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Original Research Article

Tetramethyl-O-scutellarin isolated from peels of immature Shiranuhi fruit exhibits anti-inflammatory effects on LPS-induced RAW264.7 cells

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Abstract

Purpose: *To investigate the anti-inflammatory activity of the ethanol extract of the immature fruit of a citrus, Shiranuhi, and to identify the active ingredient.*
Methods: *The immature Shiranuhi peel was extracted with 80 % ethanol, and the extract was fractionated with solvents (n-hexane, ethyl acetate and n-butanol) to afford the corresponding fractions and water residue. Among them, the EtOAc-soluble portion was subjected to medium pressure liquid chromatography (MPLC) over a reversed-phase SiO_2 column to give compound **1**. The isolated compound was identified based on the proton and carbon nuclear magnetic resonance (NMR) spectra. The release of nitric oxide, prostaglandin (PG)E2, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 secreted by mouse macrophages was measured using RAW264.7 cell culture supernatant.*
Results: *Shiranuhi (Korean name, Hallabong) is an important citrus species cultivated in Jeju Island, Korea. A polymethoxyflavonoid (PMF), tetramethyl-O-scutellarin (**1**), was isolated from the peels of immature Shiranuhi fruit. Upon the evaluation of anti-inflammatory effects, the flavonoid 1 decreased the nitric oxide production in macrophage cells with high efficiency, viz, 50 % inhibition concentration, IC_{50} of 57.4 μM. Subsequent studies demonstrated that PMF **1** effectively inhibited the generation of PGE2, TNF-α, IL-1β, and IL-6 cytokine in a dose-dependent manner.*
Conclusion: *Tetramethyl-O-scutellarin (**1**) has been successfully isolated from Shiranuhi species for the first time. Thus, Shiranuhi fruit peel extract containing PMF **1** can potentially be applied as an anti-inflammatory ingredient in food or cosmetic industries.*

Keywords: *Shiranuhi fruit, Nitric oxide, Tetramethyl-O-scutellarin, Anti-inflammatory*

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INTRODUCTION

Citrus is a genus of trees producing important fruits such as oranges, lemons, grapefruit, and limes. Mandarine oranges (*C. reticulata*) are widely cultivated in subtropical areas of China, Japan, and Korea. The dried peels of *Citrus* fruits have been used in Chinese medicine for the cure of stomachache, skin inflammation, muscle pain, cough, and high blood pressure [1]. Phytochemical studies of *Citrus* peels have led to identifying some metabolites including flavonoids [2,3], limonoids [4,5], and coumarins [6]. Polymethoxyflavones (PMFs) have attracted considerable attention as bioactive constituents in *Citrus* peels [2,7] owing to their anti-inflammatory [8], anticarcinogenic [9], and antiatherogenic properties [10]. Besides,

antioxidant [11,12] and antitumor properties [13] were also identified from the flavonoids of *Citrus* genus.

Shiranuhi is the generic name of a hybrid *Citrus* species [(*Citrus unshiu* Marc. × *C. sinensis* Osbeck) × *C. Reticulata* Blanco)], and its fruit is large size with a characteristic sweet taste. Shiranuhi fruit (Korean name, Hallabong) is produced in large quantities from orchards in Jeju Island, Korea [14]. Within the *Citrus* species, Shiranuhi is relatively unexplored as the source of phytochemical studies searching for cosmeceutical or functional food ingredients. In the process of our investigation to develop bioactive ingredients from the plants available in Jeju Island, the ethanol extracts prepared from the peels of immature Shiranuhi fruit exhibited anti-inflammatory activities. Therefore, we decided to identify the chemical constituents responsible for the activities.

Polymethoxyflavonoid compounds have been isolated from various *Citrus* plants including *C. sinensis* and *C. reticulata* [15,16]. Essential oil from the peels of Shiranuhi exhibited high radical scavenging and anti-bacterial activities [17]. So far, there is no report on the ingredients responsible for the anti-inflammatory activity from Shiranuhi plant. The anti-inflammatory activities of the extracts and the isolated compound were determined by LPS-induced RAW264.7 murine macrophage cells.

