2] [3], the preliminary experiment showed that the 80% aqueous acetone extract of the female plant of *B. laxiflora* exhibited considerable free-radical-scavenging activity in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay ( $SC_{50} = 16.4 \mu\text{g/ml}$ ). Further purification of the extract led to the isolation of two new phenolic glycosides, balaxiflorins A and B (**1** and **2**, resp.), together with 17 known phenolic compounds, **3**–**19** (Fig. 1). The isolated compounds were also examined for their DPPH radical-scavenging activities. Here, we present the details of this study.

**Results and Discussion.** – 1. *Chemistry.* The 80% aqueous acetone extract of the fresh female plant of *B. laxiflora* exhibited obvious radical-scavenging activity ( $SC_{50} = 16.4 \mu\text{g/ml}$ ) on DPPH assay. The crude extract was fractionated with a *Diaion HP20SS* column and further chromatographed on *Sephadex LH-20*, *MCI-gel CHP-20P*, and *Toyopearl HW-40F* column to afford two new phenolic glycosides **1** and **2**. In addition, 17 known phenolic compounds, including three phenylpropanoids, **3**, **17**, and **18**, four lignans, **4**–**7**, nine hydrolysable tannins, **8**–**16**, and one phenolic acid, **19**, were obtained.

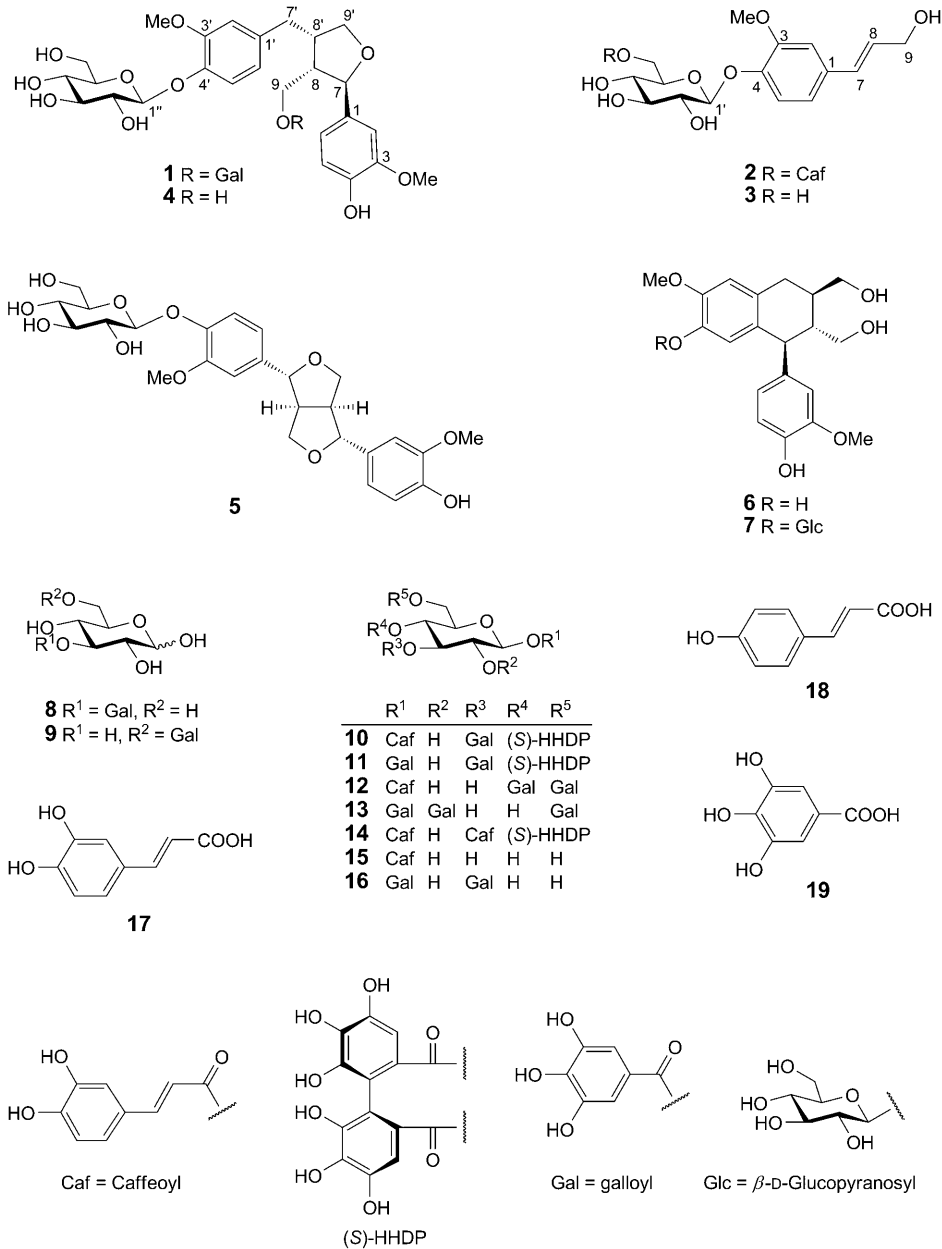


Fig. 1. Structures of **1–19** isolated from *Balanophora laxiflora*

The known compounds were identified as coniferin (**3**) [4], lariciresinol 4'-*O*- $\beta$ -D-glucopyranoside (**4**) [5], pinoresinol *O*- $\beta$ -D-glucopyranoside (**5**) [6], isolariciresinol (**6**) [7], isolariciresinol 4-*O*- $\beta$ -D-glucopyranoside (**7**) [8], 3-*O*-galloyl- $\beta$ -D-glucopyranose

(**8**), 6-*O*-galloyl- $\beta$ -D-glucopyranose (**9**), 1-*O*-[(*E*)-caffeoyl]-3-*O*-galloyl-4,6-[(*S*)-HHDP]- $\beta$ -D-glucopyranose (**10**) [9], 1,3-di-*O*-galloyl-4,6-[(*S*)-HHDP]- $\beta$ -D-glucopyranose (**11**) [9], 1-*O*-[(*E*)-caffeoyl]-4,6-di-*O*-galloyl- $\beta$ -D-glucopyranose (**12**) [9], 1,2,6-tri-*O*-galloyl- $\beta$ -D-glucopyranose (**13**) [6], 1,3-di-*O*-[(*E*)-caffeoyl]-4,6-[(*S*)-HHDP]- $\beta$ -D-glucopyranose (**14**) [9], 1-*O*-[(*E*)-caffeoyl]- $\beta$ -D-glucopyranose (**15**) [9], 1,3-di-*O*-galloyl- $\beta$ -D-glucopyranose (**16**) [9], caffeic acid (**17**), coumaric acid (**18**), and gallic acid (**19**) by direct comparison with authentic samples or comparison of the spectroscopic data with those reported in the literature.

