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Constituents of *Pyrus pyrifolia* with Inhibitory Activity on the NO Production and the Expression of iNOS and COX-2 in Macrophages and Microglia

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Abstract – It is well known that inflammation is associated with neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease and ischemia. Nitric oxide (NO), a pro-inflammatory mediator, is produced by inducible NO synthase (iNOS) in microglia as well as macrophages and appears to account for neurodegeneration. In this study, we aimed to isolate NO inhibitors from *Pyrus pyrifolia* by activity guided purification. As a result, we identified daucosterol and β-sitosterol, which have not been isolated from this plant before. This article also describes NO inhibitory activities of the methanol extract of *Pyrus pyrifolia* fruit and the isolated compounds from this, which are lupeol, betulin, betulinic acid, β-sitosterol and daucosterol, in LPS-activated RAW 264.7 and BV2 cell lines. Western blot analysis was performed to clarify the underlying mechanism of NO inhibition in the two cell lines.
Keywords – *Pyrus pyrifolia*, Nitric oxide, Macrophage, Microglia

Introduction

Macrophages and microglia are thought to contribute to the development and progress of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease and ischemia (Dheen *et al*., 2007; Mrak *et al*., 2005). Upon stimulation, macrophages in the brain release pro-inflammatory cytokines such as nitric oxide (NO) and prostaglandins. Nitric oxide (NO), a free radical, is produced from L-arginine by inducible NO synthase (iNOS), which plays a key role in the regulation of inflammatory responses. The expression of iNOS and cyclooxygenase-2 (COX-2) can be induced by lipopolysaccharide (LPS), TNF-α, and IFN-γ (Nathan, 1992).

Microglia is a type of glia cell that acts as the first and main form of active immune defense in the central nerve systems. Microglia constitutes 20% of the total glial cell population within the brain (Dobrenis *et al*., 1998; Lawson *et al*., 1990). Neuroinflammation is mainly caused by activated microglial cells, and this activation results in the induction of inflammatory enzymes such as iNOS and COX-2 as well as cytokines such as IL-1β, TNF-α, and NF-κB (Allen *et al*., 2000; Perry *et al*., 2000; Roy *et al*., 2002; Schroeter *et al*., 2002; Yoon *et al*., 2002; Klein *et al*., 2003; Mrak *et al*., 2005). Excessive production of these mediators by the activated microglia in the CNS might be involved in the pathogenesis of neurodegenerative and neuroinflammatory disorders (Block *et al*., 2005; Vilhardt, 2005).

As a part of our ongoing research on finding nitric oxide inhibitors from Korean medicinal plants, we screened inhibitory activity of *Pyrus pyrifolia* fruit in lipopolysaccharide (LPS)-stimulated murine macrophages and microglia. The methanol extract of *Pyrus pyrifolia* fruit peel showed significant inhibitory effects on NO production. *Pyrus pyrifolia* Nakai is a perennial plant belonging to the family of Rosaceae. The fruit of this plant, known as a pear, is common and highly consumed in Korea. The Korean pears, however, have been considered not only fruit but a herbal medicine in East Asia. The fruit and the roots of this plant have been used for the treatment of the fever (Yoo, 1991). Its peels of the fruit have been used to cure abscess, cough, dysentary, and indigestion (Kim, 1988). Previous researchers have reported that *P. pyrifolia* has chemopreventive effect on PAHs-induced carcinogenic mechanism (Yang, 2006), effects on heart and blood circulation (Na *et al*., 2003) and reduction activity of fat accumulation in rats (Choi *et al*., 2004). Recently, some studies on the fruits of *P. pyrifolia* have led to the isolation of several phenolic compounds such as arbutin, chlorogenic acid (Cui *et al*., 2005) and catechins (Zhang *et el*., 2003). We have previously identified three lupane triterpenoids from the fruits of *P. pyrifolia* (Yoo *et*

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al., 2012). In this research, we attempted to purify two phytosterols and evaluate NO inhibitory activity of the five compounds in LPS-activated murine macrophage RAW264.7 and microglia BV2.