EXPERIMENTAL

Plant material

Immature Shiranuhi peels were collected from the Citrus Research Institute (RDA) in Jeju Island, South Korea. An authenticated voucher specimen (no. 463) was deposited at the herbarium of Natural Product Chemistry, Department of Chemistry and Cosmetics, Jeju National University.

Extraction and isolation

Immature Shiranuhi peels (1,085 g) were dried in the shade and extracted with 80% ethanol three times by stirring using a mechanical stirrer at room temperature for 24 h. The combined ethanol extract was filtered, and the filtrate was concentrated using a vacuum evaporator to give a gummy extract (146.8 g). A part of the citrus extract (60.0 g) was suspended in distilled water (3 L) and fractionated with three solvents (*n*-hexane, ethyl acetate, and *n*-butanol), affording the corresponding solvent fractions and water residue. Among the fractions, the EtOAc-soluble fraction (5.0 g) was purified by medium-pressure liquid chromatography (MPLC, Biotage co.) instrument equipped with a C_{18} SiO_2 column (KP-C_{18}-HS, Biotage Co.). Elution of the fraction with H_2O–MeOH (10 – 100 %) solvent gradient provided 39 subfractions. Among them, subfraction 31 (126.8 mg) was identified as the pure compound. Analytical grade solvents were used in this experiment. ^{1}H and ^{13}C nuclear magnetic resonance spectra (^{1}H NMR, 500 MHz; ^{13}C NMR, 125 MHz) were obtained using an AVANCE III (FT-NMR system, Bruker co.) instrument, and the chemical shift (δ) data are reported in ppm relative to the NMR solvent used. Electrospray ionization (ESI) mass analyses were performed on a Waters Quattro micro Tandem mass system (Waters, USA).

Cell culture

Murine macrophage RAW264.7 cell line was purchased from American Type Cell Culture (Rockville, MD, USA). The cell (RAW264.7) culture was prepared in Dulbecco's modified Eagle's medium (GIBCO Inc., NY, USA) supplemented with streptomycin (100 µg/mL), penicillin (100 U/mL), and 10% fetal bovine serum (GIBCO Inc., NY, USA). The cells were incubated in 5 % CO_2 atmosphere at 37 °C.

WST-1 assay for cell viability

Tetrazolium salt WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] (Roche, Germany) was employed for the cell viability assays. The assay was performed following the manufacturer protocol. Murine RAW264.7 macrophage cells (1.5×10^5 cells/mL) were cultured in 24-well plate for 18 h, followed by treatment with 1 µg/mL of LPS in the presence of various samples. After 24 h cell incubation, WST-1 was added to the medium and it was allowed to stand for 1 h. The supernatant was measured at 440 nm with reference 600 nm. Cell viability was determined as the ratio of the sample absorbance to that of control.

Measurement of nitric oxide concentration

The NO production was determined by the Griess reagent (Sigma, USA). In this assay, the accumulated nitrite ion originated from the NO in the culture medium was monitored by a colorimetric method. Briefly, after RAW264.7 macrophage cells (1.5×10^5 cells/mL) were incubated for 18 h, the cells were treated with LPS and various concentrations of samples (total volume, 1 µg/mL) for 24 h. Then, the equal volume of Griess reagent was added to the

supernatant, and incubated at room temperature for 10 min. The produced nitrite ion was determined using a microplate reader (Sunrise™, Tecan Group Ltd., Switzerland) at a wavelength of 540 nm. The percent inhibition for each sample was calculated as.

