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Anti-inflammatory Polymethoxyflavones Isolated from the Branches of Shiranuhi Tree

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ABSTRACT. Shiranuhi is a fruit of *Citrus* species widely cultivated in Jeju Island, Korea. From an extract of Shiranuhi tree branches were identified five polymethoxyflavones possessing anti-inflammatory effects; nobiletin (**1**), sinensetin (**2**), tetramethylscutellarein (**3**), 6-hydroxy-5,7,3′,4′-tetramethoxyflavone (**4**) and 5-desmethylsinensetin (**5**). Evaluation of the activities was conducted by monitoring the production of nitric oxide (NO), prostaglandin E_2 and pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) as well as the levels of iNOS and COX-2 protein expression in LPS-induced RAW264.7 cells. Among the isolates, the compound **4** exhibited the most significant NO inhibition, and suppressed the levels of iNOS and related cytokines. Therefore, it was suggested that the extract and constituents from Shiranuhi tree branches could be useful as anti-inflammatory ingredient.

Key words: Shiranuhi, Polymethoxyflavone, Isolation, Anti-inflammation

INTRODUCTION

Shiranuhi is the general name of a fruit harvested from a hybrid *Citrus* species [(*Citrus unshiu* Marc.×*C. sinensis* Osbeck)×(*C. Reticulata* Blanco)]. It was originally introduced from Japan and then named Hallabong in Korea, as its round shape with a folded neck looks like Halla mountain. This fruit is characterized by its bulky size and sweet taste, and is one of the major crops grown in *Citrus* orchards in Jeju Island, Korea.[1]

We have investigated the peel of Shiranuhi fruit as a source of bioactive ingredients available for cosmetic formulations. This fruit is rich in polyphenols, vitamins, limonoids, carotenoids, terpene and flavonoids that are functional and valuable dietary ingredients for human health.[2] A polymethoxyflavone (PMF) component was isolated from its immature fruit and the anti-inflammatory effect was elucidated in our study.[3] The other biological effects reported in Shiranuhi peels include anti-oxidative,[4] anti-obesity[5] and anti-microbial[6] activities. PMFs are natural products, mainly found in *Citrus* species in plant kingdom, with a chemical structure bearing two or more methoxy groups in their basic benzo-γ-pyrone flavone skeleton.[7] PMFs have shown broad biological spectrum such as anti-inflammatory, anti-cancer,[8] anti-mutagenic, anti-microbial[9] and anti-aging[10] activities.

As a continuing effort, we conducted the phytochemical study using the branch parts of the Shiranuhi tree. Most of the citrus research is focused on the fruit parts with interest in the food efficacy. In terms of finding bioactive constituents from plants in Jeju Island, we became interested in not only citrus fruits but also tree stems. Here, in this study, the ethanol extract of Shiranuhi branches was screened to exhibit the inhibition activities on NO production in LPS-induced murine macrophage RAW264.7 cells. Then, five PMFs were isolated, and their anti-inflammatory effects were determined along with the analysis of structure-activity relationships.

EXPERIMENTAL

General

The medium-pressure liquid chromatography (MPLC, Biotage Co., Sweden) was performed with ODS gel column (KP-C18-HS, Biotage Co., Sweden). Column chromatography was carried out using Sephadex LH-20 (10-25 μm, GE healthcare) or silica gel (40-630 μm, Merck, Germany) as stationary phases. Nuclear magnetic resonance (NMR) spectra were recorded with a JNM-ECX 400 instrument (FT-NMR system, JEOL, Japan).

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were obtained from Gibco Co. (USA). Lipopolysaccharide (LPS), Griess reagent and RIPA lysis buffer were purchased from Sigma-Aldrich (USA). ELISA kits were provided from R&D systems (USA) for PGE_2 and IL-1β as well as Invitrogen (USA) for TNF-α and IL-6.

