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Glycerides isolated from the aerial parts of *Malva verticillata* cause immunomodulation effects via splenocyte function and NK anti-tumor activity

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Abstract A preliminary study revealed that a 10 μg/mL *n*-BuOH fraction of *Malva verticillata* aerial parts significantly enhanced splenocyte proliferation and induced significant enhancement of natural-killer (NK) cell activity against tumor cells (YAC-1). This study was initiated to identify the principal components that exhibited these activities, and four glycerides were isolated through repeated SiO_2 and ODS column chromatography. Structures of compounds **1–4** were determined to be (2*S*)-1-*O*-palmitoyl glyceride, (2*S*)-1-*O*-stearoyl glyceride, (2*S*)-1-*O*-linolenoyl glyceride, and (2*S*)-1,2-di-*O*-linoleoyl glyceride, respectively. Compounds **1–3** showed potential immune-enhancing activity in murine splenocyte and natural-killer (NK) cells at 10 μM. In contrast, compound **4** showed weak activity, indicating the monoacyl glycerides (**1–3**) are more effective than diacyl glyceride (**4**). Also, the longer the carbon number of the fatty acid in monoacyl glyceride, the better the activity, and the monoacyl glyceride including an unsaturated fatty acid (**3**) is more effective than the glycerides including the saturated fatty acids (**1–2**).

Keywords *Malva verticillata* · Glyceride · Splenocyte · Natural killer cells · Immunotherapy

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Introduction

Known as herbal therapy or phytomedicine, the therapeutic use of plants, plant parts, or plant-derived substances is generally considered a form of complementary medicine [1, 2]. Although the side effect profile of current therapeutic approaches necessitates the development of new treatments [3], the low toxicity of herbs and a long history of empirical support for the use of these herbs as immunostimulants may have therapeutic applications in integrative medicine. There is some evidence that natural immune mechanisms can be modulated to impede the development and progression of certain infectious and neoplastic diseases [4, 5]. Chemoprevention can slow, block, or reverse the disease process through the use of natural materials including foods and a variety of foods have been indicated to be potential therapeutics in past research [6]. Recently, the safety of *Malva verticillata* was verified by the Korea Ministry of Food and Drug Safety (KMFDS) and the Codex Alimentarius Commission (CAC).

Malva verticillata (Chinese mallow), is a popular leafy vegetable in East Asia that has been used as an herbal tea and as a medicine [7]. In the past few decades, the use of *M. verticillata* as a food product has spread from East Asia and consumers can easily find it in markets globally. *Malva verticillata* seeds have also been used in traditional Chinese medicinal formulae as a diuretic, laxative, and galactopoietic material [8]. Despite its medicinal uses, the chemical constituents and the biological activity of the aerial parts of the *M. verticillata* plant are not well understood. Raw vegetable oils are known to contain partial acylglycerides, such as diacylglycerols or monoacylglycerols, in high quantity [9]. Lipid droplets (LDs), such as acylglycerides, are dynamic organelles that govern the

storage and turnover of lipids [10] and they play important roles in membrane and lipid trafficking, protein storage, protein degradation, and replication of hepatitis C and dengue viruses [11–14]. Furthermore, acylglycerides were reported to have anti-cancer [15], anti-neuroinflammatory [16], and anti-tumor [17] activities.

In a preliminary study, an *n*-BuOH fraction (Fr) of *M. verticillata* aerial parts showed significant natural killer (NK) cell cytotoxicity against tumor cells (YAC-1), and TLC analysis of the *n*-BuOH Fr indicated the presence of many glycerides. NK cells play an important role in the first response against viruses and tumors and the fact that NK cells function in innate immunity is crucial to their ability to combat viral infection and destroy cancer [18, 19]. Recently, some studies have suggested that NK cells have characteristics of both the innate and adaptive immune systems. NK cells exert direct cytotoxic activity against tumor targets and can regulate the adaptive immune response by cytokine production [20–22]. Therefore, this study was focused on isolate glycerides from *M. verticillata* aerial parts to investigate their potential as immunomodulation treatments. Four glycerides were isolated from the *n*-BuOH Fr of *M. verticillata* aerial parts and identified on the basis of spectroscopic analysis. Solvent Frs and the isolated glycerides were evaluated for their immunomodulation effects.

Materials and methods

General experimental procedures

Previously, most of experimental procedures for isolation and identification were reported [23].

GC–MS spectra were recorded on a Shimadzu GC–MS-QP2010 Plus spectrometer (Kyoto, Japan).

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was purchased from Dutchefa Biochemie B.V. (Haarlem, The Netherlands). Dimethyl sulfoxide (DMSO) was purchased Daejung Chemicals (Gyeonggi-do, Korea). Dulbecco's Modified Eagle's Medium (DMEM), RPMI 1640 medium, fetal bovine serum (FBS), phosphate-buffered saline (PBS), L-glutamine, and penicillin–streptomycin were obtained from Invitrogen Life Technologies Inc. (Carlsbad, CA, USA). Unless identified otherwise, all reagents were used of analytical grade.

Plant materials

The *M. verticillata* aerial parts were obtained at a commercial farm, Namyangju City, Korea, in April 2016, and the voucher specimen (KHU20160419) is deposited at Kyung Hee University (Laboratory of Natural Products Chemistry), Yongin, Korea.

