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Anti-oxidant constituents from *Sedum takesimense*

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Abstract

As part of an ongoing search for antioxidants from medicinal plants, 14 phenolic constitutents were isolated from the Korean endemic species *Sedum takesimense* Nakai. Their structures were determined as 1-(4-hydroxyphenyl)-2-(3,5-dihydroxyphenyl)-2-hydroxyethanone (**5**), gossypetin-8-*O*-β-D-xylopyranoside (**10**), and 2,6-di-*O*-galloylarbutin (**13**) on the basis of spectroscopic analyses (IR, UV, 1D and 2D NMR, HR-MS) and chemical degradation, together with 11 previously known phenolics. Two of those (**10** and **13**) exhibited strong scavenging activities against DPPH and superoxide radicals as well as significant inhibitory effects on lipid peroxidation (IC_{50} 14.0 and 10.8 μM, respectively) and LDL oxidation induced by a metal ion Cu^{2+} (IC_{50} 5.7 and 3.3 μM, respectively).
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Keywords: *Sedum takesimense*; Crassulaceae; Phenolic compound; Anti-oxidant activity

1. Introduction

Sedum takesimense Nakai is an endemic plant, vernacularly known as 'seomkirincho', among 20 *Sedum* species in Korea (Lee, 2006). It is an edible plant common to Ulleung Island, and has light green leaves on thick stems and a yellow flower blooming in the summer. Previously, the phytochemical constituents of *Sedum* species, a large genus of family Crassulaceae, have been extensively reported (Stevens et al., 1996; Kim et al., 1996; Korul'kin, 2001), and some *Sedum* plants have been documented as either vegetables or folk medicines for treatment of many diseases (Bae, 1999). However, there has been no report regarding the chemical constituents of *S. takesimense* and its biological activities so far. In our continuous program to search for potential anti-oxidants from a natural source, a preliminary screening showed that a MeOH extract of this plant displayed significant anti-oxidant effects on scavenging free radicals (DPPH and superoxide) and anti-lipid peroxidation. Accordingly, it is well known that having anti-oxidants rich in diet, such as herbs, vegetables, fruits, and grains can prevent various diseases caused by reactive oxygen species (ROS) (Halliwell, 1994). Hence, the aim of this study was to investigate the phytochemical constituents of this plant and to evaluate their anti-oxidant activities. This paper describes the isolation, structure elucidation and evaluation of the anti-oxidant activities of principles from *S. takesimense*.

2. Results and discussion

The MeOH extract of *S. takesimense* was partitioned with *n*-hexane, EtOAc, *n*-BuOH, successively, to give hexane-, EtOAc- and BuOH-soluble fractions, respectively, and a water fraction. Of the solvent tested, the EtOAc- and BuOH-soluble fractions exhibited significant scavenging activities against free radicals (DPPH and superoxide)

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as well as remarkably inhibitory effects on lipid peroxidation (Table 1). Repeated column chromatography of these fractions resulted in the isolation of 14 compounds (**1**–**14**) including three new ones (**5**, **10**, and **13**).

2.1. Structural elucidation of isolated compounds

Among the isolates, 11 known compounds were identified as ferulic acid (**1**) (Zhang et al., 2006), caffeic acid (**2**) (Lu and Foo, 1997), gallic acid (**3**) (Korul'kina et al., 2004), methyl gallate (**4**) (Redwane et al., 2002), myricetin (**6**) (Korul'kina et al., 2004), quercetin (**7**) and luteolin (**8**) (Miyazawa and Hisama, 2003), rhodalin (**9**) and rhodalidin (**11**) (Kurkin et al., 1984), luteolin-7-*O*-β-D-glucoside (**12**) (Wang et al., 2003), and arbutin (**14**) (Chen et al., 1987; Dommisse et al., 1986), by comparison of their physicochemical and spectroscopic data with those reported in the literature (see Fig. 1).

Compound **5** was obtained as an amorphous powder and showed an m.p. 206–208 °C. Its IR spectrum indicated the presence of hydroxyl groups and a carbonyl group at 3350 and 1660 cm^{-1}, respectively. The ^1H NMR spectrum of **5** showed the signals of aromatic protons at δ 6.34 (2H, *d*, $J = 2.4$ Hz, H-2, 6), and 6.17 (1H, *t*, $J = 2.1$ Hz, H-4), characteristic of an 1,3,5-trisubstituted aromatic ring, along with two pairs of aromatic protons at δ 7.86 (2H, *d*, $J = 9.0$ Hz, H-2′, 6′) and 6.77 (2H, *d*, $J = 9.0$ Hz, H-3′, 5′), indicative of an 1,4-disubstituted aromatic ring. The ^{13}C NMR spectrum of **5** supported these benzene ring systems, a 1,3,5-trisubstituted aromatic ring [δ_C 143.2 (C-1), 107.4 (C-2, 6), 160.1 (C-3, 5) and 103.5 (C-4)], and a 1,4-disubstituted aromatic ring [δ_C 127.5 (C-1′), 132.8 (C-2′, 6′), 116.3 (C-3′, 5′), and 164.1 (C-4′)], and additionally showed oxygenated (δ_C 77.0) and carbonyl (δ_C 198.9) signals. Most of the spectroscopic data of **5** were similar to those of belamphenone (Monthakantirat et al., 2005). However, the observation of an oxymethine proton at δ 5.80 (1H, s, H-α) and a carbon at δ 77.0 suggested that the α-position of **5** was substituted with a hydroxyl group. This was further supported by long-range HMBC correlations (Fig. 2) between H-2, 6 (δ 6.34) and C-α (δ 77.0), and between H-2′, 6′ (δ 7.86) and the conjugated carbonyl carbon C-β (δ 198.9), together with the molecular formula

