FULL PAPER

Isocassiaoccidentalin B, A New *C*-Glycosyl Flavone Containing a 3-Keto Sugar, and Other Constituents from *Cassia nomame*

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A new *C*-glycosyl flavone containing a 3-keto sugar, isocassiaoccidentalin B (**1**), was isolated from whole *Cassia nomame* (Sieber) Honda plants, along with eleven known compounds, including two flavonoids (**2** and **3**), five flavonoid glycosides (**4** – **8**), two chalcones (**9** and **10**), and two phenylpropanoids (**11** and **12**). The chemical structures of all compounds were determined *via* 1D- and 2D-NMR, and ESI-MS. Among these, compounds **2**, **3**, **7**, and **8** were found to be the most potent in inhibiting nitric oxide release. Compounds **1**, **2**, **4**, **8**, **9**, and **10** showed significant free-radical scavenging activity.

Keywords: *Cassia nomame*, Flavone *C*-glycosides, Isocassiaoccidentalin B, Nitric oxide production, Anti-inflammatory activities.

Introduction

Cassia nomame (Sieber) Honda (*Cassia mimosoides* var. *nomame* (Siebold) Makino) is an annual plant in the legume family, which grows naturally in Korea, China, and Japan. The aerial parts of *C. nomame* are used as a diuretic, antidote, and tonic in folk medicine [1 – 3]. In previous studies, anthraquinones were isolated from the seeds and aerial sections of *C. nomame* [4], and this plant has also been found to contain flavan dimers [5] and other flavonoids, including luteolin, luteolin 7-glucoside, vitexin, 7,3′,4′-trihydroxyflavanone, and demethyltorosaflavones C and D [3]. Flavan dimers isolated from *C. nomame* exhibited lipase-inhibitory activities *in vitro* [5] and antiobesity effects in a high-fat diet model *via* lipase inhibition [6]. In addition, crude extracts of *C. nomame* attenuated brain damage by inhibiting apoptosis in a rat ischemic reperfusion model [2].

With the aim of searching bioactive constituents, crude extract of *C. nomame* was examined to identify bioactive constituents, and a new flavone *C*-glycoside containing a keto sugar, herein named isocassiaoccidentalin B (**1**), was isolated, along with 11 known compounds (*Fig. 1*). Flavonoid glycosides containing a deoxy-ketose have rarely been found in plants, and are usually *C*-glycosylflavones containing a 3-keto or 4-keto sugar [7]. For all compounds isolated, we determined their chemical structures as well as their anti-inflammatory activities on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 cells. In addition, free-radical scavenging activities were also tested using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays.

Results and Discussion

The AcOEt-soluble extract of *C. nomame* was subjected to repeated SiO_2, *Sephadex L-20*, and *C-18* reverse-phase column chromatography to isolate a new *C*-glycosyl flavone with a 3-keto sugar, isocassiaoccidentalin B (**1**), along with 11 known compounds (**2** – **12**). The chemical structures of the known compounds were determined by comparing their spectroscopic data (ESI-MS and NMR) with data already reported in the literature. These compounds were determined to be luteolin (**2**) [8], 7,3′,4′-trihydroxyflavanone (= butin) (**3**) [9], cassiaoccidentalin B (**4**), cassiaoccidentalin A (**5**) [10], tetrastigma B (**6**) [11], butin-7-*O*-*β*-D-glucoside (**7**) [9], luteolin 7-*O*-*β*-D-glucoside (**8**) [12], butein (**9**) [13], butein-4′-*O*-*β*-D-glucopyranoside (**10**) [9], *p*-coumaric acid (**11**), and ferulic acid (**12**) [14]. Among these known compounds, **7** and **9** were first identified from the genus *Cassia*, and **4** – **6** and **10** – **12** were initially isolated from *C. nomame*.