Experimental

General experimental procedures – The IR spectra were obtained on a Jasco FT/IR-430 Infrared spectrometer. The EI-MS spectra were obtained using a JEOL JMS-AX505WA, HP5890 Series II mass spectrometer. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were recorded using a Bruker AVANCE 500 NMR spectrometer. Column chromatography was performed using silica gel (Kieselgel 60, 70 - 230 mesh and 230 - 400 mesh, Merck).

Plant material – The fruit of *Pyrus pyrifolia* were purchased from Seoul National Agricultural Cooperation Federation in Korea, and its voucher specimen (SMU 0703) has been deposited at the Herbarium of the College of Pharmacy, Sookmyung Women's University, Korea.

Extraction and isolation – Korean pears (40.0 kg) were peeled, and the peels (5.1 kg), pulps (30.7 kg) and cores (4.2 kg) were immediately dropped into methanol, and were extracted 3 times at 80 ºC for 4 h, respectively. The MeOH extract was dissolved in distilled water, and filtered by filter paper. This process was to yield to water insoluble part (7.2, 0.9 and 0.2 g) and water soluble part (670.8, 791.2 and 81.7 g) from peel, pulp and core (5.1, 8.3 and 0.9 kg, respectively). The water insoluble part of *Pyrus pyrifolia* fruit peel (7.2 g), which showed nitrite inhibitory effect, was extracted by EtOAc to give a dark brown extract (4.5 g). The ethyl acetate extract (3.5 g) was chromatographed on silica gel (5 × 50 cm column, 120 g of silica gel, 70 - 230 mesh) eluting with a n-hexane/EtOAc gradient system (4 : 1 → 1 : 50, 4 : 1, 4 L; 2 : 1, 2 L; 1 : 1, 1 : 2, 1 : 4, 1 : 8, 1 : 15, 1 : 30, I L; 1:50, 2 L) to obtain subfractions, Fr. 1~7. Compound **4** (30.3 mg) was obtained from $CHCl_3$/MeOH (10 : 1, 1.5 L) elution of Fr. 6 - 2 (90.6 mg) using the silica gel column (2 × 30 cm column with 20 g of silica gel, 230~400 mesh). Fr.7 (650.0 mg) was further separated on a silica gel column (3 × 30 cm column with 30 g silica gel, 230~400 mesh) with a gradient elution of $CHCl_3$/EtOH (10 : 1 → 5 : 1, 10 : 1, 1 L; 7.5 : 1, 1.5 L; 5 : 1, 1 L) to afford compound **5** (16.6 mg).

β-sitosterol (4)- White powder; EI-MS; *m/z* 414 $[M]^+$; IR (NaCl neat) cm^{-1}: 3420(-OH), 1641(C = C) ; ^{1}H-NMR (500 MHz, $CDCl_3$, δ in ppm) : 5.28(1H, br d, *J* = 5.1 Hz, H-6), 3.45(1H, m, H-3), 0.94(3H, s, H-19), 0.85(3H, d, *J* = 6.5 Hz, H-21), 0.78(3H, t, *J* = 7.5 Hz, H-29), 0.77(3H, s, *J* = 6.8 Hz, H-26), 0.74(3H, d, *J* = 6.8 Hz, H-27), 0.61(3H, s, H-18); ^{13}C-NMR (125 MHz, $CDCl_3$, δ in ppm) : 141.0(C-5), 121.9(C-6), 72.0(C-3), 57.0(C-14), 56.3(C-17), 50.4(C-9), 46.1(C-24), 42.6(C-13), 42.5(C-4), 40.0(C-12), 36.7(C-1), 36.4(C-10), 34.2(C-22), 32.1(C-7), 31.9(C-8), 29.9(C-2), 29.4(C-25), 28.5(C-16), 26.3(C-23), 24.5(C-15), 23.3(C-28), 21.3(C-11), 20.0(C-26), 19.6 (C-27), 19.3(C-19), 19.0(C-21), 12.2(C-29), 12.1(C-18)

