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Original article

Anti-inflammatory Effects of Dielectric Barrier Discharge Plasma Induced Phloridzin against Lipopolysaccharide-stimulated RAW 264.7 Macrophages

So-Yeun Woo[1], Gyeong Han Jeong[1], Hanui Lee[1], Seung Sik Lee[1,2], Jin-Hong Kim[1,2], Sungbeom Lee[1,2], Kwang-Woo Jung[1], Moon-Soo Chung[1], Byung Yeoup Chung[1], and Hyoung-Woo Bai[1,2,*]

[1]Advanced Radiation Technology Institute (ARTI), Korea Atomic Energy Research Institute (KAERI), Jeongeup 56212, Republic of Korea
[2]Department of Radiation Science, University of Science and Technology (UST), Daejeon 34113, Republic of Korea

ABSTRACT Phloridzin (**1**), a natural dihydrochalcone, has been extensively studied for its antioxidant and anti-diabetic properties. In this study, we investigated the anti-inflammatory effects of three plasma-induced phloridzin derivatives such as deglucosyl-methylene-bis-phloridzin (**2**), methylene-bis-phloridzin (**3**), and phloretin (**4**) in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells. Cells were treated with various concentrations (12.5, 25, 50, and 100 μM) of compounds **1**–**4**. Among them deglucosyl-methylene-bis-phloridzin (**2**) was identified as the most potent derivative, significantly inhibiting nitric oxide (NO) production without any cytotoxicity. Furthermore, western blot analysis showed that compound **2** strongly suppressed the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). It also decreased the secretion of pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6). Additionally, compound **2** also markedly reduced intracellular reactive oxygen species (ROS) generation in a dose-dependent manner, as confirmed by DCFDA fluorescence assay, and DPPH radical scavenging activity. These results indicate that deglucosyl-methylene-bis-phloridzin (**2**) may be used as an effective anti-inflammatory and antioxidant agent for the prevention or treatment of inflammatory diseases.

Key words: Deglucosyl-methylene-bis-phloridzin, anti-inflammatory, RAW 264.7, ROS

1. Introduction

Phloridzin [phloretin-(4,2′,4′,6′-tetrahydroxy dihydrochalcone)-2′-O-glucoside] is a naturally occurring dihydrochalcone primarily isolated from the bark of apple trees. It is commonly found in apples and apple-derived products such as cider, juice, and purée. Historically, phloridzin has been used in traditional medicine for the treatment of fever, infectious diseases, and malaria, dating back approximately 180 years. In recent decades, extensive studies have reported that phloridzin exhibits a broad spectrum of biological activities, including antihyperglycemic, antioxidant, antihypertensive, antimicrobial, antitumor, anti-inflammatory, and antidiabetic effects [1].

Plasma medicine has emerged over the past two decades as a multidisciplinary field combining physics, chemistry, biology, and medicine. Plasma, often referred to as the fourth state of matter, consists of a complex mixture of electrons, ions, free radicals, reactive molecules, and photons. Among various plasma types, dielectric barrier discharge (DBD) plasma, a form of non-thermal plasma, has gained attention for its applications in biomedical and industrial fields. Its uses include dental equipment sterilization, promotion of blood coagulation, wound healing after implant treatment, and antimicrobial food packaging [2-4]. DBD plasma has also been reported to induce physiological changes by generating reactive oxygen and nitrogen species (ROS and RNS), which can interact with organic compounds, potentially leading to enhanced biological activity. Despite its growing utility, studies focusing on plasma-induced structural modifications of natural compounds and the formation of novel bioactive derivatives remain limited. As part of our ongoing research on enhancing the functionality of natural products via thermal treatment, Fe-catalyzed oxidation, and gamma irradia-

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***Corresponding author:** Hyoung-Woo Bai
Tel: +82-63-570-3334, Fax: +82-63-570-3339, E-mail: hbai@kaeri.re.kr

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tion [5-8], we previously demonstrated that DBD plasma treatment of phloridzin results in the formation of two new dimeric compounds—deglucosyl-methylene-bis-phloridzin (**2**) and methylene-bis-phloridzin (**3**) along with the known compound phloretin (**4**) [9]. These methylene-linked dimers were found to promote adipocyte differentiation in 3T3-L1 preadipocyte cells.