Compound **1** was obtained as light brown needles, and in HR-QTOF-MS, it exhibited a deprotonated molecular ion peak at an *m*/*z* of 575.1407 ($[M - H]^-$), corresponding to a molecular formula of $C_{27}H_{28}O_{14}$. The UV absorption maxima bands at 258 nm and 350 nm were attributed to a flavone skeleton. In the ^{1}H-NMR spectrum, signals at δ(H) 7.51 (*d*, *J* = 2.3, H–C(2′)), 7.50 (*dd*, *J* = 8.5, 2.3, H–C(6′)), and 6.92 (*d*, *J* = 8.5, H–C(5′)) indicated the presence of *o*-substituted OH groups on the *B* ring. This observation was confirmed by ^{13}C-NMR signals at δ (C) 122.08 (C(1′)), 113.21 (C(2′)), 145.13 (C(3′)), 150.02 (C(4′)), 115.94 (C(5′)), and 119.67 (C(6′)), which were in good agreement with a luteolin residue [10]. H-atom signals at δ(H) 6.26 (*s*, H–C(6)) on the *A* ring and 6.75 (*s*,

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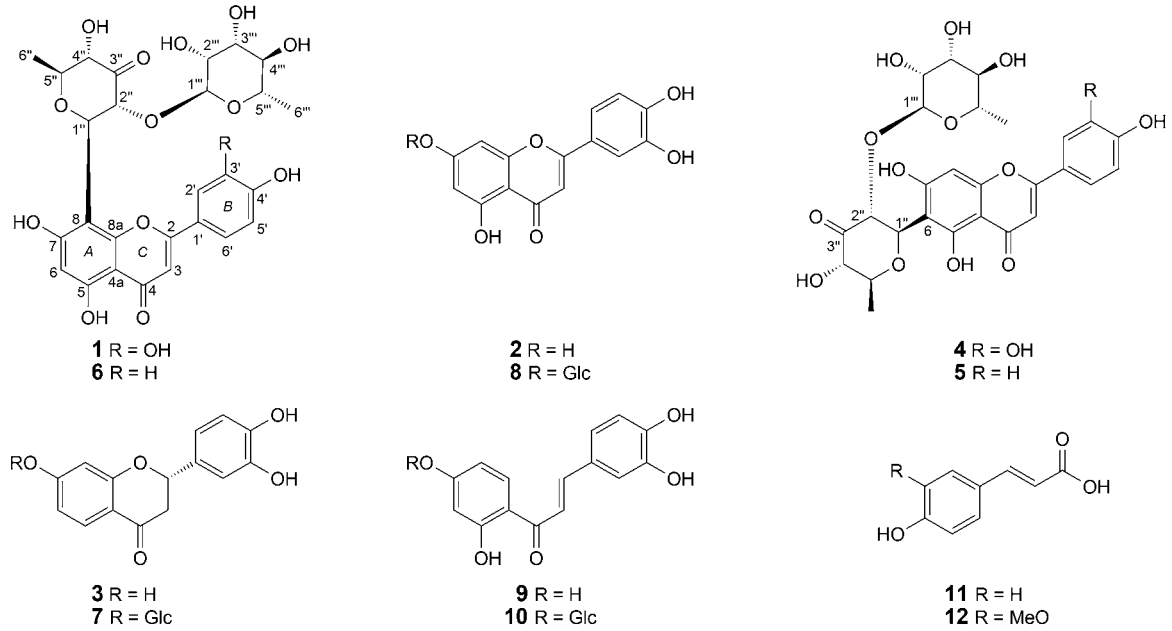

Fig. 1. Chemical structures of isolated compounds **1** – **12**.