Isolation of glycerides

The dried aerial parts of *M. verticillata* (3.1 kg) were extracted using 80% MeOH (54.0 L × 5) at room temperature (Temp) for 24 h. The extracts (Exts) were filtered through filter paper (6 μm, 70 mm) and evaporated under reduced pressure at 43 °C to yield 794 g of Ext. The obtained MeOH Exts were suspended in H_2O (2 L) and then successively extracted with ethyl acetate (EtOAc, 2 L × 4) and *n*-butanol (*n*-BuOH, 2 L × 4). Each layer was concentrated to obtain EtOAc (MVE, 80 g), *n*-BuOH (MVB, 75 g), and H_2O (MVW, 637 g) Frs. The *n*-BuOH Fr (MVB, 77 g) was applied to a SiO_2 column chromatography (SCC, φ 10 × 15 cm) and eluted with $CHCl_3$–MeOH–H_2O (25:3:1 → 22:3:1 → 20:3:1 → 18:3:1 → 15:3:1 → 12:3:1, 18.8 L of each) to yield 14 Frs (MVB-1 to MVB-14). Fr MVB-9 (4.0 g, V_e/V_t 0.384–0.557) was applied to OCC (φ 4 × 6 cm) and eluted with MeOH–H_2O (3:2 → 2:1 → 2.5:1 → 3:1 → 5:1, 3.6 L of each) to yield 16 Frs (MVB-9-1 to MVB-9-16). Fr MVB-9-5 (383.9 mg, V_e/V_t 0.116–0.263) was applied to OCC (φ 2.5 × 6 cm) and eluted with acetone-H_2O (2:3, 8.0 L) to yield 14 Frs (MVB-9-5-1 to MVB-9-5-14) along with purified compound (Com) **1** [MVB-9-5-4, 22.8 mg, V_e/V_t 0.038–0.053, R_f 0.20, TLC (Kie 60 F_{254}), $CHCl_3$–MeOH–H_2O (8:3:1), R_f 0.83, TLC (RP-18 F_{254S}), acetone-H_2O (2:1)]. Fr MVB-9-2 (115.8 mg, V_e/V_t 0.004–0.009) was applied to SCC (φ 2.5 × 15 cm) and eluted with $CHCl_3$–MeOH–H_2O (20:3:1 → 18:3:1 → 15:3:1 → 12:3:1, 1.7 L of each) to yield 17 Frs (MVB-9-2-1 to MVB-9-2-17) along with purified Com **2** [MVB-9-2-11, 16.1 mg, V_e/V_t 0.745–0.794, R_f 0.65, TLC (Kie 60 F_{254}), $CHCl_3$–MeOH–H_2O (65:35:10), R_f 0.71, TLC (RP-18 F_{254S}), MeOH–H_2O (3:1)]. Fr MVB-8 (3.5 g, V_e/V_t 0.128–0.383) was applied to ODS column chromatography (OCC, φ 4.5 × 8 cm) and eluted with MeOH–H_2O (3:1 → 4:1 → 5:1 → 6:1, 1.0 L of each) to yield 17 Frs (MVB-8-1 to MVB-8-17) along with purified Com **3** [MVB-8-5, 71.5 mg, V_e/V_t 0.055–0.647, R_f 0.81, TLC (Kie 60 F_{254}), $CHCl_3$–MeOH–H_2O (8:3:1), R_f 0.52, TLC (RP-18 F_{254S}), MeOH–H_2O (5:1)]. Fr MVB-5 [2.4 g, elution volume/total volume (V_e/V_t) 0.036–0.051] was applied to SCC (φ 3.5 × 15 cm) and eluted with EtOAc-*n*-BuOH-H_2O (30:3:1, 2.4 L) to yield 13 Frs (MVB-5-1 to MVB-5-13) along with purified Com **4** [MVB-5-12, 76.4 mg, V_e/V_t 0.766–0.874, R_f 0.19, TLC (Kieselgel (Kie) 60 F_{254}), EtOAc-*n*-BuOH–H_2O (8:3:1), R_f 0.65, TLC (RP-18 F_{254S}), acetone-H_2O (1:4)].

(2*S*)-1-*O*-palmitoyl glyceride (MVB-9-5-4, **1**), pale yellow wax; $[\alpha]_D - 1.2°$ (*c* 0.10, $CHCl_3$); positive FAB-MS *m/z* 331 $[M + H]^+$; IR (KBr, ν) 3383, 1723 cm^{-1}; ^1H-NMR (CD_3OD, 400 MHz, δ_H) 0.88 (3H, t, $J = 7.2$, H-16′),

1.27–1.34 (24H, overlapped, H-4′-15′), 1.59 (2H, m, H-3′), 2.33 (2H, t, $J = 8.0$, H-2′), 3.87 (2H, m, H-3), 3.96 (1H, m, H-2), 4.10 (1H, dd, $J = 11.4$, 6.0, H-1b), 4.17 (1H, dd, $J = 11.4$, 4.4, H-1a); ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 14.4 (C-16′), 23.6 (C-15′), 25.9 (C-3′), 29.9–30.1 (C-4′-13′), 33.0 (C-14′), 34.9 (C-2′), 66.1 (C-1), 67.4 (C-3), 69.8 (C-2), 175.1 (C-1′); GC–MS $t_R = 10.27$ min (palimic acid methyl ester).

