

Anti-inflammatory Effect of Myricetin from *Rhododendron mucronulatum* Turcz. Flowers in Lipopolysaccharide-stimulated Raw 264.7 Cells

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Received June 3, 2016 / Revised October 4, 2016 / Accepted October 5, 2016

As a research of inflammation inhibitory activity using natural resource, the inflammation inhibitory activity by purified active compound from *Rhododendron mucronulatum* flower was experimented. *Rhododendron mucronulatum* flower components were purified and separated with Sephadex LH-20 and MCI gel CHP-20 column chromatography, Purified compound was confirmed as myricetin by ¹H-NMR, ¹³C-NMR and Fast atom bombardment (FAB)-Mass spectrum to have inhibition activity on inflammatory factors secreted by Raw 264.7 cells in response to lipopolysaccharide stimulation. Myricetin inhibited nitric oxide (NO) expression in a concentration dependent manner, approximately 40% inhibition was observed at a concentration of 50 μ M. The inhibition effect of myricetin on inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 protein expression was 20% and 80%, respectively, at a concentration of 25 μ M. Myricetin also inhibited expression of the inflammatory cytokines, tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6 and prostaglandin E₂ (PGE₂) in a concentration dependent manner; a concentration of 50 μ M, 70%, 80%, 80% and 95% inhibition was observed, respectively. Therefore myricetin isolated from *Rhododendron mucronulatum* flowers is expected to have an anti-inflammatory effect in Raw 264.7 cell induced by lipopolysaccharides. The results can be expected myricetin from *Rhododendron mucronulatum* flower to use as functional resource for anti-inflammatory activity.

Key words : Anti-inflammation, lipopolysaccharide, myricetin, purification, *Rhododendron mucronulatum* flowers

Introduction

With increasing attention paid to the secondary metabolite produced by plants as a defense mechanism, several studies have investigated their potential bioactivity in humans. Most of bioactive substances present in plants are water-soluble phenolic compounds and flavonoid is one of the compounds found in several plant species. These bioactive compounds are reported to have several beneficial properties for human health, including antioxidation [4], antitumor and anticancer properties [11], and have also been used for the prevention of human disease [13].

Rhododendron mucronulatum is a member of the Rhododendraceae family and is distributed throughout the northern region of East Asia including South Korea [9]. *Rhododendron mucronulatum* flowers and leaves are known to have pharmacological effects for sthenia, diuresis [13], low blood pressure, fatigue and rheumatoid arthritis in China [7]. *Rhododendron mucronulatum* flowers contain quercetin, myricetin, afzelin, quercitrin, catechin, dihydroflavonol, and other compounds, which are flavonoid components [8]. Flavonoid compounds in plants are known to have various biological functions including antioxidant and anticancer [33], antibacterial [18], anti-inflammation [20], prevention of heart disease and diabetes, and others [24]. Recent studies have shown that *Rhododendron mucronulatum* flowers have antioxidative and anticancer effects [1], and can be used in cosmetic material development [2]. *Rhododendron mucronulatum* flowers are widely distributed, and although they have been consumed for a long time, research related to their bioactivity is scarce.

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Lipopolysaccharide (LPS) is a component of the outer membrane of gram-negative bacteria and is a known pyrogen. Macrophages are activated by a small amount of LPS to produce and release cytokines, nitric oxide (NO) and other factors [11, 17, 23, 25, 26, 28]. Macrophages are important components of the immune mechanism of the human body as phagocytes produced in the bone marrow to eliminate foreign substances [13]. Macrophages are also immunocytes and secrete hydrogen peroxide (H₂O₂) and NO, which are cytotoxic substances, and can thus eliminate heterocytes and cancer cells [22]. In addition, macrophages are involved in the removal process of viruses or foreign substances by cytophagocytosis, and secrete various cytokines and phosphatases such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)- α controlling the body's immune status. These secretions result in inflammation reactions and can be an important defense mechanism for immunity [3, 14].

