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Research article

Glycosyl glycerides from hydroponic *Panax ginseng* inhibited NO production in lipopolysaccharide-stimulated RAW264.7 cells

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ABSTRACT

Background: Although the aerial parts of hydroponic *Panax ginseng* are reported to contain higher contents of total ginsenosides than those of roots, the isolation and identification of active metabolites from the aerial parts of hydroponic *P. ginseng* have not been carried out so far.
Methods: The aerial parts of hydroponic *P. ginseng* were applied on repeated silica gel and octadecylsilane columns to yield four glycosyl glycerides (Compounds **1**–**4**), which were identified based on nuclear magnetic resonance, infrared, fast atom bombardment mass spectrometry, and gas chromatography/mass spectrometry data. Compounds **1**–**4** were evaluated for inhibition activity on NO production in lipopolysaccharide (LPS)-stimulated RAW264.7 cells.
Results and conclusion: The glycosyl glycerides were identified to be (2*S*)-1-*O*-7(*Z*),10(*Z*),13(*Z*)-hexadecatrienoyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol (**1**), (2*S*)-1-*O*-linolenoyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol (**2**), (2*S*)-1-*O*-linolenoyl-2-*O*-linolenoyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol (**3**), and 2(*S*)-1-*O*-linoleoyl-2-*O*-linoleoyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol (**4**). Compounds **1** and **2** showed moderate inhibition activity on NO production in LPS-stimulated RAW264.7 cells [half maximal inhibitory concentration (IC_{50}): 63.8 ± 6.4μM and 59.4 ± 6.8μM, respectively] without cytotoxicity at concentrations < 100μM, whereas Compounds **3** and **4** showed good inhibition effect (IC_{50}: 7.7 ± 0.6μM and 8.0 ± 0.9μM, respectively) without cytotoxicity at concentrations < 20μM. All isolated compounds showed reduced messenger RNA (mRNA) expression of interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α in LPS-induced macrophage cells with strong inhibition of mRNA activity observed for Compounds **3** and **4**.

1. Introduction

Panax ginseng Meyer is a famous traditional medicinal plant belonging to the Araliaceae family. The genus name *Panax* originates from the word *panacea*, which means "a remedy for all diseases." The 4–6-year-old roots of this perennial herbaceous plant are mainly used for medicinal purposes. *P. ginseng* leaves are palmate, and the flowers bloom in June. Ginseng has primarily been cultivated in the forest areas of East Asia including Korea, China, Russia, and Japan. Traditionally, *P. ginseng* is cultivated in soil, and numerous pharmacological and phytochemical studies of the extracts or compounds from soil-grown plants were conducted. *P. ginseng* contains ginsenosides, polyacetylenes, sugars, and some essential oils [1,2] used for enhancement of immunocompetence, nutritional fortification, improvement of liver function, and their anticancer, antioxidant, and antidiabetic effects [3–7]. More than 70 kinds of saponins have been isolated from *P. ginseng*. There is a growing interest in using safe, high-quality agricultural products, leading to hydroponic cultivation of ginseng using high-tech culture facilities. Hydroponic cultivation of ginseng takes much less

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time than soil cultivation and is accomplished in just 3–4 months in a moisture-, light-, and temperature-controlled environment without pesticide treatment. Hydroponically cultivated ginseng is mainly used in fresh and high-quality ginseng products [8,9]. The aerial parts of hydroponic *P. ginseng* are reported to contain higher contents of total ginsenosides than the roots [10]. This study was initiated to isolate active metabolites from the aerial parts of hydroponic *P. ginseng*. Of note, glycosyl glycerides have never been isolated from hydroponic *P. ginseng*. Therefore, this study is designed to isolate and identify glycosyl glycerides as well to evaluate their potential for inhibition of NO production. Monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) are commonly present in the chloroplast membrane of ginseng. The MGDG and DGDG constitute up to about 70% of chloroplast lipids. Some glycosyl glycerides were isolated from the soil-cultivated ginseng [11]. The galactolipids play roles in the photosynthesis and regulation of lipid biosynthesis during phosphate deprivation. Furthermore, glycosyl glycerides were reported to have antifilarial, anticancer, antitumor [12–14], and many anti-inflammatory [15–17] activities. Therefore, this study describes the procedure for isolation and identification of four glycosyl glycerides (Compounds **1**–**4**) from the hydroponic *P. ginseng*, and evaluation of their anti-inflammatory activities on NO production in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells.

2. Materials and methods

2.1. Aerial parts of hydroponic P. ginseng

Aerial parts of hydroponic *P. ginseng* cultivated for 4 months in an aeroponic system were obtained from the Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science, RDA, Eumseong, Korea.

2.2. Reagents and instruments

Kieselgel 60 and LiChroprep RP-18 resins were used for column chromatography (Merck, Darmstadt, Germany). Kieselgel 60 F_{254} (Merck) and RP-18 F_{254S} (Merck) were used as solid phases for TLC experiment. Spots on the TLC plate were detected by observing the plates under a UV lamp (Spectroline, model ENF-240 C/F; Spectronics Corp., New York, NY, USA) or by spraying 10% aqueous H_2SO_4 on the developed plate followed by heating. Optical rotations were measured using a JASCO P-1010 digital polarimeter (Jasco, Tokyo, Japan). A Shimadzu GCMS-QP2010 Plus (Shimadzu, Tokyo, Japan) mass spectrometer (MS) was used for gas chromatography (GC)/MS experiments. Fast atom bombardment (FAB)/MS spectrum was recorded on a spectrometer (JMS-700; JEOL, Tokyo, Japan). IR spectra were obtained from a PerkinElmer spectrum one Fourier transform-IR spectrometer (PerkinElmer, Buckinghamshire, UK). NMR spectra were recorded on a Varian Inova AS 400 spectrometer (400 MHz; Varian, Palo Alto, CA, USA).