Inflammation plays a critical role in the body's defense against infections, tissue injury, and other harmful stimuli. However, when this response becomes prolonged or dysregulated, it contributes to the development and progression of various chronic diseases, including rheumatoid arthritis, cardiovascular conditions, and malignancies. Although nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are commonly prescribed to manage inflammatory symptoms, their extended use is often linked to significant adverse effects, such as gastrointestinal irritation, hepatic dysfunction, and immunosuppressive complications. Therefore, there is a need for safer and more effective anti-inflammatory agents with fewer side effects to better support patients with inflammatory diseases [10,11]. To evaluate the anti-inflammatory effects, LPS-stimulated RAW 264.7 macrophages were selected as the in vitro model for this study. LPS-stimulated RAW 264.7 macrophages are widely used as a standardized in vitro model to evaluate the anti-inflammatory potential of natural compounds, due to their robust production of pro-inflammatory mediators such as nitric oxide (NO), necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6), upon activation [11]. Accordingly, in the present study, we aimed to investigate the anti-inflammatory effects of plasma-induced phloridzin derivatives using LPS-stimulated RAW 264.7 macrophages.

2. Materials and Methods

2.1. Preparation of phloridzin (1) derivatives (2–4)

Phloridzin (**1**) was purchased from Sigma-Aldrich and derivatives (**2**–**4**) obtains for induced by plasma treatment. Deglucosylmethylenebisphloridzin (**2**), methylenebisphloridzin (**3**) and phloretin (**4**) were obtained from the non-thermal plasma, dielectric barrier discharge (DBD) plasma-induced structural changes of phloridzin (**1**) (Fig. 1). Chemical structures of compounds **2** and **3** were elucidated novel compounds with NMR and LC/MS [9].

2.2. Cell culture

The RAW 264.7 macrophages (KCLB NO. 40071) were obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies Co., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin (Lonza, Walkersville, MD, USA). The cells were incubated under sterile conditions at 37°C in a humid environment containing 5% CO_2.

2.3. Cell viability

Cell viability studies were performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RAW 264.7 cells were seeded in 96-well plate at a density of 1×10^5 cells/well incubated with media for 24 h. After the incubation, cells were treated with varying concentrations of compounds **1**–**4** (0 to 100 μM) for 24 h. Thereafter, the 10 μL of MTT reagents (final concentration 0.5 mg/mL) were added to each well for 3 h. Next, removing the media, and the formazan crystals dissolved in 100 μL of DMSO. The absorbance of the well contents was measured at 570 nm using a scanning microplate reader. The cell viability for each compound was calculated as a percentage of the control group.

2.4. LPS-induced NO production

Cells were plated at a concentration of 1×10^5 cells/well in 96-well plates and cultured 24 h in DMEM media. Cells were treated with various concentrations (12.5, 25, 50, and 100 μM)

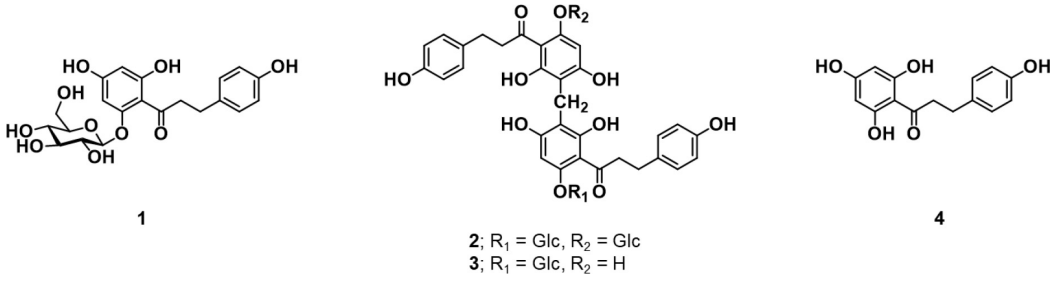

Fig. 1. Chemical structures of plasma-induced phloridzin derivatives (**1**–**4**). **1**, Phloridzin; **2**, Deglucosylmethylenebisphloridzin; **3**, methylenebisphloridzin and **4**, phloretin.