Inhibition (%) = [(Control – Sample) / Control] × 100

Measurement of prostaglandin E_2 (PGE_2) and cytokines

The production of PGE_2 and pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) was determined in the culture supernatant of RAW264.7 cells. In a 24-well cell culture plate, the macrophage cells were placed at an appropriate density (1.5×10^5 cells/mL) with culture medium (1 mL) and incubated for 18 h. Then, the cells were treated with a sample (specified concentration) and LPS (1 μg/mL) and incubated for an additional 24 h. The amount of PGE_2, TNF-α, IL-1β, and IL-6 produced by the cells was measured using an ELISA kit (PGE_2, IL-1β; R&D systems, TNF-α, IL-6; Invitrogen) and a microplate reader according to the manufacturer's instructions.

Statistical analysis

Means (± standard error of the mean) of the data are presented, and statistical analysis of the results was carried out by Student's t-test using Microsoft Excel 2013 (Microsoft Corporation, USA) for independent samples. Values of p <0.05 and p <0.01 were considered significant as appropriate.

RESULTS

At first, the ethanol extracts were examined for the anti-inflammatory activities using RAW 264.7 cells. The extract (100 μg/mL) prepared from the immature Shiranuhi peels inhibited LPS-induced NO production in the macrophage cells by 8 % (Figure 1). The ultimate goal of this study was to determine the anti-inflammatory chemical constituents in the extracts. Thus, the extract was partitioned in the order of polarity using

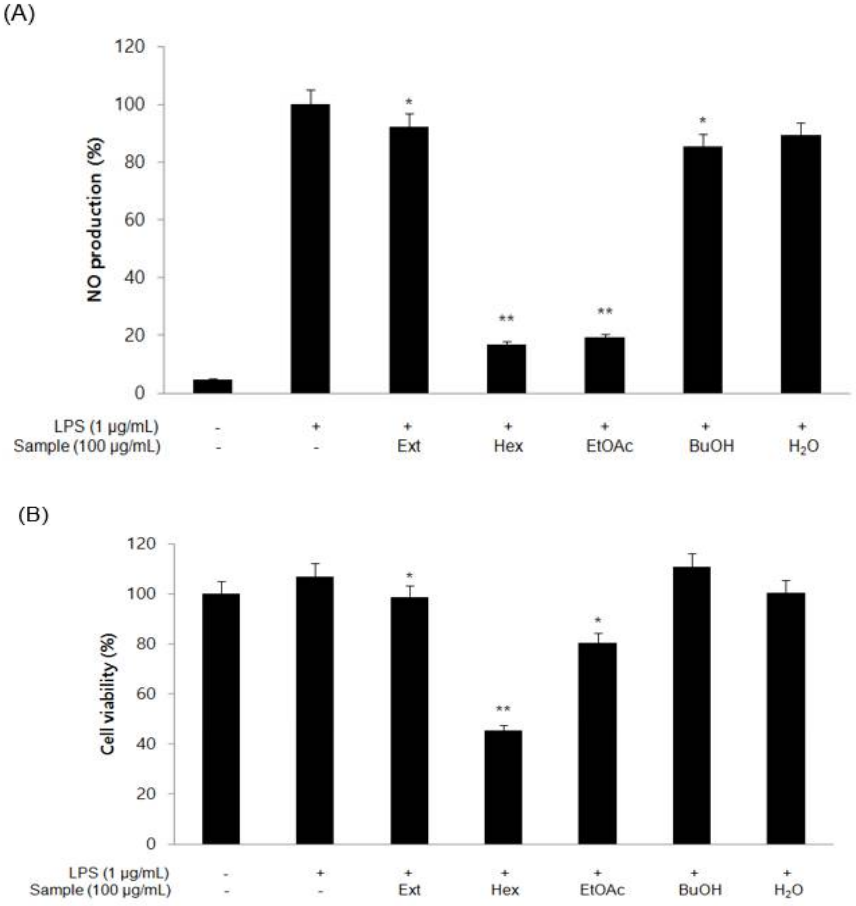

Figure 1: Effect of extract and solvent fractions from immature Shiranuhi peel on NO production (A) and cell viability (B) in LPS-induced RAW264.7 cells. The cells were stimulated with 1 μg/mL of LPS only, or with LPS plus sample (100 μg/mL) for 24 h. Values are the mean ± SEM of triplicate experiments; *p < 0.05; **p < 0.01 versus LPS alone