Compound **1** was obtained as a brown amorphous powder and gave a dark blue color with FeCl<sub>3</sub> reagent. The molecular formula was assigned as C<sub>33</sub>H<sub>38</sub>O<sub>15</sub> on the basis of the negative-ion HR-FAB-MS (*m/z* 673.2120 ([*M* – H]<sup>–</sup>)). The <sup>1</sup>H-NMR spectrum showed the presence of two 1,3,4-trisubstituted aromatic rings ( $\delta$  6.72 (br. s, H–C(2), H–C(2')), 6.70 (*d*, *J* = 8.5, H–C(6'), H–C(5)), 7.00, 6.83 (*d*, *J* = 8.5, each 1 H, H–C(5'), H–C(6)), two MeO groups ( $\delta$  3.65, 3.69 (*s*, each 3 H, MeO–C(3), MeO–C(3'))), and one glucopyranosyl unit ( $\delta$ (H) 4.85 (*d*, *J* = 8.4, H–C(1'')),  $\delta$ (C) 101.5 (C(1'')). The *J* value (8.4 Hz) of the anomeric H-atom established the  $\beta$ -configuration of the glucose moiety. In addition, the <sup>13</sup>C-NMR (DEPT) spectrum indicated the occurrence of three CH (including an O-bearing one at  $\delta$  83.2 (C(7))) and three CH<sub>2</sub> groups (including two O-bearing ones at  $\delta$  72.8 (C(9)) and 63.1 (C(9))). These NMR features resembled those of lariciresinol-4'-*O*- $\beta$ -D-glucopyranoside (**4**) [5], except for an additional set of signals arising from a galloyl group ( $\delta$ (H) 7.03 (*s*, H–C(2''), H–C(6'')) in **1**. The obvious substituted effects of C-atom signals due to downfield shift at C(9) ( $\delta$  63.1) and upfield shift at C(8) ( $\delta$  49.9) suggested that the additional galloyl group was linked to C(9) of the lariciresinol-4'-*O*- $\beta$ -D-glucopyranosyl moiety of **1**, which was further confirmed by the long-range correlations of H–C(9) ( $\delta$  4.24, 4.42) with the C-atom signal of C(7'') ( $\delta$  167.2) of the galloyl group observed in the HMBC experiment (Fig. 2). Moreover, the correlations of H–C(1'') ( $\delta$  4.85) with H–C(5') ( $\delta$  7.00), and of the two MeO H-atoms ( $\delta$  3.65, 3.69) with H–C(2') ( $\delta$  6.72) and H–C(2) ( $\delta$  6.72) in the ROESY spectrum revealed the locations of the two MeO groups at C(3') and C(3), and the glucosyl moiety at C(4') of **1** (Fig. 2). Other HMBC correlations confirmed the structure of **1**.

