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# 6-Methoxyflavonols from the aerial parts of *Tetragonia tetragonoides* (Pall.) Kuntze and their anti-inflammatory activity

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ABSTRACT

Dried aerial parts of *Tetragonia tetragonoides* were extracted with 70% EtOH, and the evaporated residue was successively separated into EtOAc, *n*-BuOH, and $H_2O$ fractions. As a result of repeated $SiO_2$, ODS, and Sephadex LH-20 column chromatography, four new 6-methoxyflavonol glycosides (**2–4**, **8**) along with four known ones (**1**, **5–7**) were isolated. Several spectroscopic data led to determination of chemical structures for four new 6-methoxyflavonol glycosides (**2–4**, **8**) and four known ones, 6-methoxykaempferol 3-*O*-*β*-D-glucopyranosyl-(1 → 2)-*β*-D-glucopyranosyl-7-*O*-(6‴′-(*E*)-caffeoyl)-*β*-D-glucopyranoside (**1**), 6-methoxyquercetin (**5**), 6-methoxykaempferol (**6**), and 6-methoxykaempferol 7-*O*-*β*-D-glucopyranoside (**7**). Methoxyflavonol glycosides **2–8** also have never been reported from *T. tetragonoides* in this study. 6-Methoxyflavonols **5** and **6** showed high radical scavenging potential in DPPH and ABTS test. Also, all compounds showed significant anti-inflammatory activities such as reduction of NO and $PGE_2$ formation and suppression of TNF-α, IL-6, IL-1β, iNOS, and COX-2 expression in LPS-stimulated RAW 264.7 macrophages. In general, the aglycones exhibited higher activity than the glycosides. In addition, quantitative analysis of 6-methoxyflavonols in the *T. tetragonoides* aerial parts extract was conducted through HPLC.

## 1. Introduction

*Tetragonia tetragonoides* (Aizoaceae), grown in Korea, China, and New Zealand, has been cultivated as a food crop [1,2]. In Korea, it also has been a conventional medicament to prevent or treat lots of diseases such as stomach cancer, inflammation, and ulcers [3–5]. Although *T. tetragonoides* is reported to contain a variety of metabolites, that is, flavonoids, alkaloids, as well as terpenes [1,6,7], there has been no report concerning their activity. This investigation concentrated upon the isolation and identification of biologically effective constituents of *Tetragonia tetragonoides*, in addition to an evaluation for the strength of obtained compounds. As a result of column chromatography, eight 6-methoxyflavonols including four new ones were isolated. 6-methoxyflavonols are examples of anti-oxidant and anti-inflammatory agents [8–10]. Therefore, the 6-methoxyflavonols isolated from *T. tetragonoides* are also anticipated to play roles as anti-oxidant and anti-inflammatory agents.

Inflammation is a protective response against bacterial pathogens, irritation, and tissue injuries [11]. The process includes the activation of macrophages, which conduct a crucial function in the immune process [12–15]. The immune response of macrophages controls cytokines such as pro-inflammatory mediators, prostaglandin E2 ($PGE_2$), nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) [15–17]. However, unregulated inflammation leading to excessive formation of inflammatory arbitrator and cytokines might induce persistence as well as evolution of undesirable chronic ailments, cancer, neurodegenerative disorders, atherosclerosis, and diabetes [11,15–18]. Also, during inflammatory processes, oxygen free radicals and reactive oxygen species (ROS) are created, which are reported as the main factors of various chronic diseases [18–20].

This article displays the isolation process for 6-methoxyflavonols from *T. tetragonoides* aerial parts, structure determination of the isolated 6-methoxyflavonols, the interaction between anti-oxidation and pro-inflammatory markers, and the structure-activity relationship for the anti-inflammatory effect. In addition, quantitative analysis of 6-methoxyflavonols in the aerial parts of *T. tetragonoides* extract was conducted

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through HPLC.

## 2. Materials and methods

### 2.1. Plant materials

*T. tetragonoides* were provided by Gwang Myeong Dang Pharm Co., Ltd, Ulsan, Republic of Korea in April 2017 and identified by Prof. Dae-Keun Kim, College of Pharmacy, Woosuk University, Jeonju, Republic of Korea. A voucher specimen (KHU-NPCL-201704) has been deposited at the Laboratory of Natural Products Chemistry, Kyung Hee University Yongin, Republic of Korea.

### 2.2. General experimental procedures

The equipments and chemicals used for isolation of compounds, structure determination of the isolated metabolites, and evaluation for anti-inflammatory activity were referred to literatures [21–24]. The stationary phases used for column chromatography (CC) were silica gel ($SiO_2$, Kiesel gel 60, Merck, Darmstadt, Germany), octadecyl $SiO_2$ (ODS) (LiChroprep RP-18, 40–60 μm, Merck), Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden), and Diaion HP-20 (Samchun Chemical Co., Seoul, Korea). Thin layer chromatography (TLC) analysis was carried out using a Kiesel gel $60F_{254}$ and a RP-18 $F_{254}$s plates (Merck), and the spots on the TLC plates were detected using a UV lamp Spectroline Model ENF-240 C/F (Spectronics Corporation, Westbury, NY, USA) and spraying a 10% $H_2SO_4$ solution followed by heating. $^1$H- and $^{13}$C NMR spectra were recorded on a Bruker Avance 600 (Billerica, MA, USA) or Varian Unity Inova AS-400 FT-NMR spectrometers (Palo Alto, CA, USA). Optical rotation was measured using a JASCO P-1010 digital polarimeter (Jasco, Tokyo, Japan). IR spectra were obtained using a Perkin Elmer Spectrum One FT-IR spectrometer (Buckinghamshire, England). HR-FAB/MS was measured on a JEOL JMSAX-700 mass spectrometer (Tokyo, Japan). HPLC-grade acetonitrile, MeOH, and water were purchased from Burdick & Jackson (Muskegon, MI, USA). Dulbecco's modified Eagles's medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco BRL Co. (Grand Island, NY, USA). Enzyme-linked immunosorbent assay (ELISA) kits for $PGE_2$, TNF-α, IL-1β, and IL-6 were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 2.3. Extraction and isolation

