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Antioxidant phenylpropanoid glycosides from the leaves of *Wasabia japonica*

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Abstract

From the MeOH extract of the leaves of *W. japonica*, seven phenylpropanoid gentiobiosides (**1**–**7**) were isolated along with eight known phenylpropanoids (**8**–**15**). Structures of **1**–**7** were determined based on spectroscopic data and chemical evidence. The activity of compounds **1**–**15** to scavenge superoxide anion radicals was investigated using an electron spin resonance (ESR) method.

Keywords: *Wasabia japonica* Matsumura; Cruciferae; Electron spin resonance (ESR); Phenylpropanoid glycosides; Superoxide anion radicals

1. Introduction

Wasabi (the root of *Wasabia japonica* Matsumura, Cruciferae), also known as ''Japanese horseradish'', is used widely in Japanese cuisine as a pungent spice to garnish traditional dishes like sushi and sashimi. The pungency is derived from volatile allylisothiocyanates via the reaction with myrosinase (Kojima et al., 1973). Allylisothiocyanate derivatives are found in the root, stem and leaf of wasabi and horseradish plants (Eto et al., 1990). They are reported to have antimicrobial (Ono et al., 1998), antimutagenic (Kinae et al., 2000), antiplatelet (Morimitsu et al., 2000), anti-lung tumorigenesis (Yano et al., 2000), anti-gastric carcinogenesis (Tanida et al., 1991), and apoptosis-inducing (Watanabe et al., 2003b) activities. Although wasabi is known to exhibit such biological activities, the leaves of the plant are usually discarded and have been little studied chemically or biologically. In the present study, we have isolated seven new phenylpropanoid glycosides (**1**–**7**) along with eight known compounds (**8**–**15**) from the fresh wasabi leaves, and have measured their antioxidant activities by an electron spin resonance (ESR) method (Yun et al., 2003). We describe herein the isolation and structure elucidation of **1**–**7** using spectroscopic data analysis and chemical evidence as well as the antioxidant activities of **1**–**15**.

2. Results and discussion

Both the *n*-BuOH- and EtOAc-soluble portions of the MeOH extract of fresh *W. japonica* leaves exhibited superoxide anion radicals ($O_2^{\cdot-}$) scavenging activity of 82% at 1 mg/mL. The *n*-BuOH extract yielded seven phenylpropanoid glycosides (**1**–**7**) (Fig. 1) and the EtOAc extract afforded eight phenylpropanoids (**8**–**15**) by repeated column chromatography and preparative reversed-phase HPLC, as described in 3. Compounds **8**–**15** were identified as *trans*-*p*-hydroxycinnamic acid, *trans*-ferulic acid, *trans*-sinapic acid, 3,4-dimethoxy-*trans*-cinnamic acid, *trans*-ferulic acid methyl ester, *trans*-sinapic acid methyl ester, 3,4-dihydroxy-5-methoxy-*trans*-cinnamic acid, and 3,4-dihydroxy-5-methoxy-*trans*-cinnamic acid methyl ester, respectively, by comparison of their spectroscopic data with those of authentic samples.

Compound **1** was obtained as a yellow amorphous powder. Its molecular formula was determined to be $C_{33}H_{40}O_{19}$ from the $[M+H]^+$ ion peak at 741.2185 (calc. for

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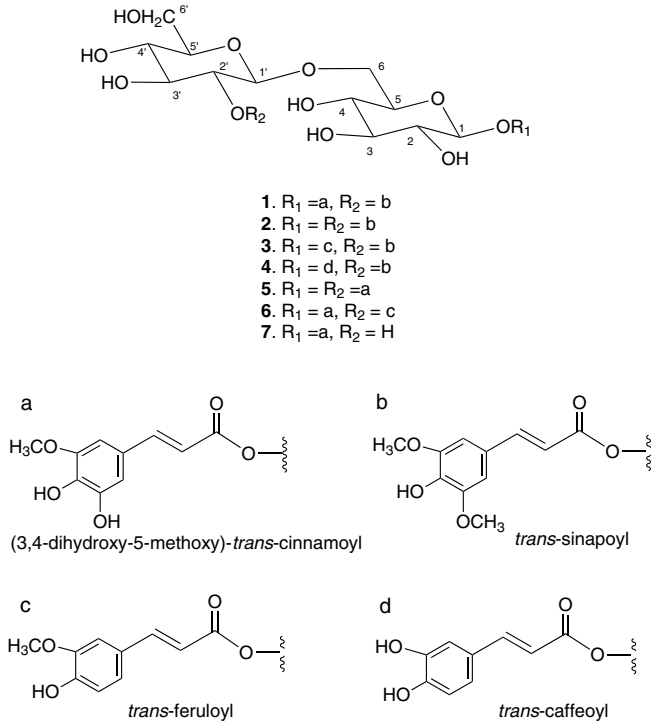

Fig. 1. Phenylpropanoid glycosides isolated from leaves of *Wasabia japonica*.