(2*S*)-1-*O*-stearoyl glyceride (MVB-9-2-11, **2**), pale yellow wax; $[\alpha]_D$ +1.5° (*c* 0.10, $CHCl_3$); positive FAB-MS *m/z* 359 $[M + H]^+$; IR (KBr, ν) 3386, 1724 cm^{-1}; ^{1}H-NMR (CD_3OD, 400 MHz, δ_H) 0.88 (3H, t, $J = 7.2$, H-18′), 1.27–1.34 (28H, overlapped, H-4′-17′), 1.59 (2H, m, H-3′), 2.34 (2H, t, $J = 8.0$, H-2′), δ: 3.90 (2H, m, H-3), 3.96 (1H, m, H-2), 4.10 (1H, dd, $J = 11.4$, 6.0, H-1b), 4.17 (1H, dd, $J = 11.4$, 4.4, H-1a); ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 14.2 (C-18′), 22.7 (C-17′), 25.1 (C-3′), 29.4–30.0 (C-4′-15′), 31.9 (C-16′), 34.3 (C-2′), 66.1 (C-1), 67.4 (C-3), 69.6 (C-2), 173.6 (C-1′); GC–MS $t_R = 14.37$ min (stearic acid methyl ester).

(2*S*)-3-*O*-linolenoyl glyceride (MVB-8-5, **3**), dark green wax; $[\alpha]_D$ +3.5° (*c* 0.10, $CHCl_3$); positive FAB-MS *m/z* 353 $[M + H]^+$; IR (KBr, ν) 3386, 1722, 1612 cm^{-1}; ^{1}H-NMR (CD_3OD, 400 MHz, δ_H) 0.96 (3H, t, $J = 7.2$, H-18′), 1.30–1.35 (8H, overlapped, H-4′-7′), 1.60 (2H, m, H-3′), 2.07 (4H, m, H-8′,17′), 2.34 (2H, t, $J = 8.0$, H-2′), 2.80 (4H, overlapped, H-11′,14′), 3.90 (2H, m, H-3), 3.96 (1H, m, H-2), 4.10 (1H, dd, $J = 11.4$, 6.0, H-1b), 4.17 (1H, dd, $J = 11.4$, 4.4, H-1a), 5.27–5.40 (6H, overlapped, H-9′,10′,12′,13′,15′,16′); ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 14.6 (C-18′), 21.5 (C-17′), 26.0 (C-3′), 26.4 (C-11′), 26.5 (C-14′), 28.1 (C-8′), 30.2–30.7 (C-4′-7′), 34.9 (C-2′), 66.2 (C-1), 67.4 (C-3), 69.9 (C-2), 128.2 (C-15′), 128.8 (C-10′), 129.2 (× 2, C-12′,13′), 131.0 (C-9′), 132.7 (C-16′), 175.3 (C-1′); GC–MS $t_R = 13.76$ min (linolenic acid methyl ester).

(2*S*)-1,2-di-*O*-linoleoyl glyceride (MVB-5-12, **4**), pale yellow wax; $[\alpha]_D$ − 2.0° (*c* 1.00, $CHCl_3$); negative FAB-MS *m/z* 615 $[M–H]^-$; IR (KBr, ν) 3389, 1725, 1610 cm^{-1}; ^{1}H-NMR (CD_3OD, 400 MHz, δ_H) 0.89 (6H, t, $J = 6.8$, H-18′, 18″), 1.28–1.32 (28H, overlapped, H-4′-7′,4″-7″,15′-17′,15″-17″), 1.60 (4H, m, H-3′,3″), 2.06 (8H, overlapped, H-8′, 8″, 14′, 14″), 2.30 (2H, t, $J = 7.6$, H-2′), 2.33 (2H, t, $J = 7.6$, H-2″), 2.80 (4H, m, H-11′,11″), 3.96 (2H, m, H-3), 4.17 (1H, dd, $J = 12.0$, 6.4, H-1b), 4.43 (1H, dd, $J = 12.0$, 2.8, H-1a), 5.20 (1H, m, H-2), 5.32–5.36 (8H, overlapped, H-9′,9″,10′,10″,12′,12″,13′,13″); ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 14.5 (× 2, C-18′,18″), 21.8 (× 2, C-17′,17″), 23.8 (× 2, C-17′,17″), 26.0 (× 2, C-3′,3″), 26.5 (× 2, C-11′,11″), 28.2 (× 4, C-8′,8″,14′,14″), 30.2–30.8 (C-4′-7′,4″-7″,15′-16′,15″-16″), 34.9 (C-2′), 35.1 (C-2″), 63.9 (C-3), 64.7 (C-1), 72.0 (C-2), 129.2 (× 4, C-10′,10″,12′,12″), 131.1 (× 4, C-9′,9″,13′,13″), 174.6 (C-1″), 174.9 (C-1′); GC–MS $t_R = 13.99$ min (linoleic acid methyl ester).

GC–MS analysis of fatty acids in glycerides

Each glyceride (1.0 mg) was dissolved in 2 mL 20% KOH/MeOH and heated in an 80 °C water bath for 60 min. After cooling to room Temp, the reaction mixture was tested for disappearance of the starting material on TLC plates (*n*-hexane: EtOAc = 1:1), and it was neutralized by adding the acidic cation-exchange resin (Dowex 50 W, H^+ form) and filtered. The filtrate was evaporated in vacuo and partitioned into EtOAc and H_2O Frs. Each EtOAc Fr was then evaporated in vacuo, dissolved in 200 µL EtOAc, and filtered, respectively, through a syringe filter (0.2 µm, 13 mm). The filtrate was stored at − 4 °C until GC–MS analysis.