The dried *T. tetragonoides* aerial parts (1.7 kg) were extracted in 70% EtOH (22 L × 4) at room temperature for one day, filtered and evaporated under reduced pressure. The concentrated EtOH residue (452 g) was suspended in $H_2O$ (2.3 L) followed by successive extraction by EtOAc (2.3 L × 3) and *n*-BuOH (1.8 L × 3). Each layer was evaporated under reduced pressure to give EtOAc (TTE, 45 g), *n*-BuOH (TTB, 35 g), and $H_2O$ (TTH, 372 g) fractions (Fr). TTE (45 g) was subjected to $SiO_2$ CC [Φ 10 × 18 cm, $CHCl_3$-MeOH-$H_2O$ (CMH) = 50:3:1 → 30:3:1 → 6:4:1, 11 L of each] with tracking by TLC to afford 10 Fr (TTE-1 to TTE-10). TTE-3 [1.5 g, elution volume/total volume (Ev/Tv) 0.074–0.194] was subjected to ODS CC [Φ 4 × 12 cm, acetone-$H_2O$ (AH) = 1:1, 1 L] to yield 16 Fr (TTE-3-1 to TTE-3-16) along with compound **6** (TTE-3-3, 122 mg, Ev/Tv 0.125–0.205, $R_f$ 0.65 on $SiO_2$ TLC, CMH = 20:3:1, $R_f$ 0.34 on ODS TLC, AH = 2:1), compound **5** (TTE-3-5, 37 mg, Ev/Tv 0.275–0.415, $R_f$ 0.60 on $SiO_2$ TLC, CMH = 20:3:1, $R_f$ 0.34 on ODS TLC, AH = 2:1), and compound **7** (TTE-3-14, 23 mg, Ev/Tv 0.865–0.915, $R_f$ 0.50 on $SiO_2$ TLC, CMH = 20:3:1, $R_f$ 0.45 on ODS TLC, AH = 2:1). TTE-9 (734 mg, Ev/Tv 0.865–0.950) was subjected to ODS CC (Φ 3 × 6 cm, AH = 2:1, 1.3 L) to afford four Fr (TTE-9-1 to TTE-9-4) and compound **8** (TTE-9-4, 32.1 mg, Ev/Tv 0.865–1.000, $R_f$ 0.50 on $SiO_2$ TLC, CMH = 10:3:1, $R_f$ 0.50 on ODS TLC, AH = 1:2). TTB (35 g) was subjected to $SiO_2$ CC (Φ 7.5 × 17 cm) and eluted with CMH = 20:3:1 → 15:3:1 → 10:3:1 → 8:3:1 → 65:35:10, 6 L of each) to afford 14 Fr (TTB-1 to TTB-14). TTB-12 (3.2 g, Ev/Tv 0.851–0.933) was subjected to ODS CC [Φ 4.5 × 8 cm, MeOH-$H_2O$ (MH) (1:2 → 1:1), 2.2 L of both] to yield 11 Fr (TTB-12-1 to TTB-12-11) and compound **2** (TTB-12-8, 176 mg, Ev/Tv 0.100–0.157, $R_f$ 0.60 on $SiO_2$ TLC, CMH = 6:4:1, $R_f$ 0.34 on ODS TLC, MH = 1:1). TTB-12-5 (66 mg, Ev/Tv 0.395–0.505) was subjected to $SiO_2$ CC (Φ 1 × 15 cm, CMH = 7:3:1, 1.4 L) to afford 12 Fr (TTB-12-5-1 to TTB-12-5-12) and compound **4** (TTB-12-5-8, 12 mg, Ev/Tv 0.324–0.468, $R_f$ 0.55 on $SiO_2$ TLC, CMH = 7:3:1, $R_f$ 0.45 on ODS TLC, MH = 1:1). TTH (372.0 g) was subjected to Diaion HP-20 CC (Φ 12 × 15 cm) and eluted with 100% $H_2O$ → 100% MeOH (2.8 L of both) to afford six Fr (TTH-1 to TTH-6). TTH-3 (5.9 g, Ev/Tv 0.248–0.505) was subjected to ODS CC (Φ 4.5 × 12 cm, MH = 1:3, 3.7 L) to yield nine Fr (TTH-3-1 to TTH-3-9). TTH-3–6 (356 mg, Ev/Tv 0.326–0.385) was subjected to $SiO_2$ CC (Φ 3 × 18 cm, CMH = 65:35:10, 2 L) to yield 12 Fr (TTH-3-6-1 to TTH-3-6-12) and compound **1** (TTH-3-6-10, 14 mg, Ev/Tv 0.905–0.945, $R_f$ 0.65 on $SiO_2$ TLC, CMH = 6:4:1, $R_f$ 0.45 on ODS TLC, MH = 1:1). TTH-5 (2.3 g, Ev/Tv 0.661–0.789) was subjected to ODS CC (Φ 4 × 15 cm, MH = 1:1, 1.6 L) to yield 10 Fr (TTH-5-1 to TTH-5-10). TTH-5-5 (758 mg, Ev/Tv 0.468–0.524) was subjected to ODS CC (Φ 2 × 7 cm, MH = 1:1, 1.3 L) to yield seven Fr (TTH-5-5-1 to TTH-5-5-7). TTH-5-5-2 (254 mg, Ev/Tv 0.375–0.525) was subjected to a Sephadex LH-20 CC (Φ 2 × 60 cm, 100% MeOH, 500 mL) to yield three Fr (TTH-5-5-2-1 to TTH-5-5-2-3) and compound **3** (TTH-5-5-2-2, 58 mg, Ev/Tv 0.325–0.400, $R_f$ 0.40 on $SiO_2$ TLC, CMH = 6:4:1, $R_f$ 0.50 on ODS TLC, MH = 1:1).

#### 2.3.1. 6-Methoxykaempferol 3-O-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl-7-O-(6‴′-(E)-caffeoyl)-β-D-glucopyranoside (1)

Yellowish gum; $[\alpha]_D^{21}$-119.7 (*c* 0.1, MeOH); UV (MeOH) $\lambda_{max}$ (nm) 271, 331; IR (KBr) $\nu_{max}$ 3299, 2940, 1655, 1598, 1511, 1462 cm$^{-1}$; positive FAB/MS *m/z* 987 $[M + Na]^+$.

#### 2.3.2. 6-methoxykaempferol 3-O-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl-(6‴′-(E)-feruloyl)-7-O-β-D-glucopyranoside (2)

Yellowish gum; $[\alpha]_D^{21}$ −110.7 (*c* 0.1, MeOH); UV (MeOH) $\lambda_{max}$ (nm) 272, 329; IR (KBr) $\nu_{max}$ 3326, 2939, 2829, 1655, 1596, 1512, 1462 cm$^{-1}$; positive HR-FAB/MS *m/z* 1001.2538 $[M + Na]^+$ (calcd. for $C_{44}H_{55}O_{25}Na$ 1001.2533); for $^1$H and $^{13}$C NMR spectroscopic data, see Tables 1 and 2.

#### 2.3.3. 6-Methoxykaempferol 3-O-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl-7-O-β-D-glucopyranoside (3)

Yellowish gum; $[\alpha]_D^{21}$ −84.4 (*c* 0.1, MeOH); UV (MeOH) $\lambda_{max}$ (nm) 269, 334; IR (KBr) $\nu_{max}$ 3352, 2926, 2835, 1651, 1596, 1449 cm$^{-1}$; positive HR-FAB/MS *m/z* 825.2065 $[M + Na]^+$ (calcd. for $C_{35}H_{44}O_{25}Na$ 825.2060); for $^1$H- and $^{13}$C NMR spectroscopic data, see Tables 1 and 2.

#### 2.3.4. 6-Methoxyquercetin 3-O-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranoside (4)

Yellowish gum; $[\alpha]_D^{21}$ −33.0 (*c* 0.1, MeOH); UV (MeOH) $\lambda_{max}$ (nm) 265, 331; IR (KBr) $\nu_{max}$ 3298, 2928, 1647, 1597, 1511 cm$^{-1}$; positive HR-FAB/MS *m/z* 679.1484 $[M + Na]^+$ (calcd. for $C_{28}H_{33}O_{19}Na$, 679.1481); for $^1$H- and $^{13}$C NMR spectroscopic data, see Tables 1 and 2.

#### 2.3.5. 6-Methoxyquercetin (5)

Yellowish gum; UV (MeOH) $\lambda_{max}$ (nm) 257, 371; IR (KBr) $\nu_{max}$ 3353, 2921, 1652, 1598, 1462 cm$^{-1}$; negative FAB/MS *m/z* 331 $[M-H]^-$.

#### 2.3.6. 6-Methoxykaempferol (6)

Yellowish gum; UV (MeOH) $\lambda_{max}$ (nm) 269, 367; IR (KBr) $\nu_{max}$ 3350, 3158, 1653, 1596, 1569, 1488 cm$^{-1}$; negative FAB/MS *m/z* 315 $[M-H]^-$.

**Table 1**
$^1$H NMR data of 6-methoxyflavonols **2**,**3**,**4**, and **8** ($\delta_H$ in ppm, coupling pattern, *J* in Hz).