Plant Material

The branches of Shiranuhi tree were collected from the Citrus Research Institute (Jeju Island, Korea) in March 2015. A voucher specimen (No. 465) was prepared and deposited at the laboratory of Natural Product Chemistry, Department of Chemistry and Cosmetics, Jeju National University.

Extraction and Isolation

The Dried branches of Shiranuhi tree (1.3 kg) were extracted three times with 70% ethanol (EtOH, 26 L) at room temperature for 24 h under stirring. The combined solutions were concentrated under reduced pressure and dried to powder (187.4 g). The extract (180.0 g) was suspended in water (6 L) and then partitioned into *n*-hexane (Hex, 18 L), ethyl acetate (EtOAc, 18 L) and *n*-butanol (BuOH, 18 L) soluble fractions. Since the EtOAc fraction exhibited potent anti-inflammatory activities in LPS-induced RAW264.7 cells, the EtOAc fraction (5.0 g) was divided into 48 fractions (Fr. MP1-Fr. MP48) by MPLC with elution of H_2O:MeOH gradient (10% to 100%). Out of these subfractions, fraction MP25 and 26 (146.8 mg) was subjected to Sephadex LH-20 column chromatography eluting with $CHCl_3$:MeOH (15:1) to afford compound **4** (33.6 mg). Fraction MP29 to 32 (285.6 mg) was further purified using a silica gel column with Hex:EtOAc (1:2) providing compounds **1** (15.4 mg), **2** (47.2 mg), **3** (11.9 mg) and **5** (12.5 mg).

Cell Culture and Viability Assay

The murine macrophage cell line RAW264.7 was purchased from American Type Cell Culture (ATCC). The cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL of penicillin and 100 μg/mL of streptomycin, and incubated in a humidified atmosphere of 5% CO_2 at 37 °C.

The cell viability was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. RAW264.7 cells (2.0×10^5 cells/well) for 18 h, were induced with LPS (1 μg/mL) and treated with various concentrations of samples. After 24 h incubation, MTT reagent (500 μg/mL) was added to the medium, and which was allowed to stand for 4 h. After removing the supernatant, the formazan crystals were dissolved in DMSO. The optical density of each well was measured at 570 nm using a microplate reader (TECAN, Austria).

Measurement of NO, PGE_2 and Cytokines Concentration

The NO production was assayed by measuring the nitrite in the supernatants of cultured cells. After RAW264.7 cells (2.0×10^5 cells/well) were incubated for 18 h, the cells were treated with LPS and samples for 24 h. Then, the equal volumes of supernatant and Griess reagent were mixed at room temperature for 10 min. Absorbance was measured at 540 nm using a microplate reader. The nitrite amounts in the test samples were calculated from $NaNO_2$ standard curve.

The production of PGE_2 and pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) was determined in the culture supernatants. The amounts of PGE_2, TNF-α, IL-1β and IL-6 produced by the cells were determined by ELISA kits.

Western Blotting

RAW264.7 cells (3.0×10^5 cells/well) were cultured in 60 mm culture dishes for 18 h and induced with LPS (1 μg/mL) in the presence or absence of samples. After 24 h incubation, the cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with RIPA lysis buffer. The cells were centrifuged for 20 min (15,000 rpm, 4 °C) and the protein concentration in the cell lysate was quantified using Bradford assay. Protein samples (20 μg) were loaded on SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After transfer, the membranes were blocked with 5% skim milk in TTBS (Tris-buffered saline and 0.1% Tween 20) overnight at 4 °C and then probed with primary antibodies (iNOS; Santa Cruz, COX-2; BD, β-actin; Sigma). After 2 h incubation followed by five washes, the membranes were incubated with appropriate secondary antibodies (iNOS; Cell signaling, COX-2, β-actin; Santa Cruz) for 1 h. After final five washes with TTBS, the protein bands were visualized by ECL reagent and the densities of the bands were detected using Chemidoc (Fusion solo, VILBER LOURMAT, Germany).