In this study, we investigated the bioactivity of myricetin isolated from *Rhododendron mucronulatum* flowers to investigate its antiinflammation effect. We used the macrophage cell line Raw 264.7 and determined the inflammation effects on basis of protein expression and cytokine expression in the cytoplasm through western blotting analysis.

Materials and Methods

Sample collection

Rhododendron mucronulatum flower used in this study were collected from Daegu, Korea, in May 2010. Collected *Rhododendron mucronulatum* flowers were frozen dried (FD-8515, Ilshin Co., Seoul, Korea), and stored after grinding, and then used as samples for protein expression analysis.

Extraction protocol

One gram of dried *Rhododendron mucronulatum* flower powder was added to 100 ml of water and 100 ml of 60% ethanol then extracted for 6 hr. The supernatant was filtered with Whatman No. 1 filter paper (Whatman, Maidstone, Kent, UK) then used as the extract sample.

Phenol compound determination

One milliliter of sample was placed in 1 ml of 95% ethanol to which 5 ml of distilled water and 0.5 ml of 1 N Folin-Ciocalteu reagent (color developing reagent) (Junsei Chemical Co., Tokyo, Japan) were added and mixed well. The color was expressed by leaving the mixture for 5 minutes and

then adding 1 ml Na₂CO₃; the optical density (Optizen 3220UV, Mecasys, Daejeon, Korea) was determined within 1 hr at 725 nm and then compared to the standard curve generated using gallic acid [10].

Purification by column chromatography

Extract samples were added to Sephadex LH-20 (Pharmacia Co., Uppsala, Sweden) columns and separated based on characteristic of absorbance. Ethanol (60%) was used as the eluting solvent at normal phase, EtOH→H₂O (100→0%), a flow velocity of 20 ml/min and then samples were concentrated and dried at the thin layer chromatography phase after identifying the presence and type of phenolic compounds. MCI-gel (Supelco, PA, USA) is a porous polystyrene gel, and was therefore used for absorbability and eluted at reverse phase, H₂O→MeOH (0→100%), and the thin layer chromatography phase. The H₂O layer, ethyl acetate layer, and *n*-BuOH layer were obtained from a 200 ml solvent fraction of H₂O/ethyl acetate/*n*-BuOH with 30 g of freeze dried *Rhododendron mucronulatum* flower 60% ethanol extract.

Melting point determination

Melting point were determined from 1 mg samples by a microelectrothermal actuator (Fisher-Jhons, Mexico City, Mexico).

Infrared spectrum (IR) determination

Halogenized alkali purification was used to generate the IR spectrum (Perkin - Elmer IR-1330, Perkin Elmer, CT, USA). One milligram of the purified and separated sample was mixed with 100 mg KBr powder, pressure was applied to obtain a pressure purified compound, and then the IR spectrum was determined.

Nuclear magnetic resonance (NMR) spectrum determination

¹H and ¹³C-NMR (ARX-250, Burkert Co., Billerica, MA, USA) spectra were obtained, using the pulse fourier transform method by eluting 10 mg of the pure, refined sample and determining the solvent CDCl₃+DMSO-D₆+D₂O at a 5-20%(w/v) ratio with tetramethylsilane [(CH₃)₄Si] as standard. Then, spectra were determined at a proton magnetic resonance of 300 MHz.

Fast atom bombardment (FAB)-mass spectrum determination

With a 1 mg solid sample at decompressed phase (10^4 - 10^6 mmHg), the negative ion FAB-mass spectrum (Jeol JMS-PX 300, Tokyo, Japan) was determined by using the chemical analysis method; thioglycerol (Sigma Chemical Co., MO, USA) was used as the determining solvent with an emitter electric current of 22-28 eV. Then, mass spectrometric analysis was performed with an accelerative pressure of the ion source at 6-7 kV.