2.3. Isolation of glycosyl glycerides

The dried and powdered aerial parts of hydroponic *P. ginseng* (6.27 kg) were extracted with 80% MeOH (30 L × 3) at room temperature for 24 h. The extracts were filtered through a filter paper and evaporated under reduced pressure at 45°C to yield 1.4 kg of extract. The extract was poured into H_2O (3 L) and then extracted with ethyl acetate (EtOAc; 3 L × 3) and *n*-butanol (*n*-BuOH; 2.6 L × 3) successively. Each layer was concentrated under reduced pressure to obtain EtOAc (75 g), *n*-BuOH (470 g), and H_2O (855 g) fractions. The EtOAc fraction (75 g) was applied on a silica gel column (φ 14 × 16 cm) and eluted with $CHCl_3$–MeOH (30:1, 60 L) and $CHCl_3$–MeOH–H_2O (15:3:1, 136 L) to obtain 24 fractions (HPE1 to HPE24). Fraction HPE9 (9.28 g; *Ve/Vt* = 0.10–0.16, where *Ve* refers to the volume of eluent for the corresponding fraction and *Vt* represents the total elution volume) was applied on a silica gel column (φ 7 × 15 cm) using *n*-hexane–EtOAc (1:2, 28 L) as eluent to obtain 13 fractions (HPE9-1 to HPE-9-13). Fraction HPE9-10 (4.47 g, *Ve/Vt* = 0.24–0.98) was further fractionated on an octadecyl silica gel (octadecylsilane or ODS) column (φ 4.5 × 5 cm, MeOH–H_2O = 15:1, 4 L) to produce nine fractions (HPE9-10-1 to HPE9-10-9) including 2(*S*)-1-*O*-linoleoyl-2-*O*-linoleoyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol [**4**, HPE9-10-4, 141.6 mg; *Ve/Vt* = 0.24–0.29, TLC R_f = 0.25 (RP-18 F_{254S}, MeOH–H_2O = 50:1), R_f = 0.50 (Kieselgel 60 F_{254}, *n*-hexane–EtOAc = 1:30)]. Fraction HPE9-10-2 (3.14 g, *Ve/Vt* = 0.06–0.14) was further fractionated on the ODS column (φ 4 × 6 cm, acetone–acetonitrile–H_2O = 2:2:1, 3.6 L) to yield 10 fractions (HPE9-10-2-1–HPE9-10-2-10) including (2*S*)-1-*O*-linolenoyl-2-*O*-linolenoyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol [**3**, HPE9-10-2-9, 446.0 mg, *Ve/Vt* = 0.38–0.55, TLC R_f = 0.50 (RP-18 F_{254S}, acetone–acetonitrile–H_2O = 7:3:1), R_f = 0.55 (Kieselgel 60 F_{254}, CH_2Cl_2–MeOH = 10:1)]. Fraction HPE15 (5.49 g, *Ve/Vt* = 0.34–0.36) was further fractionated on the ODS column [φ 4.5 × 12 cm, MeOH–H_2O = 3:2 (1.0 L) → 2:1 (2.5 L) → 3:1 (5.2 L) → 5:1 (2.0 L)] to yield 25 fractions (HPE15-1–HPE15-25). Fraction HPE15-12 (135.2 mg, *Ve/Vt* = 0.34–0.40) was further fractionated on the ODS column (φ 2.5 × 7 cm, MeOH–H_2O = 3:1, 800 mL) to yield eight fractions (HPE15-12-1 to HPE15-12-8) including (2*S*)-1-*O*-7(*Z*),10(*Z*),13(*Z*)-hexadecatrienoyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol [**1**, HPE-15-12-6, 29.4 mg, *Ve/Vt* = 0.14–0.28, TLC R_f = 0.30 (RP-18 F_{254S}, MeOH–H_2O = 4:1), R_f = 0.50 (Kieselgel 60 F_{254}, $CHCl_3$–MeOH–H_2O = 10:3:1)]. Fraction HPE15-18 (142.7 mg, *Ve/Vt* = 0.50–0.58) was further fractionated on the ODS column (φ 3 × 8 cm, MeOH–H_2O = 4:1, 900 mL) to yield seven fractions (HPE15-18-1–HPE15-18-7) including (2*S*)-1-*O*-linolenoyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol [**2**, HPE15-18-5, 34.5 mg, *Ve/Vt* = 0.46–0.59, TLC R_f = 0.50 (RP-18 F_{254S}, MeOH–H_2O = 6:1), R_f = 0.40 (Kieselgel 60 F_{254}, $CHCl_3$–MeOH–H_2O = 10:3:1)].

(2*S*)-1-*O*-7(*Z*),10(*Z*),13(*Z*)-hexadecatrienoyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol (panaxcerol A, **1**): pale yellow wax; IR (CaF_2, cm^{-1}) 3,386, 2,932, 1,732, 1,610; positive FAB/MS *m/z* 487 $[M+H]^+$ for $C_{25}H_{43}O_9$; $[\alpha]_D$ −2.22° (*c* = 0.35, MeOH); ^{1}H-NMR (400 MHz, pyridine-d_5, δ_H) 5.37–5.46 (6H, m, overlapped, H-7″, 8″, 10″, 11″, 13″, 14″), 4.83 (1H, d, *J* = 7.6 Hz, H-1′), 4.46–4.50 (3H, br s, overlapped, H-1, 4′), 4.40–4.44 (2H, overlapped, H-2, 2′), 4.36 (2H, overlapped, H-6′a, H-6′b), 4.31 (1H, overlapped, H-3a), 4.11 (1H, br d, *J* = 9.6 Hz, H-3′), 4.05 (1H, overlapped, H-3b), 4.02 (1H, br. dd, *J* = 6.4, 6.0 Hz, H-5′), 2.87 (4H, br s, overlapped, H-9″, 12″), 2.27 (2H, t, *J* = 7.6 Hz, H-2″), 2.02 (2H, m, H-15″), 1.58 (2H, m, H-3″), 1.26 (4H, br s, H-4″, 5″), 0.88 (3H, t, *J* = 7.6 Hz, H-16″); ^{13}C-NMR (100 MHz, pyridine-d_5, δ_C) 173.4 (C-1″), 127.5, 128.2, 128.5, 128.6, 130.1, 132.1 (C-7″, 8″, 10″, 11″, 13″, 14″), 105.8 (C-1′), 77.0 (C-5′), 75.2 (C-3′), 72.5 (C-2′), 72.2 (C-3), 70.1 (C-4′), 69.0 (C-2), 66.5 (C-1), 62.3 (C-6′), 34.2 (C-2″), 29.4 (C-6″), 28.9 (C-5″), 27.2 (C-4″), 25.9 (C-12″), 25.9 (C-9″), 25.0 (C-3″), 20.7 (C-15″), and 14.3 (C-16″).