Statistical Analysis

All experiments were performed in triplicate. The experimental data were expressed as the mean ± standard deviation (S.D.). Statistical analysis of the results was performed using the student's t-test for independent samples. Values of $^{*}p<0.05$ and $^{**}p<0.01$ were considered significant.

RESULTS AND DISCUSSION

EtOH extract and solvent fractions were prepared using Shiranuhi tree branch parts, and their inhibitory activities on NO production was screened using LPS-stimulated

RAW264.7 cells. The cells were incubated in the presence or absence of the samples (100 μg/mL), and NO generation was analyzed by Griess reagent. In order to determine the toxicity of the fractions to the cells, MTT assay was simultaneously conducted. As shown in *Fig*. 1A, among the tested samples, the EtOAc fraction exhibited most significant NO inhibition with little loss of cell viability. Within a concentration range from 20 to 60 μg/mL, the EtOAc fraction decreased NO generation in a dose dependent manner (IC_{50} 30.3 μg/mL) without causing cell toxicity (*Fig*. 1B).

Based on the observed activity data, the EtOAc fraction was selected for further purification to identify the active chemical components. Use of column chromatography with silica gel or Sephadex LH-20 as well as MPLC with a reversed phase ODS gel led to the isolation of five constituents. All of the isolates were chemical species of PMF, whose structure were identified by comparative analysis of the NMR data to the literature values;[11–14] nobiletin (**1**), sinensetin (**2**), tetramethylscutellarein (**3**), 6-hydroxy-5,7,3',4'-tetramethoxyflavone (**4**) and 5-desmethylsinensetin (**5**). The PMFs **1-3** have earlier been identified in Shiranuhi[2, 15] while the compounds **4** and **5** are reported here for the first time from this plant.

Compounds **4** and **5** are isomers in which hydroxy (-OH) and methoxy ($-OCH_3$) groups are replaced each other at C-5 and C-6 positions. The compound **5** has a hydroxy at C-5 position inducing intramolecular hydrogen bond with carbonyl group at C-4. Therefore, the compound **5** shows a characteristic down field chemical shift of 182.7 ppm for C-4 in ^{13}C NMR spectrum. In comparison, δ_C for carbonyl carbon (C-4) is within the range of 175.6-177.5 ppm for the compounds **1-4** all bearing methoxy groups at C-5.

The effects of isolated PMFs (**1-5**) on NO levels were measured using LPS-induced macrophage cells. As shown in *Table* 1, all of the compounds showed inhibitory activities under the reaction conditions. Among the isolates, the compound **4** showed the most potent inhibition on NO generation with IC_{50} value of 18.2 μM. With the isolated