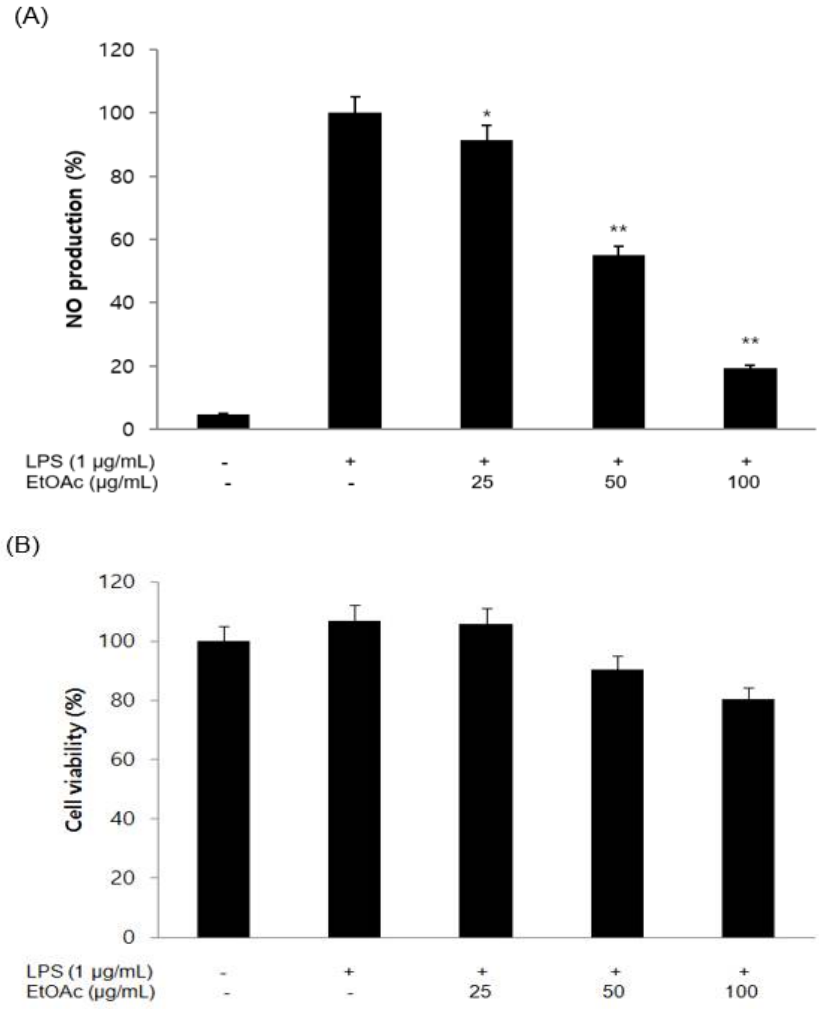

Figure 2: Effect of ethyl acetate (EtOAc) fractions on NO production (A) and cell viability (B) in LPS-induced RAW264.7 cells. The cells were stimulated with 1 μg/mL of LPS only, or with LPS plus various concentrations (25, 50, and 100 μg/mL) of samples for 24 h. Values are the mean ± SEM of triplicate experiments; *$p < 0.05$; **$p < 0.01$ versus LPS alone

solvents (*n*-hexane, EtOAc, and *n*-butanol) to afford corresponding fractions as well as the remaining water residue. Each solvent fraction was again subjected to the activity test for the generation of NO. Figure 1A shows that both the *n*-hexane and EtOAc fractions possess strong NO inhibition activities. However, the WST-1 assay indicated that the *n*-hexane fraction have considerable cell toxicity. In contrast, the EtOAc fraction mostly maintained cell viability at a concentration up to 100 μg/mL, indicating that the cell-destruction effect is rarely involved in the observed NO inhibition. Figure 2 shows the NO inhibition and cell toxicity data of the EtOAc fraction under various concentrations (25 – 100 μg/mL), indicating that the EtOAc fraction decreased the NO generation in a dose-dependent manner. The EtOAc fraction was selected for further purification to isolate the active ingredient, because it strongly inhibited NO synthesis without causing cell toxicity at 100 μg/mL (Figure 2B).