of samples and 0.1 μg/mL of LPS (Sigma-Aldrich, USA). After treatment, all samples were incubated for 24 h. Next, 50 μL of supernatant without cells were mixed with 50 μL of Griess reagent solution in 96-well plate during 15 min. The NO production was measured the absorbance at 548 nm using spectrophotometer.

2.5. Measurement of PGE2 and proinflammatory cytokine production

RAW 264.7 cells were seeded into 24 well plate at a density of 1×10^5 cells/well and incubated for 24 h. Deglucosyl-methylene-bis-phloridzin (**2**) was treated with LPS (0.1 μg/mL) for 24 h and the concentrations of PGE2, TNF-α, and IL-6 in the supernatant were determined using the enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences).

2.6. Western blot analysis

RAW 264.7 macrophages were seeded at a density of 1×10^5 cells per well in 6-well plates and incubated for 24 hours under standard culture conditions. Following this, the cells were treated with lipopolysaccharide (LPS, 0.1 μg/mL) in the presence or absence of amber extract at concentrations of 10, 25, and 50 μg/mL for an additional 24 hours. After treatment, cells were washed twice with cold phosphate-buffered saline (PBS, 0.1 M) and lysed using 100 μL of ice-cold radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, and a protease inhibitor cocktail. The cell lysates were collected into pre-chilled 1.5 mL microcentrifuge tubes and subjected to centrifugation at 1,300 × g for 40 minutes at 4°C to remove cellular debris. Protein concentrations in the supernatants were determined using the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein samples were resolved on 10% SDS-polyacrylamide gels and subsequently transferred onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Darmstadt, Germany). The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hour at room temperature and incubated overnight at 4°C with primary antibodies specific for iNOS (1 : 1,000), COX-2 (1 : 1,000), and GAPDH (1 : 1,000) (all from Cell Signaling Technology, Danvers, MA, USA). After washing three times for 7 minutes each with TBST, membranes were incubated for 2 hours at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit IgG, #7074, Cell Signaling Technology) diluted 1 : 2,000. Protein bands were visualized using enhanced chemiluminescence (ECL) detection reagents (Millipore Corp., Billerica, MA, USA) and imaged using a chemiluminescent detection system.

2.7. DPPH radical scavenging assay

The free radical scavenging activity of plasma-induced phloridzin derivatives (**1**–**4**) was evaluated using 1, 1-diphenyl-2-picryl hydrazyl (DPPH) assay [18]. In brief, 0.1 mM DPPH solution was prepared in ethanol, and 100 μL of this solution was mixed with 100 μL of each test sample (**1**–**4**) at various concentrations (12.5, 25, 50, and 100 μM). The mixtures were incubated at room temperature in the dark for 15 minutes and absorbance was then measured at 517 nm using a spectrophotometer.

2.8. Measurement of reactive oxygen species (ROS) production

RAW 264.7 cells were seeded on a 6 well plate (5×10^5 cells/well) and cultured for 24 h. The cells were added compound 2 (25, 50, and 100 M) and cultured with 0.1 μg/mL LPS for 24 h. The wells were washed 3 times with PBS and each well was exposed with 20 μM 2′,7′-Dichlorodihydrofluorescien diacetate (DCFH-DA) (ab cam). and left 30 min. After an additional 30-minute incubation, fluorescence intensity was measured. Intracellular reactive oxygen species (ROS) levels were quantified by measuring DCF fluorescence using a microplate reader (INFINITE F200 PRO, TECAN) with excitation and emission wavelengths set at 485 nm and 530 nm, respectively. The accumulate the ROS were expressed as relative fluorescence intensity compared to the control group, based on the amount of DCF-DA.