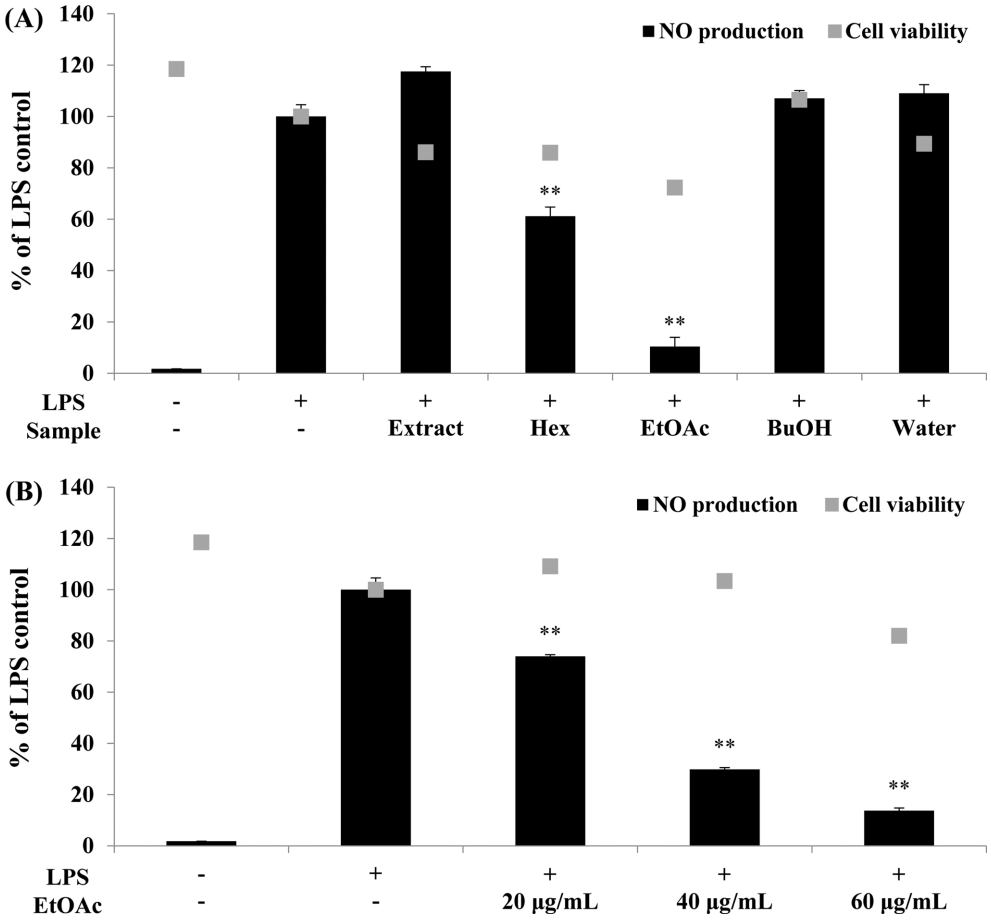
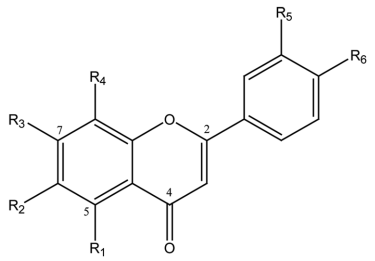


***Figure* 1.** Effects of EtOH extract, solvent fractions (100 μg/mL) (A) and EtOAc fraction (B) on NO production and cell viability in LPS-induced RAW264.7 cells.



Compound	R_1	R_2	R_3	R_4	R_5	R_6
1	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3
2	OCH_3	OCH_3	OCH_3	H	OCH_3	OCH_3
3	OCH_3	OCH_3	OCH_3	H	H	OCH_3
4	OCH_3	OH	OCH_3	H	OCH_3	OCH_3
5	OH	OCH_3	OCH_3	H	OCH_3	OCH_3

***Figure* 2.** Chemical structures of isolated compounds **1-5** from Shiranuhi tree branches.

***Table* 1.** IC_{50} values of the isolated compounds **1-5**

Compound	IC_{50} (μM)
Nobiletin (**1**)	97.9 ± 2.4
Sinensetin (**2**)	116.5 ± 1.1
Tetramethylscutellarein (**3**)	95.9 ± 1.5
6-Hydroxy-5,7,3',4'-tetramethoxyflavone (**4**)	18.2 ± 1.4
5-Desmethylsinensetin (**5**)	148.8 ± 0.5

PMFs **1-5** at hand, it seems meaningful to analyze the relationship between the structure and activity in NO production. Compared with the nobiletin (**1**, IC_{50} 98 μM), the sinensetin (**2**, IC_{50} 116 μM) and tetramethylscutellarein (**3**, IC_{50} 96 μM) exhibit similar activity. This suggests that even without methoxy groups in position R_4 or R_5, there is no significant difference on the activity. In terms of chemical structure, the most active compound **4** could be compared to the compound **2**. The only difference between **2** and **4** lies at R_2 position, where **2** and **4** have methoxy and hydroxy group respectively. Since the activity of **4** was observed more than six times higher than that of **2**, this implies that the effect of hydroxy group at R_2 position is important in the NO inhibition. Compared to **2**, the compound **5** has the structure bearing hydroxy instead of methoxy at R_1. Given **5**'s activity is no higher than **2**'s, the hydroxy group at R_1 is considered to have little effect on the activity. At this point, analysis of given compounds **1-5** suggests that hydroxy group at R_2 is important to exhibit higher activity, while changes of functional groups in other position have no significant impact.