EtOAc fraction was separated by MPLC and silica gel column chromatography, leading to the isolation of compound **1**. The chemical structure of compound **1** was elucidated by the interpretation of the ^{1}H and ^{13}C NMR spectra. Compound **1** showed 13 carbon peaks in the ^{13}C NMR spectrum, a characteristic indication of the presence of a flavone skeleton. The signal at δ 177.4 ppm in the ^{13}C NMR spectrum indicates the presence of a carbonyl group at C-4 position. The ^{13}C signals at δ 62.4, 61.7, 56.4, and 55.6 ppm correspond to the four methoxy groups at the C-5, C-6, C-7, and C-4′ positions, respectively.

The ^{1}H NMR spectrum of compound **1** revealed that it has an A_2B_2 system (H-2′/H-6′ and H-3′/H-5′), as demonstrated by the coupling constant

signals at δ 7.81 (d, H-2′, H-6′) and δ 6.99 (d, H-3′, H-5′), and one methoxy group at position C-4′. The aromatic proton singlet signals at δ 6.56 and 6.77 ppm were assigned to the H-3 and H-8 in the structure. Four proton signals for the methoxy groups at the H-5, H-6, H-7 and H-4′ were observed at δ_H 3.86, 3.89, 3.95, and 3.96, respectively.

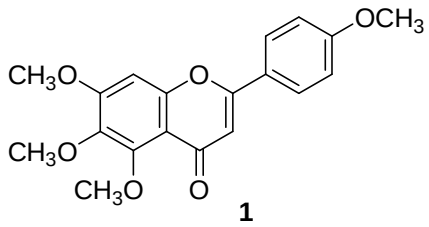

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Figure 3: Structure of tetramethyl-*O*-scutellarin (**1**) isolated from immature Shiranuhi peel

By careful examination of the NMR data discussed above, compound **1** was identified as a polymethoxyflavone, tetramethyl-*O*-scutellarin (Figure 3). The ^{1}H NMR and ^{13}C NMR data are as follows: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.81 (2H, d, *J* = 9.0, H-2′, 6′), 6.99 (2H, d, *J* = 9.0, H-3′, 5′), 6.77 (1H, s, H-8), 6.56 (1H, s, H-3), 3.96 (3H, s, 4′-OCH_3), 3.95 (3H, s, 7′-OCH_3), 3.89 (3H, s, 6-OCH_3), 3.86 (3H, s, 5-OCH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 177.4 (C-4), 162.3 (C-2), 161.3 (C-4′), 157.8 (C-7), 154.6 (C-5), 152.7 (C-9), 140.4 (C-6), 127.8 (C-2′, 6′), 124.0 (C-1′), 114.5 (C-3′, 5′), 113.0 (C-10), 107.2 (C-3), 96.4 (C-8), 62.4 (5-OCH_3), 61.7 (6-OCH_3), 56.4 (7-OCH_3), 55.6 (4′-OCH_3). ESIMS (positive-ion mode): *m/z* 343 $(M+H)^+$. The suggested structure was finally confirmed by the comparison of the observed data to the literature values [18]. As far as we know, tetramethyl-*O*-scutellarin (**1**) was isolated for the first time from Shiranuhi fruit.