(2*S*)-1-*O*-linolenoyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol (panaxcerol B, **2**): pale yellow wax; IR (CaF_2, cm^{-1}) 3,364, 2,931, 1,730, 1,585; positive FAB/MS *m/z* 515 $[M+H]^+$ for $C_{27}H_{47}O_9$; $[\alpha]_D$ +3.89° (*c* = 0.38, MeOH); ^{1}H-NMR (400 MHz, pyridine-d_5, δ_H) 5.39–5.46 (6H, m, overlapped, H-9″, 10″, 12″, 13″, 15″, 16″), 4.82 (1H, d, *J* = 7.6 Hz, H-1′), 4.51 (2H, d, *J* = 6.0 Hz, H-1), 4.50 (1H, overlapped, H-4′), 4.43 (1H, m, H-2), 4.42 (1H, overlapped, H-6′a), 4.36 (1H, overlapped, H-6′b), 4.33 (1H, dd, *J* = 10.0, 5.2 Hz, H-3a), 4.11 (1H, dd, *J* = 9.6, 3.2 Hz, H-3′), 4.06 (1H, dd, *J* = 10.0, 3.6 Hz, H-3b), 4.05 (1H, overlapped, H-5′), 2.86–2.89 (4H, m, overlapped, H-11″, 14″), 2.28 (2H, t, *J* = 7.6 Hz, H-2″), 2.03–2.06 (4H, m, overlapped, H-8″, 17″), 1.54–1.57 (4H, m, overlapped, H-3″, 4″), 1.04–1.27 (6H, m, overlapped, H-5″, 6″, 7″),

0.90 (3H, t, J = 7.6 Hz, H-18″); ^{13}C-NMR (100 MHz, pyridine-d_5, δ_C) 173.5 (C-1″), 127.5, 128.0, 128.6, 128.6, 130.5, 132.0 (C-9″, 10″, 12″, 13″, 15″, 16), 105.8 (C-1′), 77.0 (C-5′), 75.2 (C-3′), 72.5 (C-2′), 72.1 (C-3), 70.1 (C-2), 69.0 (C-4′), 66.5 (C-1), 62.3 (C-6′), 34.2 (C-2″), 29.8 (C-4″), 29.3 (C-5″), 29.2 (C-6″), 29.2 (C-7″), 27.4 (C-8″), 25.9 (C-14″), 25.8 (C-11″), 25.1 (C-3″), 20.8 (C-17″), and 14.5 (C-18″).

(2*S*)-1-*O*-linolenoyl-2-*O*-linolenoyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol (panaxcerol C, **3**): pale yellow wax; IR (CaF_2, cm^{-1}) 3,399, 2,929, 1,737, 1,590; positive FAB/MS *m/z* 775 $[M+H]^+$ for $C_{45}H_{75}O_{10}$; $[\alpha]_D$ +11.6° (c = 0.34, MeOH); ^{1}H-NMR (400 MHz, pyridine-d_5, δ_H) 5.61 (1H, m, H-2), 5.37–5.49 (12H, m, overlapped, H-9″, 9‴, 10″, 10‴, 12″, 12, 13″, 13‴, 15″, 15‴, 16″, 16‴), 4.75 (1H, d, J = 7.6 Hz, H-1′), 4.64 (1H, dd, J = 11.6, 2.0, H-1a), 4.45–4.50 (2H, m, overlapped, H-1b, 4′), 4.37 (3H, m, H-2′, 6′), 4.07 (1H, overlapped, H-3′), 4.03 (1H, overlapped, H-3), 3.99 (1H, dt, J = 11.6, 6.0 Hz, H-5′), 2.88 (8H, overlapped, H-11″, 11‴, 14″, 14‴), 2.32 (4H, t, J = 6.8 Hz, overlapped, H-2″, 2‴), 2.05 (8H, m, overlapped, H-8″, 8‴, 17″, 17‴), 1.61 (4H, m, overlapped, H-3″, 3‴), 1.30 (4H, m, overlapped, H-4″, 4‴), 1.23 (12H, m, overlapped, H-5″, 5‴, 6″, 6‴, 7″, 7‴), 0.92 (6H, t, J = 7.6 Hz, H-18″, 18‴); ^{13}C-NMR (100 MHz, pyridine-d_5, δ_C) 172.6, 172.8 (C-1″, C-1‴), 127.2 × 2, 127.7 × 2, 128.2 × 2, 128.2 × 2, 130.2 × 2, 131.7 × 2 (C-9″, 9‴, 10″, 10‴, 12″, 12, 13″, 13‴, 15″, 15‴, 16″, 16‴), 105.2 (C-1′), 76.7 (C-5′), 74.8 (C-3′), 71.9 (C-2′), 70.6 (C-2), 69.7 (C-4′), 67.7 (C-3), 62.9 (C-1), 61.9 (C-6′), 34.4 (C-2‴), 34.1 (C-2″), 29.8 × 2 (C-4″, 4‴), 29.4 × 2 (C-7″, 7‴), 29.3 × 2 (C-6″, 6‴), 29.2 × 2 (C-5″, 5‴), 27.4 × 2 (C-8″, 8‴), 25.9 × 2 (C-14″, 14‴), 25.8 × 2 (C-11″, 11‴), 25.1 × 2 (C-3″, 3‴), 20.7 × 2 (C-17″, 17‴), and 14.3 × 2 (C-18″, 18‴).