H–C(3)) on the *C* ring were also observed. In addition, two doublet anomeric H-atom signals at δ(H) 4.99 (*d*, J = 10.4, H–C(1″)) and 4.60 (*d*, J = 1.3, H–C(1‴)), and two Me signals at δ(H) 1.40 (*d*, J = 6.0, H–C(6″)) and 0.51 (*d*, J = 6.2, H–C(6‴)) suggested the existence of two 6-deoxy-monosaccharides, such as rhamnose. Coupling constants of the two anomeric H-atoms (J = 10.4 and J = 1.3) indicate that these two monosaccharides have relative configurations of β and α, respectively. The ^{13}C-NMR signals at δ(C) 98.9 (C(1‴)), 71.1 (C(4‴)), 70.1 (C(2‴)), 70.0 (C(3‴)), 68.9 (C(5‴)), and 17.4 (C(6‴)) confirmed that one of the two sugar moieties is a rhamnose unit with an *O*-glycosidic linkage. The other sugar moiety included a C=O group at δ(C) 205.3 (C(3″)), four oxygenated C-atoms at δ(C) 73.5 (C(1″)), 76.0 (C(2″)), 78.5 (C(4″)), and 78.7 (C(5″)), and a Me group at δ(C) 19.3 (C(6″)), which are assignable to the 3-keto sugar, 6-deoxy-*ribo*-hexos-3-ulose [10]. Taken together (*Table 1*), these data suggested that compound **1** is similar to cassiaoccidentalin B (**4**) [10]. However, the chemical shifts at δ(C) 98.38 (C(6)) and 104.12 (C(8)) indicated that the 3-keto sugar moiety was connected at the C(8) position of luteolin [10], in a similar manner to orientin [15], which was further confirmed by the 2D-NMR data (COSY, HSQC, and HMBC). The HMBC correlations from the anomeric H-atom at δ(H) 4.99 (H–C(1″)) with C-atoms at δ(C) 104.1 (C(8)), 162.4 (C(7)), and 156.2 (C(8a)) confirmed the *C*-glycosylation at C(8) (*Fig. 2*). Correlations of δ(H) 4.92 (H–C(2″)) at δ(C) 98.90 (C(1‴)) and 4.60 (H–C(1‴)) at δ(C) 76.04 (C(2″)) in HMBC revealed that a rhamnose residue was attached to the *O*-2″ position of 6-deoxy-*ribo*-hexos-3-ulose. Based on these data,

Table 1. ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) Data (in (D_6) DMSO) and HMBC correlations of Compound **1**. δ in ppm, J in Hz

Position	δ(H)	δ(C)	HMBC
2		164.14	
3	6.75 (*s*)	103.11	C(2), C(4), C(4a), C(1′)
4		181.94	
4a		103.72	
5		161.20	
6	6.26 (*s*)	98.38	C(5), C(7), C(8), C(4a)
7		162.38	
8		104.12	
8a		156.24	
1′		122.08	
2′	7.50 (*d*, J = 2.3)	113.21	C(1′), C(3′), C(6′), C(2)
3′		145.13	
4′		150.02	
5′	6.92 (*d*, J = 8.5)	115.94	C(4′), C(6′), C(3′)
6′	7.50 (*dd*, J = 8.5, 2.3)	119.67	C(1′), C(2′), C(5′), C(2)
6-Deoxy-*ribo*-hexos-3-ulose			
1″	4.99 (*d*, J = 10.4)	73.48	C(7), C(8), C(8a), C(2″), C(3″), C(5″)
2″	4.92 (*dd*, J = 10.4, 1.3)	76.04	C(1″), C(1‴)
3″		205.29	
4″	4.09 (*d*, J = 9.7)	78.46	C(3″), C(5″), C(6″)
5″	3.54 – 3.50 (*m*)	78.72	C(1″), C(4″)
6″	1.40 (*d*, J = 6.0)	19.61	C(4″), C(5″)
Rhamnose			
1‴	4.60 (*d*, J = 1.1)	98.90	C(2″), C(2‴), C(3‴), C(5‴)
2‴	3.65 (*s*)	70.12	C(1‴), C(3‴)
3‴	3.01 – 2.98 (*m*)	70.03	C(2‴), C(4‴)
4‴	2.96 – 2.91 (*m*)	71.10	C(3‴), C(5‴)
5‴	2.12 (*dd*, J = 8.8, 6.3)	69.45	C(4‴), C(6‴)
6‴	0.51 (*d*, J = 6.3)	17.09	C(4‴), C(5‴)