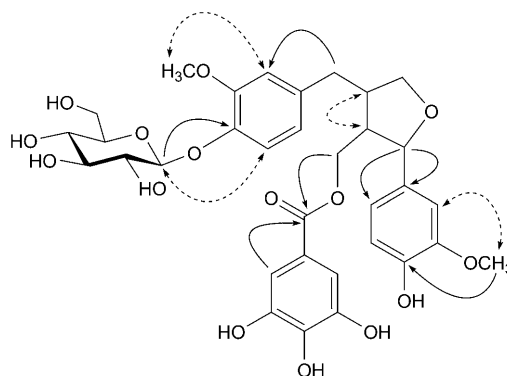


Fig. 2. Key HMBC (H → C) and NOESY (←---→) correlations of **1**

The relative configuration of **1** was determined by 1D-NMR and ROESY spectroscopic data. The NMR signals due to the aliphatic part of **1** are closely similar to those of larciresinol-4'-*O*- $\beta$ -D-glucopyranoside (**4**) [5]. Furthermore, the ROESY correlations of H–C(8) ( $\delta$  2.54) with H–C(8') ( $\delta$  2.76) established the *cis*-configuration between H–C(8) and H–C(8'), and *trans*-configuration between H–C(7) and H–C(8) in **1**. Therefore, compound **1** was assigned as (7*S*\*,8*R*\*,8'*R*\*)-9-*O*-galloyllarciresinol-4'-*O*- $\beta$ -D-glucopyranoside and named as balaxiflorin A.

Compound **2** was obtained as a pale brown amorphous powder. The molecular formula C<sub>25</sub>H<sub>28</sub>O<sub>11</sub> was deduced from negative-ion HR-FAB-MS ( $m/z$  503.1516 ( $[M-H]^-$ )) in combination with NMR experiments. The <sup>1</sup>H- and <sup>13</sup>C-NMR data of **2** were closely related to those of coniferin (**3**), in which the signals due to a 1,2,4-trisubstituted phenyl ( $\delta$  6.97 (br. *s*, H–C(2)), 6.90–6.95 (*m*, H–C(5)), 6.72 (*d*,  $J=8.4$ , H–C(6))), a glucopyranosyl ( $\delta$ (H) 4.92 (*d*,  $J=8.2$ , H–C(1')),  $\delta$ (C) 100.9 (C(1')), a hydroxypropenyl ( $\delta$  6.32 (*d*,  $J=15.9$ , H–C(7)), 6.00 (*d*,  $J=15.9$ , H–C(8)), 4.09 (*d*,  $J=5.9$ , 2 H–C(9))), and a MeO group ( $\delta$  3.78 (*s*, MeO–C(3))) were detected. The  $J$  value (8.2 Hz) of the anomeric H-atom established the  $\beta$ -configuration of the glucose moiety. In addition, the occurrence of a caffeoyl group ( $\delta$  7.10 (br. *s*, H–C(2'')), 6.90–6.95 (*m*, H–C(5'')), 6.83 (*d*,  $J=8.0$ , H–C(6'')), 7.45 (*d*,  $J=15.5$ , H–C(7'')), 6.18 (*d*,  $J=15.5$ , H–C(8''))) was evidenced for **2**, suggesting that **2** was a caffeoyl-coniferin. The downfield chemical shift of C(6') of the glucopyranosyl unit at  $\delta$  64.1 indicated that the additional caffeoyl group was linked at C(6'), which was further confirmed by the HMBC correlations of H–C(6') ( $\delta$  4.47 and 4.29) of the glucosyl unit with the carboxy C-atom at  $\delta$  167.8 (C(9'')) of the caffeoyl group. On the basis of the above evidence, the structure of balaxiflorin B (**2**) was elucidated as 6'-*O*-*E*-caffeoyl coniferin.