2.9. Statistical analysis

Experiment results were performed at least three times and expressed as the mean ± Standard deviation. For multiple comparisons, One-way analysis of variance (ANOVA) was used followed by Tukey's multiple comparisons test. Differences with $p \leq 0.05$ were statistically significant result.

3. Results and Discussion

3.1. Cell viability and NO production in RAW 264.7 macrophages

The MTT assay was performed to evaluate the cell viability of the phloridzin derivatives (**1**-**4**) in RAW 264.7 macrophages.

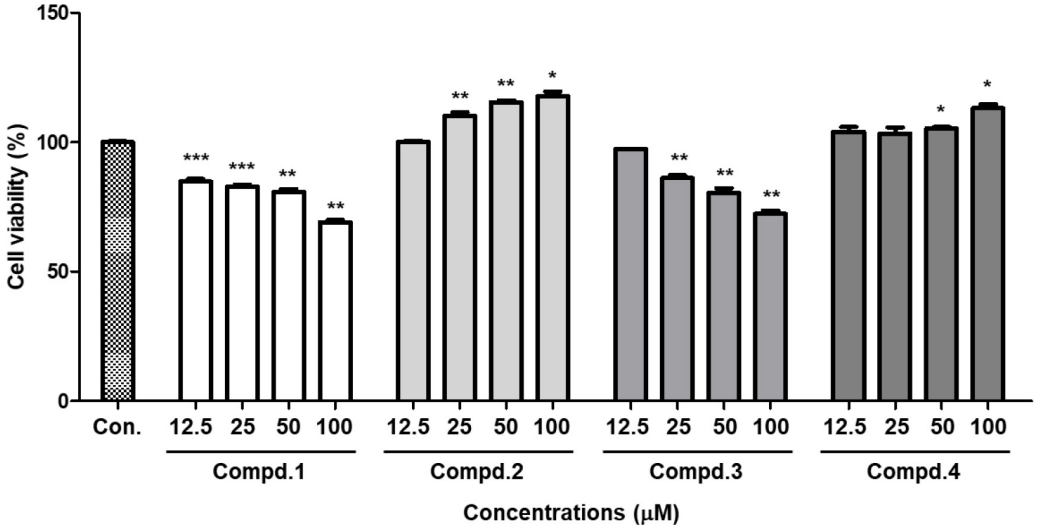

Fig. 2. Cell viability of RAW 264.7 macrophages on phloridzin derivatives (**1**–**4**). RAW 264.7 cell were seeded in 96-well plate and treated different concentrations of compounds for 24 h. The cell viability was determined by MTT assay. Quantitative results are presented as the mean ± standard deviation from three independent experiments (n = 3). Statistical significance was assessed as follows: *$p < 0.1$, **$p < 0.01$, ***$p < 0.001$ vs. the control group.