A DB-5 column (0.25-µm film thickness × 0.25-mm diameter × 30-m length) was used for GC–MS experiments. Helium was used as the Mobile phase at a flow rate of 24.2 mL/min. The injector and detector Temps were both set at 280 °C. The oven Temp was programmed as follows: from 160 to 320 °C (rate of 4 °C/min) and held for 15 min. Sample solution (1 µL) was 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 the retention time with those of authentic fatty acids and their mass spectra with those of a library (Wiley Library, version 2008; John Wiley & Sons Inc., Hoboken, NJ, USA).

Splenocyte isolation

All of the experimental procedures were performed in accordance with the Principles of Laboratory Animal Care (NIH publication, #80-23, revised 1996) and the Animal Care and Use Guidelines of Kyung Hee University, Korea. Adult male ICR mice (7–10 weeks old) were obtained from Young Bio Lab Co. (Osan, Korea). The mice were maintained in a standard laboratory animal facility under 12-h light/dark cycles with food and water ad libitum.

For splenocyte isolation, each spleen was removed aseptically from ICR mice and kept in cooled HBSS. A single cell suspension was prepared by gently homogenizing the spleens with the distal end of a syringe in a cell strainer and washed with HBSS. The cell suspension was centrifuged at 400×*g* and further incubated with red blood cell lysis buffer for 15 min. The cells were then centrifuged at 400×*g*, washed, and cultured in RPMI 1640 medium (supplemented with 10% FBS). Primary mouse splenocytes were cultured in RPMI 1640 media with 10% FBS at 37 °C in 5% CO_2 atmosphere incubator.

Cell culture

YAC-I (KCLB no. 40160) cell lines were purchased from the Korean Cell Line Bank (Seoul, Korea). The cell lines were grown in RPMI 1640 medium or DMEM with 10% FBS and 1% penicillin–streptomycin, and incubated at 37 °C in 5% CO_2. Cell cultures with less than 5 passages were used in all experiments.

In vitro splenocyte proliferation assay

The MTT assay was used to measure splenocyte proliferation. In summary, 50 μg of 4×10^6 cells/well were seeded in 96-well culture plates. After 2 h, cells were treated with 10 μg/mL of each Fr and 10 μM of each Com, respectively, for 48 h. MTT solution was added and the cells were incubated for another 4 h. After removing the media, DMSO was added to each well to dissolve MTT-formazan product. The resulting absorbance was measured using a microtiter plate reader (Synergy HT, Multi-mode microplate reader, BioTek Instruments, Inc., Winooski, VT, USA) at 570 and 630 nm. Cell proliferation is expressed as the percentage of viable treated sample cells to control cells. All tests were performed in quadruplicate.

Cytotoxicity NK cell assay

To evaluate NK cell activity, the non-adherent splenocytes were used as the effector cells and YAC-1 as the target cells [50 μL effector (E) at 4×10^6 cell/well was added to 100 μL of 2×10^5 target (T) cells/well to give an E:T ratio of 10:1] were cultured in 96-well plates in the presence or absence of the Frs (10 μg/mL) or the Coms (10 μM/mL) and incubated at 37 °C in a 5% CO_2 incubator for 24 h. Afterwards, tumor killing activity of NK cell was evaluated by the MTT assay. NK cell activity is shown as cell viability compared to control according to the following equation: cell viability = $(OD_{sample} - OD_{effector\ control})/OD_{target\ control} \times 100$.

Statistical analysis

Data were analyzed using the Prism 5 Statistical Software package (GraphPad, San Diego, CA, USA). All data are expressed as the mean ± standard error mean (SEM). Statistical comparisons between the groups were performed using one-way repeated measures ANOVA with Tukey's post hoc test. Values of $p < 0.05$, 0.01, and 0.001 were considered statistically significant.

Results and discussion

Repeated column chromatography for the *n*-BuOH Fr of *M. verticillata* aerial parts led to the isolation of four glycerides (**1–4**), which were identified as (2*S*)-1-*O*-palmitoyl glyceride [24], (2*S*)-1-*O*-stearoyl glyceride [25], (2*S*)-1-*O*-linolenoyl glyceride [26], and (2*S*)-1,2-di-*O*-linoleoyl glyceride [27], respectively, based on NMR, IR, FAB-MS, GC–MS, and FAB-MS analyses in addition to comparison of the data with those in literatures (Table 1).

Com **1** was detected as a yellow color on TLC plates by spraying with 10% sulfuric acid (SA) and heating. The molecular weight (MW) and the molecular formula (MF) were determined to be 330 and $C_{19}H_{38}O_4$, respectively, from the molecular ion peak (MIP) *m/z* 331 $[M + H]^+$ in the positive FAB-MS. In the IR spectrum, Com **1** showed the hydroxyl (3383 cm^{-1}) and ester (1723 cm^{-1}) absorbance bands. The ^{1}H-NMR spectrum showed one terminal methyl (δ_H 0.88, 3H, t, $J = 7.2$), one allyl methylene (δ_H