| No. | **2**[a] | **3**[b] | **4**[c] | **8**[b] |
|---|---|---|---|---|
| 2 | – | – | – | – |
| 3 | – | – | – | – |
| 4 | – | – | – | – |
| 5 | – | – | – | – |
| 6 | – | – | – | – |
| 7 | – | – | – | – |
| 8 | 7.15, s | 6.94, s | 6.69, s | 6.50, s |
| 9 | – | – | – | – |
| 10 | – | – | – | – |
| 1′ | – | – | – | – |
| 2′ | 7.95, d, 8.0 | 8.11, d, 8.0 | 7.70, d, 2.0 | 7.96, d, 8.0 |
| 3′ | 7.53, d, 8.0 | 6.92, d, 8.0 | – | 6.78, d, 8.0 |
| 4′ | – | – | – | – |
| 5′ | 7.53, d, 8.0 | 6.92, d, 8.0 | 7.03, d, 8.0 | 6.78, d, 8.0 |
| 6′ | 7.95, d, 8.0 | 8.11, d, 8.0 | 7.77, dd, 8.0, 2.0 | 7.96, d, 8.0 |
| 3-*O*-glc | | | | |
| 1″ | 5.80, d, 7.6 | 5.51, d, 7.6 | 5.84, d, 7.6 | |
| 2″ | 3.91, overlapped | 3.85, overlapped | 3.87, overlapped | |
| 3″ | 3.90, overlapped | 3.78, overlapped | 3.84, overlapped | |
| 4″ | 3.89, overlapped | 3.94, overlapped | 3.99, overlapped | |
| 5″ | 3.89, overlapped | 3.87, overlapped | 3.74, overlapped | |
| 6″a | 3.90, overlapped | 3.98, overlapped | 3.80, overlapped | |
| 6″b | 3.80, overlapped | 3.79, overlapped | 3.60, overlapped | |
| 2″-*O*-glc | | | | |
| 1‴ | 4.90, d, 7.6 | 5.13, d, 7.6 | 4.77, d, 7.6 | |
| 2‴ | 3.69, overlapped | 3.81, overlapped | 3.87, overlapped | |
| 3‴ | 3.72, overlapped | 3.75, overlapped | 3.45, overlapped | |
| 4‴ | 3.68, overlapped | 3.66, overlapped | 3.60, overlapped | |
| 5‴ | 3.84, overlapped | 3.84, overlapped | 3.45, overlapped | |
| 6‴a | 3.90, overlapped | 3.98, overlapped | 3.80, overlapped | |
| 6‴b | 3.80, overlapped | 3.79, overlapped | 3.60, overlapped | |
| 7-*O*-glc | | | | |
| 1⁗ | 5.51, d, 7.6 | 4.78, d, 7.6 | | 5.10, d, 7.6 |
| 2⁗ | 3.91, overlapped | 3.85, overlapped | | 3.81, overlapped |
| 3⁗ | 3.89, overlapped | 3.78, overlapped | | 3.50, overlapped |
| 4⁗ | 3.68, overlapped | 3.73, overlapped | | 3.47, overlapped |
| 5⁗ | 3.90, overlapped | 3.95, overlapped | | 3.53, overlapped |
| 6⁗a | 5.12, br.d, 12.0 | 3.98, overlapped | | 4.22, dd, 12.0, 1.6 |
| 6⁗b | 4.87, overlapped | 3.79, overlapped | | 3.87, overlapped |
| Ferulic acid moiety | | | | |
| 1⁗′ | – | | | – |
| 2⁗′ | 7.13, d, 1.6 | | | 6.50, s |
| 3⁗′ | – | | | – |
| 4⁗′ | – | | | – |
| 5⁗′ | 7.27, d, 8.0 | | | 6.68, d, 8.0 |
| 6⁗′ | 7.04, dd, 8.0, 1.6 | | | 6.68, br.d, 8.0, 2.0 |
| 7⁗′ | 7.81, d, 16.0 | | | 7.39, d, 16.0 |
| 8⁗′ | 6.54, d, 16.0 | | | 6.10, d, 16.0 |
| 9⁗′ | – | | | – |
| OMe-6 | 3.93, s | 3.90, s | 3.90, s | 3.87, s |
| OMe-3⁗′ | 3.67, s | | | 3.61, s |

[a] Pyridine-$d_5$, 400 MHz.
[b] $CD_3OD$, 600 MHz.
[c] DMSO-$d_6$, 400 MHz.

*2.3.7. 6-Methoxykaempferol 7-O-β-D-glucopyranoside (7)*

Yellowish gum; $[\alpha]_D^{21}$ −43.0 (*c* 0.1, MeOH); UV (MeOH) $\lambda_{max}$ (nm) 257, 366; IR (KBr) $\nu_{max}$ 3268, 2939, 1652, 1595, 1478 cm$^{-1}$; positive FAB/MS *m/z* 501 $[M + Na]^+$.

*2.3.8. 6-Methoxykaempferol 7-O-(6‴′-(E)-feruloyl)-β-D-glucopyranoside (8)*

Yellowish gum; $[\alpha]_D^{21}$ −34.0 (*c* 0.1, MeOH); UV (MeOH) $\lambda_{max}$ (nm) 270, 328; IR (KBr) $\nu_{max}$ 3354, 2926, 1626, 1594, 1514, 1473 cm$^{-1}$; positive HR-FAB/MS *m/z* 655.1660 $[M + H]^+$ (calcd. for $C_{32}H_{32}O_{16}$, 655.1663); for $^1$H- and $^{13}$C NMR spectroscopic data, see Tables 1 and 2.

*2.4. 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) and 2,2′-Azino-Bis(3-Ethylbenzothiazoline-6-Sulfonic acid) Diammonium Salt (ABTS) radical scavenging assays*

DPPH and ABTS radical scavenging assay was carried out according to the previously described method [19,25]. Briefly, 0.9 mL of each flavonoid (6.25, 12.5, 25, 50, 100, and 200 ppm) was added to 1.9 mL DPPH solution (0.1 mM in MeOH). After incubating at 37 °C for 30 min, optical density was evaluated at 517 nm against a blank of DPPH solution including 100% MeOH using the UV spectrophotometer. For ABTS assay, 20 μL of each sample (6.25, 12.5, 25, 50, 100, and 200 ppm) was added to ABTS solution (980 μL). Optical density was

**Table 2**
$^{13}$C NMR data of 6-methoxyflavonols **2**,**3**,**4**, and **8** ($\delta_C$ in ppm).

| No. | 2[a] | 3[b] | 4[c] | 8[b] |
|---|---|---|---|---|
| 2 | 145.4 | 152.1 | 148.4 | 145.8 |
| 3 | 135.6 | 133.5 | 132.7 | 135.7 |
| 4 | 178.3 | 178.7 | 177.5 | 175.6 |
| 5 | 156.3 | 156.4 | 155.3 | 155.8 |
| 6 | 133.6 | 132.5 | 131.1 | 132.1 |
| 7 | 156.6 | 158.2 | 152.3 | 159.0 |
| 8 | 94.0 | 92.6 | 93.5 | 93.8 |
| 9 | 151.3 | 152.5 | 157.2 | 151.4 |
| 10 | 106.7 | 106.8 | 104.3 | 105.4 |
| 1′ | 123.5 | 121.3 | 121.7 | 122.4 |
| 2′ | 132.8 | 131.1 | 115.8 | 131.6 |
| 3′ | 115.6 | 114.9 | 144.7 | 114.8 |
| 4′ | 161.0 | 160.4 | 151.2 | 159.1 |
| 5′ | 115.6 | 114.9 | 115.2 | 114.8 |
| 6′ | 131.3 | 131.1 | 121.0 | 131.8 |
| 3-*O*-glc | | | | |
| 1″ | 99.2 | 96.8 | 97.8 | |
| 2″ | 83.4 | 82.4 | 82.5 | |
| 3″ | 77.6 | 76.6 | 76.9 | |
| 4″ | 70.4 | 69.6 | 69.9 | |
| 5″ | 77.4 | 76.6 | 76.2 | |
| 6″ | 61.6 | 60.8 | 60.6 | |
| 2″-*O*-glc | | | | |
| 1‴ | 105.4 | 103.4 | 99.6 | |
| 2‴ | 75.0 | 74.4 | 74.7 | |
| 3‴ | 78.3 | 77.5 | 77.5 | |
| 4‴ | 70.1 | 69.5 | 69.9 | |
| 5‴ | 77.9 | 77.0 | 77.5 | |
| 6‴ | 61.2 | 60.5 | 60.6 | |
| 7-*O*-glc | | | | |
| 1⁗ | 101.5 | 99.4 | | 100.1 |
| 2⁗ | 73.8 | 73.1 | | 73.0 |
| 3⁗ | 77.4 | 76.3 | | 78.4 |
| 4⁗ | 70.1 | 69.2 | | 70.9 |
| 5⁗ | 77.4 | 73.9 | | 76.5 |
| 6⁗ | 63.5 | 62.9 | | 63.1 |
| Ferulic acid moiety | | | | |
| 1⁗′ | 125.5 | | | 126.0 |
| 2⁗′ | 113.9 | | | 113.2 |
| 3⁗′ | 148.1 | | | 147.2 |
| 4⁗′ | 150.3 | | | 148.8 |
| 5⁗′ | 116.0 | | | 113.9 |
| 6⁗′ | 121.1 | | | 122.0 |
| 7⁗′ | 149.4 | | | 147.4 |
| 8⁗′ | 110.6 | | | 109.4 |
| 9⁗′ | 166.9 | | | 167.4 |
| OMe-6 | 60.2 | 59.9 | 60.6 | 60.3 |
| OMe-3⁗′ | 55.2 | | | 54.6 |

[a] Pyridine-$d_5$, 400 MHz.
[b] $CD_3OD$, 600 MHz.
[c] DMSO-$d_6$, 400 MHz.

measured at 734 nm after incubating at 37 °C for 10 min against a blank of ABTS solution including 100% MeOH using the UV spectrophotometer. Kaempferol and quercetin were used as positive controls in both DPPH and ABTS assay. Electron donating ability (EDA, %) of DPPH and ABTS assay was calculated using the following equation: EDA (%) = {(Ac − As)/Ac} × 100 (%), As: the absorbance in the presence of the sample, Ac: the absorbance in the absence of the sample.