Daucosterol (5)- White powder; EI-MS; *m/z* 414 [M-Glucose]$^+$; IR (NaCl neat) cm^{-1}: 3350(-OH), 1734(C = C) : ^{1}H-NMR (500 MHz, C_5D_5N, δ in ppm) : 5.37(1H, d, *J* = 5.0 Hz, H-6), 5.08(1H, d, *J* = 7.7 Hz, H-1'), 4.59(1H, dd, *J* = 11.8, 2.3 Hz, H-6'a), 4.44(1H, dd, *J* = 11.8, 5.2 Hz, H-6'b), 4.31(1H, dd, *J* = 8.2 Hz, H-4'), 4.31(1H, m, H-5'), 4.08(1H, dd, *J* = 8.2 Hz, H-2'), 4.01(1H, dd, *J* = 8.2 Hz, H-3'), 4.01(1H, m, H-3), 2.75(1H, dd, *J* = 13.2, 2.5 Hz, H-4a), 2.50(1H, dd, *J* = 13.2 Hz, H-4b), 1.01(3H, d, *J* = 6.4 Hz, H-21), 0.96(3H, s, H-19), 0.92(3H, t, *J* = 7.4 Hz, H-29), 0.90(3H, d, *J* = 6.8 Hz, H-27), 0.89(3H, d, *J* = 6.8 Hz, H-26), 0.68(3H, s, H-18); ^{13}C-NMR (125 MHz, C_5D_5N, δ in ppm) : 141.4(C-5), 122.4(C-6), 103.1 (C-1'), 79.1(C-3), 79.0(C-3'), 78.6(C-5'), 75.8(C-2'), 72.2 (C-4'), 63.3(C-6'), 57.3(C-14), 56.8(C-17), 50.8(C-9), 46.5(C-24), 43.0(C-13), 40.5(C-12), 39.8(C-4), 38.0(C-1), 37.4(C-10), 36.9(C-20), 34.7(C-22), 32.7(C-7), 32.6(C-8), 30.8(C-2), 30.4(C-25), 30.0(C-16), 26.9(C-23), 25.0(C-15), 23.9(C-28), 21.8(C-11), 20.5(C-27), 19.9(C-26), 19.7 (C-19), 19.5(C-21), 12.7(C-29), 12.5(C-18)

Cell culture – Mouse macrophage cell line (RAW 264.7) and murine microglial cell line (BV2) were cultured in DMEM containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin. Cells were maintained at 37 ºC with 5% CO_2 in a fully humid atmosphere.

Nitrite Assay – RAW 264.7 and BV2 were plated at a density of 1.5×10^5 cells/mL and 1.0×10^5 cells/mL (respectively) in a 48-well cell culture plate with 400 μL of culture medium and incubated for 24h. The cells were treated with LPS (1 μg/mL in RAW 264.7 and 0.1 μg/mL in BV2) and various concentrations of test samples for 20 h. Nitrite was measured by adding 150 μL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 100 μL culture medium. Absorbance at 570 nm was measured using a microplate reader, and the nitrite concentration was calculated by comparison with the absorbance of standard solutions of sodium nitrite.

Western blot analysis – Cells were plated in 60 mm culture dishes, and incubated for 24 h. The cells were then treated with various concentrations of samples with LPS

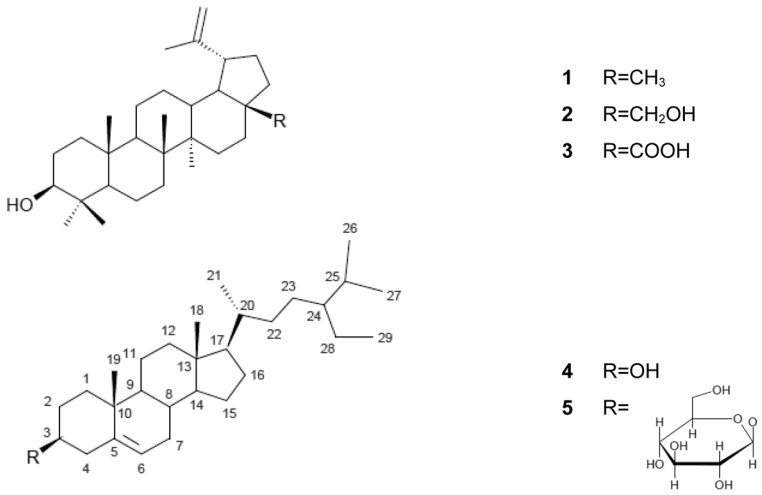

Fig. 1. Isolated compounds **1 - 5** isolated from *Pyrus pyrifolia*.