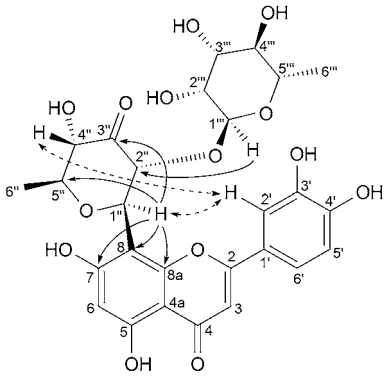

Fig. 2. Key HMBC (H → C) and NOESY (H ⇢ H) correlations of compound **1**.

compound **1** was confirmed to be 2″-*O*-α-rhamnosyl-8-*C*-(6-deoxy-*ribo*-hexos-3-ulosyl) luteolin, and named isocassiaoccidentalin B (**1**).

All of the 12 isolated compounds (**1** – **12**) were tested for their anti-inflammatory activities on LPS-induced NO production in RAW 264.7 cells. Several compounds modulated the release of NO, and the hierarchy of anti-inflammatory potential of the compounds was found to be **2** > **3** > **7** > **8** (*Fig. 3a*). Although compounds **9** and **10** were found to be the most potent inhibitors of NO, they were also significantly cytotoxic, while the other compounds did not show any significant cytotoxicity (*Fig. 3b*). Due to the potential inhibitory activity of these compounds against NO production, we expect that our data can be further expanded to evaluate the underlying mechanisms of their anti-inflammatory effects. The free-radical scavenging activities of compounds (**1** – **12**) were also investigated using DPPH and ABTS assays. Compounds **1**, **2**, **4**, **8**, **9**, and **10** exhibited potent free-radical scavenging activities in both the DPPH and ABTS assays, with IC_{50} values of 12.8, 17.8, 12.9, 11.8, 11.8, 11.3 and 26.0, 24.0, 23.7, 23.1, 22.2, and 25.6 μM, respectively, which were comparable to ascorbic acid (IC_{50} = 12.9 and 19.0 μM) (*Table 2*). In each of these compounds, the presence of a catechol moiety in the *B* ring (C(3)′, C(4′)) and a OH group at the C(5) position on the *A* ring is the key factor for their high radical scavenging abilities. Among the *C*-glycosides containing a keto sugar, compounds **1** and **4** showed lower IC_{50} values than **5** and **6** in both DPPH and ABTS assays, which is due to the presence of a catechol moiety on the *B* ring of the flavone nucleus. Compounds **3** and **7** exhibited comparatively weak radical scavenging activity due to their lack of a OH group at the C (5) position on the *A* ring, and a C(2)–C(3) unsaturated bond on the *C* ring. In general, flavonoids are potent free-radical scavengers, due to the presence of OH groups, which can trap free radicals. The extent of this antioxidative capacity depends on the number and position of the OH groups on the flavonoid nucleus. The presence of OH groups at the C(3′) and C(4′) positions on the *B* ring (the catechol moiety) notably enhances their scavenging capability [16].

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Supplementary Material

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/hlca.201600131.