The bioactivity test flavonoid **1** was also tested using RAW 264.7 cells under under varying concentrations (25, 50, and 100 μM). When activated by LPS, RAW 264.7 cells increased the nitric oxide (NO) production by 12 fold (Figure 4A). The treatment of tetramethyl-*O*-scutellarin (**1**) to the activated macrophage cells decreased

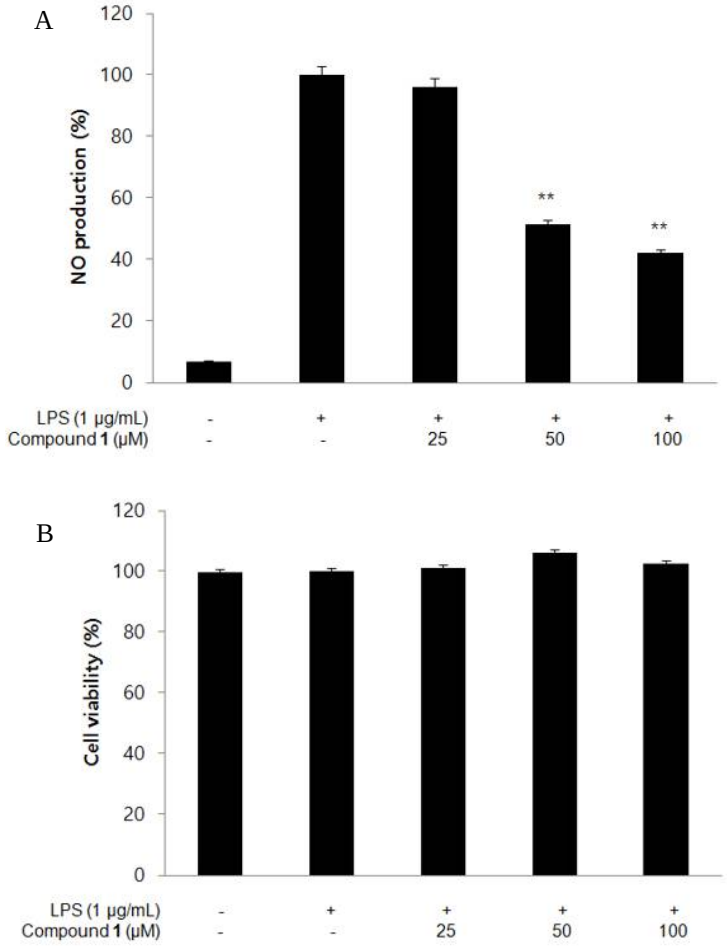

Figure 4: Effect of tetramethyl-*O*-scutellarin (**1**) on NO production (A) and cell viability (B) in LPS-induced RAW264.7 macrophage cells. The cells were stimulated with 1 μg/mL of LPS only, or with LPS plus various concentrations (25, 50, and 100 μM) of samples for 24 h. Values are the mean ± SEM of triplicate experiments *$p < 0.05$; **$p < 0.01$ versus LPS alone

the NO production with high efficiency (IC_{50} = 57.4 μM)) in a dose dependent manner. The potential cytotoxicity of the isolate **1** was determined by the WST-1 assay, where the cells were incubated for 24 h with or without LPS (Figure 4B). In this test, cell viability mostly unchanged at the employed concentrations (25, 50, and 100 μM), suggesting that the NO inhibitory effect of isolate **1** had nothing to do with its cell toxicity. To further elucidate the anti-inflammatory mechanism of PMF **1**, inflammation-related substances such as PGE_2, IL-6, IL-1β, and TNF-α were monitored in culture supernatants by ELISA. As shown in Figure 5, LPS-activated RAW 264.7 cells significantly produced the inflammatory cytokines (Figure 5). This experiment clearly revealed that flavonoid **1** reduced the production of the cytokines (PGE_2, TNF-α, IL-1β, and IL-6) with high to medium degree in a concentration dependent manner (Figure 5 and Figure 6).

DISCUSSION

If body tissue is exposed to a variety of stimuli, inflammation occurs as a biological response. Inflammation is commonly characterized by blood flow increase to the damaged tissue inducing temperature rise, redness, swelling and pain. It is mediated by different types of signaling molecules produced by various cells. Macrophages play an important role in inflammation-related disease through the secretion of molecular mediators such as NO, PGE_2 as well as relevant cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 [19].