2(*S*)-1-*O*-linoleoyl-2-*O*-linoleoyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol (panaxcerol D, **4**): pale yellow wax; IR (CaF_2, cm^{-1}) 3,417, 2,927, 1,736, 1,595; positive FAB/MS *m/z* 779 $[M+H]^+$ for $C_{45}H_{79}O_{10}$; $[\alpha]_D$ +0.70° (c = 0.40, MeOH); ^{1}H-NMR (400 MHz, pyridine-d_5, δ_H) 5.60 (1H, m, H-2), 5.40–5.50 (8H, m, overlapped, H-9″, 9‴, 10″, 10‴, 12″, 12, 13″, 13‴), 4.77 (1H, d, J = 7.6 Hz, H-1′), 4.65 (1H, dd, J = 12.0, 3.2 Hz, H-1a), 4.46–4.51 (2H, overlapped, H-1b, 4′), 4.38 (3H, overlapped, H-2′, 6′), 4.31 (1H, dd, J = 10.8, 5.2 Hz, H-3a), 4.08 (1H, dd, J = 9.6, 3.2 Hz, H-3′), 4.05 (1H, dd, J = 10.8, 5.6 Hz, H-3b), 4.00 (1H, m, overlapped, H-5′), 2.90 (4H, m, overlapped, H-11″, 11‴), 2.30 (4H, t, J = 7.2 Hz, H-2″, 2‴), 2.08 (8H, m, overlapped, H-8″, 8‴, 14″, 14‴), 1.62 (4H, m, H-3″, 3‴), 1.24–1.35 (28H, m, overlapped, H-4″,4‴, 5″, 5‴, 6″, 6‴, 7″, 7‴, 15″, 15‴, 16″, 16‴, 17″,17‴), 0.92 (3H, t, J = 7.6, H-18″), 0.84 (3H, t, J = 6.8, H-18‴); ^{13}C-NMR (100 MHz, pyridine-d_5, δ_C) 173.1, 173.2 (C-1″, C-1‴), 128.4 × 2, 128.7 × 2, 130.4 × 2, 130.5 × 2 (C-9″, 9‴, 10″, 10‴, 12″, 12, 13″, 13‴), 105.7 (C-1′), 77.1 (C-5′), 75.3 (C-3′), 72.3 (C-2′), 72.1 (C-2), 70.1 (C-4′), 68.1 (C-3), 63.3 (C-1), 62.3 (C-6′), 34.5 (C-2‴), 34.2 (C-2″), 31.7 × 2 (C-16″, 16‴), 29.9 × 2 (C-15″, 15‴), 29.9 × 2 (C-8″, 8‴), 29.6 × 2 (C-4″, 4‴), 29.5 × 2 (C-14″, 14‴), 29.4 × 2 (C-7″, 7‴), 29.3 × 2 (C-6″, 6‴), 29.3 × 2 (C-5″, 5‴), 26.1 × 2 (C-11″, 11‴), 25.2 × 2 (C-3″, 3‴), 22.8 × 2 (C-17″, 17‴), and 14.2 × 2 (C-18″, 18‴).

2.4. Alkaline hydrolysis and methylation of compounds 1–4

Each compound (1.5 mg) was dissolved in 0.5 mL of 1M KOH/MeOH and heated at 80°C in a heating block for 30 min. After cooling in an icebox, 0.5 mL of 14% methanol BF_3 (Aldrich Chemistry, St. Louis, MO, USA) was added and the mixture was heated at 80°C in the heating block for 30 min. After cooling, 1 mL of *n*-hexane high performance liquid chromatography (HPLC) grade] and 0.5 mL of H_2O (HPLC grade) were added to the reaction mixture. The supernatants were collected and a small amount of Na_2SO_4 was added to remove the H_2O. The solutions were filtered using a syringe filter (0.2 μm, 13 mm) and stored at −4°C until GC/MS analysis.

2.5. GC/MS analysis

A DB-5 column (0.25-μm film thickness × 0.25 mm diameter × 30 m length) was used for the GC/MS experiment. Helium was used as the carrier gas at a flow rate of 23.3 mL/min. The oven temperature was programmed as follows: 80°C for 2 min, increased to 320°C at a rate of 15°C/min and held for 10 min. The injector and detector temperatures were set at 280°C and 250°C, respectively. Sample solutions (1 μL) were injected into the GC column with a 10:1 split ratio. Detection was performed by electron ionization (70 eV) and quadrupole mass spectrometry. Fatty acids were identified by comparing their mass spectra with those of a library (Wiley Library, version 2008; John Wiley & Sons Inc., Hoboken, NJ, USA).

2.6. Cell culture

Murine macrophage RAW264.7 cells (Korea Cell Line Bank, Seoul, Korea) were cultured at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2mM glutamate, 100 units/mL of penicillin, and 100 μg/mL of streptomycin (WelGENE Inc., Seoul, Korea) in a humidified incubator with 5% CO_2.

2.7. Measurements of nitric oxide

The amount of NO was calculated by measuring the amount of nitrite, an oxidized product, in the cell culture supernatants as previously explained [18]. RAW264.7 cells were seeded in 96-well cell culture plates at a density of 1 × 10^4 cells/well and incubated for 12–18 h. After discarding the growth medium, cells were stimulated with 1 μg/mL LPS (Sigma-Aldrich Co., St. Louis, MO, USA) in the presence of various concentrations of each compound in a serum-free medium for 20 h. Next, 100 μL of cell culture supernatant was mixed with 100 μL of Griess reagent (Sigma-Aldrich Co.) in a new 96-well plate, followed by spectrophotometric measurement at 550 nm according to the manufacturer's instructions (BioTek Instruments, Inc., Winooski, VT, USA). Nitrite concentrations were determined by comparison with a sodium nitrite standard curve.

2.8. Viability of RAW264.7 macrophage cells

RAW264.7 cells were seeded in 96-well cell culture plates at a density of 1 × 10^4 cells/well and incubated for 12–18 h. After discarding the growth medium, cells were treated with various concentrations of each compound in a serum-free medium for 20 h. After treatment, 10 μL of 10 μg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich Co.) solution was added to each well (except for the blank well), and the sample was reincubated in an incubator (at 37°C, 5% CO_2) in darkness. After 30–60 min, culture supernatants were removed, and 100 μL dimethyl sulfoxide (Sigma-Aldrich Co.) was added to completely dissolve formazan crystals. The absorption was read at 550 nm with a spectrophotometer.

2.9. Reverse transcriptase-polymerase chain reaction analysis

RAW264.7 macrophages were treated with various concentrations of samples with 1 μg/mL of LPS for 24 h. Total RNA was prepared from RAW264.7 cells using a TRIzol Reagent kit (Invitrogen, Carlsbad, CA, USA). The total RNA (5 μg) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Thermo Scientific, Pittsburgh, PA, USA). The following primers were used for polymerase chain reaction amplification: interleukin-1β (IL-1β): 5′-TTC ACA GAG GAT ACC ACT CC-3′ (sense) and 5′-GAA GCT GTG GCA GCT ACC TAT GTC T-3′ (antisense); IL-6: 5′-

GAG GAT ACC ACT CCC AAC AG-3′ (sense) and 5′-TTC ACA GAG GAT ACC ACT CC-3′ (antisense); tumor necrosis factor-α (TNF-α): 5′-ATG AGC ACA GAA AGC ATG ATC-3′ (sense) and 5′-TAC AGG CTT GTC ACT CGA ATT-3′ (antisense); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5′-CGA CTT CAA CAG CAA CTC CCA CTC TTC C-3′ (sense) and 5′-TGG GTG GTC CAG GGT TTC TTA CTC CTT-3′ (antisense). GAPDH messenger RNA (mRNA) levels were used as internal controls.