### 2.5. Macrophage RAW 264.7 culture, measurement of nitrite (NO) and $PGE_2$, IL-1β, IL-6, and TNF-α, western blot analysis

Macrophage RAW 264.7 culture, measurement of nitrite (NO), $PGE_2$, TNF-α, IL-1β, and IL-6 assays and western blot analyses were carried out according to a previously described method [26]. Briefly, RAW 264.7 cells ($1 \times 10^4$ cells/well), grown in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin (Gibco, Gaithersburg, MD, USA) for 12–18 h, were added by LPS (1 μg/mL added to cultures to yield concentrations of 0, 20, 40, or 80 μM) for 24 h. The amount of nitrite was determined based on the Griess analysis using the supernatant, and the quantity of $PGE_2$, IL-1β, IL-6 and TNF-α were calculated in the medium by an ELISA kit (R&D Systems, Minneapolis, MN, USA). Pellets of RAW 264.7 macrophages were obtained through centrifugation at 1200 rpm (3 min, 4 °C). Sequentially, cells were washed using phosphate-buffered saline and lysed in 20 mM Tris-HCl buffer (pH 7.4) containing a protease inhibitor mixture (5 mg/mL pepstatin A, 0.1 mM phenylmethanesulfonyl fluoride, and 1 mg/mL chymostatin). The quantity of protein was decided by a Lowry protein assay kit (Sigma Chemical Co.). Thirty milligrams of protein was resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto a hybond-enhanced chemiluminescence nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with a blocking solution (5% skim milk), a primary antibody was bound to the membrane (iNOS, COX-2, HO-1) at 4 °C overnight followed by a secondary antibody (anti-rabbit, anti-goat, anti-mouse) binding to each primary antibody at 4 °C 2 h, and then luminescence was generated by incubating the membrane with ECL buffer (Amersham Pharmacia Biotech, Piscataway, NJ, USA) at room temperature 5 min. Butein was used as a positive control.

### 2.6. HPLC analysis of 6-methoxyflavonols in T. tetragonoides

Calibration curves for each standard were made using six concentrations (31.25–1000 μg/mL). The 70% EtOH extracts were filtered (0.22-μm membrane filter, Woongki Science Co., Ltd., Seoul, Korea) and concentrated under reduced preessure. A 10-μL aliquot of each extract solution in 100% MeOH (10.0 mg/mL) was injected (a Waters 600S with Waters 2487 UV detector, 330 nm, Milford, MA, USA). The column was a Kinetex C18 column (Phenomenex, 5 μm, 250 × 4.6 mm). The mobile phase (0.1% FA in $H_2O$, solvent A; acetonitrile, solvent B) was eluted at a flow rate of 1.0 mL/min with the following elution gradient of B: 20% (0.01 min) → 30% (7 min) → 50% (15 min) → 50% (16 min) → 80% (37 min) → 100% (40 min) → 100% (45 min). Quantitative analysis was replicated three times.

### 2.7. Statistical analysis

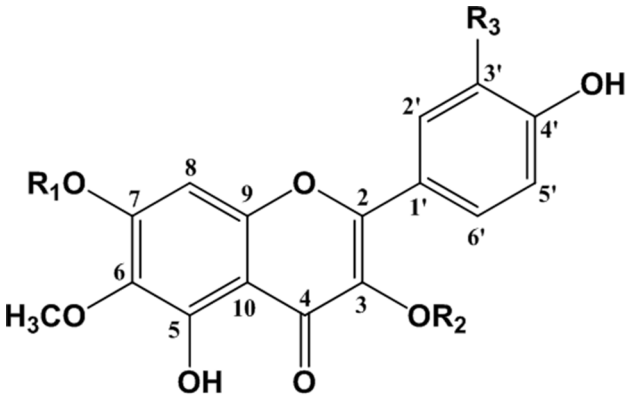
Statistical analysis was performed using GraphPad Prism software version 3.03 and 5 (GraphPad Software, Inc., San Diego, CA, USA). Data were presented as mean ± standard error. Significance was estimated using repeated one-way ANOVA followed by Tukey's test.

## 3. Results and discussion

### 3.1. Isolation and identification of 6-methoxyflavonols from T. tetragonoides

Dried aerial parts of *T. tetragonoides* were extracted in aqueous EtOH, and the concentrate was divided in EtOAc, *n*-BuOH, and $H_2O$ Fr. The repeated $SiO_2$, ODS, Sephadex LH-20, and Diaion HP-20 CC on each Fr gave the isolation of four new 6-methoxyflavonols and four known ones (Fig. 1), 6-methoxykaempferol 3-*O*-β-D-glucosyl-(1 → 2)-β-D-glucopyranosyl-7-*O*-(6⁗-(*E*)-caffeoyl)-β-D-glucopyranoside (**1**), 6-methoxyquercetin (**5**), 6-methoxykaempferol (**6**), and 6-methoxykaempferol 7-*O*-β-D-glucopyranoside (**7**) based on various spectroscopic data but also affirmed by comparing the data with those in reports [6,27–29].

Compound **2**, a yellowish gum (MeOH), showed IR absorbance bands of hydroxyl (3326 $cm^{-1}$) and aromatic groups (1655, 1596, 1512, 1462 $cm^{-1}$). Positive HR-FAB/MS analysis of **2** gave a molecular formula (MF) of $C_{44}H_{55}O_{25}$ from the molecular ion peak (MIP) $[M + Na]^+$ *m/z* 1001.2538 (calcd. for $C_{44}H_{55}O_{25}Na$ 1001.2533). The $^1$H NMR spectrum (Table 1) showed the proton signals of eight aromatic methines [$\delta_H$ 7.04, dd, *J* = 8.0, 1.6 Hz (*J* in Hz), H-6⁗′; 7.13, d,




| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| **1** | 6-caf-glc | glc (1→2) glc | H |
| **2** | 6-fer-glc | glc (1→2) glc | H |
| **3** | glc | glc (1→2) glc | H |
| **4** | H | glc (1→2) glc | OH |
| **5** | H | H | OH |
| **6** | H | H | H |
| **7** | glc | H | H |
| **8** | 6-fer-glc | H | H |