$C_{33}H_{41}O_{19}$, 741.2242) in the high-resolution ESI MS (HR ESI MS). The IR spectrum showed absorptions for hydroxyl (3416 cm^{-1}) and conjugated carbonyl (1704 cm^{-1}) groups and aromatic rings (1633 and 1518 cm^{-1}). The UV absorption maxima occurred at 239 and 329 nm, implying the presence of conjugated double bond systems in the molecule. Analysis of the ^1H NMR spectrum (Table 1) showed signals for four aromatic ring protons (δ 6.70, 6.71 and 6.93 × 2), four olefinic protons (δ 6.27, 6.54, 7.60 and 7.69), three methoxy groups (δ 3.86 × 3), and 14 sugar-derived protons (δ 3.28–5.54). The ^1H NMR spectrum of **1** indicates the presence of two 1,3,4,5-tetrasubstituted aromatic rings [δ 6.70 (1H, *d*, $J = 1.6$ Hz), 6.71 (1H, *d*, $J = 1.6$ Hz), and 6.93 (2H, *s*)]. The ^{13}C NMR spectrum (Table 2) exhibited signals for two ester carbonyl carbons (δ 167.4 and 168.5), 14 aromatic and olefinic carbons (δ 105.1–149.7), three methoxy groups (δ 56.7 and 56.8 × 2), and 12 sugar-derived carbons (δ 62.6 69.2, 71.2, 71.6, 73.9, 75.2, 76.2, 77.9, 78.0, 78.7, 95.6, and 102.6). Moreover, the four large J values of 15.8 Hz each suggested the presence of two pairs of *trans*-olefinic protons (Cuendet et al., 2001). Acid hydrolysis of **1** gave D-glucose as the sugar component, which was confirmed by comparing the HPLC and optical rotation data with those of an authentic sample, as described in 3 (Watanabe et al., 2003a). Analysis of the HMQC and HMBC spectra of **1** established the presence of a (3″,4″-dihydroxy-5″-methoxy)-cinnamoyl group, a sinapoyl group, and two glucoses. An HMBC NMR correlation between the H-1 (δ 5.54) signal of one of the glucose and the C-9″ (δ 167.4) resonance of the 3″,4″-dihydroxy-5″-methoxy-cinnamoyl group and the characteristic chemical shift of the anomeric carbon (δ 95.6) suggested that this glucose unit has an *O*-glucosidic linkage at C-9″ of the acyl group. The second glucose unit was found to be linked to the hydroxyl group at C-6 by observation of an HMBC correlation between the H-1′ (δ 4.70) and C-6 (δ 69.2) resonances. In the ^1H NMR spectrum of **1** in CD_3OD, the signal for H-2′ of the glucose unit was shifted to a lower field (δ 4.80), which was considered to be caused by the aromatic acid ester bond. The HMBC experiment for **1** revealed a correlation between the ester carbonyl carbon C-9‴ (δ 168.5) and the H-2′ proton, confirming that the sinapoyl group is located at the C-2′ hydroxyl group. Thus, the structure of **1** was determined as 1-(3″,4″-dihydroxy-5″-methoxy)-*O*-*trans*-cinnamoyl-2′-*O*-*trans*-sinapoyl gentiobiose.

Compound **2**, a yellow amorphous solid, gave the molecular formula $C_{34}H_{42}O_{19}$, as determined by its HRESIMS (*m*/*z* 755.2362 $[M+H]^+$). As described in Section 3, the IR, UV, ^1H and ^{13}C NMR spectroscopic features of **2** were generally similar to those of **1**, implying that **2** also consists of two sinapoyl groups and two sugars. Acidic hydrolysis and HPLC analysis of **2** were used to deduce that the two sugars are D-glucose units. In the HMBC studies, two sinapoyl groups were shown to be connected to the OH-1 and OH-2′ of glucose, since correlations were observed between H-1 (δ 5.56) and a carbonyl carbon (δ 167.4), and between H-2′ (δ 4.80) and a carbonyl carbon (δ 168.5). Thus, the HMBC spectrum of **2** provided evidence that, like **1**, there were linkages between the two sinapoyl groups and the two glucoses. On analysis of all of the available data, the structure of **2** was elucidated as 1,2′-di-*O*-*trans*-sinapoyl gentiobiose.