2.10. Statistical analysis

Unless otherwise stated, all experiments were performed with triplicate samples and repeated at least three times. The data were presented as means ± standard deviation, and statistical comparisons between groups were performed using a one-way analysis of variance test followed by a Student *t* test using SigmaPlot software Ver.11 (San Joe, California, USA).

3. Results and discussion

Detection of Compound **1** (pale yellow wax) involved spraying the plate with 10% sulfuric acid followed by heating. Formation of a dark purple color confirms the presence of Compound **1**. The molecular weight was determined to be 486 from the molecule ion peak *m/z* 487 $[M+H]^+$ in the positive FAB/MS. Compound **1** showed absorbance bands due to the hydroxyl (3,386 cm^{-1}), carbonyl (1,732 cm^{-1}), and double bond (1,610 cm^{-1}) groups in the IR spectrum. The ^{1}H-NMR spectrum showed six olefinic proton signals at δ_H 5.37–5.46, a terminal methyl proton signal at δ_H 0.88, and several methylene proton signals at δ_H 1.20–2.87 due to an unsaturated fatty acid with three double bonds. A hemiacetal proton signal at δ_H 4.83 (d, $J = 7.6$ Hz) and several oxygenated methine and methylene proton signals at δ_H 4.00–4.50 were also observed as the signals of a sugar moiety. The proton signals of an oxygenated methine at δ_H 4.42 (H-2), and two oxygenated methylenes at δ_H 4.48 (H-1), δ_H 4.31 (H-3a), and δ_H 4.05 (H-3b) due to a glycerol moiety were also observed. Based on these results, Compound **1** was assumed to be a monoglycosyl monoglyceride. The ^{13}C-NMR spectrum showed the carbon signals of a hexose at δ_C 105.8, 77.0, 75.2, 72.5, 70.1, and 62.3, which were identified as those of a β-galactopyranose from the chemical shifts. In addition, an oxygenated methine carbon signal at δ_C 72.2 and two oxygenated methylene carbon signals at δ_C 69.0 and 66.5 were confirmed as the signals of a glycerol moiety. An ester carbonyl (δ_C 173.4), six olefin methine (δ_C 127.5, 128.2, 128.5, 128.6, 130.1 × 2, 132.1), a terminal methyl (δ_C 14.3), and eight methylene (δ_C 20.7, 25.0, 25.9, 25.9, 27.2, 28.9, 29.4, 34.2) carbon signals were observed indicating that Compound **1** contains a hexadecatrienoic acid as a fatty acid moiety. The fatty acid methyl ester obtained by alkaline hydrolysis, methyl esterification, and solvent fractionation appeared as a clear peak at 13′86″ on the GC/MS spectrum, which was identified as 7(*Z*),10(*Z*),13(*Z*)-hexadecatrienoic acid methyl ester by comparing the mass spectrum of the peak with that of the library (Wiley Library, version 2008). In the gradient heteronuclear multiple-bond correlation gHMBC (Gradient Heteronuclear Multiple Bond Correlation) spectrum, long-range correlations were observed between the oxygenated methylene proton signal of glycerol H-1 (δ_H 4.46) and the ester carbonyl carbon signal C-1″ (δ_C 173.4), and between the anomeric proton signal H-1 (δ_H 4.83) and the oxygenated methine carbon signal of glycerol C-3 (δ_C 72.2), indicating that the fatty acid and the galactose moieties were linked to the hydroxyls of C-1 and C-3 of glycerol, respectively. Therefore, the chemical structure of Compound **1** was determined to be (2*S*)-1-*O*-7(*Z*),10(*Z*),13(*Z*)-hexadecatrienoyl-3-*O*-β-D-galactopyranosyl-*sn*-glycerol, named as panaxcerol A (Fig. 1) [19].

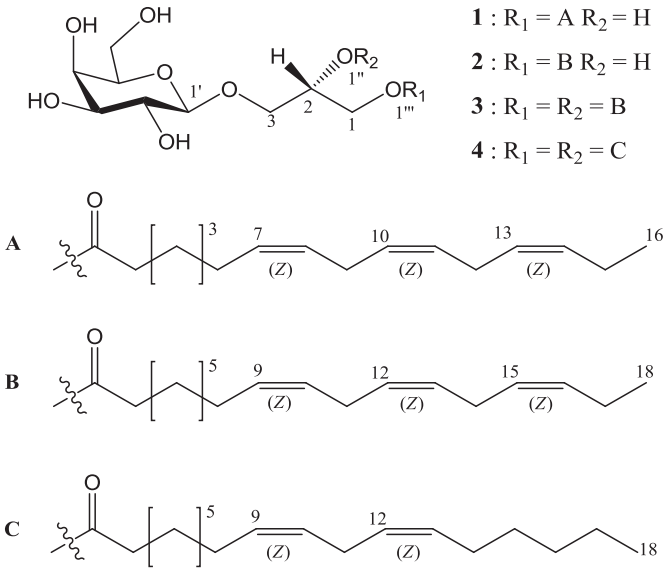

Fig. 1. Chemical structure of Compounds **1**–**4** isolated from the aerial parts of hydroponic *Panax ginseng*.