Experimental Part

General

TLC: SiO_2 *60* F_{254} precoated plates (*Merck,* Germany). Column chromatography (CC): SiO_2 *60* (230 – 400 mesh; *Merck,* Germany). *Sephadex LH-20* (*GE Healthcare,* Uppsala, Sweden). HPLC: anal. HPLC *Agilent 1260 system (Agilent Technologies,* Waldbronn, Germany)*, G1322A 1260* degasser*,* a *G1312C 1260* Binary Pump*,* a *G1329B* autosampler*,* a *G1316A* column oven*,* a *G1315D DAD* detector*,* an *INNO C-18* column (4.6 × 250 mm, 5 μm; *Youngjin Biochrome*, Korea). Semiprep. HPLC: *Gilson* HPLC system (*Gilson,* USA) *321* pump, *157* UV/VIS detector, *INNO C-18* column (20 × 250 mm, 5 μm; *Youngjin Biochrome*, Korea). UV Spectra: *EnSpire* multimode spectrophotometer (*Perkin–Elmer*, Norwalk, USA); λ_{max} (logε) in nm. ^{1}H-, ^{13}C-, and 2D-NMR spectra: *Bruke*r model digital *AVANCE III 400* (*Bruker*, Germany); δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. ESI-MS: *Advion* compact mass spectrometer; in *m*/*z*. HR-ESI-MS: *Agilent 6530 LC Q-TOF/MS* (*Agilent Tech*, Santa Clara, CA, USA); in *m*/*z*.

Plant Material

Whole *C. nomame* plants were collected in Ansan province, Korea, in August of 2013 and were identified by one of the authors (Dr. *C. Y. K.*). A voucher specimen (HYUP-CN-001) was deposited with the herbarium of the College of Pharmacy at Hanyang University.

Extraction and Isolation

Dried whole *C. nomame* plants (7 kg) were extracted with MeOH (3 × 10 l) by sonication at r.t. After filtration, the soln. was concentrated using a vacuum rotary evaporator and yielded 850 g of extract. This crude extract was suspended in 4.5 l of H_2O, and successively partitioned with the same volume of hexane, AcOEt, and BuOH to give hexane (51 g), AcOEt (71 g), and BuOH (45 g) fractions. The AcOEt-soluble fraction was subjected to column chromatography (CC; SiO_2 *60*, 230 – 400 mesh; *Merck,* Germany), using a hexane/AcOEt gradient from 8:1 to 0:1, to yield six fractions, *Frs. F1 – F6*. *Fr. F5* (7.5 g) was further subjected to CC (SiO_2; hexane/AcOEt gradient from 10:1 to 0:1) to obtain