Compound **3** was obtained as a yellow amorphous solid. The molecular formula was determined to be $C_{33}H_{40}O_{18}$ from the $[M+Na]^+$ ion peak at *m*/*z* 747.2128 in the HRESIMS (Calc. for $C_{33}H_{40}O_{18}Na$, 747.2112). The IR, UV, and ^1H and ^{13}C NMR spectra of **3** were very similar to those of **1** and **2**, suggesting that **3** consists of two glucoses, a feruloyl group, and a sinapoyl group. Long-range correlations were observed between the H-1 (δ 5.55) and C-9″ (δ 167.4), and between the H-2′ (δ 4.89) and C-9‴ (δ 168.5) in their HMBC spectra, showing that the feruloyl group was located at C-1 and the sinapoyl group at C-2′. Therefore, the structure of **3** was proposed as 1-*O*-*trans*-feruloyl-2′-*O*-*trans*-sinapoyl gentiobiose.

Compound **4**, a yellow amorphous solid, gave the molecular formula $C_{32}H_{38}O_{18}$ by HRESIMS (*m*/*z* 733.2011, $[M+Na]^+$). Its IR, UV, and ^1H and ^{13}C NMR data were also generally similar to those of **1**–**3** and suggested the presence of two glucoses, a caffeoyl group, and a sinapoyl group. From the HMBC spectrum of **4**, the position of the caffeic acid was deduced as C-1 of glucose, and the location of the sinapoyl group was found to be at C-2′ from the long-range correlations between H-1 (δ 5.54) and H-2′ (δ 4.80) and the carbonyl carbons of caffeic acid