Detection of Compound **2** involved spraying the plate with 10% sulfuric acid followed by heating. Formation of a dark purple color confirms the presence of Compound **2**. The molecular weight was determined to be 514 $[M]^+$ from the molecule ion peak *m/z* 515 $[M+H]^+$ in the positive FAB/MS. Compound **2** showed absorbance bands due to the hydroxyl (3,364 cm^{-1}), carbonyl (1,730 cm^{-1}), and double bond (1,585 cm^{-1}) groups in the IR spectrum. The ^{1}H-NMR and ^{13}C NMR spectra of Compound **2** were very similar to that of Compound **1** with the exception of the number of methylene units. The ^{1}H-NMR showed six olefinic proton signals at δ_H 5.39–5.46, a terminal methyl proton signal at δ_H 0.90, and several methylene proton signals at δ_H 1.19–2.89 due to an unsaturated fatty acid with three double bonds. A hemiacetal proton signal at δ_H 4.82 (d, $J = 7.6$ Hz), several oxygenated methine and methylene proton signals at δ_H 4.01–4.52 due to a sugar moiety, an oxygenated methine proton signal at δ_H 4.43 (H-2), and two oxygenated methylene proton signals at δ_H 4.48 (H-1), δ_H 4.31 (H-3b), and δ_H 4.05 (H-3a) due to a glycerol moiety were observed. The ^{13}C-NMR spectrum showed hexose carbon signals at δ_C 105.8, 77.0, 75.2, 72.5. 69.0, and 62.3 owing to a β-galactopyranose derivative and three oxygenated carbon signals at δ_C 72.1, 70.1, and 66.5 of a glycerol, indicating that Compound **2** was a monogalactosyl monoacylglyceride. An ester carbonyl (δ_C 173.5), six olefin methine (δ_C 127.5, 128.0, 128.6, 128.6, 130.5 × 2, 132.0), a terminal methyl (δ_C 14.5), and 10 methylene (δ_C 20.8, 25.1, 25.8, 25.9, 27.4, 29.2, 29.2, 29.3, 29.8, 34.2) carbon signals were observed indicating that the fatty acid was an octadecatrienoic acid. The alkaline hydrolysis, methyl esterification, and solvent partition of Compound **2** gave the fatty acid methyl ester, which was identified to be 9(*Z*),12(*Z*),15(*Z*)-octadecatrienoic acid (methyl linolenoate, RT (Retention Time) = 15′20″) by GC/MS analysis. In the gHMBC spectrum, long-range correlations were observed between the oxygenated methylene proton signal of the glycerol H-1 (δ_H 4.51) and the ester carbonyl carbon signal C-1″ (δ_C 173.5), and between the anomeric proton signal H-1′ (δ_H 4.82) and the oxygenated methylene carbon signal of the glycerol C-3 (δ_C 72.1). These indicated that the fatty acid and the galactose moieties were connected to the hydroxyls of C-1 and C-3 of the glycerol, respectively. Consequently, Compound **2** was identified to be (2*S*)-1-*O*-9(*Z*),12(*Z*),15(*Z*)-octadecatrienoyl-3-*O*-β-D-galactopyranosyl-*sn*-glycerol, named as panaxcerol B (Fig. 1) [20].

Detection of Compound **3** involved spraying the plate with 10% sulfuric acid followed by heating. Formation of a dark brown color confirms the presence of Compound **3**. The molecular weight was determined to be 774 from the molecule ion peak *m/z* 775 $[M+H]^+$ in the positive FAB/MS. Compound **3** showed absorbance bands due to the hydroxyl (3,399 cm^{-1}), carbonyl (1,737 cm^{-1}), and double bond (1,590 cm^{-1}) groups in the IR spectrum. ^{1}H-NMR and ^{13}C-NMR spectra of Compound **3** were similar to those of Compound **2**, with the exception of the integration value of the fatty acid moiety. Two ester carbonyl (δ_C 172.6, 172.8), 12 olefin methine (δ_C 127.2 × 2, 127.7 × 2, 128.2 × 2, 128.2 × 2, 130.2 × 2, 131.7 × 2, δ_H 5.37–5.49), two terminal methyl (δ_C 14.3 × 2, δ_H 0.92), and 20 methylene (δ_C