Cell culture for determination of anti-inflammation effects

Raw 264.7 cell of a murine macrophage cell line, were purchased from the Korean Cell Line Research Foundation. Cell were cultured for 72 hr at 37°C and 5% CO₂ with Dulbecco's modified Eagle's medium (DMEM) (Hyclone, GE Healthcare Life Sciences, UT, USA) containing 10% fetal bovine serum (FBS) (Hyclone, GE Healthcare Life Sciences), 100 unit/ml penicillin, and 100 µg/ml streptomycin (Hyclone, GE Healthcare Life Sciences). The cells were maintained at 5% CO₂ and sub-cultured at a $2-3 \times 10^6$ cell/ml cell density on a cell culture dish. Cells at 80% confluency from passage 20 were used for experiments. The cells were cultured for 12 hr with FBS and removed from the medium before the experiment [6].

NO determination

The NO content in the supernatant of the cells was determined from the nitrite and nitrate levels. Griess reagent (Sigma Chemical Co.) was used to stabilize nitrite reduced to nitrate. At 80% confluence, 5×10^4 cells were transferred to a 96 well plate and washed 2 times with phosphate buffered saline, and then cultured with serum-free medium for over 18 hr. Cells were stimulated by adding 10 µM/ml LPS to all wells except for the control group. The experiment proceeded with different concentrations of myricetin after 1 hr. NO production levels were determined by measuring absorbance at 540 nm of the supernatant reacted with Griess reagent (Sigma Chemical Co.) for 10 minutes, which were collected hourly [6, 30]. The inhibition of NO production was determined using the following formula: inhibition ratio (%) = $[1 - \text{absorbance of sample} / \text{absorbance of control}] \times 100$.

Inducible NO synthase (iNOS) and cyclooxygenase (COX-2) activity determination with western blotting

To determine the activity of iNOS protein, Raw 264.7 cells (macrophage) were stabilized by culturing for 24 hr in a 6

well plate with a suspension of 5×10^5 cells/ml in each well. After removing the medium, different concentration (10 µg/ml, 100 µg/ml) of treated medium was used to cultivate the cells for 24 hr. Then, the medium was removed and cells were washed with phosphate buffered saline 2 times. A 100 µl cell suspension was eluted with lysis buffer (complete Mini 1 tablet added to 10 ml radioimmuno precipitation assay [RIPA] buffer) and centrifuged (Fleta 40, Hanil scientific INC., Gimpo, Korea) for 20 minutes at 12,000 rpm at 4°C. The supernatant was collected, transferred to a new tube, and stored at -20°C for further use. Protein (20 µl) were separated by 10% sodium sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Separated protein were transferred to a polyvinylidenedifluoride membrane (Millipore Corp., Bedford, MA, USA) using a semidry transfer cell machine (Bio Rad, Hercules, CA, USA) and incubated for 1 hr with blocking buffer (5% skim milk in Tris-buffered saline with Tween-20 [TBST]) at room temperature. The membrane was washed with TBST 3 times every 10 minutes and incubated overnight with diluted primary antibody against, iNOS (1:1,000; BD Biosciences, San Jose, CA, USA), COX-2 (1:1,000; Cayman, Ann arbor, MI, USA), and GAPDH (1:1,000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4°C. The membrane was washed with TBST 3 times every 10 minutes once more, and incubated for 2 hr at room temperature with secondary antibodies, mouse anti-rabbit IgG HRP (1:1,000; Santa Cruz Biotechnology) and bovine anti-goat IgG horseradish peroxidase (HRP; 1:1,000; Santa Cruz Biotechnology). After washing 3 more times membranes were reacted with ECL (Millipore Corp.) solution in a darkroom and exposed it to X-ray film. The intensity of each band was determined using Molecular Imager (Bio-Rad) [6, 30].