Table 1 GC–MS analysis for fatty acids in glycerides **1–4**

Fatty acids	Retention time (min)	Molecular weight	Molecular formula	MS product ions (*m/z*)	Glycerides[a]
Palmitic acid methyl ester	10.27	270	$C_{17}H_{34}O_2$	270 $[M]^+$, 227 $[M\text{-}(CH_2)_2CH_3]^+$, 143 $[M\text{-}(CH_2)_8CH_3]^+$, 87 $[M\text{-}(CH_2)_{12}CH_3]^+$, 74 $[M\text{-}(CH_2)_{13}CH_3 + H]^+$	1
Linolenic acid methyl ester	13.76	292	$C_{19}H_{32}O_2$	236 $[M–H(CH)_2CH_2CH_3]^+$, 108 $[M\text{-}(CH)(CH_2)_8COOCH_3]^+$, 95 $[C_7H_{11}]^+$, 79 $[C_6H_7]^+$, 67 $[C_5H_7]^+$	3
Linoleic acid methyl ester	13.99	294	$C_{19}H_{34}O_2$	294 $[M]^+$, 263 $[M\text{-}OCH_3]^+$, 109 $[M–H(CH)(CH_2)_8COOCH_3]^+$, 95 $[C_7H_{11}]^+$, 81 $[C_6H_9]^+$, 67 $[C_5H_7]^+$	4
Stearic acid methyl ester	14.37	298	$C_{19}H_{38}O_2$	298 $[M]^+$, 255 $[M\text{-}(CH_2)_2CH_3]^+$, 199 $[M\text{-}(CH_2)_6CH_3]^+$, 143 $[M\text{-}(CH_2)_{10}CH_3]^+$, 87 $[M\text{-}(CH_2)_{14}CH_3]^+$, 74 $[M\text{-}(CH_2)_{15}CH_3 + H]^+$	2

[a]Glycerides include the corresponding fatty acids. Each glyceride (1.0 mg) was dissolved in 2 mL of 20% KOH/MeOH and heated 80 °C in water bath for 60 min. After cooling at room temperature, the reaction mixture was neutralized by adding the acidic cation-exchange resin (Dowex 50 W, H^+ form) and filtered. The filtrate was evaporated in vacuo and partitioned into EtOAc and H_2O fractions. Each EtOAc fraction was then evaporated in vacuo, dissolved in EtOAc 200 μL and filtered through a syringe filter (0.2 μm, 13 mm). The filtrate was used to GC–MS analysis

2.33, 2H, t, $J = 8.0$), and thirteen methylene (δ_H 1.27–1.34, 24H) proton signals due to a hexadecanoic acid. The oxygenated methine (δ_H 3.96, 1H, H-2), and two oxygenated methylene (δ_H 4.17, 1H, H-1a; δ_H 4.10, 1H, H-1b; δ_H 3.87, 2H, H-3) proton signals due to a glycerol moiety were also observed. Observation of the oxygenated methylene (δ_H 4.17, 1H, H-1a; δ_H 4.10, 1H, H-1b) proton signals in the lower magnetic field compared with that of the glycerol confirmed Com **1** to have an ester bond at C-1. Based on these results, Com **1** was assumed to be a monoglyceride with a saturated fatty acid. The ^{13}C-NMR spectrum showed an oxygenated methylene (δ_C 66.1, C-1), an oxygenated methine (δ_C 69.8, C-2), and an oxygenated methylene (δ_C 67.4, C-3) carbon signals were confirmed as the signals of a glycerol moiety. An ester (δ_C 175.1), one terminal methyl (δ_C 14.4), an allyl methylene (δ_C 34.9) and thirteen methylene (δ_C 25.9–30.1) carbon signals were observed as signals of a hexadecanoic acid moiety. The fatty acid methyl ester obtained by alkaline hydrolysis and solvent fractionation appeared as a clear peak at 10.27 min on the GC–MS spectrum, which was identified as a palmitic acid methyl ester by comparing the mass spectrum of the peak with the reference value (Wiley 9 Library). In the gradient heteronuclear multiple-bond connectivity (gHMBC) spectrum, ^3J correlations were observed between the oxygenated methylene proton signal of the glycerol H-1a, 1b (δ_H 4.17, 4.10) and the ester carbonyl carbon signal C-1′ (δ_C 175.1) confirming that palmitic acid was linked to the C-1 of the glycerol. On the basis of the positive optical rotation value of Com **1** ($[\alpha]_D - 1.2°$, *c* 0.10, $CHCl_3$), which was similar to that of (2*S*)-1-*O*-palmitoyl glyceride ($[\alpha]_D - 1.2°$, *c* 0.10, $CHCl_3$) [28], and a biogenetic perspective of the plant glyceride, the absolute configuration of C-2 in Com **1** was identified to be *S*. Therefore, the chemical structure (CS) of Com **1** was identified to be (2*S*)-1-*O*-palmitoyl glyceride (Fig. 1).

Com **2** was appeared yellow in color on TLC plates after spraying the plate with 10% SA and heating. The MW and the MF were determined to be 358 and $C_{21}H_{42}O_4$, respectively, from the MIP *m/z* 359 $[M + H]^+$ in the positive FAB-MS. In the IR spectrum, Com **2** showed the hydroxyl (3386 cm^{-1}) and ester (1724 cm^{-1}) absorbance bands. The ^{1}H-NMR spectrum showed one terminal methyl (δ_H 0.88, 3H, t, $J = 7.2$), one allyl methylene (δ_H 2.34, 2H, t, $J = 8.0$), and fifteen methylene (δ_H 1.27–1.34, 28H) proton signals due to an octadecanoic acid. The oxygenated methine (δ_H 3.96, 1H, H-2) and two oxygenated methylene (δ_H 4.17, 1H, H-1a; δ_H 4.10, 1H, H-1b; δ_H 3.90, 2H, H-3) proton signals due to a glycerol moiety were also observed. Observation of the oxygenated methylene (δ_H 4.17, 1H, H-1a; δ_H 4.10, 1H, H-1b) proton signals in the lower magnetic field compared with that of glycerol confirmed Com **2** to have an ester bond at C-1. Based on these results,