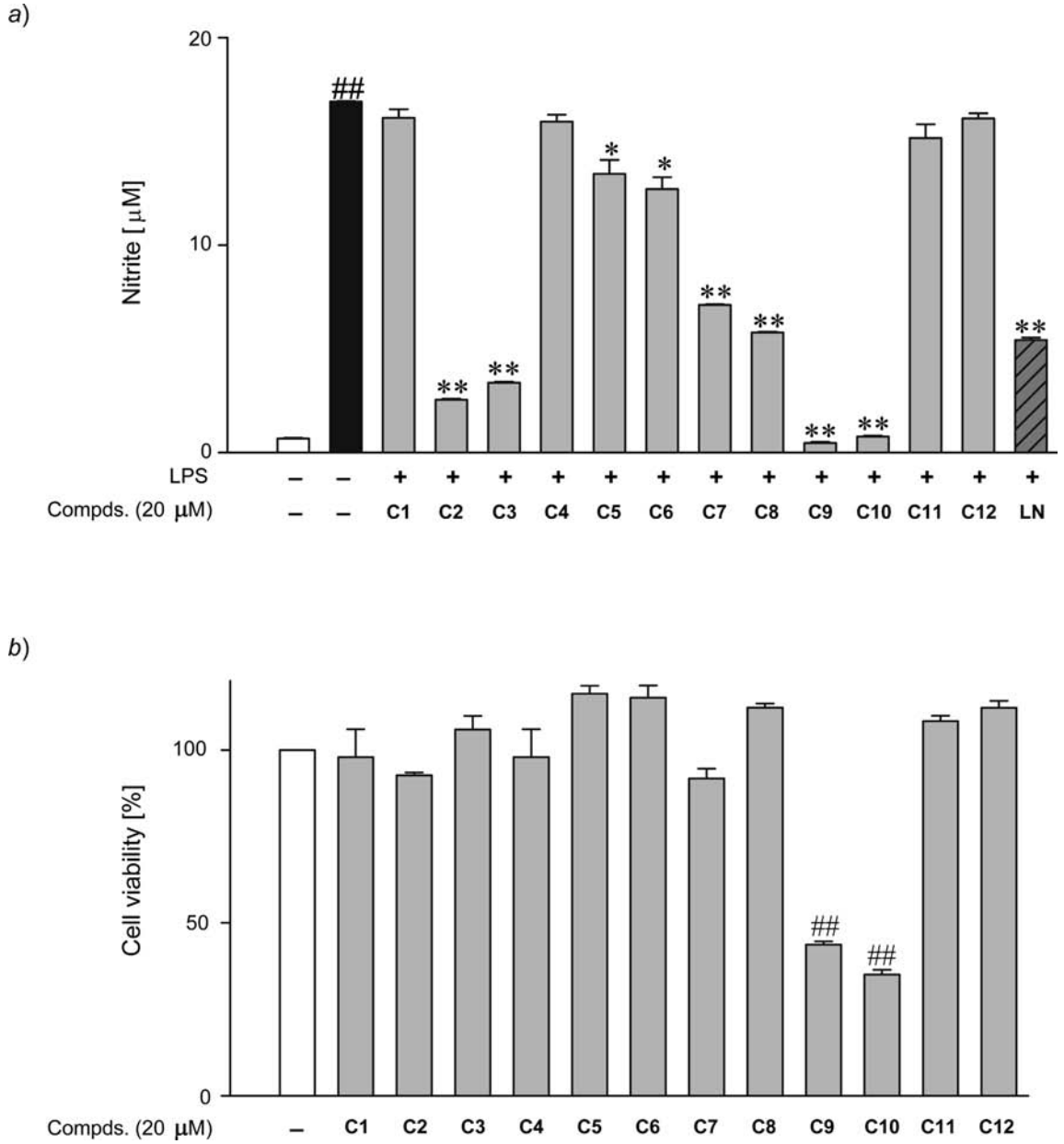

Fig. 3. Effects of isolated compounds **1** – **12** on generation of nitric oxide *a*) and cell viability *b*). NO production was determined by the *Griess* reaction following 24 h of stimulation by lipopolysaccharide (LPS; 200 ng/ml) in RAW 264.7 cells. Cell viability was determined by the MTT assay. *N*-Nitro-L-arginine methyl ester (LN; 100 μM) was used as a positive control. Values are represented as the mean ± SEM. Significance was determined using the *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$ *vs*. LPS-treated group; $^{\#\#}P < 0.01$ *vs*. control group. $N = 03$.

four subfractions, *Frs. F5.1 – F5.4*. *Fr. F5.3* (2.5 g) was subjected to CC (*Sephadex LH-20*; MeOH), followed by semiprep. HPLC ($MeCN/H_2O$, 20:80, 0.1% HCOOH) to give compounds **2** (24.2 mg) and **9** (17.7 mg). *Fr. F6* (41 g) was subjected to CC (SiO_2; CH_2Cl_2/MeOH 8:1) to produce five subfractions, *Frs. F6.1 – F6.5*. *Fr. F6.2* (5.2 g) was subjected to CC (*Sephadex LH-20*; MeOH) to produce four additional subfractions, *Frs. F6.2.1 – F6.2.4*. *Frs. F6.2.2 – F6.2.4* were further subjected to semi prep. HPLC (as described above) to yield the following compounds: from *Fr. F6.2.2* (835 mg) – compounds **11** (10.4 mg) and **12** (33.1 mg); from *Fr. F6.2.3* (2.3 g) – compounds **1** (32.4 mg), **4** (151.3 mg), **5** (85.1 mg), and **6** (20.9 mg); from *Fr. F6.2.4* (1.7 g) – compounds **3** (12.7 mg), **7** (8.3 mg), **8** (41.3 mg), and **10** (26.7 mg).