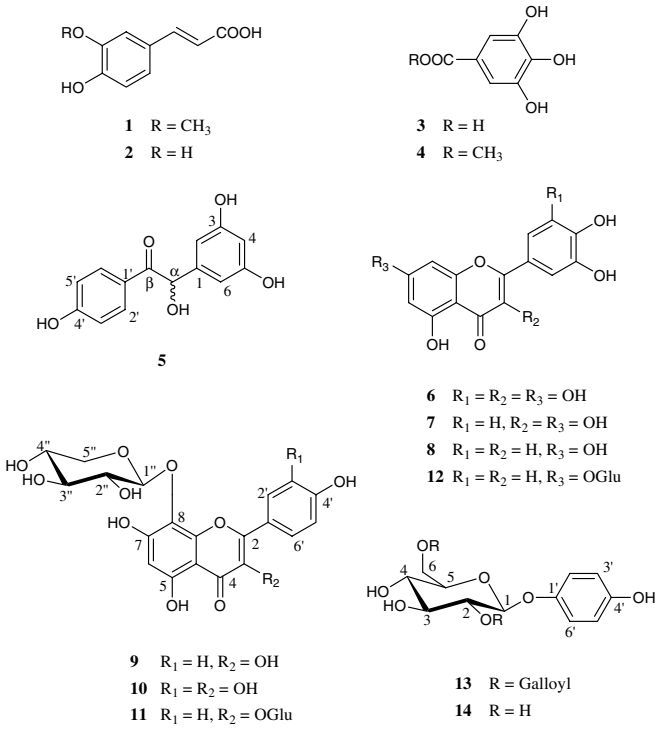

Fig. 1. Chemical structures of isolated compounds from *S. takesimense*.

Table 1
Anti-oxidant activities of fractions and compounds isolated from *Sedum takesimense*

Sample	Radical scavenging activities			Inhibition of TBARS	
	DPPH (IC_{50})[a]	$O_2^{\cdot-}$ (IC_{50})	$NO^{\cdot}$ (% inhibition)[b]	Mitochondrial (IC_{50})	LDL (IC_{50})
MeOH ex.	12.7 ± 2.1	2.2 ± 0.3	ND[c]	59.6 ± 4.7	ND
Hexane fr.	>100	>100	ND	>100	ND
EtOAc fr.	4.6 ± 0.3	0.9 ± 0.2	ND	34.3 ± 7.3	ND
BuOH fr.	5.2 ± 0.3	0.7 ± 0.2	ND	46.2 ± 5.9	ND
5	>100	>100	31.0 ± 10.5	>100	>100
9	>100	17.7 ± 0.8	39.0 ± 13.6	>100	ND
10	35.3 ± 4.1	5.5 ± 0.5	41.7 ± 0.2	14.0 ± 2.6	5.7 ± 1.0
11	>100	40.1 ± 6.6	43.4 ± 10.8	>100	ND
13	3.6 ± 0.2	14.0 ± 1.2	0	10.8 ± 1.7	3.3 ± 0.5
14	>100	>100	0	>100	>100
Caffeic acid	35.6 ± 3.3	45.6 ± 6.1	50.1 ± 3.7	23.1 ± 4.0	9.3 ± 1.4
Gallic acid	26.5 ± 1.6	88.3 ± 9.2	10.6 ± 4.6	>100	36.5 ± 6.1
Resveratrol[d]	>100	>100	ND	11.2 ± 1.7	6.2 ± 1.1
BHT[d]	50.8 ± 3.6	120.8 ± 7.5	ND	3.6 ± 0.6	4.2 ± 0.7

[a] IC_{50} values express the concentration in μg/ml for solvent fractions and μM for pure compounds. Values are mean ± SD of three separated experiments.
[b] Test compounds were treated at 250 μM as the final concentration.
[c] ND: not determined.
[d] Reference compounds.