**glc: *β*-D-glucopyranosyl**

**glc (1→2) glc: *β*-D-glucopyranosyl (1→2)-*β*-D-glucopyranosyl**

**6-caf-glc: (6-(*E*)-caffeoyl)-*β*-D-glucopyranosyl**

**6-fer-glc: (6-(*E*)-feruloyl)-*β*-D-glucopyranosyl**

**Fig. 1.** 6-Methoxyflavonols **1–8** isolated from the aerial parts of *Tetragonia tetragonoides*.

1.6, 2‴″; 7.15, s, H-8; 7.27, d, 8.0, 5‴″; 7.53, 2H, d, 8.0, H-3′,5′; 7.95, 2H, d, 8.0, H-2′,6′] owing to a *p*-disubstituted, a 1,2,4-trisubstituted, and a 1,2,3,4,5-pentasubstituted benzene moiety. The olefin methine signals with *trans* configuration were also observed at $\delta_H$ 6.54 (d, 16.0, H-8‴″) and $\delta_H$ 7.81 (d, 16.0, H-7‴″). The above-mentioned data suggested the aglycone of **2** as a flavonol with a caffeic acid. In the $^1$H NMR spectrum (Table 1), three hemiacetals, twelve oxygenated methines, and three oxygenated methylene proton signals were observed as the proton signals of three hexoses. The coupling constants of the anomer proton signals of three sugars ($J$ = 7.6 Hz) confirmed the anomer hydroxys to have *β*-configurations. The $^{13}$C NMR data (Table 2) exhibited 26 carbon signals comprising two methoxies ($\delta_C$ 60.2; 55.2) as the aglycone signals and 18 carbons derived from three hexoses, indicating **2** to be a flavonol glycoside with one phenylpropanoid and three hexoses. Based on the chemical shifts of the sugar carbon signals, three hemiacetals ($\delta_C$ 101.5, C-1‴; 105.4, C-1‴; 99.2, C-1″), twelve oxygenated methines ($\delta_C$ 70.1, C-4‴; 70.4, C-4″; 70.1, C-4‴; 73.8, C-2‴; 75.0, C-2‴; 77.4, C-5″; 77.4, C-3‴; 77.4, C-5‴; 77.6, C-3″; 78.3, C-3‴; 77.9, C-5‴; 83.4, C-2″), and three oxygenated methylenes ($\delta_C$ 61.2, C-6‴; 61.6, C-6″; 63.5, C-6‴), all the sugars were determined to be *β*-glucopyranoses. An oxygenated methylene resonance of glucose (C-2″) was detected at a lower magnetic field, $\delta_C$ 83.4, than the commonly detected chemical shift, $\delta_C$ 75, due to glycosidation shift, which confirmed the position of the glycosidic linkage. In the gHMBC spectrum, two methoxy proton signals ($\delta_H$ 3.67 and 3.93) showed cross-peaks with the oxygenated olefin quaternary carbon signals ($\delta_C$ 133.6, C-6; 148.1, C-3‴″), indicating that two methoxy groups were located at C-3‴″ and C-6. Accordingly, the aglycone of **2** is composed of a 6-methoxykaempferol and a ferulic acid. Also the three anomer proton signals of three glucoses ($\delta_H$ 4.90, H-1‴; 5.51, H-1‴″; 5.80, H-1″) showed cross-peaks with two oxygenated aromatic quaternaries ($\delta_C$ 135.6, C-3; 156.6, C-7) and an oxygenated methine ($\delta_C$ 83.4, C-2″) carbon signal, indicating that glucoses were located at the C-3 and C-7 positions of the flavonol moiety and the C-2 position of the glucopyranose moiety, respectively. Attachment of the caffeoyl group at C-6‴″ of the glucopyranose was proven by a downfield shift of the oxygenated methine proton signal (H-6″) to $\delta_H$ 5.12, which usually occurs at $\delta_H$ 3.8. Also, the connection between the glucopyranosyl unit (H-6‴″) and the C-9‴″ of the caffeoyl group was confirmed by a cross-peak between the oxygenated methine proton signal at $\delta_H$ 5.12 (H-6‴″) and the conjugated ester carbon signal at $\delta_C$ 166.9 (C-9‴″) in the gHMBC spectrum. Taken together, the chemical structure of **2** was determined to be 6-methoxykaempferol 3-*O*-*β*-D-glucopyranosyl-(1 → 2)-*β*-D-glucopyranosyl-7-*O*-(6‴″-(*E*)-feruloyl)-*β*-D-glucopyranoside.

Compound **3**, a yellowish gum (MeOH), showed IR absorbance bands of hydroxyl (3352 cm$^{-1}$) and aromatic groups (1651, 1596, 1449 cm$^{-1}$). The MF of **3**, $C_{35}H_{44}O_{25}$, was deduced from the MIP *m/z* 825.2065 $[M + Na]^+$ (calcd. for $C_{35}H_{44}O_{25}Na$ 825.2060) in the positive HR-FAB/MS. The molecular weight of **3**, 802 Da, was 176 amu less than that of **2** (978 Da), which suggested the absence of a ferulic acid moiety in **3**. Also, the NMR spectra of **3** were very similar to those of **2,** with the exception of the absence of a ferulic acid moiety. In the gHMBC, the correlations of all anomer proton signals ($\delta_H$ 4.78, H-1‴; 5.13, H-1‴; 5.51, H-1″) with two oxygenated aromatic quaternaries ($\delta_C$ 133.5, C-3; 158.2, C-7) and an oxygenated methine ($\delta_C$ 83.4, C-2″) carbon signal, respectively, confirmed that the three glucoses connect at C-3 and C-7 of 6-methoxyflavonol and C-2″ of glucopyranose. Taken together, the chemical structure of **3** was determined to be 6-methoxykaempferol 3-*O*-*β*-D-glucopyranosyl-(1 → 2)-*β*-D-glucopyranosyl-7-*O*-*β*-D-glucopyranoside.

Compound **4**, a yellowish gum (MeOH), showed IR absorbance bands of hydroxyl (3298 cm$^{-1}$) and aromatic groups (1647, 1597, and 1511 cm$^{-1}$). The MF of **4**, $C_{28}H_{33}O_{19}$, was deduced from the MIP *m/z* 679.1484 $[M + Na]^+$ (calcd. for $C_{28}H_{33}O_{19}Na$, 679.1481) in the positive HR-FAB/MS. $^1$H- and $^{13}$C NMR spectra (Tables 1 and 2) of **4** were very similar to those of **3** with the exception of the absence of a glucopyranose moiety and a difference in the B ring of flavonol aglycone. The $^1$H NMR signals, observed as three aromatic methines ($\delta_H$ 7.03, d, 8.0, H-5′; 7.70, d, 2.0, H-2′; 7.77, dd, 8.0, 2.0, H-6′), suggested that the B ring had a 1,2,4-trisubstituted benzene moiety. In addition, the molecular weight of **4**, 656 Da, was 146 amu less than that of **3** (802 Da), which confirmed the absence of a glucopyranose moiety and the presence of an additional hydroxyl group. In the gHMBC spectrum, the correlations of two anomer proton signals ($\delta_H$ 4.77, H-1‴; 5.84, H-1″) with one oxygenated olefin methine ($\delta_C$ 132.7, C-3) and one oxygenated methine ($\delta_C$ 82.5, C-2″), respectively, confirmed two glucoses: one at C-3 of 6-methoxyflavonol and the other at C-2″ of 3-O-*β*-D-glucopyranose. Taken together, the chemical structure of **4** was determined to be 6-methoxyquercetin 3-*O*-*β*-D-glucopyranosyl (1 → 2)-*β*-D-glucopyranoside.

Compound **8**, a yellowish gum (MeOH), showed IR absorbance bands of hydroxyl (3354 cm$^{-1}$) and aromatic groups (1626, 1594, 1514, 1473 cm$^{-1}$). The MF of **8**, $C_{32}H_{31}O_{16}$, was deduced from the MIP *m/z* 655.1660 $[M + H]^+$ (calcd. for $C_{32}H_{32}O_{16}$, 655.1663) in the positive HR-FAB/MS. The molecular weight of **8**, 645 Da, was 324 amu less than that of **2** (978 Da), which suggested the absence of a disaccharide composed of two hexoses. Also, the NMR spectra of **8** were very similar to those of **2**, except for the absence of *O*-*β*-D-glucopyranosyl-(1 → 2)-*β*-D-glucopyranosyl moieties [6]. In the gHMBC spectrum, two methoxy proton signals ($\delta_H$ 3.61 and 3.87) showed cross-peaks with two oxygenated olefin quaternary carbon signals ($\delta_C$ 132.1, C-6; 159.0, C-3‴″). In addition, cross-peaks were detected between an anomer proton signal of glucose ($\delta_H$ 5.10, H-1‴) and an oxygenated olefin quaternary ($\delta_C$ 159.0, C-7), as well as between the oxygenated

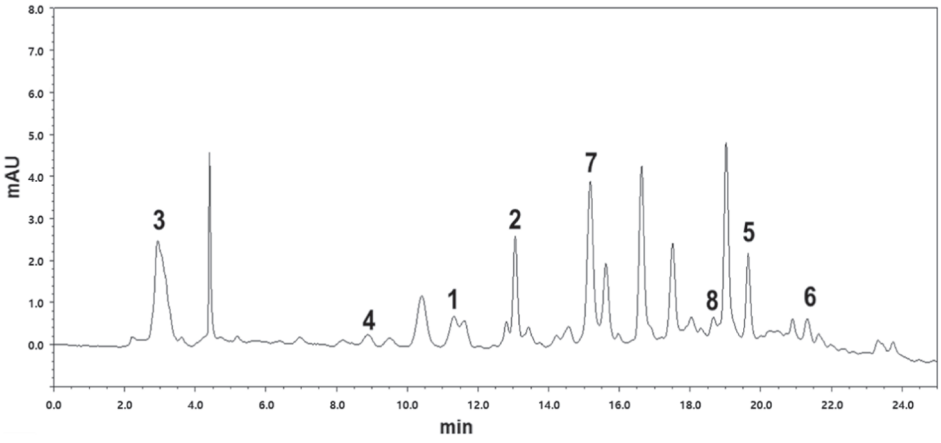



**Fig. 2.** HPLC chromatogram and contents of 6-methoxyflavonols **1–8** in 70% EtOH extracts from the aerial parts of *Tetragonia tetragonoides* (10,000 ppm). HPLC analysis was determined as described in the Materials and Methods section. Calibration curves for compounds **1–8** were made using six concentrations (31.25, 62.5, 125, 250, 500, and 1000 μg/mL). Analysis was achieved using a Waters 600S with Waters 2487 UV detector at 330 nm. The column was a Kinetex C18 column with the mobile phase consisted of 0.1% FA in $H_2O$ (solvent A) and acetonitrile (solvent B) eluted at a flow rate of 1.0 mL/min with the following gradient elution; 20% (0.01 min) → 30% (7 min) → 50% (15 min) → 50% (16 min) → 80% (37 min) → 100% (40 min) → 100% (45 min). Quantitative analysis was replicated three times. Chromatogram shown represents the best of three experiments.