2. *DPPH Radical-Scavenging Assay.* All of the isolated compounds were tested for the free-radical-scavenging activity by DPPH assay, with ascorbic acid as positive control. The results are shown in the *Table*. In this assay, the hydrolyzable tannins, **8**–

Table. *Radical-Scavenging Activity of Compounds 1–19 from B. laxiflora on DPPH Assay*

Samples	SC <sub>50</sub> [ $\mu$ M] <sup>a)</sup> b)	Samples	SC <sub>50</sub> [ $\mu$ M] <sup>a)</sup> b)
Lignans		<b>10</b>	4.2 ± 0.2
<b>1</b>	21.1 ± 0.6	<b>11</b>	4.6 ± 0.2
<b>4</b>	242 ± 4	<b>12</b>	6.1 ± 0.3
<b>5</b>	119 ± 2	<b>13</b>	5.1 ± 0.2
<b>6</b>	143 ± 2	<b>14</b>	8.8 ± 0.2
<b>7</b>	304 ± 5	<b>15</b>	4.7 ± 0.1
		<b>16</b>	5.1 ± 0.2
Phenylpropanoids		Phenolic acids	
<b>2</b>	10.3 ± 0.3	<b>17</b>	15.7 ± 0.3
<b>3</b>	389 ± 6	<b>18</b>	234 ± 3
Hydrolyzable tannins		<b>19</b>	10.7 ± 0.5
<b>8</b>	8.8 ± 0.2	Ascorbic acid <sup>c)</sup>	10.5 ± 0.2
<b>9</b>	10.4 ± 0.2		

a) SC<sub>50</sub>: Radical-scavenging activity (concentration in  $\mu$ M required for 50% reduction of DPPH radical).

b) Values represent means ± SD ( $n=3$ ). c) Positive control.

**16**, exhibited higher activities than the other kinds of the phenolic compounds, and the new phenolic glycosides, **1** and **2**, with additional galloyl or caffeoyl groups attached showed stronger activity than their corresponding phenol compounds (**4** and **3**, resp.). In case of the phenolic acids, **17–19**, the number of phenolic OH groups is important for their free-radical-scavenging activity. The result was in accord with the trend that compounds with more adjacent phenolic OH groups (galloyl, pyrogallol, or catechol group) had higher radical-scavenging activities on DPPH reported previously [10]. The above results suggest that these phenolic compounds may play an important role for the radical-scavenging activity of this plant.

### Experimental Part

*General.* Column chromatography (CC): *Diaion HP20 SS* (Mitsubishi Chemical Co.), *Sephadex LH-20* (Pharmacia Fine Chemical Co. Ltd.), MCI-gel *CHP20P* (Mitsubishi Chemical Co.), and *Toyopearl HW-40F* (Tosoh Co.). TLC: was carried on silica-gel *G* pre-coated plates (*Qingdao Haiyang Chemical Co.*) with benzene/HCOOEt/formate/HCOOH 2:7:1; detection of spots by spraying with 2% FeCl<sub>3</sub> in EtOH. Optical rotations: *P-1020* Polarimeter (*JASCO*, Tokyo, Japan). IR Spectra: *IR-450* spectrometer (*Shimadzu*, Kyoto, Japan); KBr pellets. <sup>1</sup>H- and <sup>13</sup>C-NMR, HMQC, HMBC, and NOESY spectra: in (D<sub>6</sub>)acetone with *Bruker AM-400* and *DRX-500* spectrometers, at 500 and 400 MHz for <sup>1</sup>H, and 125 and 100 MHz for <sup>13</sup>C, resp. FAB-MS and HR-FAB-MS: *AutoSpec 3000* spectrometer (*VG*, Manchester, UK); glycerol as the matrix. DPPH (*Aldrich Chem. Co.*) radical-scavenging assay was performed on *Emax* precision microplate reader.