(1 and 0.1 μg/mL in RAW 264.7 and BV2, respectively). After 20 h incubation, cells were lysed with lysis buffer and centrifuged at 12,000 rpm at 4 °C for 20 min. The protein concentration of each sample was determined based on the method of Bradford using a Bio-Rad Protein Assay reagent. The proteins (50 μg) were separated by electrophoresis on 8% SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked in 5% nonfat milk in TBS-T (25 mM Tris, 137 mM NaCl and 0.1% Tween 20) for 1h at room temperature, and incubated with indicated primary antibodies (1000 : 1 for iNOS and COX-2 and 2000 : 1 for β-actin in 5% nonfat milk in TBS-T) for overnight at 4 °C. The membranes were washed with TBS-T five times for 10 min, and continuously incubated with specific anti-mouse or anti-rabbit HRP-conjugated secondary antibodies (2000 : 1 in 5% nonfat milk in TBS-T) for 2h, and detected by ECL system.

Statistical Analysis – The results were expressed as a mean ± S.E.M. (standard error of mean) of three independent experiments. The statistical analysis was performed by the Student's *t*-test, and *p* values less than 0.01 were considered statistically significant.

Results and Discussion

Lupeol, betulin and betulinic acid, which were reported to be isolated in our previous article (Yoo *et al*., 2012), were used as compounds **1 - 3**, respectively.

The EI-MS of **4** indicated a molecular ion peak at *m/z* = 414, in the agreement with the formula of $C_{29}H_{50}O$. The IR spectrum of **4** showed absorption bands due to a hydroxy group at 3420 cm^{-1} and an olefinic bond at 1641 cm^{-1}. In the ^{1}H-NMR spectrum (500 MHz in $CDCl_3$, δ in ppm), an olefinic methine and an oxygenated methine peaks were observed at δ 5.28 and 3.45, respectively. The three singlet methyl (δ 0.94, 0.77 and 0.61), two doublet methyl (δ 0.85 and 0.74) and a triplet methyl (δ 0.78) were also observed. The ^{13}C-NMR spectrum (125 MHz in $CDCl_3$, δ in ppm) suggested 29 carbons including an olefinic quarternary carbon (δ 141.0), an olefinic methine (δ 121.9), an oxygenated methine (δ 72.0) and six methyl carbons (δ 20.0, 19.6, 19.3, 19.0, 12.2 and 12.1). Based on the above spectroscopic data, the structure of **4** was determined as β-sitosterol. The spectroscopic literature values were examined to assist in the elucidation of compound **4** (Lavoie *et al*., 2005; Lee *et al*., 2005; Lee *et al*, 2003; Yu *et al*., 2007).

Compound **5**, white powder, showed absorption bands due to a hydroxyl group at 3350 cm^{-1} and an olefinic bond at 1734 cm^{-1} in the IR spectrum. The EI-MS indicated a [M-Glucose] $^+$ peak at 414. The ^{1}H-NMR spectrum (500 MHz in C_5D_5N, δ in ppm) showed the presense of an olefinic proton at δ 5.37, an anomeric proton at δ 5.08 and six methyl group at δ 1.01, 0.96, 0.92, 0.90, 0.89 and 0.68. The ^{13}C-NMR (125 MHz in C_5D_5N, δ in ppm) spectrum showed 35 signals, of which 29 signals were originated from the aglycone and the other 6 signals from a sugar moiety containing an anomeric carbon at δ 103.07. The carbon signals from the aglycone including an olefinic quaternary carbon at δ 141.4, an olefinic methine carbon at δ 122.4 and six methyl carbons at 20.5, 19.9, 19.7, 19.5, 12.7 and 12.5 were observed. All assignments in the ^{1}H-NMR and ^{13}C-NMR were based on the measurement of HSQC, HMBC and ^{1}H-^{1}H COSY. The final structure was confirmed by