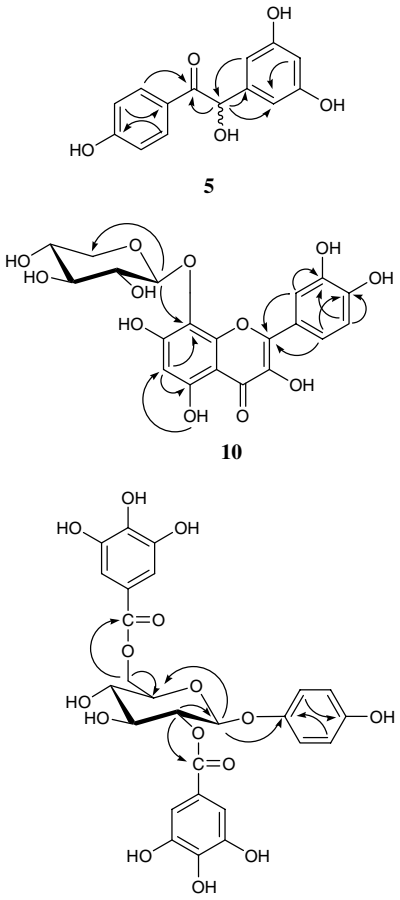

Fig. 2. Key HMBC correlations (H → C) for new compounds **5**, **11**, and **13**.

$C_{14}H_{12}O_5$ derived from the molecular ion $[M+H]^+$ peak at *m/z* 261.0765 in HRFAB-MS. Hence, the structure of **5** was elucidated as 1-(4-hydroxyphenyl)-2-(3,5-dihydroxyphenyl)-2-hydroxyethanone. This is the first isolation of this compound from a natural source.

Compound **10** was obtained as a yellowish powder with an m.p. 325–327 °C and gave a positive ferric chloride reaction. The IR spectrum of **10** showed the presence of hydroxyl and carbonyl groups at 3300 and 1660 cm^{-1}, respectively, and the UV spectrum displayed two maximum bands at 260 and 381 nm, characteristic of a flavon-3-ol. In comparison, the ^1H and ^{13}C NMR spectroscopic patterns of **10** and **9** were similar, each having a flavonoid aglycone and a sugar moiety. The significant differences in the ^1H NMR data of **10**, as compared to **9**, were the patterns of substitution in the B-ring, in which proton ABX-type signals were observed at δ 8.00 (1H, *d*, $J = 2.4$ Hz), 7.88 (1H, *dd*, $J = 2.4$, 8.8 Hz), and 6.88 (1H, *d*, $J = 8.8$ Hz), indicative of H-2′, H-6′, and H-5′, respectively. Except for a sugar unit, most of the ^1H and ^{13}C NMR spectroscopic data matched those of gossypetin-8-*O*-α-D-xylopyranoside isolated from *Orostachys japonicus* (Sung et al., 2002). Similar to **9**, acid hydrolysis of **10** yielded β-D-xylose that was confirmed by GC–MS analysis using an authentic sample, and by a coupling constant $J = 7.6$ Hz of the proton H-1″ (δ 4.70). The molecular formula $C_{20}H_{18}O_{12}$ of **10**, determined by a quasimolecular ion $[M+Na]^+$ peak at *m/z* 473.0695 in the HRFAB-MS, supported this. The linkage of the β-D-xylose to the C-8 of the aglycone was determined by the HMBC correlation (Fig. 2) from the anomeric proton at δ 4.70 (H-1″) to C-8 (δ 124.4). Therefore, **10** was elucidated as a new compound, gossypetin-8-*O*-β-D-xylopyranoside.

Compound **13** was isolated as a white amorphous powder with a negative optical rotation. Its IR spectrum presented the absorbance of hydroxyl and carbonyl groups at 3300 and 1690 cm^{-1}, respectively. Interpretation of the ^1H and ^{13}C NMR spectroscopic data for **13** established the existence of an A_2B_2-type benzene ring [δ_H 6.77 (2H, *d*, $J = 8.8$ Hz, H-2′, 6′) and 6.56 (2H, *d*, $J = 8.8$ Hz, H-3′, 5′); δ_C 119.8 (C-2′, 6′), and 116.7 (C-3′, 5′)], along with a sugar moiety [δ_H 4.95 (1H, *d*, $J = 8.4$ Hz, H-1), 4.62 (1H, *dd*, $J = 2.0$, 12.0 Hz, H-6a), 4.48 (1H, *dd*, $J = 6.4$, 12.0 Hz, H-6b), 3.79 (1H, *m*, H-5), 3.75 (1H, *t*, $J = 9.6$ Hz, H-3), 3.59 (1H, *t*, $J = 9.6$ Hz, H-4); δ_C 102.6 (C-1), 75.7 (C-2), 76.1 (C-3), 71.9 (C-4), 75.3 (C-5), 64.7 (C-6)]. In addition, acid hydrolysis of this compound yielded β-D-glucose, which suggested that **13** was a derivative of arbutin (Chen et al., 1987). Two singlet protons at δ 7.14 and 7.11, together with carbon signals at δ 168.2 and 167.6 suggested two galloyl moieties in the structure, which were closely related to those of 4,6-di-*O*-galloylarbutin (Chen et al., 1987). The molecular formula of **13** assigned as $C_{26}H_{24}O_{15}$ from the quasimolecular ion $[M+Na]^+$ peak at *m/z* 599.1021 in HRFAB-MS indicated a digalloyl derivative of arbutin. The position of the two galloyl groups was established by analyses of the HMBC data (Fig. 2); thus, the structure of **13** was elucidated as 2,6-di-*O*-galloylarbutin. Although arbutin and 6-*O*-galloylarbutin have been reported as major compounds in the Crassulaceae plant *Rhodiola coccinea* (Krasnov et al., 1975), as well as galloyltannins have been also documented (Britton and Haslam, 1965; Chen et al., 1987), this is the first isolation of **13** from a natural source.