Isocassiaoccidentalin B (= **(1*R*)-1,5-Anhydro-6-deoxy-2-*O*-(6-deoxy-α-L-mannopyranosyl)-1-[2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4*H*-1-benzopyran-8-yl]-L-*ribo*-hex-3-ulose**; **1**). Light brown needles. UV (MeOH): 258, 350. ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-TOF-MS: 575.1407 ($[M - H]^-$, $C_{27}H_{27}O_{14}^-$; calc. 575.1401).

Table 2. Free-radical scavenging activities of isolated compounds **1** – **12** in DPPH and ABTS assays

Compound	IC_{50} [μM][a])	
	DPPH	ABTS
1	12.8 ± 0.5	26.0 ± 0.6
2	17.8 ± 0.4	24.0 ± 0.9
3	28.4 ± 0.6	38.5 ± 1.1
4	12.9 ± 0.2	23.7 ± 0.6
5	19.7 ± 0.3	40.6 ± 0.4
6	19.0 ± 0.5	36.2 ± 0.5
7	28.6 ± 0.5	40.6 ± 1.5
8	11.8 ± 0.3	23.1 ± 0.7
9	11.8 ± 0.2	22.2 ± 0.6
10	11.3 ± 0.2	25.6 ± 1.2
11	31.9 ± 0.8	62.5 ± 0.4
12	21.5 ± 1.1	30.7 ± 0.9
Ascorbic acid[b])	12.9 ± 0.9	19.0 ± 0.4

[a]) IC_{50} are represented as mean ± SD based on three independent experiments. [b]) Ascorbic acid is used as a positive control.

Cell Culture, Cell Viability and NO Determination Assay

RAW 264.7 macrophages were procured from *ATCC* (VA, USA) and maintained in DMEM (*Gibco*, NY, USA) supplemented with 10% heat-inactivated FBS (*Gibco*), 100 units/ml of penicillin, and 100 μg/ml of streptomycin (*Welgene*, South Korea) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were treated with isolated compounds (20 μM) and cell viability was determined by MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay as reported previously [17]. To determine the isolated compounds' ability to block NO release, the cells were challenged with LPS (200 ng/ml) for 24 h in the presence or absence of compounds (20 μM). *N*-Nitro-L-arginine methyl ester was used as a positive control. The conditioned medium was collected and assayed for nitrite, a stable end product of NO, using the *Griess* reaction [18]. The absorbance was measured at 540 nm using an *EnSpire* multimode spectrophotometer (*Perkin–Elmer*). Nitrite concentration was determined using a standard curve calibrated with sodium nitrite.

DPPH˙ Radical Scavenging Assay

DPPH˙ activity was determined as previously described [19] with only slight modifications. Briefly, 100 μl of DPPH˙ soln. (0.1 mM) was mixed with 100 μl of sample/standard soln. (at varying concentrations) in a 96-well plate. Absorbance was measured at 517 nm after 10 min of incubation in the dark. Scavenging ability of DPPH˙ radical was calculated as follows: Scavenging ability (%) $=[(A_0 - A_S)/A_0] \times 100$, where A_0 is the absorbance of the control (containing all reagents except the tested samples) and A_S is the absorbance of the tested sample. The concentration of sample required to cause a 50% inhibition of free radicals (IC_{50}) was calculated from an inhibition graph against concentration. Ascorbic acid was used as a positive control.

ABTS Radical Cation Decolorization Assay

ABTS radical scavenging activity was determined as described previously [20]. $ABTS^+$ radical was produced by incubating an aqueous stock soln. of ABTS (7 mM) and $K_2S_2O_8$ (2.45 mM) in the dark for 14 – 16 h. Immediately before use, the $ABTS^+$ radical solution was diluted with MeOH until an absorbance at 735 nm of 0.7 ± 0.4 was attained. In a 96-well plate, 10 μl of sample (various concentrations, in MeOH) was reacted with 200 μl of $ABTS^+$ solution for 5 min (r.t., in the dark) and absorbance was measured at 735 nm. Scavenging capacity was calculated using the equation described above for the DPPH assay. Ascorbic acid was used as a positive control.

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