Determination of cytokine production

Cultured macrophages (1×10^6 cell/ml) were plated in 6 well plates and then treated with an agonist (cell stimulator) at different concentrations every hour. After a hour, cells were treated with LPS (1 µg/ml), and cytokine levels were determined from the cell culture medium hourly for 24 hr after LPS treatment. The collected medium was stored at -70°C before determination. An enzyme immunoassay (EIA) kit (R&D systems Inc., Minneapolis, MN, USA) was used for determining the contents of TNF-α, IL-1β, IL-6, IL-8, and prostaglandin E₂ (PGE₂). Each cytokine amount was determined using a standard curve obtained from the reaction of standard material [3, 6, 14, 30].

Statistical analysis

All tests were repeated 3 times and statistical analyses were performed by using the SAS program (SAS Institute, Cary, North Carolina, USA).

Results and Discussion

Purification and identification of the chemical structure of anti-inflammation compounds

The purified compound was a yellow powder with melting point at 324°C. The molecular weight was determined to be 319 based on positive FAB-MS. Based on the KBr-IR spectrum (cm^{-1}), groups of 3315 (OH), 1662 ($>\text{C}=\text{O}$), 1594 (conjugated diene), 1551 (phenyl), 1518, 1457, and 1228 (C-O-C) were identified. The $^1\text{H-NMR}$ (CDCl_3 , 300 MHz) spectrum showed a profile of 7.37 ppm (2H, s, H-2' & H-6'), 6.38 ppm (1H, d, $J=1.8$, H-8), and 6.17 ppm (1H, d, $J=1.7$, H-6) and $^{13}\text{C-NMR}$ (CDCl_3 , 300 MHz) spectrum showed a profile of 147.4 (s, C-2), 137.1 (s, C-3), 177.7 (s, C-4), 157.9 (s, C-5), 99.1 (s, C-6), 166.1 (s, C-7), 94.2 (s, C-8), 163.2 (s, C-9), 104.3 (s, C-10), 123.7 (s, C-1'), 109.1 (d, C-2' & C-5'), 146.9 (s, C-3' & C-5'), and 137.8 (s, C-4'), therefore identified with Myricetin (Fig. 1) [15].

Cell viability by myricetin treatment

An MTT assay [5] was used to determine cell viability in response to the addition of myricetin at concentrations of 6.25–50 μM . As shown in Fig. 2, no obvious changes in viability were found at 6.25–12.5 μM , but cell viability decreased at 20–50 μM , although the decrease was relatively small.

Inhibition of NO production by myricetin

NO is a type of free radical and is thus a very unstable molecule. NO is converted to NO_2 , N_2O_3 , N_2O_4 , nitrite (NO_2^-) or (NO_3^-) by oxygen or superoxide, which are stable nitrogen

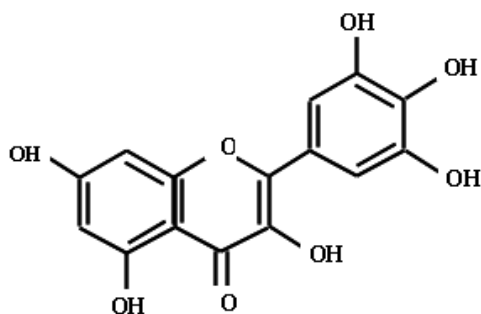


Fig. 1. Chemical structure of purified myricetin from *Rhododendron mucronulatum* flowers.