2.2. Anti-oxidant properties of isolates

Among the isolates, ferulic acid (**1**), caffeic acid (**2**), gallic acid (**3**), methyl gallate (**4**), myricetin (**6**), quercetin (**7**), luteolin (**8**), and luteolin-7-*O*-β-D-glucoside (**12**) are widely distributed compounds in the plant kingdom, and extensively known to have anti-oxidant activity. In this study, we evaluated the anti-oxidant properties of six phenolics, including 1-(4-hydroxyphenyl)-2-(3,5-dihydroxyphenyl)-2-hydroxyethanone (**5**), rhodalin (**9**), gossypetin-8-*O*-β-D-xylopyranoside (**10**), rhodalidin (**11**), 2,6-di-*O*-galloylarbutin (**13**), and arbutin (**14**). As presented in Table 1, two new compounds **10** and **13** were able to efficiently scavenge both the DPPH and superoxide radicals as well as inhibit lipid peroxidation. Consistent with this, these compounds also exhibited significant inhibitory effects on LDL oxidation using a TBARS assay (Table 1); in particular, the activity

of **13** was much higher than BHT in the REM analysis (Fig. 3b), while **10** was similar to the positive control (Fig. 3a). For structure–activity relationships, the anti-lipid peroxidation ability of **5** was much less than that of resveratrol, suggesting that a modification of the double bond reduced the activity of **5**. It was clearly demonstrated that the anti-oxidant activity of the flavonol glycoside **10** was significantly stronger than that of **9** and **11** due to the *ortho*-hydroxyl group in the B-ring (Hou et al., 2004a,b). Interestingly, 2,6-di-*O*-galloylarbutin (**13**) displayed remarkably free radical quenching capacities as well as inhibitory effects on both lipid peroxidation and LDL oxidation, as compared to either gallic acid or arbutin (**14**). This indicated that the addition of galloyl groups significantly increased the anti-oxidant activity of compound **13**.

Studies have provided strong evidence that the generation of free radicals ($OH^{\bullet}$, $O_2^{\bullet-}$, $NO^{\bullet}$, $LOO^{\bullet}$, $LO^{\bullet}$,...) in biological systems may initiate oxidative stress, such as lipid peroxidation, LDL oxidation, protein degeneration, and DNA alteration (Halliwell and Gutteridge, 2000; Vendemiale et al., 1999). These lead to biochemical damage of cells, and are therefore associated with pathogenesis of many diseases, for example, atherosclerosis, cancer, inflammation, neurodegenerative diseases, and aging (Halliwell and Gutteridge, 2000). Fortunately, the oxidative damage can be reduced or prevented by anti-oxidants, characterized as compounds that either decrease the formation of free radicals or react with to neutralize them (Sies, 1991; Halliwell and Gutteridge, 2000). It has been reported that plant polyphenols, because of their anti-oxidant properties, are of great interest in the development of therapeutic agents that have beneficial effects on oxidative stress-related diseases (Sies, 1991; Duthie and Crozier, 2000).

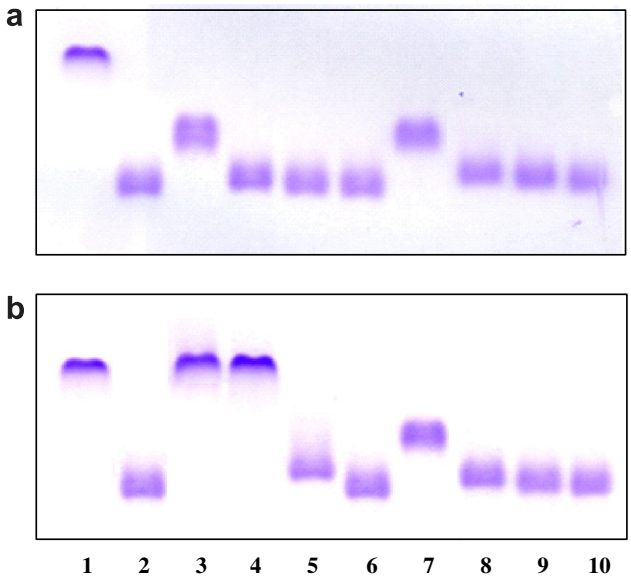

Fig. 3. Effects of compounds **10** (a) and **13** (b) on changes in REM of LDL. Lane 1: native LDL; lane 2: oxidized LDL; lane 3: test compound, 10 μM, lane 4: test compound, 5 μM; lane 5: test compound, 3 μM; lane 6: test compound, 1 μM; lane 7: BHT, 10 μM; lane 8: BHT, 5 μM; lane 9: BHT, 3 μM; and lane 10: BHT, 1 μM.