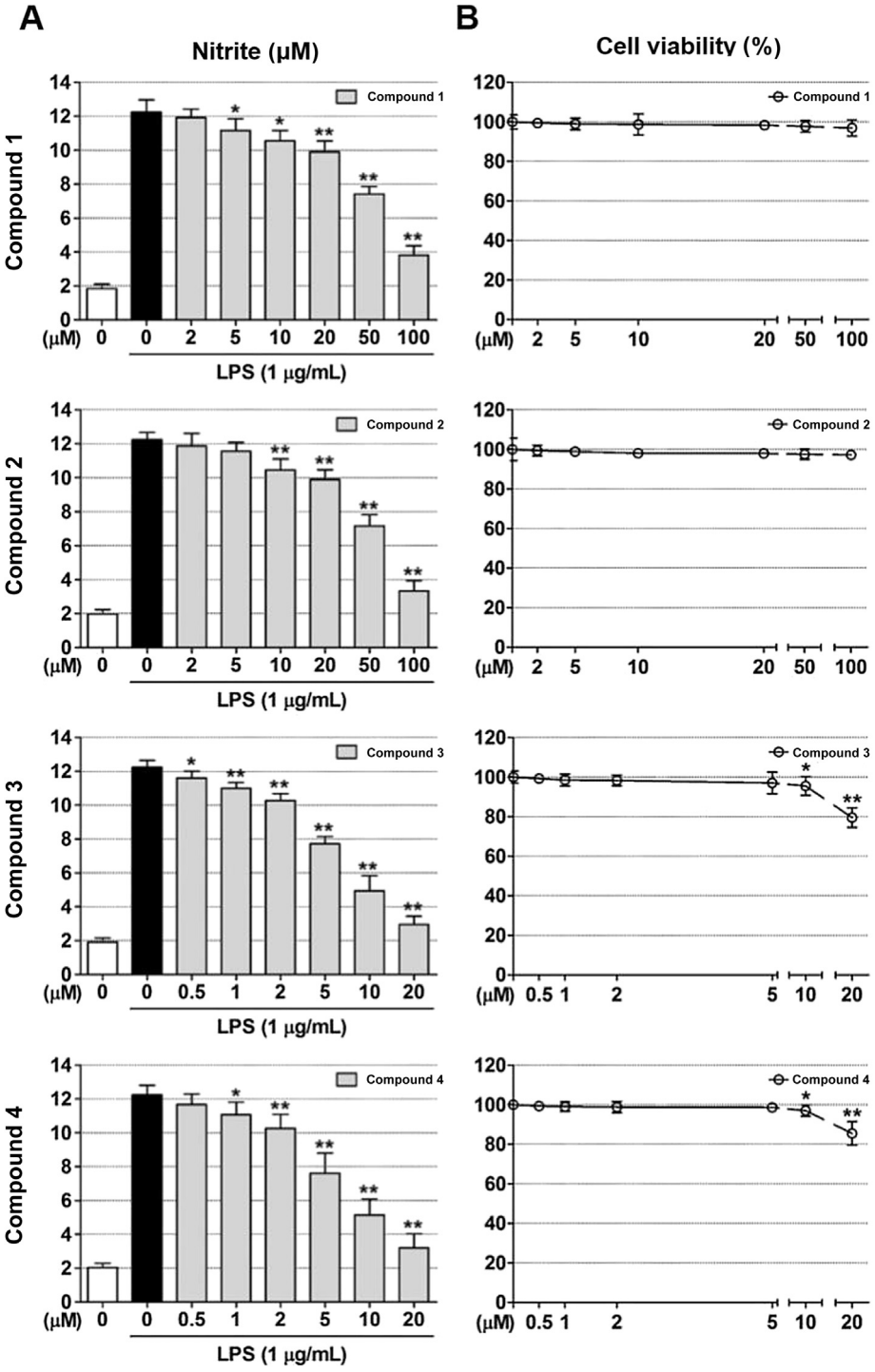

Fig. 2. Effects of Compounds **1**–**4** isolated from the aerial parts of hydroponic *Panax ginseng* on LPS-stimulated NO production and cell viability. (A) RAW264.7 cells were treated with various concentrations of each compound with 1 μg/mL of LPS for 20 h. Nitrite levels were measured in the culture media of LPS-stimulated cells by the Griess reaction. (B) Cell viability was measured by the MTT assay. Data are presented as mean ± SD of at least three independent experiments with triplicate samples (*$p < 0.01$ and **$p < 0.001$ vs. control). LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Table 1
Effect of IC_{50} values of compounds from hydroponic *Panax ginseng* on LPS-stimulated no production and cell viability

Compound	NO production and cell viability[1)]	
	IC_{50} (μM)	LD_{50} (μM)
1	63.8 ± 6.4	>100
2	59.4 ± 6.8	>100
3	7.7 ± 0.6	>20
4	8.0 ± 0.9	>20

Data are presented as mean ± SD ($n = 9$)
IC_{50}, concentrations inhibiting NO production by 50%; LD_{50} values, concentrations inhibiting cell growth by 50%; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
[1)] RAW264.7 cells were treated with various concentrations of each compound with 1 μg/mL of LPS for 20 h. Nitrite levels were measured in the culture media of LPS-stimulated cells by the Griess reaction. Cell viability was measured by the MTT assay

20.7 × 2, 25.1 × 2, 25.8 × 2, 25.9 × 2, 27.4 × 2, 29.2 × 2, 29.3 × 2, 29.4 × 2, 29.8 × 2, 34.1, 34.4, δ_H 1.15–2.91) carbon signals were observed, indicating Compound **3** to be a monogalactosyl diacylglyceride including two octadecatrienoic acids as fatty acid moieties. The observation of oxygenated methylene (δ_H 4.47, 4.64) and oxygenated methine (δ_H 5.61) proton signals in the lower magnetic field compared with those of Compound **2** confirmed Compound **3** to have two ester bonds at C-1 and C-2. In the gHMBC spectrum, glycerol proton signals from oxygenated methylenes, H-1a and 1b (δ_H 4.64, 4.47), and oxygenated methine, H-2 (δ_H 5.61), showed correlations with two ester carbonyl carbon C-1″ and C-1‴ (δ_C 172.6, 172.8) signals of fatty acids. The anomeric proton signal (δ_H 4.64) and the oxygenated methylene carbon signal (δ_C 67.7) of glycerol showed correlation with each other, indicating the two fatty acids and the galactose to be linked to the hydroxyls of C-1 (δ_C 62.9), C-2 (δ_C 70.6), and C-3 (δ_C 67.7) of glycerol, respectively. The fatty acid methyl ester obtained by chemical reaction was identified to be 9(*Z*),12(*Z*),15(*Z*)-octadecatrienoic acid methyl ester (methyl linolenoate, RT = 15′20″) by the GC/MS analysis. Based on the aforementioned data, Compound **3** was identified as a monogalactosyl diacylglyceride, (2*S*)-1-*O*-linolenoyl-2-*O*-linolenoyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol, and the compound was named panaxcerol C (Fig. 1) [21].

Detection of Compound **4** involved spraying the plate with 10% sulfuric acid followed by heating. Formation of a dark brown color