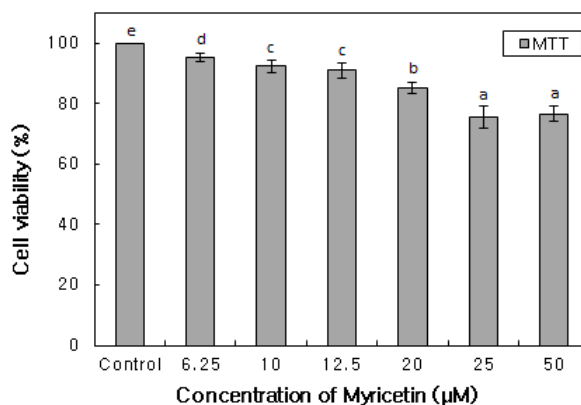


Fig. 2. Cell viability (MTT) following treatment with purified myricetin from *Rhododendron mucronulatum* flowers extracts. Raw 264.7 cells were incubated with various concentrations (6.25–50 μM) of *Rhododendron mucronulatum* flowers extracts for 1 hr and were then treated with 1 $\mu\text{g}/\text{ml}$ of LPS for 24 hr. ^{a-c}Mean \pm standard deviation ($n=3$), means with different superscript letters within column are significantly different at $p<0.05$ by Duncan's multiple range tests.

oxide species. NO is produced by NOS from L-arginine. NOS can be classified into endothelial NOS (eNOS), which produces NO for maintaining homeostasis, neuronal NOS (nNOS) and inducible NOS (iNOS), which is induced by inflammatory factors. An excess amount of inflammatory factors such as NO and prostaglandin E_2 (PGE_2) is produced by iNOS and COX-2 during inflammation reaction processes in the body. NO production is therefore important for eliminating bacteria or removing tumors [18], but in excess amounts can cause inflammation [20, 26], organ damage, gene mutation or pathological neural damage [31].

To determine inhibition effect of myricetin on NO production of Raw 264.7 cells, cells were treated with various concentrations 6.25–50 μM of myricetin and LPS, and the amount of NO produced was determined. As shown in Fig. 3, the LPS treated group showed 3 times higher NO expression than the group without LPS treatment. Inhibition of NO production was observed at all myricetin concentrations, including the lowest concentration (6.25 μM). By inhibiting the expression of transcription factors that interact with NO, is expected to show an excellent anti-inflammation effect in Raw264.7 macrophages induced by LPS.

Inhibition of iNOS and, COX-2 protein expression by myricetin

The NO synthase (NOS) enzyme is involved in the production of NO. There are two types of NOS, constitutive

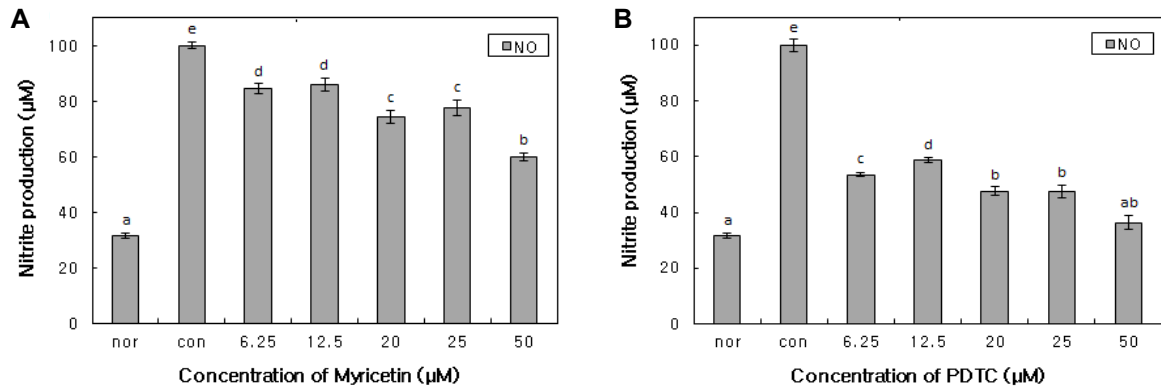


Fig. 3. Effect of myricetin from *Rhododendron mucronulatum* flowers on the production of NO in LPS stimulated Raw 264.7 cells. (A) myricetin, (B) PDTC as positive control. ^{a-c}Mean±standard deviation (n=3), means with different superscript letters within column are significantly different at $p<0.05$ by Duncan’s multiple range tests.

