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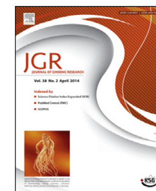

Research article

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**.
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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″),

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