*Plant Material.* The female plant of *B. laxiflora* was collected from Wenshan County, Yunnan Province, China, in September 2004, and identified by Prof. X. W. Li. The voucher specimen (No. 0409111) was deposited with the KUN Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences.

*Extraction and Isolation.* The whole plant (4.0 kg) was cut into small pieces and extracted with 80% aq. acetone (10 l × 3, 24 h each) at r.t. The combined extracts were filtered and concentrated under vacuum to obtain a crude extract (300 g). The extract was subjected to *Diaion HP20SS* CC eluted with H<sub>2</sub>O/MeOH 1:0–0:1 in step gradient to give nine fractions. *Fr. 2* (36 g) was subjected to *Sephadex LH-20*, MCI-gel *CHP20P*, and *Toyopearl HW-40F* CCs eluted with H<sub>2</sub>O/MeOH to give **7** (75 mg), **8** (18 mg), and **15** (19 mg). With same chromatographic procedures, **3** (19 mg) from *Fr. 4* (8 g), **1** (12 mg), **2** (23 mg), and **4** (20 mg) from *Fr. 5* (42 g), **5** (15 mg) and **6** (16 mg) from *Fr. 6* (16 g), **13** (9 mg), **18** (30 mg), and **19** (70 mg) from *Fr. 7* (49 g), **11** (900 mg) and **12** (199 mg) from *Fr. 8* (52 g), and **10** (69 mg), **14** (9 mg), and **16** (16 mg) from *Fr. 9* (41 g) were obtained.

*Balaxiflorin A* (= (-)-(7*S*,8*R*,8'*R*)-9-*O*-Galloyllariciresinol-4'-*O*-β-D-glucopyranoside; **1**). Brown amorphous powder.  $[\alpha]_D^{25} = -29.5$  ( $c = 0.01$ , acetone). IR (KBr): 3431, 1774, 1708, 1625, 1394, 1227. <sup>1</sup>H-NMR (400 MHz): 2.50–2.53 (*m*, H–C(8)); 2.56–2.58 (*m*, H–C(7'')); 2.74–2.78 (*m*, H–C(8'')); 2.83 (*dd*,  $J = 5.0, 15.9$ , H–C(7'')); 3.45–3.53 (*m*, H–C(2''), H–C(3''), H–C(4'') H–C(5'')); 3.60–3.64 (*m*, H<sub>a</sub>–C(6'')); 3.65 (*s*, MeO–C(3)); 3.69 (*s*, MeO–C(3'')); 3.80 (*d*,  $J = 12.0$ , H<sub>b</sub>–C(6'')); 4.00 (*t*,  $J = 7.2$ , H–C(9'')); 4.24 (*dd*,  $J = 6.6, 11.3$ , H–C(9)); 4.42 (*dd*,  $J = 7.1, 11.3$ , H–C(9)); 4.79 (*d*,  $J = 6.8$ , H–C(7'')); 4.85 (*d*,  $J = 8.4$ , H–C(1'')); 6.70 (*d*,  $J = 8.5$ , H–C(5), H–C(6'')); 6.72 (*br. s*, H–C(2'), H–C(2'')); 6.83 (*d*,  $J = 8.5$ , H–C(6)); 7.00 (*d*,  $J = 8.5$ , H–C(5'')); 7.03 (*s*, H–C(2''), H–C(6'')). <sup>13</sup>C-NMR (100 MHz): 33.1 (*t*, C(7'')); 42.9 (*d*, C(8'')); 49.9 (*d*, C(8)); 55.8 (*q*, MeO–C(3'')); 56.0 (*q*, MeO–C(3)); 61.4 (*t*, C(6'')); 63.1 (*t*, C(9)); 70.1 (*d*, C(4'')); 72.8 (*t*, C(9'')); 73.6 (*d*, C(2'')); 76.3 (*d*, C(5'')); 76.8 (*d*, C(3'')); 83.2 (*d*, C(7)); 101.5 (*d*, C(1'')); 109.6 (*d*, C(2''), C(6'')); 110.1 (*d*, C(2)); 113.2 (*d*, C(2)); 115.8 (*d*, C(5)); 116.5 (*d*, C(5'')); 118.9 (*d*, C(6)); 120.0 (*s*, C(1'')); 121.5 (*s*, C(6'')); 134.7 (*s*, C(1)); 135.7 (*s*, C(1'')); 138.9 (*d*, C(4'')); 145.1 (*s*, C(4'')); 145.6 (*s*, C(3''), C(5'')); 145.9 (*s*, C(4)); 148.0 (*s*, C(3)); 149.2 (*s*, C(3'')); 167.2 (*s*, C(7'')). HR-FAB-MS (*neg.*): 673.2120 (C<sub>33</sub>H<sub>37</sub>O<sub>15</sub>; calc. 673.2132). FAB-MS (*neg.*): 673 ([*M*–H]<sup>–</sup>), 503 ([*M*–H–galloyl]<sup>–</sup>).