NOS (cNOS) and inducible NOS (iNOS). In particular, when stimulated, iNOS continues to produce NO, which in turn activates guanyl cyclase, resulting in cytotoxicity. Therefore by decrease in iNOS protein levels in Raw 264.7 cells induced by LPS and treated with myricetin, would indicate an anti-inflammation effect. In addition, in macrophages, COX-2 protein increase expression of proinflammatory cytokines such as TNF- α and IL-6 in monocytes, leading to an inflammation reaction [16, 29]. COX-2 expression in monocytes is increased by proinflammatory agents such as IL-1 β , TNF- α , phosphatidic acid, and fibroblast growth factor; COX-2 inhibition is induced by glucocorticoid, IL-4, and

IL-13 [34]. Therefore, development of a selective inhibitor for COX-2 would serve as a target molecule for inflammation treatment.

iNOS is known to function in the defense response against contagious pathogens including viruses associated with various inflammatory diseases, circulatory disorders, and cancer [32]. To identify the relationship between iNOS protein and the mechanism of myricetin NO inhibition, iNOS protein expression in the cytoplasm was quantified with a western blot analysis. As shown in Fig. 4A, compared to the control group, the myricetin treated group showed 20% inhibition of iNOS at a concentration of 25 μ M. With increasing myr-

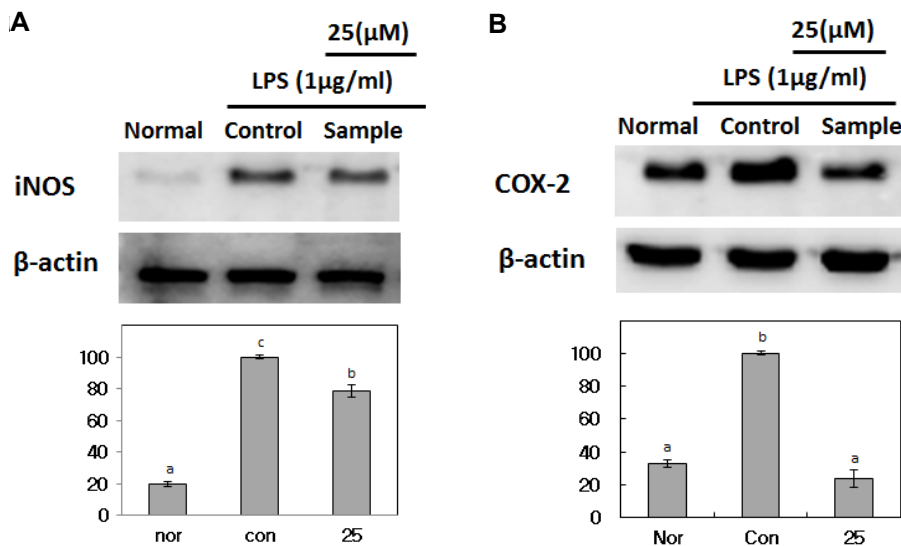


Fig. 4. Effect of myricetin from *Rhododendron mucronulatum* flowers on the production of iNOS (A) and COX-2 (B) in LPS stimulated Raw 264.7 cells. Raw 264.7 cells were treated with 25 μ M myricetin from *Rhododendron mucronulatum* flowers dissolved in distilled water for 1 hr prior to the addition of LPS (1 μ g/ml), and the cells were further incubated for 24 hr. Control cells were incubated with incubator alone. ^{a-c}Mean±standard deviation (n=3), means with different superscript letters within column are significantly different at $p<0.05$ by Duncan’s multiple range tests.