Polymethoxyflavones (PMFs) are a class of compounds, with flavones as a base structure and methoxy substituents in different positions. Interestingly, PMFs exist mainly in *Citrus* genus in the plant kingdom. More than 30 PMFs have

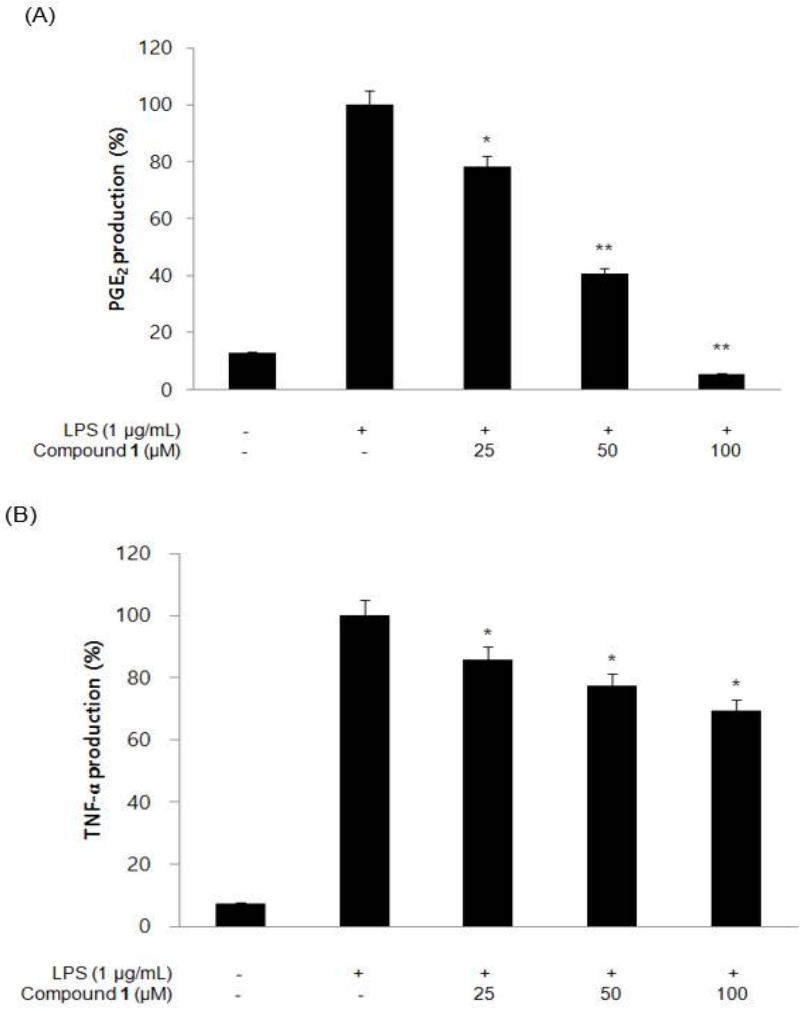

Figure 5: Effect of tetramethyl-*O*-scutellarin (**1**) on PGE_2 (A) and TNF-α (B). The cells were stimulated with 1 μg/mL of LPS only, or with LPS plus various concentrations (25, 50, and 100 μM) of samples for 24 h. Values are the mean ± SEM (n = 3); *p <0.05; **p <0.01 versus LPS alone