Table 1
^{1}H NMR (400 MHz) spectroscopic data for **1**–**7** in CD_3OD

	1	**2**	**3**	**4**	**5**	**6**	**7**
β-D-*glucose*							
1	5.54 (1H, *d*, 7.9)	5.56 (1H, *d*, 7.9)	5.55 (1H, *d*, 7.9)	5.54 (1H, *d*, 8.0)	5.55 (1H, *d*, 7.7)	5.54 (1H, *d*, 7.7)	5.56 (1H, *d*, 7.5)
2	3.36 (1H, *m*[a])	3.36 (1H, *m*[a])	3.37 (1H, *m*[a])	3.37 (1H, *m*[a])	3.37 (1H, *m*[a])	3.36 (1H, *m*[a])	3.45 (1H, *m*[a])
3	3.30 (1H, *m*[a])	3.31 (1H, *m*[a])	3.30 (1H, *m*[a])	3.30 (1H, *m*[a])	3.30 (1H, *m*[a])	3.30 (1H, *m*[a])	3.32 (1H,*m*[a])
4	3.28 (1H, *m*[a])	3.27 (1H, *m*[a])	3.27 (1H, *m*[a])	3.27 (1H, *m*[a])	3.27 (1H, *m*[a])	3.28 (1H, *m*[a])	3.47 (1H,*m*[a])
5	3.51 (1H, *m*[a])	3.52 (1H, *m*[a])	3.51 (1H, *m*[a])	3.49 (1H, *m*[a])	3.52 (1H, *m*[a])	3.52 (1H, *m*[a])	3.59 (1H, *m*[a])
6	3.79 (1H, *dd*, 6.2, 11.3)	3.79 (1H, *dd*, 6.3, 11.1)	3.78 (1H, *dd*, 6.2, 11.1)	3.81 (1H, *dd*, 6.3, 11,2)	3.80 (1H, *dd*, 6.0, 11.4)	3.79 (1H, *dd*, 6.1, 11.1)	3.78 (1H, *dd*, 5.4, 11.3)
	4.08 (1H, *brd*, 11.3)	4.10 (1H, *brd*, 11.1)	4.09 (1H, *brd*, 11.1)	4.08 (1H, *brd*, 11.2)	4.07 (1H, *brd*, 11.4)	4.08 (1H, *brd*, 11.1)	4.17 (1H, *brd*, 11.3)
β-D-*glucose (at C-6)*							
1′	4.70 (1H, *d*, 8.1)	4.71 (1H, *d*, 8.0)	4.71 (1H, *d*, 8.0)	4.71 (1H, *d*, 8.0)	4.71 (1H, *d*, 8.0)	4.70 (1H, *d*, 8.0)	4.34 (1H, *d*, 7.7)
2′	4.80 (1H, *dd*, 8.1, 9.4)	4.80 (1H, *m*[a])	4.80 (1H, *m*[a])	4.80 (1H, *m*[a])	4.80 (1H, *m*[a])	4.80 (1H, *m*[a])	3.22 (1H, *dd*, 7.7, 8.8)
3′	3.56 (1H, *dd*, 9.2, 9.4)	3.58 (1H,*dd*, 9.1, 9.1)	3.57 (1H, *dd*, 9.1, 9.1)	3.56 (1H, *dd*, 9.1, 9.1)	3.57 (1H, *dd*, 9.1, 9.2)	3.57 (1H, *dd*, 9.1, 9.2)	3.32 (1H, *m*[a])
4′	3.42 (1H, *m*[a])	3.41 (1H, *m*[a])	3.41 (1H, *m*[a])	3.41 (1H, *m*[a])	3.40 (1H, *m*[a])	3.40 (1H, *m*[a])	3.50 (1H, *m*[a])
5′	3.36 (1H, *m*[a])	3.36 (1H, *m*[a])	3.36 (1H, *m*[a])	3.36 (1H, *m*[a])	3.36 (1H, *m*[a])	3.36 (1H, *m*[a])	3.26 (1H, *m*[a])
6′	3.68 (1H, *dd*, 5.7, 11.9)	3.69 (1H, *dd*, 5.5, 11.9)	3.69 (1H, *dd*, 5.6, 12.0)	3.68 (1H, *dd*, 5.5, 12.2)	3.69 (1H, *dd*, 5.5, 12.1)	3.70 (1H, *dd*, 5.5, 12.1)	3.65 (1H, *dd*, 5.5, 11.9)
	3.88 (1H, *m*[a])	3.88 (1H, *m*[a])	3.90 (1H, *brd*, 12.0)	3.89 (1H,*m*[a])	3.89 (1H, *m*[a])	3.89 (1H, *m*[a])	3.84 (1H, *brd*, 11.9)
Aglycon (at C-1)							
2″	6.70 (1H, *d*, 1.6)	6.85 (1H, *s*)	7.12 (1H, *d*, 1.7)	7.02 (1H, *d*, 1.8)	6.71 (1H, *d*, 1.4)	6.72 (1H,*s*)	6.77 (1H, *d*, 1.5)
5″			6.80 (1H, *d*, 8.1)	6.77 (1H, *d*, 8.1)			
6″	6.71 (1H, *d*, 1.6)	6.85 (1H, *s*)	7.01 (1H, *dd*, 1.7, 8.1)	6.89 (1H, *dd*, 1.8, 8.1)	6.72 (1H, *d*, 1.4)	6.73 (1H, *s*)	6.78 (1H, *d*, 1.5)
7″	7.60 (1H, *d*, 15.8)	7.65 (1H, *d*, 15.8)	7.67 (1H, *d*, 15.8)	7.62 (1H, *d*, 15.8)	7.61 (1H, *d*, 15.8)	7.61 (1H, *d*, 15.8)	7.74 (1H, *d*, 15.8)
8″	6.27 (1H, *d*, 15.8)	6.34 (1H, *d*, 15.8)	6.30 (1H, *d*, 15.8)	6.23 (1H, *d*, 15.8)	6.28 (1H, *d*, 15.8)	6.28 (1H, *d*, 15.8)	6.42 (1H, d, 15.8)
OMe-3″		3.81 (3H, *s*)					
OMe-5″	3.86 (3H, *s*)	3.81 (3H, *s*)	3.87 (3H, *s*)		3.82 (3H, *s*)	3.86 (3H, *s*)	3.87 (3H, *s*)
Aglycon (at C-2′)							
2‴, 6‴	6.93 (2H, *s*)	6.95 (2H, *s*)	6.96 (2H, *s*)	6.99 (2H, *s*)	6.78 (1H, *d*, 1.4)	7.27 (1H, *d*, 1.5)	
5‴						6.79 (1H, *d*, 8.1)	
6‴					6.82 (1H, *d*, 1.4)	7.08 (1H, *dd*, 1.5, 8.1)	
7‴	7.69 (1H, *d*, 15.8)	7.69 (1H, *d*, 15.8)	7.70 (1H, *d*, 15.8)	7.71 (1H, *d*, 15.8)	7.63 (1H, *d*, 15.8)	7.69 (1H, *d*, 15.8)	
8‴	6.54 (1H, *d*, 15.8)	6.53 (1H, *d*, 15.8)	6.54 (1H, *d*, 15.8)	6.56 (1H, *d*, 15.8)	6.45 (1H, *d*, 15.8)	6.51 (1H, *d*, 15.8)	
OMe-3″						3.86 (3H, *s*)	
OMe-3‴,5‴	3.86 (6H, *s*)	3.85 (6H, *s*)	3.84 (6H, *s*)	3.86 (6H, *s*)	3.85 (3H, *s*)		

[a] Multiplicity was not determined due to overlapping of the signals.