*Balaxiflorin B* (= 4-*O*-[6-*O*-[(*E*)-Caffeoyl]-β-D-glucopyranosyl] coniferin; **2**). Brown amorphous powder.  $[\alpha]_D^{25} = -5.9$  ( $c = 0.07$ , H<sub>2</sub>O). <sup>1</sup>H-NMR (400 MHz): 3.62–3.42 (*m*, H–C(2'), H–C(3'),

H–C(4'), H–C(5')); 3.78 (s, MeO–C(3)); 4.09 (d,  $J=5.9$ , H–C(9)); 4.29 (dd,  $J=4.7, 12.4$ , H<sub>a</sub>–C(6')); 4.47 (dd,  $J=2.4, 12.4$ , H<sub>b</sub>–C(6')); 4.92 (d,  $J=8.2$ , H–C(1')); 6.00 (d,  $J=15.9$ , H–C(8)); 6.18 (d,  $J=15.5$ , H–C(8'')); 6.32 (d,  $J=15.9$ , H–C(7)); 6.72 (d,  $J=8.4$ , H–C(6)); 6.83 (d,  $J=8.0$ , H–C(6'')); 6.90–6.95 (m, H–C(5), H–C(5'')); 6.97 (br. s, H–C(2)); 7.10 (br. s, H–C(2'')); 7.45 (d,  $J=15.5$ , H–C(7'')).  
<sup>13</sup>C-NMR (125 MHz): 56.1 (q, MeO); 62.6 (t, C(9)); 64.1 (t, C(6')); 70.9 (d, C(4')); 73.7 (d, C(2)); 74.5 (d, C(5')); 76.4 (d, C(3')); 100.9 (d, C(1')); 110.7 (d, C(2)); 114.5 (d, C(2'')); 114.8 (d, C(8'')); 116.2 (d, 2 C, C(5), C(5'')); 119.7 (d, C(6)); 122.6 (d, C(6'')); 126.8 (s, C(1'')); 128.8 (d, C(8)); 129.6 (d, C(7)); 132.4 (s, C(1)); 145.9 (s, C(3'')); 146.2 (d, C(7'')); 146.3 (s, C(4)); 148.8 (s, C(4'')); 149.2 (s, C(3)); 167.8 (d, C(9')).  
 HR-FAB-MS (neg.): 503.1516 ([M–H]<sup>–</sup>, C<sub>25</sub>H<sub>27</sub>O<sub>11</sub>; calc. 503.1553). FAB-MS (neg.): 503 ([M–H]<sup>–</sup>).

**DPPH Radical-Scavenging Assay.** The DPPH assay was performed as described in [2], and ascorbic acid was used as positive control. Scavenging activity was determined by the following equation:

$$\text{Scavenging activity [\%]} = 100 \times (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}$$

The SC<sub>50</sub> value was obtained through extrapolation from linear regression analysis and denoted the concentration of sample required to scavenge 50% of DPPH radicals.

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