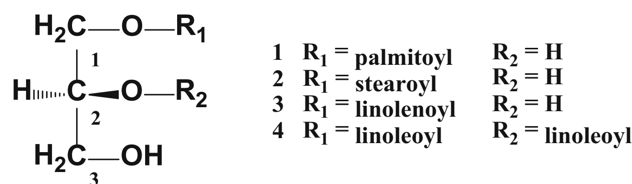

Fig. 1 Strutures of compounds **1**–**4** isolated from the aerial parts of *M. verticillata*. **1**: (2*S*)-1-*O*-palmitoyl glyceride, **2**: (2*S*)-1-*O*-stearoyl glyceride, **3**: (2*S*)-1-*O*-linolenoyl glyceride, **4**: (2*S*)-1,2-di-*O*-linoleoyl glyceride

Com **2** was assumed to be a monoglyceride with a saturated acid. The ^{13}C-NMR spectrum showed an oxygenated methylene (δ_C 66.1, C-1), an oxygenated methine (δ_C 69.6, C-2), and an oxygenated methylene (δ_C 67.4, C-3) carbon signals were confirmed as the signals of a glycerol moiety. An ester (δ_C 173.6), one terminal methyl (δ_C 14.2), an allyl methylene (δ_C 34.3) and fifteen methylene (δ_C 25.1-30.0) carbon signals indicated the presence of an octadecanoic fatty acid moiety. The fatty acid methyl ester obtained by alkaline hydrolysis and solvent fractionation appeared as a clear peak at 14.37 min on the GC–MS spectrum, which was identified as a stearic acid methyl ester by comparing the mass spectrum of the peak with that in the library (Wiley 9 Library). In the gHMBC spectrum, ^3J correlations were observed between the oxygenated methylene proton signal of the glycerol H-1a, 1b (δ_H 4.17, 4.10) and the ester carbonyl carbon signal C-1′ (δ_C 173.6) confirming that stearic acid was linked to the C-1 of the glycerol. On the basis of the positive optical rotation value of Com **2** ($[\alpha]_D +$ 1.5°, *c* 0.10, $CHCl_3$), which was similar to that of (2*S*)-1-*O*-stearoyl glyceride ($[\alpha]_D +1.3°$, *c* 0.10, $CHCl_3$) [27], and a biogenetic perspective of the plant glyceride, the absolute configuration of C-2 in Com **2** was identified to be *S*. Therefore, the CS of Com **2** was identified to be (2*S*)-1-*O*-stearoyl glyceride.

Com **3** was dark yellow in color on TLC plates after spraying the plate with 10% SA and heating. The MW and the MF were determined to be 352 and $C_{21}H_{36}O_4$, respectively, from the MIP *m/z* 353 $[M + H]^+$ in the positive FAB-MS. In the IR spectrum, Com **3** showed the hydroxyl (3386 cm^{-1}), ester (1722 cm^{-1}), and double bond (1612 cm^{-1}) absorbance bands. The ^{1}H-NMR spectrum showed one terminal methyl (δ_H 0.96, 3H, t, $J = 7.2$), five allyl methylene (δ_H 2.07–2.80, 10H), five methylene (δ_H 1.30–1.60, 10H), six olefin methine (δ_H 5.27–5.40, 6H) proton signals due to an octadecatrienoic acid. The oxygenated methine (δ_H 3.96, 1H, H-2) and two oxygenated methylene (δ_H 4.17, 1H, H-1a; δ_H 4.10, 1H, H-1b; δ_H 3.90, 2H, H-3) proton signals due to a glycerol moiety were also observed. Observation of the oxygenated methylene (δ_H 4.17, 1H, H-1a; δ_H 4.10, 1H, H-1b) proton signals in the lower magnetic field compared with that of glycerol

confirmed Com **3** to have an ester bond at C-1. Based on these results, Com **3** was assumed to be a monoglyceride with an unsaturated fatty acid. The ^{13}C-NMR spectrum showed an oxygenated methylene (δ_C 66.2, C-1), an oxygenated methine (δ_C 69.9, C-2), and an oxygenated methylene (δ_C 67.4, C-3) carbon signals as the signals of a glycerol moiety. An ester (δ_C 175.3), one terminal methyl (δ_C 14.6), six olefin methine (δ_C 128.2–132.7), five allyl methylene (δ_C 21.5–34.9), and five methylene (δ_C 26.0–30.0) carbon signals indicated the presence of an octadecatrienoic acid moiety. The fatty acid methyl ester obtained by alkaline hydrolysis and solvent fractionation appeared as a clear peak at 13.76 min on the GC–MS spectrum, which was identified as a linolenic acid methyl ester by comparing the mass spectrum of the peak with that in the library (Wiley 9 Library). In the gHMBC spectrum, ^3J correlations were observed between the oxygenated methylene proton signal of the glycerol H-1a, 1b (δ_H 4.17, 4.10) and the ester carbonyl carbon signal C-1′ (δ_C 175.3) indicating that the linolenic acid was linked to the C-1 of the glycerol. On the basis of the positive optical rotation value of Com **3** ($[\alpha]_D$ +3.5°, *c* 0.10, $CHCl_3$), which was similar to that of (2*S*)-1-*O*-linolenoyl glyceride ($[\alpha]_D$ +3.8°, *c* 0.42, $CHCl_3$) [25], and a biogenetic perspective of the plant glyceride, the absolute configuration of C-2 in Com **3** was identified to be *S*. Therefore, the CS of Com **3** was identified to be (2*S*)-1-*O*-linolenoyl glyceride.