(δ 167.5) and sinapic acid (δ 168.6). Thus, the structure of **4** was determined as 1-*O*-*trans*-caffeoyl-2′-*O*-*trans*-sinapoyl gentiobiose.

Compound **5**, a yellow amorphous solid, was founded to have the molecular formula, $C_{32}H_{38}O_{19}$ on the basis of the HRESIMS (*m*/*z* 749.1880 $[M+Na]^+$). The IR spectrum of **5** showed strong absorption bands at 3335 (OH) and 1627(aromatic) cm^{-1}. Signals in the ^{1}H and ^{13}C NMR spectra suggested that **5** has a structure similar to **1**–**4**, consisting of two (3,4-dihydroxy-5-methoxy)-*trans*-cinnamoyl groups and two glucoses. The HMBC spectrum of **5** provided evidence for the substitution of the two (3,4-dihydroxy-5-methoxy)-cinnamoyl groups at C-1 (δ 95.5) and at C-2′ (δ 75.1). Accordingly, the structure of **5** was determined as 1,2′-di-(3″,4″-dihydroxy-5″-methoxy)-*O*-*trans*-cinnamoyl gentiobiose.

Compound **6** was assigned the molecular formula, $C_{32}H_{38}O_{18}$, from the HRESIMS (*m*/*z* 733.1955 $[M+Na]^+$). The IR and UV data were generally similar to those of **1**–**5**. It was established that **6** has two glucoses, a feruloyl group, and a (3,4-dihydroxy-5-methoxy)-cinnamoyl group. Long-range HMBC NMR studies showed that the (3,4-dihydroxy-5-methoxy)-cinnamoyl unit is located at the oxygen atom of C-1 (δ 95.6), and the feruloyl moiety is bonded at the oxygen atom of C-2′ (δ 75.2). Therefore, the structure of **6** was determined as 1-(3″, 4″-dihydroxy-5″-methoxy)-*O*-*trans*-cinnamoyl-2′-*O*-*trans*-feruloyl gentiobiose.

Compound **7**, a yellow amorphous solid, was shown to have the molecular formula, $C_{22}H_{30}O_{15}$, as determined by HRESIMS (*m*/*z* 557.1493 $[M+Na]^+$). The IR and UV data were the same as those of compound **6**. Analysis of the ^{1}H

Table 2
^{13}C NMR (100 MHz) spectroscopic data for **1**–**7** in CD_3OD

	1	2	3	4	5	6	7
β-D-glucose							
1	95.6	95.6	95.6	95.6	95.5	95.6	95.7
2	73.9	74.0	73.9	73.9	73.8	73.9	73.9
3	77.9	78.0	78.0	78.0	77.9	78.0	77.9
4	71.2	71.3	71.3	71.2	71.1	71.2	71.5
5	78.7	78.7	78.7	78.8	78.4	78.6	77.9
6	69.2	69.3	69.3	69.3	69.1	69.2	69.5
β-D-glucose (at C-6)							
1′	102.6	102.6	102.6	102.6	102.4	102.6	104.5
2′	75.2	75.2	75.2	75.2	75.1	75.2	75.1
3′	76.2	76.3	76.3	76.2	76.1	76.3	77.7
4′	71.6	71.7	71.6	71.6	71.5	71.7	70.9
5′	78.0	78.1	78.1	78.1	77.9	78.0	77.8
6′	62.6	62.6	62.6	62.6	62.5	62.6	62.6
Aglycon (at C-1)							
1″	126.5	126.4	127.5	127.5	126.4	126.5	126.5
2″	110.5	106.9	111.8	115.3	110.5	110.5	110.5
3″	146.8	149.3	149.4	146.8	146.5	146.7	146.7
4″	138.6	139.4	150.9	149.9	138.6	138.6	138.7
5″	149.7	149.3	116.3	116.6	149.7	149.7	149.8
6″	105.1	106.9	124.2	123.2	105.0	105.2	105.3
7″	148.5	148.3	148.2	148.2	148.5	148.5	148.8
8″	114.8	115.1	114.7	114.4	114.8	114.8	114.7
9″	167.4	167.4	167.4	167.5	167.4	167.3	167.6
OMe-3″		56.8					
OMe-5″	56.7	56.8	56.4		56.7	56.7	56.7
Aglycon (at C-2′)							
1‴	126.9	126.9	126.9	127.0	126.9	128.0	
2‴	106.9	106.9	107.0	107.0	110.6	111.7	
3‴	149.5	149.4	149.3	149.4	146.5	149.3	
4‴	139.4	139.8	139.4	139.4	138.1	150.4	
5‴	149.3	149.4	149.3	149.4	149.7	116.4	
6‴	106.9	106.9	107.0	107.0	105.2	124.3	
7‴	147.3	147.3	147.3	147.4	147.5	147.1	
8‴	116.3	116.3	116.5	116.3	115.9	115.8	
9‴	168.5	168.5	168.5	168.6	168.5	168.6	
OMe-3‴	56.8	56.8	56.8	56.8		56.5	
OMe-5‴	56.8	56.8	56.8	56.9	56.7		