| Compound | Retention time | Regression equation | $r^2$ | Contents (%)* |
|---|---|---|---|---|
| 1 | 11.33 | y=1154.10x+8842.50 | 1.000 | 0.11±0.01 |
| 2 | 13.05 | y=1818.50x–12534.00 | 0.999 | 0.16±0.01 |
| 3 | 2.94 | y=641.67x+15413.00 | 0.999 | 0.53±0.02 |
| 4 | 8.17 | y=260.39x-4079.70 | 0.998 | 0.20±0.03 |
| 5 | 19.65 | y=2164.50x-8709.20 | 1.000 | 0.12±0.01 |
| 6 | 21.32 | y=4880.90x+107981.00 | 0.996 | > 0.01±0.00 |
| 7 | 15.18 | y=2294.00x-3466.40 | 1.000 | 0.19±0.02 |
| 8 | 18.66 | y=581.68x+2905.90 | 0.998 | 0.02±0.00 |

* Contents of compounds (%) in 70% EtOH extract of the aerial parts of *Tetragonia tetragonoides*

methylene proton signal of the glucopyranosyl unit ($\delta_H$ 4.22, H-6‴) and the ester carbon signal of the ferulic acid ($\delta_C$ 167.4, C-9⁗). Taken together, the chemical structure of **8** was determined to be 6-methoxykaempferol 7-*O*-(6‴-(*E*)-feruloyl)-*β*-D-glucopyranoside. Compounds **2–4** and **8** were revealed to be new compounds and were named tetragonosides A-D, respectively.

### 3.2. Quantitative analysis of 6-methoxyflavonols in T. tetragonoides

The quantification of 6-methoxyflavonols in 80% aqueous MeOH extract was performed from the areas of the peak recorded at 330 nm compared with calibration curves obtained using standard solutions for the 6-methoxyflavonols under the analysis conditions described in the Materials and Methods. The correlation coefficient ($r^2$) in the regression curves of the compounds ranged from 0.996 to 1.000, indicating this method was reliable. 6-methoxyflavonols **1–8** were eluted at 11.33, 13.05, 2.94, 8.17, 19.65, 21.32, 15.18, and 18.66 min, respectively. The amounts of the 6-methoxyflavonols **1–8** in the 70% EtOH extract of *T. tetragonoides* were determined to be 0.11 ± 0.01, 0.16 ± 0.01, 0.53 ± 0.02, 0.20 ± 0.03, 0.12 ± 0.01, < 0.01 ± 0.00, 0.19 ± 0.02, and 0.02 ± 0.00%, respectively (Fig. 2).

### 3.3. DPPH and ABTS radical scavenging activities of 6-methoxyflavonols from T. tetragonoides

Inflammation has been associated with various chronic diseases such as liver disorders, atherosclerosis, cancer, arthritis, and diabetes mellitus [18]. ROS and oxygen free radicals in the inflammatory process have been reported to be the main factors in chronic diseases [20,30,31]. For this reason, suppression of ROS could be important to prevent chronic disease. Anti-oxidant effects of 6-methoxyflavonols **1–8** from *T. tetragonoides* were evaluated in DPPH and ABTS assays. Kaempferol (**9**) and quercetin (**10**) were used as positive controls. As shown in Table 3, all compounds showed radical scavenging activity except compounds **3** and **7** in the DPPH assay. In particular, compounds **1**, **5**, **6**, and **8–10** exhibited high radical scavenging activity, and compounds **5–7**, **9**, and **10** showed high ABTS radical scavenging activity. As shown in Table 3, flavonols with a catechol structure in the B ring (**5** and **10**) showed strong anti-oxidant activity compared to the *p*-substituted benzene ring (**6** and **9**) in both the DPPH and ABTS assays. In addition, as the number of glucoses increased, especially when glucopyranosyl moieties were located at C-3, the activity decreased. In general, the aglycones exhibited higher activity than the glycosides in both the DPPH and ABTS assays. In particular, flavonols with a methoxyl group in the C-6 position showed slightly weaker DPPH and ABTS radical scavenging activities than those without it. Also, 6-methoxyflavonols with a phenylpropanoid moiety at glu-6″ (**1**, **2**, **8**) showed higher radical scavenging activity than those without it (**3**, **7**) in the DPPH assay. However, the presence of phenylpropanoid moieties rarely affected ABTS radical scavenging activity. The previous literatures [32,33] also assured the correlation between chemical structure and DPPH and ABTS radical scavenge activity as described by authors.

### 3.4. Inhibitory effects of 6-methoxyflavonols from T. tetragonoides on the production of pro-inflammatory mediators and enzymes in LPS-stimulated RAW 264.7 macrophages

$PGE_2$ and NO are representative pro-inflammatory mediators, and are involved in various inflammatory diseases [34,35]. Also, IL-6, IL-1β, and TNF-α play key roles in promoting and triggering the inflammatory process in macrophages [35]. For this reason, suppression of pro-inflammatory mediators and cytokines plays an important role in regulating the immune system. The inhibitory effects of 6-methoxyflavonols **1–8**, kaempferol (**9**), quercetin (**10**), and butein (a positive control) on NO and $PGE_2$ production in RAW 264.7 macrophages were evaluated. Cells were pretreated with flavonols **1–10** 3 h before 24-h LPS treatment. As shown in Fig. 3, compounds **5**, **6**, **9**, and **10** highly inhibited NO production, while compounds **1–4**, **7**, and **8** showed moderate inhibition. Flavonol aglycones had a higher inhibition effect on NO production compared with the glycosides. The number of

**Table 3**
ABTS and DPPH radical scavenging activities of 6-methoxyflavonols **1–8** from the aerial parts of *Tetragonia tetragonoides*, kaempferol (**9**), and quercetin (**10**).

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| DPPH $IC_{50}$ (μM) | 22.7 ± 0.6 | 64.7 ± 1.0 | > 100 | 64.1 ± 1.0 | 15.4 ± 0.2 | 31.0 ± 0.5 | 92.3 ± 1.1 | 23.3 ± 0.4 | 16.3 ± 0.3 | 14.5 ± 0.1 |
| ABTS $IC_{50}$ (μM) | > 250 | 176.6 ± 9.5 | > 250 | 208.8 ± 8.7 | 13.7 ± 0.1 | 21.1 ± 0.1 | 31.6 ± 2.0 | 123.7 ± 6.9 | 10.5 ± 0.2 | 8.7 ± 0.1 |

glucose moieties, the presence of phenylpropanoid moieties, and the presence of a 6-methoxyl group rarely affected NO production in flavonol glycosides.

Compounds **4–7**, **9**, and **10** significantly inhibited $PGE_2$ production in LPS-stimulated RAW 264.7 macrophages in a dose-dependent manner. Compounds **1**, **2**, and **5** exhibited a slight inhibitory effect on $PGE_2$ production. As shown in Fig. 3., flavonols with a *p*-hydroxyl phenyl moiety in the B ring (**6** and **9**) more effectively suppressed $PGE_2$ production compared to those with a catechol structure (**5** and **10**). As the number of glucoses increased, $PGE_2$ production was slightly decreased. In general, the aglycones exhibited higher activity than the glycosides. However, glucopyranosyl moieties located at C-3 rarely affected its production. In addition, phenylpropanoids, especially those with the (*E*)-feruloyl moiety at glu-6″ (**1**, **2**, **8**), showed a weaker inhibitory effect on $PGE_2$ production compared to those without the phenylpropanoid moiety (**3**, **7**).