2.3. Concluding remarks

In this study, *S. takesimense* was demonstrated to contain many phenolic compounds. Moreover, we also indicated that this edible plant and its constituents possess significant anti-oxidant activity. The finding therefore suggests that *S. takesimense* provides a source of natural anti-oxidants and can be used for the treatment of oxidative stress-related diseases.

3. Experimental

3.1. General experimental procedure

Melting points were determined using a Kofler micro-hotstage. Optical rotations were measured on a JASCO DIP-370 polarimeter. IR spectra (KBr) were obtained on a Bruker spectrometer. FAB-MS and HR-FAB-MS (high resolution FAB-MS) were registered using a JEOL JMS-DX 300 spectrometer. NMR spectra including ^{1}H and ^{13}C NMR, HMBC (heteronuclear multiplebond connectivity), and HMQC (^{1}H-detected heteronuclear multiplequantum coherence), were recorded on Bruker DRX-300 or DRX-600 NMR spectrometers. Analytical TLC was performed on pre-coated silica gel 60 F_{254} plates (Merck) or RP-18 F_{254} (Merck). For column chromatography, silica gel (Kieselgel 60, Merck), Sephadex LH-20 (Amersham Biosciences), and ODS (Merck) were used. Medium pressure liquid chromatography (MPLC) was applied on a Shimazu MPLC system. GC–MS was performed on an Agilent 6890N with 5973N MSD detector and ultra-2 capillary column (25 m × 0.32 mm) to identify sugars.

3.2. Plant material

The whole plant of *Sedum takesimense* Nakai was collected in June, 2004 at Ulleung Island, Kyungbook Province, Korea, and properly identified by Prof. KiHwan Bae. A voucher specimen has been deposited at the herbarium of College of Pharmacy, Chungnam National University, Daejeon, Korea.

3.3. Extraction, fractionation, and isolation

A suspension of fresh whole plant tissue of *S. takesimense* (2.5 kg) in MeOH (20 l) was heated until reflux began, this being maintained for 4 h. The MeOH extraction procedure was then repeated (3×), with the MeOH extracts combined and evaporated under reduced pressure to give a dry residue (460 g). The residue was suspended into H_2O (2 l) to give a suspension that was partitioned consecutively with *n*-hexane, EtOAc, and *n*-BuOH (each 2 l), then exhaustively concentrated to yield a hexane fraction

(81 g), an EtOAc fraction (103 g), a BuOH fraction (132 g), and a H_2O fraction (144 g), respectively.

The EtOAc fraction was subjected to silica gel CC (10.5 × 20 cm) eluted with *n*-hexane–EtOAc (5:1, 4:1, 3:1, 2:1, 1:1, and 0:1, each 10 l) to give six fractions (E1: 5.3 g, E2: 10.5 g, E3: 7.6 g, E4: 20 g, E5: 20 g, and E6: 36 g). Fraction E3 was applied to a silica gel column (5.5 × 20 cm) eluted with a gradient of *n*-hexane–EtOAc (3:1, 2:1, and 1:1, each 5 l) to yield six subfractions (E3.1–E3.6). Subfraction E3.2 was purified on a silica gel column (4 × 30 cm), and eluted with *n*-hexane–EtOAc (2:1) to give compound **1** (123 mg) and compound **2** (281 mg). Subfraction E3.3 was purified by preparative MPLC [ODS column (2.6 × 30 cm), mobile phase MeOH–H_2O (1:1), UV detection at 254 nm, flow rate 4 ml/min)] to give compound **3** (2.0 g, $t_R = 29$ min) and compound **4** (1.1 g, $t_R = 55$ min). Fraction E4 was applied to a Sephadex LH-20 column (5.5 × 20 cm) eluted with MeOH and separated into seven fractions (E4.1–E4.7), with fraction E4.6 purified on a silica gel column (3 × 25 cm) eluted with EtOAc–MeOH (10:1) to give compound **5** (98 mg). Fraction E5 was subjected to Sephadex LH-20 CC using MeOH as eluent to give seven fractions (E5.1–E5.7). Selected fractions E5.5 (MeOH, 3–3.5 l) were purified by preparative MPLC [ODS column (2.6 × 30 cm), mobile phase MeOH–H_2O (1:1), UV detection at 254 nm, flow rate 4 ml/min] to give compounds **6** (21 mg, $t_R = 82$ min), **7** (300 mg, $t_R = 116$ min), and **8** (11 mg, $t_R = 129$ min).