and ^{13}C NMR spectra showed the occurrence of one acyl group and a gentiobiose moiety. An HMBC experiment displayed a correlation between the carbonyl carbon at δ 167.6 and the anomeric proton at δ 5.56, confirming that the acyl group is located at the C-1 position of the glucose unit in **7**. Thus, the structure of **7** was determined as 1-(*O-trans*-3″,4″-dihydroxy-5″-methoxy)-*O-trans*-cinnamoyl gentiobiose.

The radical scavenging activities of all phenylpropanoid derivatives (**1**–**15**) isolated in this study were evaluated against superoxide anion radicals ($O_2^{\cdot-}$) using ESR, in order to determine their antioxidative ability. The results showed that **1**, **4**–**6**, **14**, and **15** exhibited IC_{50} values of 28.5, 84.5, 8.4, 17.1, 36.0, and 31.3 μM, respectively, when compared with an IC_{50} value of 140 μM for ascorbic acid as a positive control. The scavenging ability of hydroxycinnamic acids against superoxide anion radicals was dependent on the numbers of hydroxy groups on the benzene and *ortho* substitutions with the electron donor methoxy group. The results were in accordance with the radial scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH), which decreased in the order caffecic acid > sinapic acid > ferulic acid > *p*-coumaric acid (Kikuzaki et al., 2002). Therefore, the leaves of *W. japonica* may be considered a promising lead for future antioxidants.

3. Experimental

3.1. General

Optical rotations were measured with a JASCO DIP-360 automatic digital polarimeter. UV and IR absorption spectra were recorded on a Hitachi U-2001 spectrophotometer and a JASCO FT/IR-620 spectrophotometer, respectively. ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker DPX-400 spectrometer in CD_3OD at 300 K. Standard pulse sequences and parameters were used for the experiments. ^1H chemical shifts in CD_3OD were referenced to residual CD_2HOD (δ 3.31); ^{13}C chemical shifts were referenced to the solvent (CD_3OD, δ 49.0). The chemical shift values are reported in δ (ppm) units and the coupling constants (*J*) are in Hz. High-resolution ESI MS were obtained on a Micromass LCT (Manchester, UK) spectrometer. HPLC was carried out using a Shimadzu LC-10AT pump equipped with a SPE-10vp detector (λ 254 nm) and Mightysil RP-18 columns (for analytical HPLC, 250 × 4.6 mm i.d.; 5 μm particle size and for preparative HPLC, 250 × 20 mm i.d.; 5 μm particle size, Kanto Kagaku Co. Ltd., Japan), using a CH_3CN/H_2O solvent system. The HPLC analysis of the sugar component was carried out using an optical rotation detector, SHODEX OR-2 (Showadenko Co. Ltd.), a Capcell Pak NH_2 UG80 column (250 × 4.6 mm i.d.; 5 μm particle size, Shiseido, Tokyo, Japan), and CH_3CN/H_2O (15:85) solvent system (0.9 mL/min).

3.2. Plant materials

The leaves of *W. japonica* Matsumura (4.6 kg) cultivated in Okutama, Tokyo, Japan were harvested in December 2001. The plant was identified by Mr. Tsuguo Saito, pharmacist, Sankyotou Pharmacy. A voucher specimen has been deposited at Sankyotou Pharmacy, Okutama, Tokyo, Japan.

3.3. Extraction and isolation

The fresh leaves of *W. japonica* (4.6 kg) were extracted with MeOH (36 L × 3, a week each) at room temperature. After filtration and removal of the solvent by evaporation in vacuo, a residue (179.8 g) was obtained, which was suspended in water (2 L) and then extracted successively with *n*-hexane, EtOAc, and *n*-BuOH (each 2 L × 3). Each organic layer was evaporated in vacuo to give *n*-hexane (42.4 g), EtOAc (2.3 g), and *n*-BuOH (14.1 g) extracts and an H_2O-soluble portion.