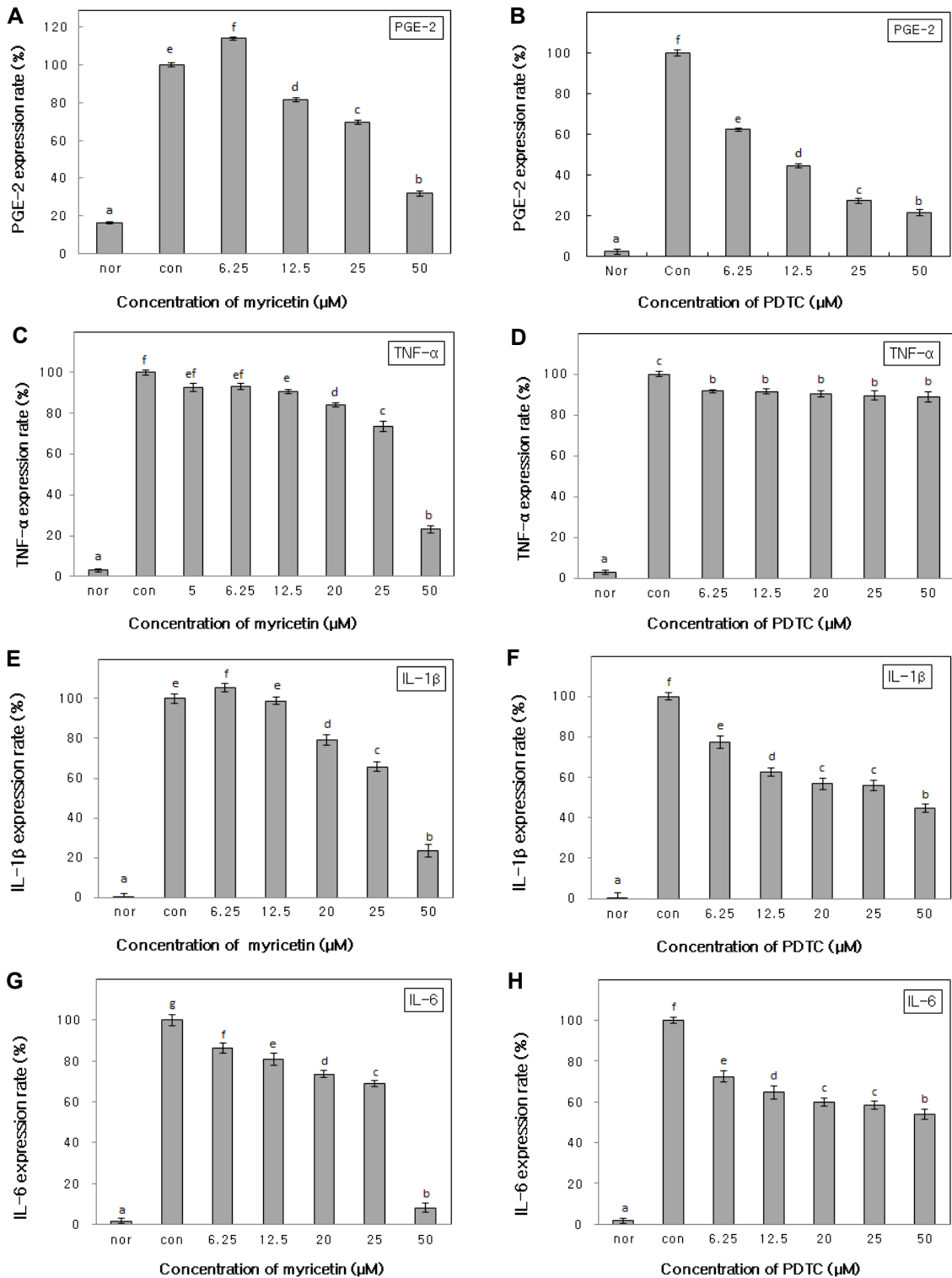


Fig. 5. Inhibitory effect of myricetin from *Rhododendron mucronulatum* flowers on cytokine expression. Raw 264.7 cells were incubated with various concentrations (6.25-50 μM) of myricetin for 1 hr and then treated with 1 μg/ml of LPS for 24 hr. A: PGE₂ expression of myricetin treated cells, B: PGE₂ expression of PDTC treated cells (positive control), C: TNF-α expression of myricetin treated cells, D: TNF-α expression of PDTC treated cells, E: IL-1β expression of myricetin treated cells, F: IL-1β expression of PDTC treated cells, G: IL-6 expression of myricetin treated cells, H: IL-6 expression of PDTC treated cells. ^{a-f}Mean±standard deviation (n=3), means with different superscript letters within column are significantly different at *p*<0.05 by Duncan's multiple range tests.

icetin concentration, the anti-inflammation effect and immune function are expected to increase in macrophage Raw 264.7 cells induced with LPS via inhibition of the interaction between iNOS and NO. Fig. 4B shows the effect of myricetin on another inflammatory factor, COX-2. Compared to the control group, high inhibition of COX-2 (80%) was found when cells were treated with 25 μ M myricetin. Therefore, a high anti-inflammation effect of myricetin is expected in this macrophage cell line (Raw 264.7 cells) through the inhibition of COX-2 protein expression.

Effect of myricetin on anti-inflammation cytokines

The inhibitory effect of myricetin on the production of PGE₂ and pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6 was evaluated in Raw 264.7 cells. As shown in Fig. 5A, cells treated with myricetin showed inhibition of PGE₂ expression in a concentration dependent manner, with 70% inhibition observed with 50 μ M myricetin. As shown in