Com **4** was detected as dark yellow in color on TLC plates by spraying the plate with 10% SA and heating. The MW and the MF were determined to be 616 and $C_{39}H_{68}O_5$, respectively, from the MIP *m/z* 615 $[M–H]^-$ in the negative FAB-MS. In the IR spectrum, Com **4** showed the hydroxyl (3389 cm^{-1}), ester (1725 cm^{-1}), and double bond (1610 cm^{-1}) absorbance bands. The ^{1}H-NMR spectrum showed two terminal methyl (0.89, 6H, t, $J = 6.8$), eight allyl methylene (δ_H 2.06–2.80, 16H), eleven methylene (δ_H 1.28–1.60, 22H), eight olefin methine (δ_H 5.32–5.36, 8H) proton signals due to two octadecadienoic acids. The oxygenated methine (δ_H 5.20, 1H, H-2), and two oxygenated methylene (4.43, 1H, H-1a; δ_H 4.17, 1H, H-1b; δ_H 3.96, 2H, H-3) proton signals due to a glycerol moiety were also observed. Observation of the oxygenated methylene (δ_H 4.17, 1H, H-1a; δ_H 4.10, 1H, H-1b) and oxygenated methine (δ_H 5.20, 1H, H-2) proton signals in the lower magnetic field compared with those of the glycerol confirmed Com **4** to have ester bonds at C-1 and C-2. Based on these results, Com **4** was assumed to be a diacylglyceride with two unsaturated fatty acids. The ^{13}C-NMR spectrum showed two oxygenated methylene (δ_C 64.7, C-1; δ_C 63.9, C-3), an oxygenated methine (δ_C 72.0, C-2) carbon signals were confirmed to be the signals of a glycerol moiety. Two ester (δ_C 174.9, 174.6), two terminal methyl (δ_C 14.5, × 2), eight olefin methine (δ_C 129.2–131.1), eight allyl methylene (δ_C 21.8–35.1), and eleven methylene (δ_C 21.8–30.8) carbon signals were observed indicating the presence of two octadecadienoic acids moieties. The fatty acid methyl ester obtained by alkaline hydrolysis and solvent fractionation appeared as a clear peak at 13.99 min on the GC–MS spectrum, which was identified as linoleic acid methyl ester by comparing the mass spectrum of the peak with that in the library (Wiley 9 Library). In the gHMBC spectrum, ^3J correlations were observed between the oxygenated methylene proton signal of the glycerol H-1a, 1b (δ_H 4.43, 4.17) and the ester carbonyl carbon signal C-1′ (δ_C 174.6), as well between the oxygenated methine proton signal of glycerol H-2 (δ_H 5.20) and the ester carbonyl carbon signal C-1″ (δ_C 174.9) indicating that two linoleic acids were linked to the C-1 and C-2 of the glycerol, respectively. On the basis of the positive optical rotation value of Com **4** ($[\alpha]_D$ − 2.0°, *c* 1.00, $CHCl_3$), which was similar to that of (2*S*)-1,2-di-*O*-linoleoyl glyceride ($[\alpha]_D$ − 2.0°, *c* 1.10, $CHCl_3$) [29], and a biogenetic perspective of the plant glyceride, the absolute configuration of C-2 in Com **4** was identified to be *S*. Therefore, the CS of Com **4** was identified to be (2*S*)-1,2-di-*O*-linoleoyl glyceride.

All Frs and Coms **1–4** obtained from *M. verticillata* aerial parts showed no significant toxicity on splenocytes at 10 µg/mL or 10 µM/mL, respectively, therefore, these concentrations were used for all assays. Treatment with some Frs and a glyceride showed a significant effect on splenocyte proliferation. As shown in Fig. 2, the EtOAc Fr, the *n*-BuOH Fr, and Com **3** induced splenocyte proliferation approximately 1.5- and 1.2-fold versus the negative (only cells) control. Splenocyte cytotoxicity was also examined against NK-sensitive tumor cells (YAC-1). NK cell cytotoxicity was significantly increased after exposure to all Frs (10 µg/mL) and Coms **1–3** (10 µM/mL), at approximately 65 and 50% versus the control, which indicated that the all Frs and Coms **1–3** can modulate the innate immune response (Fig. 3).