The *n*-BuOH extract (14.1 g) was loaded onto a Diaion HP-20 column (5 × 60 cm) and eluted sequentially with 2 L each of H_2O/MeOH mixtures (100:0, 80:20, 60:40, 40:60, 20:80 and 0:100, v/v) and acetone to give seven fractions (frs. 1-7). The H_2O/MeOH (60:40) eluate was concentrated, and the residue (4.9 g) was subjected to ODS column chromatography using H_2O/MeOH mixtures (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 25:75 and 0:100, v/v), to afford five fractions (frs. 3A-3E). Fr. 3A (1.1 g) was separated by preparative HPLC using 10% aqueous CH_3CN as solvent to give **1** (73.0 mg), **4** (9.7 mg), **5** (37.3 mg), and **7** (23.2 mg). Fr. 3B (2.3 g) gave **1** (252.4 mg) and **4** (77.5 mg) by preparative HPLC with 13% aqueous CH_3CN as solvent and **2** (36.6 mg) and **3** (4.3 mg) by preparative HPLC using 15% aqueous CH_3CN for elution, respectively. Similarly, Frs. 3C (715.8 mg) and 3D (222.4 mg) were subjected to preparative HPLC using H_2O/CH_3CN (87:13, v/v for 3C and 85:15, v/v for 3D, respectively) to give compounds **2** (30.2 mg) and **6** (25.5 mg), respectively.

The EtOAc extract (2.3 g) was placed on a silica gel column (10 × 60 cm) and eluted sequentially with 1 L each of $CHCl_3$ and $CHCl_3$/MeOH mixtures (60:1, 30:1, 10:1, 9:1, 8:2, 7:3, and 1:1, v/v) to give five fractions (frs. 1-5). By preparative HPLC with aqueous 35% CH_3CN containing 1% CH_3COOH, fr. 1 gave **12** (6.0 mg) and **13** (22.0 mg), and fr. 2 gave **11** (5.6 mg) and **15** (12.6 mg) using 30% aqueous CH_3CN containing 1% CH_3COOH. After chromatography of fr. 3 on an ODS column (4 × 50 cm) with H_2O/MeOH mixtures (60:40,40:60, 20:80 and 0:100, v/v), the second eluate among four fractions was subjected to preparative HPLC with 25% aqueous CH_3CN containing 1% CH_3COOH to give **9** (12.2 mg) and **10** (24.4 mg). Frs. 4 and 5 were subjected to preparative HPLC eluting with 20% aqueous CH_3CN containing 1% CH_3COOH and with 25% aqueous CH_3CN containing 1% CH_3COOH, to give **14** (48.3 mg) and **8** (4.0 mg), respectively.

3.4. Characterization data

3.4.1. 1-(3″,4″-Dihydroxy-5″-methoxy)-O-trans-cinnamoyl-2′-O-trans-sinapoyl gentiobiose (1)

Yellow amorphous solid; $[\alpha]_D^{25}$ − 5.6(MeOH; *c*0.50); *IR*ν_{max}^{neat}cm^{-1} : 3416, 2927, 1704, 1633, 1518; UVλ_{max}^{MeOH}nm (log ε) : 239(4.42), 329(4.50); For ^{1}H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2, respectively; HRESIMS *m/z* 741.2185 $[M+H]^+$ (Calc. for $C_{33}H_{41}O_{19}$, 741. 2242).

3.4.2. 1,2′-Di-O-trans-sinapoyl gentiobiose (2)

Yellow amorphous solid; $[\alpha]_D^{25}$ − 8.6(MeOH;*c*0.50); IRν_{max}^{neat}cm^{-1} : 3451, 2759, 1698, 1632, 1517; UVλ_{max}^{MeOH}nm (log ε) : 239(4.41), 331(4.50); For ^{1}H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2, respectively; HRESIMS *m/z* 755.2362 $[M+H]^+$ (Calc. for $C_{34}H_{43}O_{19}$, 755.2399).

3.4.3. 1-O-Trans-feruloyl-2′-O-trans-sinapoyl gentiobiose (3)

Yellow amorphous solid; $[\alpha]_D^{25}$ − 10.2(MeOH;*c*0.41); IRν_{max}^{neat}cm^{-1} : 3425, 2780, 1702, 1633, 1518; UVλ_{max}^{MeOH}nm (log ε) : 238(4.37), 327(4.51); For ^{1}H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2, respectively; HRESIMS *m/z* 747.2128 $[M+Na]^+$ (Calc. for $C_{33}H_{40}O_{18}Na$, 747.2112).