The BuOH fraction (132 g) was applied to a dianion column (10 × 40 cm) and eluted with H_2O–MeOH (1:0, 5:1, 4:1, 3:1, 2:1, 1:1, and 0:1, each 3 l) to afford a selected fraction [26 g, H_2O–MeOH (1:1 to 0:1)]. This fraction was subjected to Sephadex LH-20 CC eluting with MeOH to give six fractions (B1: 6.3 g, B2: 3.5 g, B3: 2.7 g, B4: 5.6 g, B5: 2.0 g, and B6: 3.8 g). Fraction B3 was purified by preparative MPLC [ODS column (2.6 × 30 cm), mobile phase MeOH–H_2O (1:1.5), UV detection at 254 nm, flow rate 4 ml/min] to give compound **9** (85 mg, $t_R = 65$ min). Fraction B4 was purified by preparative MPLC under the same conditions to yield compounds **10** (49 mg, $t_R = 77$ min), **11** (136 mg, $t_R = 103$ min), and **12** (12 mg, $t_R = 120$ min). Finally, purification of fraction B5 using preparative MPLC [ODS column (2.6 × 30 cm), mobile phase MeOH–H_2O (1:1), UV detection at 254 nm, flow rate 4 ml/min] afforded compounds **13** (180 mg, $t_R = 17$ min) and **14** (120 mg, $t_R = 29$ min).

3.3.1. 1-(4-Hydroxyphenyl)-2-(3,5-dihydroxyphenyl)-2-hydroxyethanone (5)

Brown amorphous powder; $FeCl_3$ test: positive; m.p. 206–208 °C; $[\alpha]_D^{22}$ −143.0 (*c* 0.8, MeOH); UV (MeOH) λ_{max} nm (log ε): 218 (4.34), 274 (4.29); IR (KBr) ν_{max} cm^{-1}: 3350 (OH), 1660 (C=O), 1580, 1520 (C=C), 1460; ^1H NMR (300 MHz, CD_3OD) δ: 7.86 (2H, *d*, *J* = 9.0 Hz, H-2′, 6′), 6.77 (2H, *d*, *J* = 9.0 Hz, H-3′, 5′), 6.34 (2H, *d*, *J* = 2.4 Hz, H-2, 6), 6.17 (1H, *t*, *J* = 2.1 Hz, H-4), 5.80 (1H, *s*, H-α); ^{13}C NMR (75 MHz, CD_3OD) δ: 198.9 (C-β), 164.1 (C-4′), 160.1 (C-3, 5), 143.2 (C-1), 132.8 (C-2′, 6′), 127.5 (C-1′), 116.3 (C-3′, 5′), 107.4 (C-2, 6), 103.5 (C-4), 77.0 (C-α); HRFAB-MS *m/z* 261.0765 $[M+H]^+$ (calcd for $C_{14}H_{13}O_5$, 261.0763).

3.3.2. Herbacetin-8-O-β-D-xylopyranoside (9)

Yellowish amorphous powder; $FeCl_3$ test: positive; m.p. 312–314 °C; $[\alpha]_D^{22}$ −16.1 (*c* 0.5, MeOH); UV (MeOH) λ_{max} nm (log ε): 273 (4.11), 375 (4.01); IR (KBr) ν_{max}, cm^{-1}: 3300 (OH), 1650 (C=O), 1610, 1560, 1510 (C=C), 1180, 1070; ^1H NMR (300 MHz, DMSO-d_6) δ: 12.27 (1H, *s*, 5-OH); 8.27 (2H, *d*, *J* = 9.0 Hz, H-2′, 6′), 6.91 (2H, *d*, *J* = 9.0 Hz, H-3′, 5′), 6.26 (1H, *s*, H-6), 4.71 (1H, *d*, *J* = 7.5 Hz, H-1″), 3.78 (1H, *dd*, *J* = 5.1, 11.4 Hz, H-5″), 3.42 (2H, m, H-2″, 4″), 3.24 (1H, *t*, *J* = 11.2 Hz, H-3″), 3.11 (1H, *t*, *J* = 11.1 Hz, H-5″); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 176.0 (C-4), 159.3 (C-4′), 156.4 (C-5), 156.2 (C-7), 148.3 (C-2), 146.8 (C-9), 135.7 (C-3), 129.9 (C-2′, 6′), 124.3 (C-8), 121.8 (C-1′), 115.4 (C-3′, 5′), 105.9 (C-1″), 102.8 (C-10), 98.3 (C-6), 75.9 (C-3″), 73.7 (C-2″), 69.4 (C-4″), 66.1 (C-5″); FAB-MS *m/z* 435 $[M+H]^+$.