To confirm the effects of flavonols **1–10** on the production of pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α, cells were pre-treated with each compound or butein, and then stimulated with LPS. As shown in Fig. 4, compounds **5–10** significantly suppressed the levels of IL-6 and IL-1β, and compounds **3** and **4** also showed a slight inhibitory effect, while compounds **1** and **2** did not suppress the levels of IL-6 and IL-1β. These results showed that 6-methoxyflavonols with glucopyranosyl moieties at C-3 (**1–4**) rarely inhibited the levels of IL-6 and IL-1β. Also, compounds **5–10** suppressed the level of TNF-α and compounds **1–4** showed a slight inhibitory effect. Therefore, the 3-hydroxy group in the A ring of flavonols is the key structure in the inhibition of pro-inflammatory cytokines, while flavonol glycosides with glucose, phenylpropanoid, and 6-methoxy moieties rarely suppressed pro-inflammatory cytokines. The TNF-α level slightly recovered after treatment with compounds **1–4**, which had glucopyranosyl moieties at C-3. The previous literatures [32,36–40] also assured the correlation between chemical structure and inhibitory effects as pro-inflammatory mediators and enzymes described by authors.

### 3.5. Effects of 6-methoxyflavonols from T. tetragonoides on iNOS and COX-2 expression in LPS-stimulated RAW 264.7 macrophages

iNOS and COX-2 are involved in the synthesis of NO and $PGE_2$, respectively [41,42]. Their products, $PGE_2$ and NO, produce inflammatory symptoms with pain and fever, and excess NO causes various chronic diseases [34,35]. For this reason, suppression of iNOS and COX-2 is essential to control immune responses. The effects of 6-methoxyflavonols **1–8**, kaempferol (**9**), and quercetin (**10**), and butein (a positive control) were investigated on LPS-induced iNOS and COX-2 protein upregulation in RAW 264.7 cells. RAW 264.7 macrophages

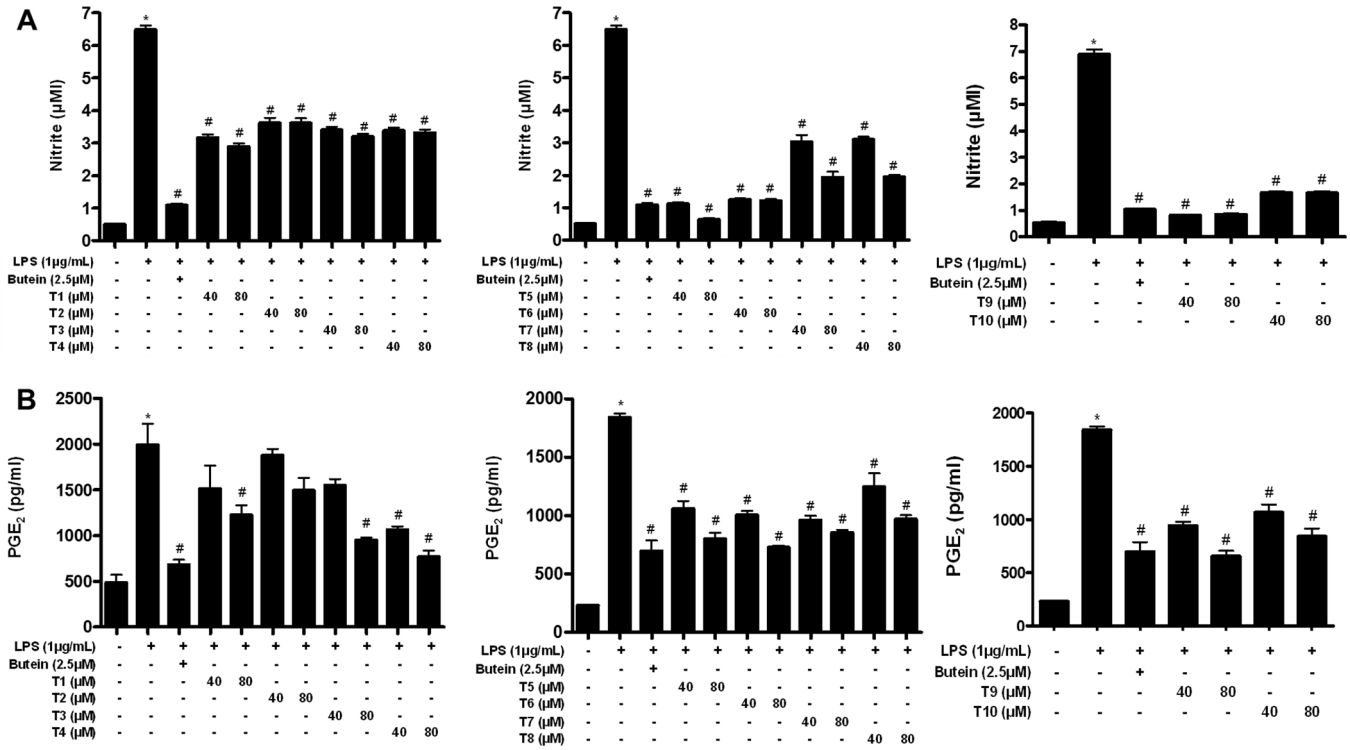



**Fig. 3.** Inhibitory effects of 6-methoxyflavonols **1–8** from the aerial parts of *Tetragonia tetragonoides*, kaempferol (**9**), and quercetin (**10**) on nitrite (**A**) and $PGE_2$ (**B**) production in LPS-induced RAW264.7 cells. The cells were pre-treated with 40 and 80 μM of each compounds for 12 h, and then stimulated with LPS (1 μg/mL) for 18 h. The production of nitrite and $PGE_2$ was determined as described in the Materials and Methods section. Data shown represent the mean ± SD of three expriments. $^{*}p < 0.05$ and $^{\#}p < 0.05$ as compared with the normal group and group treated with LPS alone, respectively.