3.4.4. 1-O-Trans-caffeoyl-2′-O-trans-sinapoyl gentiobiose (4)

Yellow amorphous solid; $[\alpha]_D^{25}$ − 5.4(MeOH;*c*0.35); IRν_{max}^{neat}cm^{-1} : 3423, 2703, 1703, 1631, 1516; UVλ_{max}^{MeOH}nm (log ε) : 240(4.31), 331(4.50); For ^{1}H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2, respectively; HRESIMS *m/z* 733.2011 $[M+Na]^+$ (Calc. for $C_{32}H_{38}O_{18}Na$, 733.1956).

3.4.5. 1,2′-Di-(3″,4″-dihydroxy-5″-methoxy)-O-trans-cinnamoyl gentiobiose (5)

Yellow amorphous solid; $[\alpha]_D^{25}$ − 7.8(MeOH;*c*0.55); IRν_{max}^{neat}cm^{-1} : 3335, 2903, 1706, 1627, 1516; UVλ_{max}^{MeOH}nm (log ε) : 239(4.38), 331(4.46); For ^{1}H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2, respectively; HRESIMS *m/z* 749.1880 $[M+Na]^+$ (Calc. for $C_{32}H_{38}O_{19}Na$, 749.1950).

3.4.6. 1-(3″,4″-Dihydroxy-5″-methoxy)-O-trans-cinnamoyl-2′-O-trans-feruloyl gentibiose (6)

Yellow amorphous solid; $[\alpha]_D^{25}$ − 9.0(MeOH;*c*0.15); IRν_{max}^{neat}cm^{-1} : 3633, 2927, 1704, 1602, 1518; UVλ_{max}^{MeOH}nm (log ε) : 234(4.28), 329(4.41); For ^{1}H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2, respectively; $C_{32}H_{38}O_{18}$ HRESIMS *m/z* 733.1956 $[M+Na]^+$ (Calc. for $C_{32}H_{38}O_{18}$ Na, 733.1956).

3.4.7. 1-(3″,4″-Dihydroxy-5″-methoxy)-O-trans-cinnamoyl gentiobiose (7)

Yellow amorphous solid; $[\alpha]_D^{25}$ − 12.3(MeOH;*c*0.70); IRν_{max}^{neat}cm^{-1} : 3378, 2733, 1703, 1632, 1517; UVλ_{max}^{MeOH}nm (log ε) : 239(4.15), 328(4.30); For ^{1}H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2, respectively; HRESIMS *m/z* 557.1493 $[M+Na]^+$ (Calc. for $C_{22}H_{30}O_{15}Na$, 557.1482).

3.5. Acid hydrolysis of 1–7

A solution of compound **1** (10.0 mg) in 2 M HCl was heated at 100 °C for 2.5 h. The reaction mixture was neutralized by passage through an Amberlite IRA-93ZU column and then subjected to Diaion HP-20 chromatography eluted sequentially with H_2O and MeOH, to give a sugar fraction and an aglycone fraction. The H_2O extract was shown to be D-glucose by HPLC retention time (t_R 14.8 min), and sign (positive) of the optical rotation. Compounds **2**–**7** were also investigated in the same

manner, and their sugar units were all identified as D-glucose by HPLC analysis.

3.6. Superoxide radical anion scavenging activity

3.6.1. Chemicals

Diethylenetriaminepentaacetic acid (DETAPAC) and phosphate buffer powder were purchased from Wako Pure Chemical Industries (Osaka, Japan). The trapping agent, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was purchased from Dojin Chemical (Kumamoto, Japan), and hypoxanthine (HPX) was purchased from Sigma–Aldrich. Xanthine oxidase (XOD) from Roche (IN, USA) was used for active oxygen generation.

3.6.2. General procedure

ESR spectra were recorded on a JEOL JES-FR30. The conditions of the ESR measurements were as follows: magnetic field: 335.5 ± 5 mT; power: 4 mW, 9.4 GHz; sweep time: 1 min; modulation: 100 kHz; mod. wid.: 0.1 mT; amplitude: 160; time constant: 0.1 s; temperature: 293 K.

3.6.3. ESR spin-trapping for measuring $O_2^{\cdot-}$ scavenging activity

The HPX-XOD reaction system was used for the evaluation of the superoxide anion radical scavenging activity. First, 35 μL of 5.5 mmol/L DETAPAC, 15 μL of 9.2 mmol/L DMPO, 50 μL of 2 mmol/L HPX, and 50 μL of each of the compounds were mixed in a test tube. The mixture was transferred to the ESR spectrometry cell and the DMPO-$O_2^{\cdot-}$ spin-adduct was quantified for 45 s after the addition of 50 μL of 0.4 unit/mL XOD from cow's milk. The signal intensities were evaluated in terms of the peak height of the first signal of the DMPO-$O_2^{\cdot-}$ spin-adduct.

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