3.3.3. Gossypetin-8-O-β-D-xylopyranoside (10)

Yellowish amorphous powder; $FeCl_3$ test: positive; m.p. 325–327 °C; $[\alpha]_D^{22}$ −15.7 (*c* 0.2, MeOH); UV (MeOH) λ_{max} nm (log ε): 260 (4.46), 381 (4.34); IR (KBr) ν_{max} cm^{-1}: 3300 (OH), 1660 (C=O), 1620, 1560, 1520 (C=C), 1170, 1080; ^1H NMR (400 MHz, DMSO) δ: 12.74 (1H, *s*, 5-OH), 8.00 (1H, *d*, *J* = 2.4 Hz, H-2′), 7.88 (1H, *dd*, *J* = 2.4, 8.8 Hz, H-6′), 6.88 (1H, *d*, *J* = 8.8 Hz, H-5′), 6.24 (1H, *s*, H-6), 4.70 (1H, *d*, *J* = 7.6 Hz, H-1″), 4.01 (1H, *dd*, *J* = 5.6, 11.6 Hz, H-5″), 3.65 (1H, *m*, H-4″), 3.60 (1H, *dd*, *J* = 7.2, 8.4 Hz, H-2″), 3.43 (1H, *t*, *J* = 8.8 Hz, H-3″), 3.23 (1H, *dd*, *J* = 5.6, 12.0 Hz, H-5″); ^{13}C NMR (100 MHz, DMSO) δ: 177.5 (C-4), 158.5 (C-5, C-7), 150.3 (C-2), 149.0 (C-4′), 148.4 (C-9), 146.2 (C-3′), 137.4 (C-3), 126.4 (C-8), 124.4 (C-1′), 122.4 (C-6′), 117.0 (C-5′), 116.3 (C-2′), 108.3 (C-1″), 104.5 (C-10), 99.5 (C-6), 77.6 (C-3″), 75.4 (C-2″), 71.1 (C-4″), 67.5 (C-5″); HRFAB-MS *m/z* 473.0695 $[M+Na]^+$ (calcd for $C_{20}H_{18}O_{12}Na$, 473.0696).

3.3.4. Herbacetin-3-O-β-D-glucopyranosyl-8-O-β-D-xylopyranoside (11)

Yellowish amorphous powder; $FeCl_3$ test: positive; m.p. 247–249 °C; $[\alpha]_D^{22}$ −52.6 (*c* 0.3, MeOH); UV (MeOH) λ_{max} nm (log ε): 261 (4.40), 381 (4.28); IR (KBr) ν_{max}, cm^{-1}: 3300 (OH), 1660 (C=O), 1600, 1560, 1510 (C=C), 1165, 1070; ^1H NMR (300 MHz, DMSO-d_6) δ: 12.40 (1H, *s*, 5-OH), 8.25 (2H, *d*, *J* = 9.0, H-2′, 6′), 6.87 (2H, *d*, *J* = 9.0, H-3′, 5′), 6.29 (1H, *s*, H-6), 5.46 (1H, *d*, *J* = 7.8, H-1‴), 4.71 (1H, *d*, *J* = 7.2, H-1″); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 177.6 (C-4), 160.2 (C-4′), 157.0 (C-2), 156.7 (C-5), 156.2 (C-7), 148.5 (C-9), 133.2 (C-3), 131.3 (C-2′, 6′), 124.4 (C-8), 121.0 (C-1′), 115.1 (C-3′, 5′), 105.9 (C-1″), 103.9 (C-10), 101.1 (C-1‴), 98.9 (C-6), 77.6 (C-5‴), 76.5 (C-3‴), 75.8 (C-3″), 74.2 (C-2″), 73.7 (C-2‴), 69.9 (C-4‴),

69.4 (C-4″), 66.1 (C-5″), 60.9 (C-6‴); FAB-MS *m/z* 619 $[M+Na]^+$.

3.3.5. 2,6-Di-O-galloylarbutin (*13*)

White amorphous powder; $FeCl_3$ test: positive; $[\alpha]_D^{24}$ −17.2 (*c* 1.0, MeOH); UV (MeOH) λ_{max}, nm (log ε): 220 (4.79), 279 (4.41); IR (KBr) ν_{max}, cm^{-1}: 3300 (OH), 1690 (C=O), 1610, 1540, 1500 (C=C), 1080; ^1H NMR (400 MHz, CD_3OD) δ: 7.14 and 7.11 (each 2H, *s*, galloyl H), 6.77 (2H, *d*, *J* = 8.8 Hz, H-2′, 6′), 6.56 (2H, *d*, *J* = 8.8 Hz, H-3′, 5′), 5.14 (1H, *dd*, *J* = 8.0, 9.2 Hz, H-2), 4.95 (1H, *d*, *J* = 8.4 Hz, H-1), 4.62 (1H, *dd*, *J* = 2.0, 12.0 Hz, H-6a), 4.48 (1H, *dd*, *J* = 6.4, 12.0 Hz, H-6b), 3.79 (1H, *m*, H-5), 3.75 (1H, *t*, *J* = 9.6 Hz, H-3), 3.59 (1H, *t*, *J* = 9.6 Hz, H-4); ^{13}C NMR (100 MHz, CD_3OD) δ: 168.2 and 167.6 (OCO), 154.2 (C-4′), 152.2 (C-1′), 146.5 (C-3″, 5″), 139.9 (C-4″), 121.5 and 121.4 (C-1″), 119.8 (C-2′, 6′), 116.7 (C-3′, 5′), 110.3 and 110.2 (C-2″, 6″), 102.6 (C-1), 76.1 (C-3), 75.7 (C-2), 75.3 (C-5), 71.9 (C-4), 64.7 (C-6); HRFAB-MS *m/z* 599.1021 $[M+Na]^+$ (calcd for $C_{26}H_{24}O_{15}Na$, 599.1013).