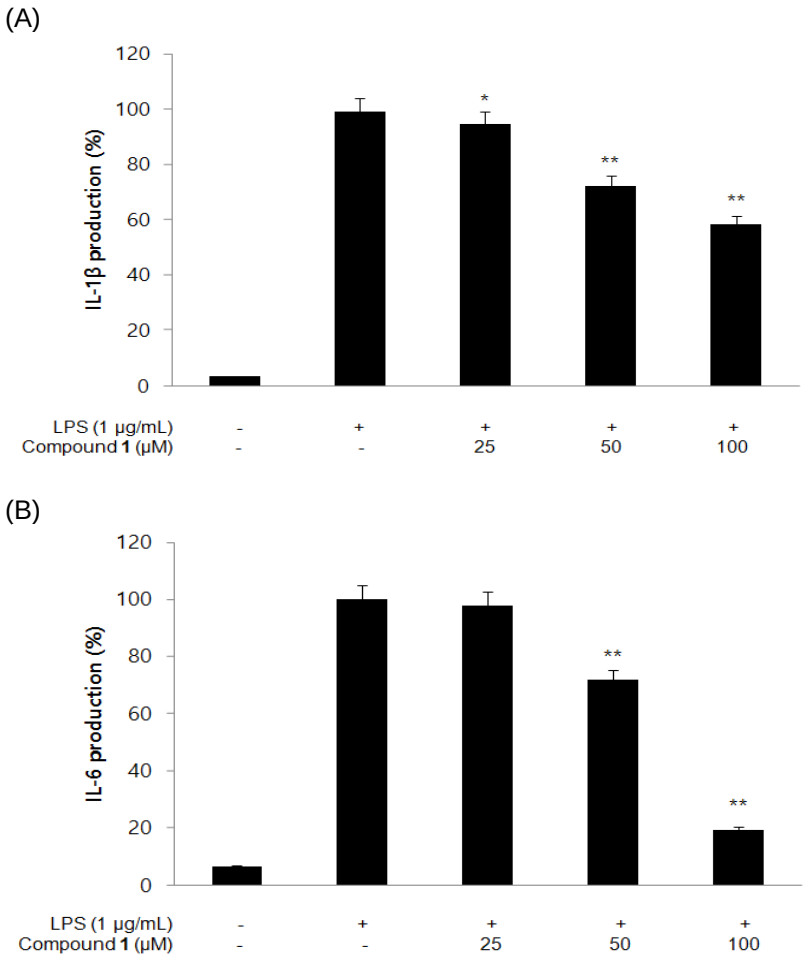

Figure 6: Effect of tetramethyl-*O*-scutellarin (**1**) on IL-1β (A) and IL-6 (B) production in LPS-induced RAW264.7 macrophage cells. The cells were stimulated with 1 μg/mL of LPS only, or with LPS plus various concentrations (25, 50, and 100 μM) of samples for 24 h. Values are the mean ± SEM (n = 3); *p <0.05; **p <0.01 versus LPS alone

been isolated from different parts of the citrus species including mandarin (*C. reticulata* Blanco)and sweet orange (*C. sinensis*) [20]. The structural types and ingredient contents of PMFs vary between different varieties of citrus plants. Among those isolated PMF derivatives, nobiletin and tangeretin have been intensively explored owing to their availability and interesting pharmacological activities. Numerous studies suggest that PMF compounds isolated from citrus display a broad spectrum of biological activity [21].

In this study, we isolated a polymethoxy-flavonoid, tetramethyl-*O*-scutellarin (**1**) as an active constituent from immature Shiranuhi peel and examined its anti-inflammatory activities. Shiranuhi is a hybrid *Citrus* species, and its fruit is produced in large quantities in Jeju Island of Korea. The isolated PMF **1** strongly inhibited the NO production with a high efficiency (IC_{50} 57.4 μM). The subsequent studies demonstrated that the flavonoid **1** effectively reduced the secretion of prostaglandin E_2 as well as the production of pro-inflammatory cytokines such as tumor necrosis factor-alpha, interleukin (IL)-1β, and IL-6 in a dose dependent manner.

CONCLUSION

Tetramethyl-*O*-scutellarin (**1**) has successfully been isolated for the first time from Shiranuhi citrus species. PMF compound **1** possesses anti-inflammatory properties as indicated by the suppression of NO, PGE_2, IL-6, IL-1β, and TNF-α production in LPS-triggered macrophage RAW 264.7 cells. The control of pro-inflammatory mediators (NO, TNF-α, and IL-6) has been an effective strategy for the development of novel anti-inflammatory materials. Thus, the results suggest that tetramethyl-*O*-scutellarin (**1**) derived from a citrus might be a potential agent for the treatment of inflammation-associated human health problems.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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