Previously, nobiletin (**1**) and its metabolites have been investigated for the anti-inflammatory activity. A research group has identified that the demethylated nobiletin metabolites (e.g., 3'- or 4'-demethylnobiletin) isolated in mouse urine possess more potent activity than the parent compound (nobiletin, **1**) in the study using RAW264.7 cells.[16] This observation is in agreement of our result that the presence of hydroxy group in PMFs can affect the anti-inflammatory activity.

The compound **4** was investigated in more detail searching for the mechanism related to the anti-inflammation. Therefore, production of prostagladin E_2 and pro-inflammatory cytokines were monitored by treating the PMF **4** on macrophage cells (*Fig.* 3). As shown in *Fig.* 3, while the com-

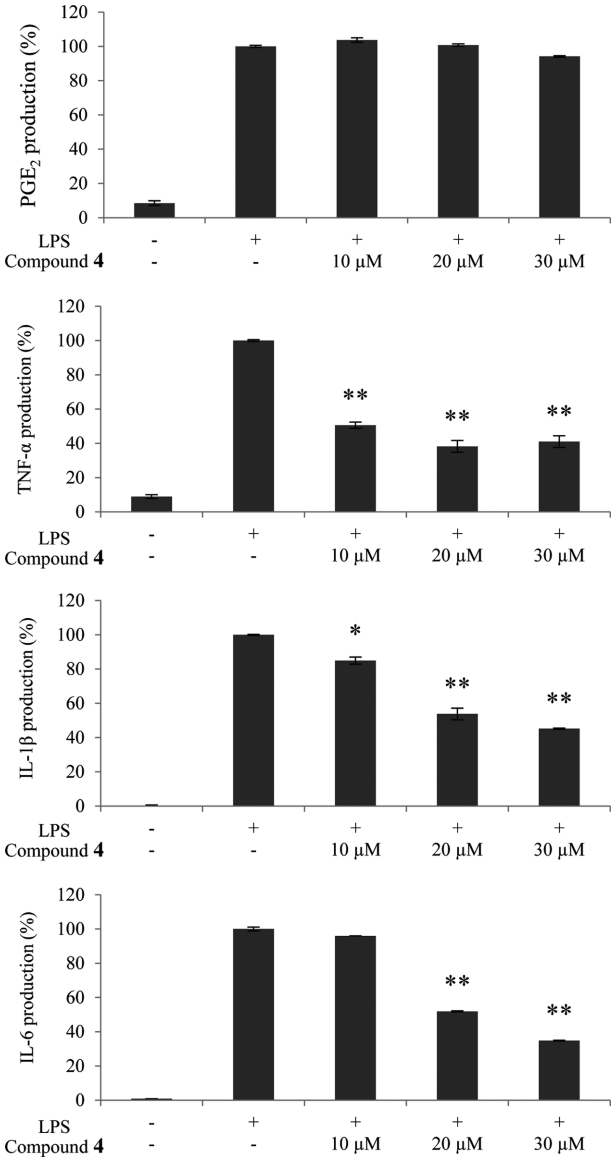


***Figure* 3.** Effects of compound **4** on PGE_2, TNF-α, IL-1β and IL-6 production.

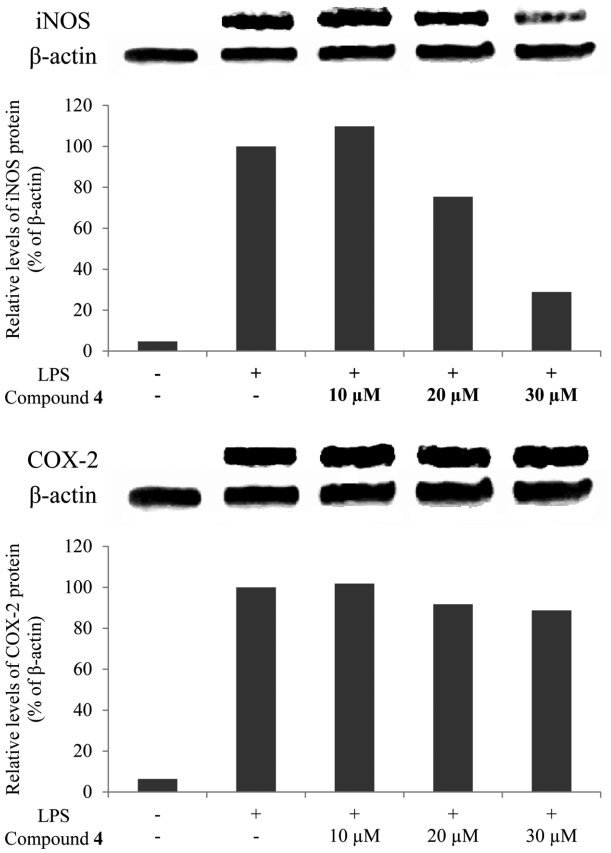


***Figure* 4.** Effects of compound **4** on iNOS and COX-2 protein levels in LPS-induced RAW264.7 cells.

pound **4** did not affect PGE_2 production, it effectively decreases the levels of TNF-α (IC_{50} 10.1 μM), IL-1β (IC_{50} 22.3 μM) and IL-6 (IC_{50} 20.4 μM) in dose dependent manner.

Since iNOS and COX-2 are major enzymes inducing inflammatory mediators such as NO and PGE_2,[17] the effect of their protein expression by PMF **4** was investigated (*Fig.* 4). The compound **4** was observed to significantly decrease iNOS level in a dose dependent mode with 71.1% inhibition at 30 μM concentration. As presented in Figure, on the while, COX-2 level was slightly decreased (11.3%) at 30 μM.