In this study, the immune modulation activity of *M. verticillata* Frs and Coms **1–4** were investigated. Several studies have investigated and indicated the immunomodulatory and antitumor activity of some medicinal plants [30, 31]. The immunomodulatory effect of *M. verticillata* Frs and Coms **1–4** were evaluated for their effects on splenocyte proliferation. The results indicated that the Frs (10 µg/mL) and Coms **1–4** (10 µM/mL) did not show any significant toxicity in the splenocytes for 48 h. In addition, NK cell cytotoxic activity was evaluated to determine the immune modulation effect of the Frs and Coms **1–4** on innate immunity. The results indicated that all tested Frs and Coms **1–3** significantly increased NK cell cytotoxic activity. The NK cell cytotoxicity against NK-sensitive tumor cells (YAC-1) significantly increased after exposure to all Frs and Coms **1–3**, indicating increased the innate

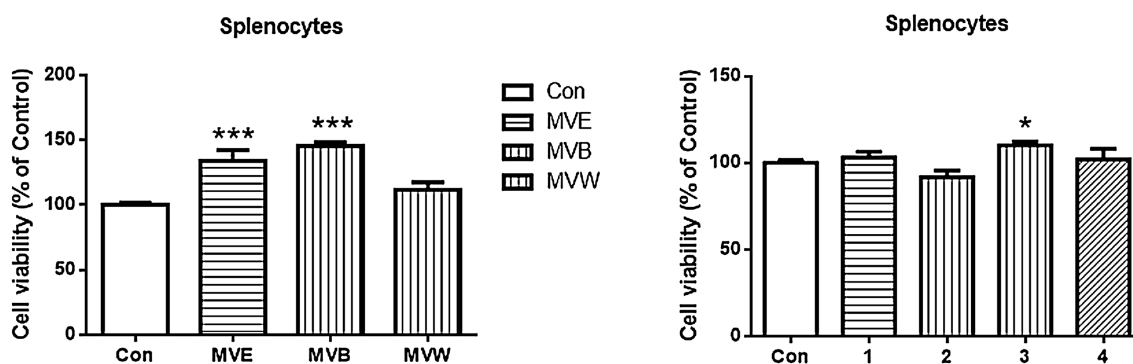

Fig. 2 The effect of *M. verticillata* fractions and compounds **1–4** on splenocyte proliferation. *$p < 0.05$ and ***$p < 0.001$ indicate significant differences from normal group using one-way ANOVA). The results shown are representative of four independent experiments. MVE: the EtOAc fraction of *M. verticillata* aerial parts, MVB: the *n*-BuOH fraction of *M. verticillata* aerial parts, MVW: the H_2O fraction of *M. verticillata* aerial parts. **1**: (2*S*)-1-*O*-palmitoyl glyceride, **2**: (2*S*)-1-*O*-stearoyl glyceride, **3**: (2*S*)-1-*O*-linolenoyl glyceride, **4**: (2*S*)-1,2-di-*O*-linoleoyl glyceride

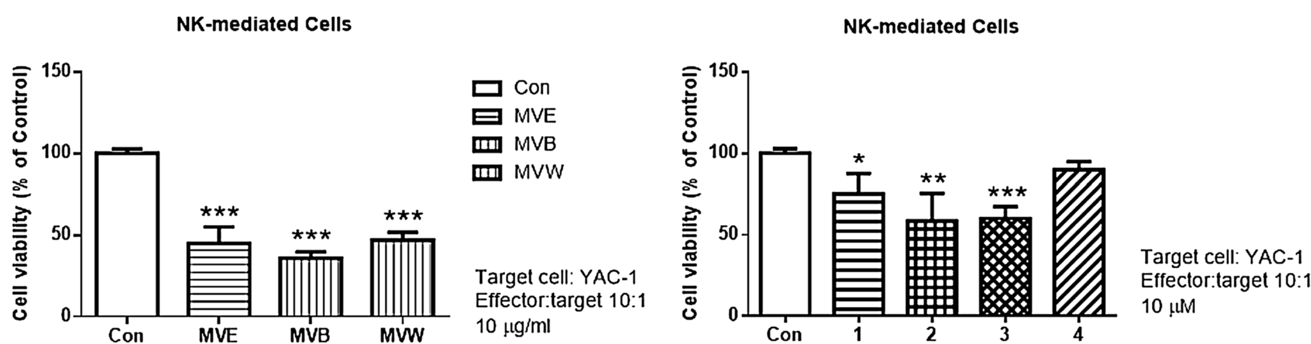

Fig. 3 The effect of *M. verticillata* fractions and compounds **1–4** on the NK cell activity (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). The results shown are representative of four independent experiments. MVE: the EtOAc fraction of *M. verticillata* aerial parts, MVB: the *n*-BuOH fraction of *M. verticillata* aerial parts, MVW: the H_2O fraction of *M. verticillata* aerial parts

immunity response against tumor cells in the presence of these Coms.

In conclusion, we isolated four glycerides from the *n*-BuOH Fr of *M. verticillata* aerial parts, and it was found that monoacyl glycerides significantly enhanced splenocyte proliferation and induced significant enhancement of NK cell activity against tumor cells (YAC-1). In particular, the *n*-BuOH Fr is the most effective in immune modulation, and Coms **1–3**, which were isolated from the *n*-BuOH Fr, also showed immune modulation effects. In contrast, Com **4** showed very weak activity, indicating the monoacyl glycerides are more effective than diacyl glycerides. Also, the longer the carbon number of the fatty acid in monoacyl glyceride, the better the activity, and the monoacyl glyceride including an unsaturated fatty acid (**3**) is more effective than the glycerides including the saturated fatty acids (**1–2**). When monoacylglycerides and diacylglyceride were structurally compared with each other, it was confirmed that the activity varies greatly depending on the presence or absence of fatty acid bond in 2-OH of glycerol moiety. The fact that natural killer cells were very stable to monoglycerides induced death was reported [32], the role of NK cell activator against tumor cells (YAC-1) was first introduced in this study. These findings suggest that *M. verticillata* could inhibit cancer cell growth as well as generate an effective cell-mediated immune response through the activation of immune cell function.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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