3.4. Acid hydrolysis of compounds (*9*, *10*, *11*, and *13*)

Compounds **9–11** and **13** (5 mg each) 10% HCl–dioxane (2 ml, 1:1, v/v) was heated at 100 °C for 2 h in a water bath. After neutralization by adding 10% NH_4OH, the reaction solution was filtered and then extracted twice with EtOAc. The aqueous fraction was subjected to GC–MS analysis with sugar standards.

3.5. Identification of sugars

For GC–MS analyses, 20 μl of two silylating agents (hexamethyldisilazane and chlorotrimethylsilane) and 20 μl of aqueous fraction were vortexed and allowed to stand for 30 min at 60 °C. This solution was then subjected to GC analysis using He as a carrier gas to identify the sugars. GC conditions were as follows: the column temperature was initiated at 150 °C for 1 min and then increased by the rate of 5°C/min, injector temperature of 200 °C, detector temperature of 280 °C, and a flow rate of 10 ml/min. Sugars were identified by comparison of their retention times with those of standard samples: t_R (min) 7.37 for D-xylose and 10.85 for D-glucose.

3.6. Free radical scavenging assays

3.6.1. DPPH radical scavenging activity

DPPH radical scavenging activity was determined by the described method (Hung et al., 2006). Briefly, each MeOH extract (5 μl) was added to 150 μM DPPH (195 μl) solution in 96 well plates. The resulting solution was mixed for 1 min and incubated at room temperature for 30 min. Then the absorbance of the reaction mixture was measured at 520 nm on a microplate reader.

3.6.2. Superoxide radical scavenging activity

Superoxide radical scavenging activity was assayed by NBT reduction method (Valentao et al., 2002) with some modification. The 495 μl assay mixture consisted of 50 mM sodium carbonate buffer (pH 7.8), 50 μM xanthine, 50 μM nitro blue tetrazolium (NBT), and 0.1 mM EDTA in the presence or absence of tested compound. The reaction was initiated by addition of 5 μl xanthine oxidase. The increase in absorbance at 560 nm was read after 5 min on a spectrophotometer.

3.6.3. $NO^{\cdot}$ radical scavenging activity

$NO^{\cdot}$ scavenging activity of isolated compounds was determined by the method of Sreejayan and Rao (1997). In brief, test sample was added to sodium nitroprusside (5 mM) in phosphate-buffered saline (pH 7.4) then incubated under light at 25 °C for 150 min. The reaction solution was added an equal volume of Griess reagent and further incubated for 10 min at room temperature. The amount of $NO^{\cdot}$ produced was assayed by a spectrophotometric method measuring at 520 nm.

3.7. Anti-lipid peroxidation assay

3.7.1. Preparation of mitochondria

Mitochondria were prepared from liver of male Sprague–Dawley rats by the method of Ham and Liebler with slight modification (Ham and Liebler, 1995; Wei et al., 2006). Rat livers were removed and washed with 0.9% of ice-cold NaCl solution. The livers were homogenated in 9-fold volumes of ice-cold buffer (0.25 M sucrose in 5 mM phosphate buffer, pH 7.4) and centrifuged at 10,000 rpm at 4 °C for 15 min. The mitochondria pellets were washed three times and finally suspended in the 0.15 M KCl in 20 mM phosphate buffer (pH 7.4) and stored at −70 °C until needed. The protein concentration was determined by BCA method using BSA as standard (Brenner and Harris, 1995).

3.7.2. Anti-lipid peroxidation activity

The inhibitory effect on lipid peroxidation was measured spectroscopically by the TBARS method (Wei et al., 2006) with some modification. In brief, a reaction mixture containing 390 μl of 20 mM K_2HPO_4–HCl buffer (pH 7.4), 50 μl of liver mitochondria (3 mg protein/1 ml, final concentration 0.3 mg protein/1 ml), 10 μl of test compounds in MeOH, and 25 μl of 2.5 mM $FeSO_4$/25 μl of 2.5 mM ascorbic acid. The reaction mixtures were incubated at 37 °C for 1 h, and then 250 μl of 20% TCA was added to stop oxidation. After adding 250 μl of 1% TBA, the mixture was heated at 95–100 °C for 15 min, cooled and centrifuged at 5000 rpm for 10 min. The absorbance of supernatant was measured at 532 nm against a blank (without $FeSO_4$ and ascorbic acid).

3.8. Inhibitory effect on low density lipoprotein (LDL) oxidation assay

3.8.1. LDL preparation

Human LDL was prepared from healthy and normalipidemic volunteers' blood by preparative ultracentrifugation as previously described (Hung et al., 2006).

3.8.2. Cu^{2+}-induced LDL oxidation

Human LDL in PBS (pH 7.4) was incubated with 5 μM $CuSO_4$ at 37 °C in the presence or absence of test compounds. After incubation, the reaction was stopped by addition of 1 mM EDTA and 500 μM BHT. The extent of LDL oxidation was assayed by the thiobarbituric acid reactive substances (TBARS) (Hou et al., 2004a; Hung et al., 2006) and relative electrophoretic mobility (REM) methods (Lee et al., 2005).

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