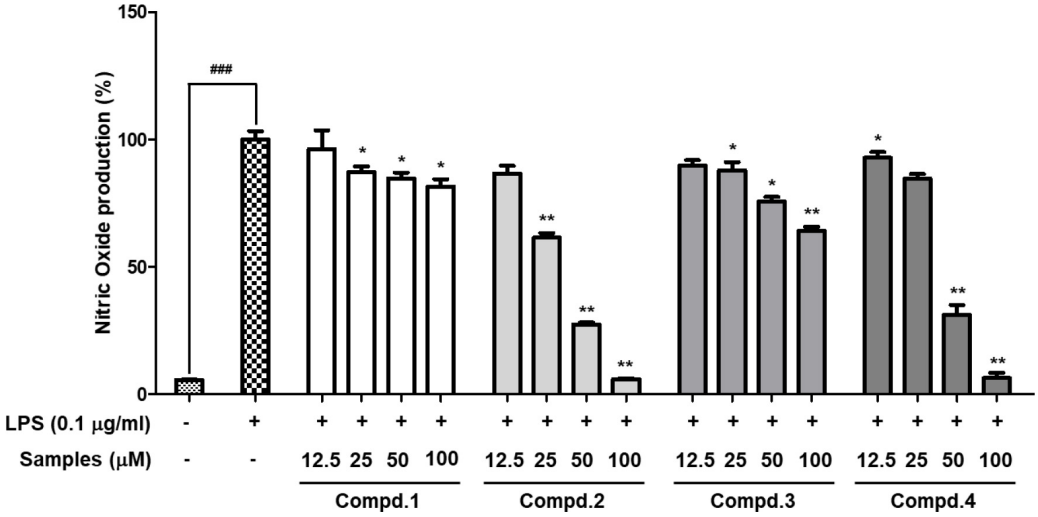

Fig. 3. Effect of phloridzin derivatives (**1**–**4**) on nitric oxide(NO) production. Cells were stimulated with 0.1μg/ml of LPS and various concentrations (12.5, 25, 50, and 100 μM) of compounds (**1**–**4**) for 24 h. NO production was measured by using the Griess reagent method. Quantitative results are presented as the mean ± standard deviation from three independent experiments (n = 3). Statistical significance was assessed as follows: $^{###}p < 0.001$ vs. the control group; *$p < 0.1$, **$p < 0.01$ vs. the LPS-treated group.

The results of cell viability following treatment with phloridzin derivatives at concentrations of 12.5, 25, 50, and 100 μM are shown in Fig. 2. At the highest concentration (100 μM), compound **1** exhibited the cell viability of 70.9%, compound **2** of 117.2%, compound **3** of 68.3%, and compound **4** of 114.3%, respectively. Compounds **1** and **3** showed a dose-dependent decrease in cell viability, while compound **2** demonstrated an opposite trend, with increased cell viability at higher concentrations. Furthermore, We also investigated NO expression were then assessed using Griess reagent. Nitric oxide (NO) production was measured as a primary indicator of anti-inflammatory activity. Compounds **1**-**4** were screened for their ability to inhibit LPS-induced NO production in RAW 264.7 macrophages at various concentrations of 12.5, 25, 50, and 100 μM. The results in Fig. 3 showed that **2** exhibited the most potent inhibitory effect on NO production compared to the LPS-control group.