Fig. 5C, TNF- α expression was inhibited in cells treated with 6.25-50 μ M myricetin, with 70-80% inhibition observed at 40-50 μ M myricetin concentration. Compared to pyrrolidinedithiocarbamate (PDTC) (Fig. 5D), which was used as a positive control, myricetin showed increased inhibition of TNF- α expression at 20 μ M. Similarly, treatment of 6.25-50 μ M myricetin inhibited the expression of IL-1 β in a concentration dependent manner (Fig. 5E), with 50 μ M myricetin showing \geq 80% inhibition. Compared to PDTC (Fig. 5F), myricetin showed an increased inhibitory effect at a concentration of 50 μ M.

Myricetin also inhibited IL-6 expression in a concentration dependent manner, as shown in Fig. 5G. 50 μ M myricetin showed 95% inhibition, and the inhibitory effect was higher than that of PDTC, at concentrations of 40 μ M or higher (Fig. 5H). These results are similar to the observations for iNOS and COX-2 protein expression inhibition. Therefore, the application of myricetin is expected to show anti-inflammation effect in macrophage Raw264.7 cells induced by LPS.

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초록 : Lipopolysaccharide로 유도된 Raw264.7 cell에서 *Rhododendron mucronulatum* Turcz. Flower으로부터 분리한 myricetin에 의한 염증 억제효과

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진달래꽃으로부터 Sephadex LH-20 및 MCI gel CHP-20 column chromatography로 정제한 결과 항염증 활성을 가지는 myricetin을 분리, 동정하였다. Myricetin은 농도 의존적으로 NO 발현을 억제하였고, 50 μ M 농도에서 약 40%의 억제효과를 나타내었다. Myricetin의 iNOS와 COX-2의 발현억제력은, 25 μ M 농도에서 각각 20% 및 80%의 protein 발현 억제효과를 나타내었다. 또한 myricetin의 염증반응의 cytokine을 측정하여 TNF- α , IL-1 β , IL-6 및 PGE₂의 억제력을 살펴본 결과, 농도 의존적으로 발현억제 효과를 나타내었으며, 50 μ M 농도에서 각각 70%, 80%, 80% 및 95%의 발현 억제효과를 나타내었다. 따라서 진달래 꽃잎에서 분리한 myricetin은 LPS로 유도되어진 대식세포주인 Raw 264.7 세포에서 염증반응의 억제효과를 기대할 수 있었다.