PMFs exist almost exclusively in *Citrus* genus[18] and more than 20 PMFs have been identified from different tissues of the citrus plants.[19] One of the most significant biological properties of the PMFs is their anti-inflammatory activity.[20] Flavonoids with methoxy groups have been identified to inhibit the enzymes like iNOS and NADPH oxidase that generate free radicals like NO and superoxide anion.[21] The anti-inflammatory effect of the 6-hydroxy-5,7,3',4'-tetramethoxyflavone (**4**) has not been released yet. The results obtained in this study are well in line with the anti-inflammatory effects of the reported PMFs.

CONCLUSION

This study revealed that the EtOAc fraction of Shiranuhi tree branches significantly inhibited NO production in RAW264.7 cells. Further purification process identified five polymethoxyflavons (**1-5**) as active constituents. Among the isolates from Shiranuhi tree branches, 6-hydroxy-5,7,3',4'-tetramethoxyflavone (**4**) exhibited the most potent inhibitory effect on NO production. From the careful analysis of the structure, it is suggested that hydroxy group at R_2 (*Fig.* 2) exerts important effect on the NO inhibition activity. In addition, PMF **4** suppressed the levels of iNOS protein and pro-inflammatory cytokines. Isolation of the compounds **4** and **5** from Shiranuhi as well as anti-inflammatory activities of **4** were reported for the first time in this study. Based on these results, it was suggested that the extracts possessing PMFs from Shiranuhi tree branches could be potentially applicable as a functional ingredient against inflammation.

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Supporting Information. Additional supporting information (NMR data for the compounds **1-5**) is available in the online version of this article.

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