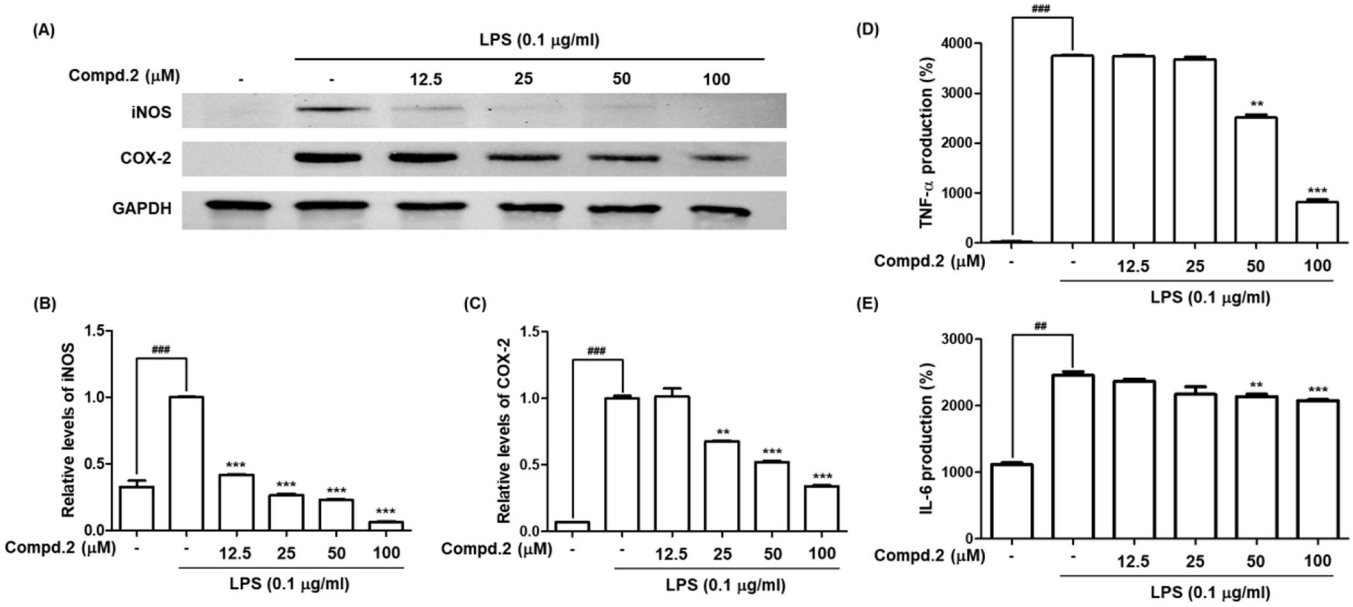

Fig. 4. The inhibitory effect of 2 on the LPS-induced the inflammatory proteins of iNOS and COX-2 were determined by western blot analysis. RAW 264.7 were treated with 2 and lipopolysaccharide (LPS; 0.1 μg/mL) for 24 hours. Protein lysates were subsequently collected, and Western blotting was performed using anti-iNOS and anti-COX-2 antibodies (A–C). GAPDH served as a positive control. Effect of 2 on IL-6 and TNF-α production in RAW 264.7 macrophages. Compound 2 on the secretion of cytokines was evaluated in RAW 264.7 macrophages. RAW 264.7 were treated with 2 and lipopolysaccharide (LPS; 0.1 μg/mL) for 24 hours. Culture supernatants were collected, and levels of TNF-α (D), and IL-6 (E) were quantified using enzyme-linked immunosorbent assay (ELISA). Quantitative results are presented as the mean ± standard deviation from three independent experiments (n = 3). Statistical significance was assessed as follows: $^{###}p< 0.001$ vs. the untreated control; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the LPS-treated group.

Among the tested compounds, compound **2**, which inhibited NO production without any cytotoxicity, exhibited a strong inhibitory effect on NO production compared to the LPS-control group.

3.2. Effect of Deglucosyl-methylene-bis-phloridzin (2) on iNOS and COX-2 protein expression

Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were found to be upregulated in response to LPS stimulation, consistent with their known roles as inflammation related proteins. These enzymes are typically induced by pro-inflammatory mediators, growth factors, and tumor-promoting stimuli, suggesting their involvement in both inflammatory processes and cellular proliferation [12-14]. Therefore, to investigate the effects of compound **2** on the expression of iNOS and COX-2 proteins, Western blot analysis was performed. Western blot analysis of the inflammatory marker iNOS and COX-2 revealed that its expression was markedly suppressed by compound **2** (Fig. 4A, B and C). Consistent with the NO inhibition assay, **2** significantly reduced iNOS and COX-2 expression even at a low concentration of 12.5 and 25 μM, respectively. These findings indicate that compound **2** suppresses the LPS-stimulated inflammatory responses by suppressing iNOS and COX-2 protein expression in RAW 264.7 cells.