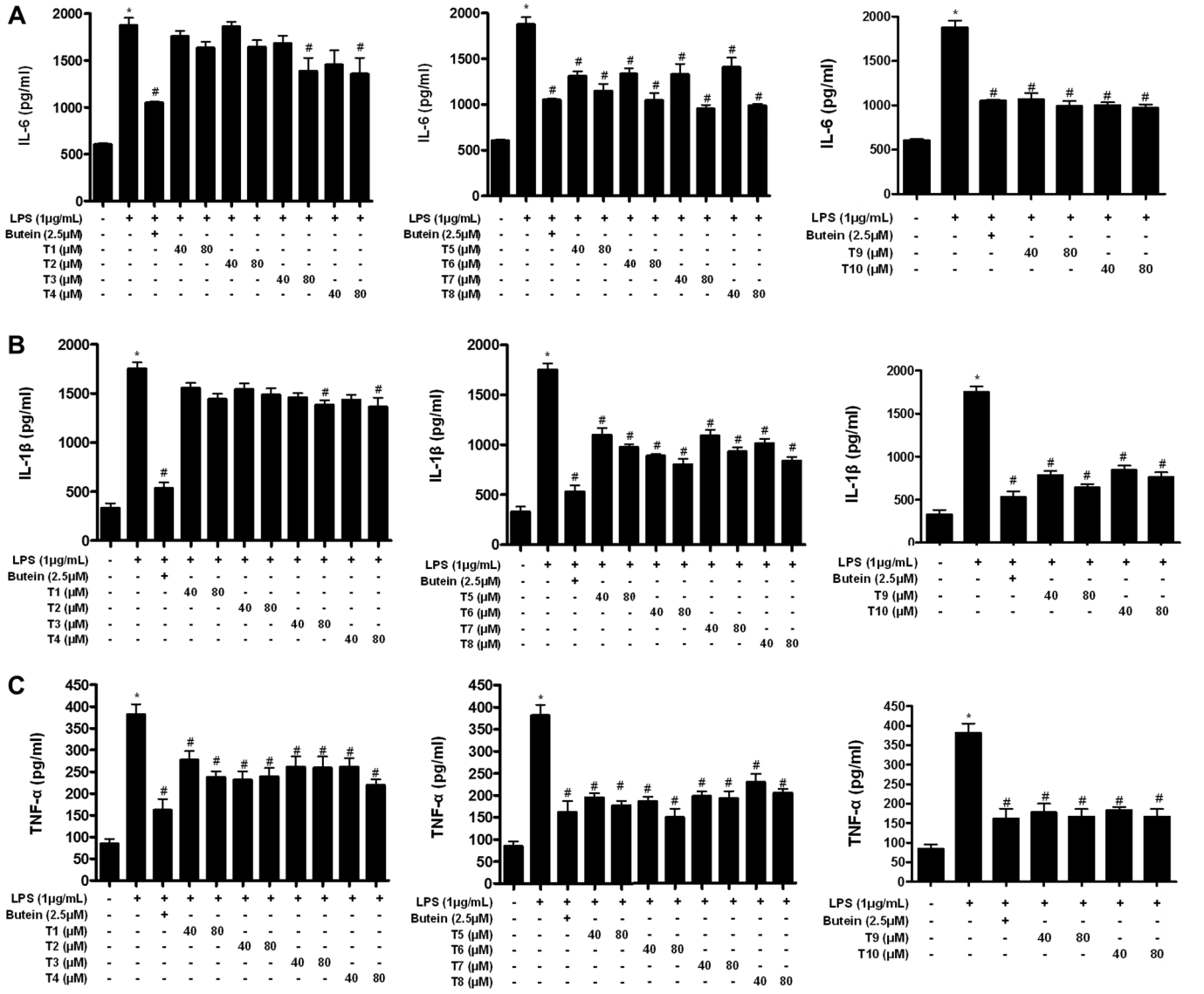



**Fig. 4.** Inhibitory effects of 6-methoxyflavonols **1**–**8** from the aerial parts of *Tetragonia tetragonoides*, kaempferol (**9**), and quercetin (**10**) on IL-6 (**A**), IL-1β (**B**), and TNF-α (**C**) productions in LPS-induced RAW264.7 cells. The cells were pre-treated with 40 and 80 μM of each compounds for 3 h, and then stimulated with LPS (1 μg/mL) for 24 h. The production of IL-6, IL-1β, and TNF-α was determined as described in the Materials and Methods section. Data shown represent the mean ± SD of three expriments. $^{*}p < 0.05$ and $^{\#}p < 0.05$ as compared with the normal group and group treated with LPS alone, respectively.

were treated with 20, 40, and 80 μM of flavonols **1**–**10** or with butein for 3 h prior to LPS treatment for 24 h, and the expression of iNOS and COX-2 were measured. As shown in Fig. 5, all compounds suppressed iNOS expression, and compounds **3**–**10** also strongly suppressed COX-2 expression. Compounds **1** and **2**, which had three glucopyranosyl and phenylpropanoid moieties, barely suppressed iNOS expression. In general, the flavonol aglycones (**5**, **6**, **9**, and **10**) exhibited higher activity than the glycosides. In addition, flavonols with a catechol structure in the B ring and a methoxyl group in the C-6 position more strongly suppressed COX-2 expression compared with flavonols without them. The previous literatures [32,36–39] also assured the correlation between chemical structure and inhibitory effects iNOS and COX-2 expression described by authors.

## 4. Conclusion

In conclusion, eight 6-methoxyflavonols, including four newly discovered types, were isolated from the aerial parts of *T. tetragonoides*. The NMR, IR, and FAB/MS data led to the determination of the chemical structures for four new 6-methoxyflavonol glycosides, tetragonosides A-D (**2**–**4**, **8**), and four known ones, 6-methoxykaempferol 3-*O*-*β*-D-glucopyranosyl-(1 → 2)-*β*-D-glucopyranosyl-7-*O*-(6‴′-(*E*)-caffeoyl)-*β*-D-glucopyranoside (**1**), 6-methoxyquercetin (**5**), 6-methoxykaempferol (**6**), and 6-methoxykaempferol 7-*O*-*β*-D-glucopyranoside (**7**). Compounds **2**–**8** were isolated for the first time from *T. tetragonoides* in this study (Fig. 1). Quantitative analysis of 6-methoxyflavonols in the aerial parts of *T. tetragonoides* extract was also conducted through HPLC. The contents of the 6-methoxyflavonols in the extracts of *T. tetragonoides* were determined to be 15.305 ± 0.041 mg/g (Fig. 2), indicating that 6-methoxyflavonols are major components of this plant. Most compounds showed significant anti-oxidant (DPPH and ABTS radical scavenging activities) and anti-inflammatory (inhibition effect of NO, $PGE_2$, IL-6, IL-1β, and TNF-α production and iNOS and COX-2 expression) activities. Further studies are needed to demonstrate that 6-methoxyflavonols from the aerial parts of *T. tetragonoides* can treat chronic diseases caused by inflammation.

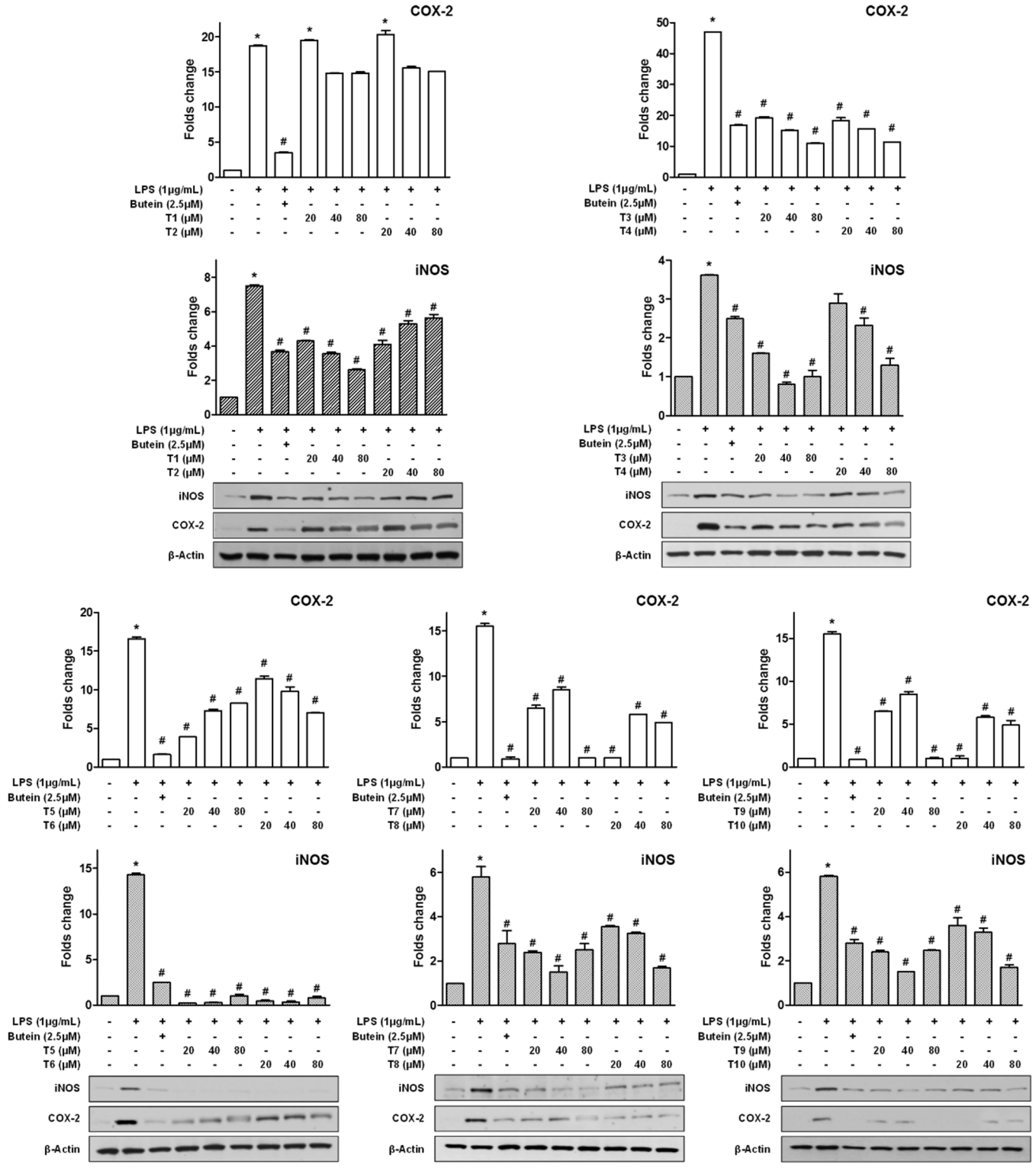



**Fig. 5.** Effects of 6-methoxyflavonols **1–8** from the aerial parts of *Tetragonia tetragonoides*, kaempferol (**9**), and quercetin (**10**) on iNOS and COX-2 protein expression in LPS-induced RAW264.7 cells. The cells were pre-treated with 20, 40, and 80 μM of each compounds for 3 h, and then stimulated with LPS (1 μg/mL) for 24 h. Western blot analysis was performed as described in the Materials and Methods section. Data shown represent the mean ± SD of three expriments. $^{*}p < 0.05$ and $^{\#}p < 0.05$ as compared with the normal group and group treated with LPS alone, respectively.

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## Appendix A. Supplementary material

Spectroscopic data ($^{1}$H and $^{13}$C NMR, FAB/MS, and IR) of 6-

methoxyflavonols **1–8** are available as supporting information. Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.102922.

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