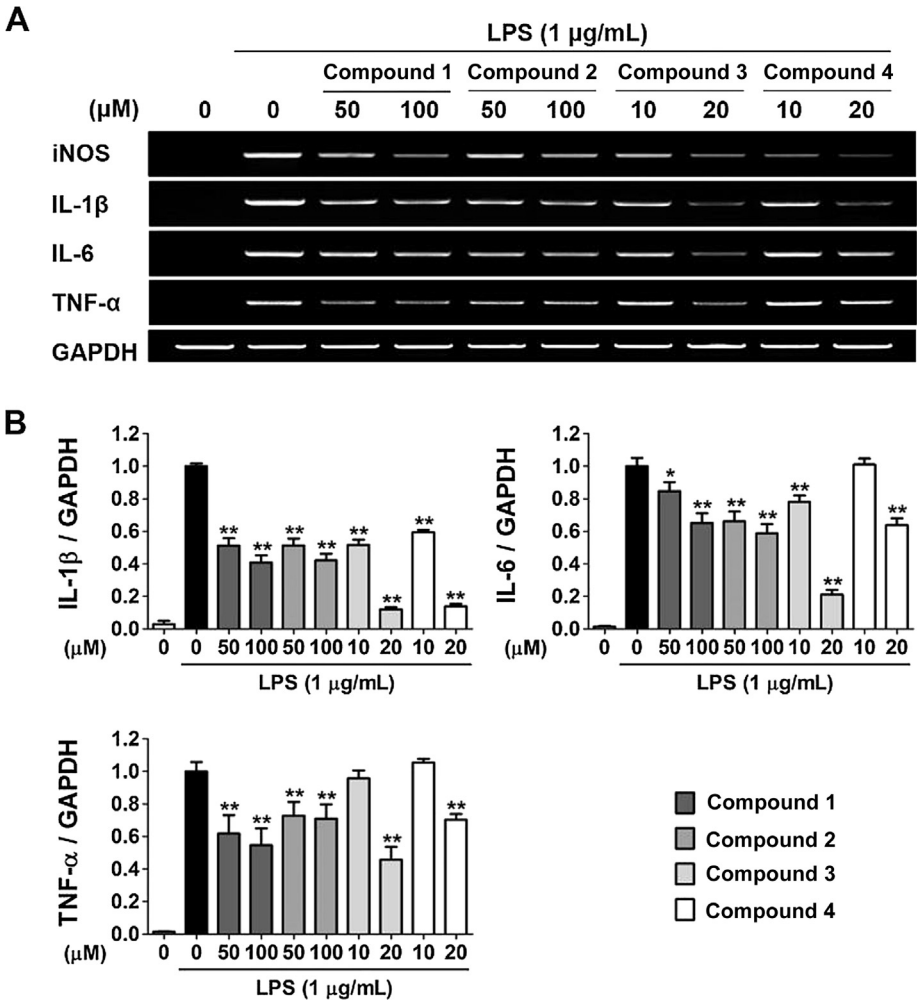

Fig. 3. Effects of four compounds on production of proinflammatory cytokines in LPS-stimulated RAW264.7 cells. (A) For the RT-PCR of proinflammatory cytokine gene expressions, total RNA was prepared from RAW264.7 cells treated with LPS (1 μg/mL) at various concentrations (50μM, 100μM or 10μM, 20μM) of each compounds for 24 h. The mRNA levels of IL-1β, IL-6, and TNF-α were determined by RT-PCR analysis. PCR of GAPDH was performed to verify that the initial complementary DNA contents of samples were similar. (B) mRNA expression levels of IL-1β, IL-6, and TNF-α were determined by RT-PCR analysis. The expression level of GAPDH mRNA served as the internal control for the normalization of IL-1β, IL-6, and TNF-α mRNA expression. Data are presented as mean ± SD ($n = 3$). $^*p < 0.05$ and $^{**}p < 0.01$ compared with treatment with LPS alone. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; IL, interleukin; LPS, lipopolysaccharide; mRNA, messenger RNA; RT-PCR, reverse transcriptase-polymerase chain reaction; TNF-α, tumor necrosis factor-α.

confirms the presence of Compound **4**. The molecular weight was determined to be 778 from the molecule ion peaks at *m/z* 779 $[M+H]^+$ in the positive FAB/MS. Compound **4** exhibited absorbance bands due to the hydroxyl (3,417 cm^{-1}), carbonyl (1,736 cm^{-1}), and double bond (1,595 cm^{-1}) groups in the IR spectrum. ^{1}H-NMR and ^{13}C-NMR spectra of Compound **4** were similar to those of Compound **3**, with the exception of fatty acids moieties. Compound **4** showed eight olefin methine signals (δ_C 128.4 × 2, 128.7 × 2, 130.4 × 2, 130.5 × 2, δ_H 5.40–5.50) instead of the 12 olefin methine signals of Compound **3**. Therefore, both the fatty acids of Compound **4** were identified as octadecadienoic acid. The fatty acid methyl ester obtained by chemical reaction was identified as 9(*Z*), 12(*Z*)-octadecadienoic acid methyl ester (methyl linoleate, RT = 14′50″) by the GC/MS analysis. Based on these results, the chemical structure of Compound **4** was determined to be 2(*S*)-1-*O*-linoleoyl-2-*O*-linoleoyl-3-*O*-*β*-D-galactopyranosyl-*sn*-glycerol, named panaxcerol D (Fig. 1) [21].

In this study, four glycosyl glycerides were isolated from the aerial parts of hydroponic *P. ginseng* and their structures were identified. The isolated glycosyl glycerides were evaluated for potential inhibition of NO production in LPS-stimulated RAW264.7 macrophage cells (Fig. 2). Compounds **1** and **2** showed half maximal inhibitory concentration (IC_{50}) values of 63.8 ± 6.4μM and 59.4 ± 6.8μM and lethal concentration, 50% (LD_{50}) values > 100μM and > 100μM, respectively (Table 1). Compounds **3** and **4** showed IC_{50} values of 7.7 ± 0.6μM and 8.0 ± 0.9μM and LD_{50} values > 20μM and > 20μM, respectively (Table 1). Compounds **3** and **4** exhibited a greater effect than L-N^G-monomethyl arginine, a well-known inhibitor (IC_{50}: 25.5μM). Compounds **3** and **4** also exhibited a greater effect than the naturally derived active compounds, muqubilone (IC_{50}: 23.8μM), sigmosceptrellin A (IC_{50}: 9.9μM), and ginsenoside Rh2 (IC_{50} > 50μM) from a marine sponge (*Latrunculia* sp.) and *P. ginseng*. [22,23]. Compounds **3** and **4** have two fatty acids in the molecule, whereas Compounds **1** and **2** have one fatty acid. This molecular structure is responsible for the decrease in the polarity of Compounds **3** and **4** compared with that of Compounds **1** and **2**. Because of this variation, the permeability of Compounds **3** and **4** to the cell membrane is increased and the activity or cytotoxicity to the cells is also increased. Compounds **1** and **2** had moderate inhibition on NO production in LPS-stimulated RAW264.7 cells (IC_{50}: 63.8 ± 6.4μM and 59.4 ± 6.8μM) without cytotoxicity at concentrations lower than 100μM, whereas Compounds **3** and **4** showed good inhibition (IC_{50}: 7.7 ± 0.6μM and 8.0 ± 0.9μM) without cytotoxicity at concentrations < 20μM (Table 1). Proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, are also induced as part of the inflammatory process in LPS-stimulated RAW264.7 cells. Therefore, the effects of Compounds **1**–**4** were investigated on the expressions of IL-1β, IL-6, and TNF-α in RAW264.7 macrophage cells treated with 1 μg/mL LPS for 24 h in the presence of various concentrations (50μM, 100μM or 10μM, 20μM) of each compounds. The anti-inflammatory activities of Compounds **1**–**4** were evaluated, and the results indicated that Compounds **3** and **4** inhibited the expressions of IL-1β, IL-6, and TNF-α mRNA in a concentration-dependent manner in LPS-stimulated cells, without affecting the expression of the control gene GAPDH (Fig. 3).

Our results suggest that Compounds **1**–**4** from the hydroponic *P. ginseng* may be used as potential anti-inflammatory agents in the skin drugs or functional cosmetics industry.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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