3.3. Deglucosyl-methylene-bis-phloridzin (2) suppresses the production of pro-inflammatory cytokines in RAW 264.7 macrophages

Pro-inflammatory cytokines are essential mediators within the immune system, contributing significantly to the initiation and progression of inflammatory responses [15,16]. However, excessive secretion of cytokines such as TNF-α and IL-6 can compromise epithelial integrity, trigger apoptosis in epithelial cells and exacerbate inflammatory conditions [17]. To assess the impact of compound **2** on these pro-inflammatory mediators, RAW 264.7 macrophages were co-treated with LPS and compound **2**. As shown in Fig. 4D and E, treatment with the compound **2** led to a reduction in TNF-α and IL-6 levels in the culture supernatant, as measured by enzyme-linked immunosorbent assay (ELISA). Compound **2** significantly inhibited the production of TNF-α and IL-6 in a dose-dependent manner compared to the LPS-only group. These result suggest that

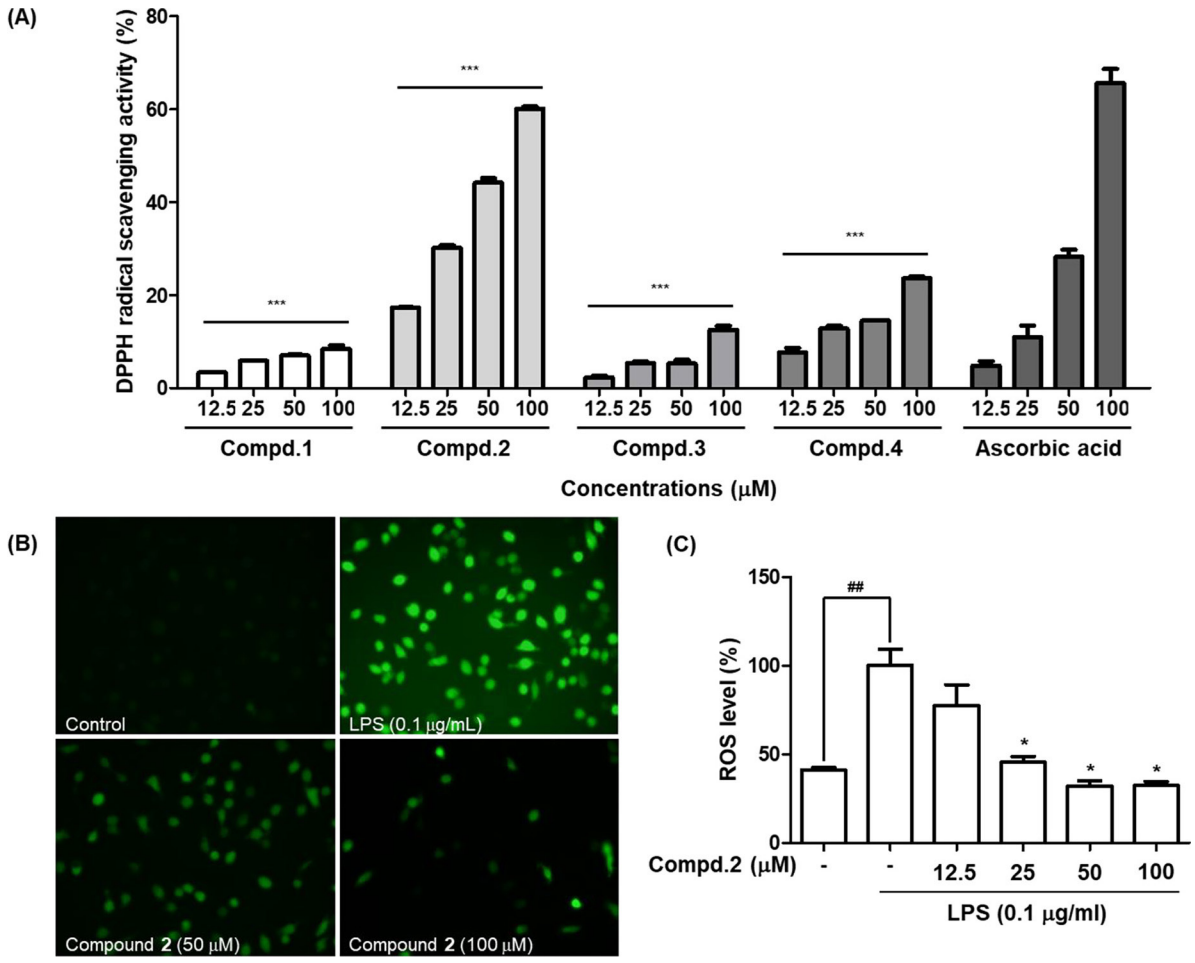

Fig. 5. Effects of phloridzin derivatives (**1**–**4**) on thr DPPH radical scavenging activity and intracellular ROS levels in LPS-induced RAW 264.7 macrophages. RAW 264.7 were treated with 2 and lipopolysaccharide (LPS; 0.1 μg/mL) for 24 hours. The DPPH radical scavenging activity of phloridzin derivatives (**1**–**4**) were evaluated 100 μL of 0.2 mM DPPH solution mixed with 100 μL of various concentrations of samples (A). Quantitative results are presented as the mean ± standard deviation from three independent experiments (n = 3). Statistical significance was assessed as follows: ***$p < 0.001$ vs. the control group. Intracellular reactive oxygen species (ROS) were visualized following incubation with 20 μM DCFH-DA for 45 min; cells were washed with phosphate-buffered saline (PBS) and observed microscopically in the presence or absence of compound 2 (magnification, ×20) (B) and for quantitative analysis, cells were treated with DCFH-DA for 45 minutes and measured by microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm (C). Quantitative results are presented as the mean ± standard deviation from three independent experiments (n = 3). Statistical significance was assessed as follows: $^{\#\#}p < 0.01$ vs. the untreated control; *$p < 0.1$, vs. the LPS-treated group.

compound **2** anti-inflammatory properties by suppressing the production of key pro-inflammatory cytokines in macrophages activated by LPS.

3.4. Deglucosyl-methylene-bis-phloridzin (2) inhibited ROS generation

The effects of compound **2** at concentrations of 12.5, 25, 50, and 100 μM on intracellular reactive oxygen species (ROS) production induced by LPS in RAW 264.7 macrophages was investigated. As shown in Fig. 5B, LPS treatment markedly increased ROS levels compared to the untreated control. In contrast, treatment with compound **2** effectively attenuated this LPS-induced ROS generation in a concentration-dependent manner.

The free radical scavenging capacity of the synthesized compounds against 1,1-diphenyl-2-picrylhydrazyl (DPPH) was evaluated using a modified version of the method originally developed by Blois [18]. In this assay, a 0.2 mM DPPH solution was prepared in ethanol (EtOH), and 100 μl of this solution was combined with 100 μl of each phloridzin derivatives (**1**–**4**) diluted to final concentrations 12.5, 25, 50, and 100 μM. The

mixtures were then incubated for 15 minutes at room temperature in a dark. After that, the absorbance was measured at 517 nm using a microplate (ELISA) reader to determine radical scavenging activity.

4. Conclusion

In this study, we investigated the anti-inflammatory and antioxidant properties of phloridzin derivatives (**1**–**4**), particularly focusing on deglucosyl-methylene-bis-phloridzin (**2**), in LPS-stimulated RAW 264.7 macrophages. Among the four compounds, compound **2** demonstrated the most favourable profile, showing no cytotoxicity and significantly suppressing nitric oxide (NO) production in a dose-dependent manner. Western blot analysis revealed that **2** effectively inhibited the expression of key inflammatory enzymes, iNOS and COX-2, even at low concentrations. Furthermore, **2** markedly reduced the production of pro-inflammatory cytokines, TNF-α and IL-6, suggesting its role in modulating inflammatory signalling cascades. In addition, **2** was shown to attenuate intracellular ROS levels in LPS-induced macrophages and exhibited notable free radical scavenging activity in the DPPH assay (IC_{50} = 70.5 ± 0.5 μM). Collectively, these findings highlight the potential of deglucosyl-methylene-bis-phloridzin (**2**) as a promising anti-inflammatory and antioxidant agent. Its ability to suppress LPS-induced inflammatory mediators and oxidative stress without cytotoxic effects suggests its potential therapeutic application in the management of inflammation-related diseases.

Acknowledgements

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