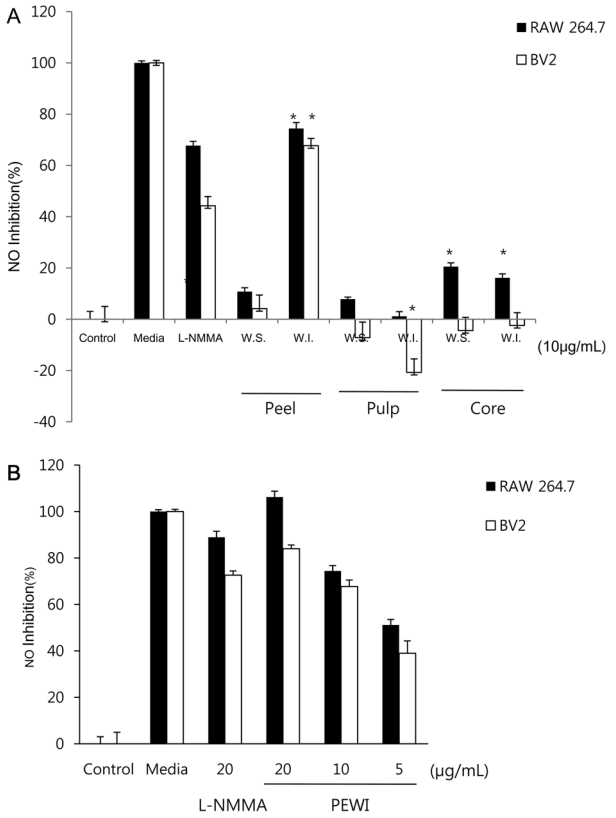

Fig. 2. A, Effects of water soluble (W.S.) and insoluble (W.I.) parts of methanol extract of *Pyrus pyrifolia* fruit on nitric oxide production in RAW 264.7 macrophages and BV2 microglia. B, Inhibition of NO production by PEWI (water insoluble part of the MeOH extract of *P. pyrifolia* fruit peel). Cells were treated for 20 h with LPS (1 μg/mL in RAW 264.7 and 0.1 μg/mL in BV2) with/without extracts. The amount of NO in the supernatant was determined by the Griess reaction. Data are means ± S.E.M. from three independent experiments. L-NMMA, NO synthase inhibitor, was used as a positive control. In each experiment, triplicate determinations were made for each treatment. A, $*p < 0.01$, significantly different from control (LPS alone), analyzed by Student's *t*-test. B, All values were significantly different from control (LPS alone).

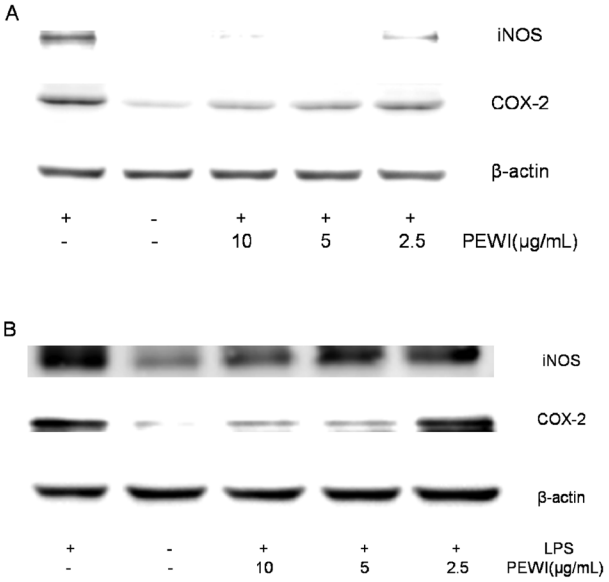

Fig. 3. Effect of PEWI on the expression of iNOS and COX-2 protein in LPS-activated RAW 264.7 macrophages (A) and BV2 microglia (B). Cells were treated for 20 h with PEWI (10, 5 and 2.5 μg/mL) in the presence of 1 μg/mL LPS in RAW 264.7 and 0.1 μg/mL LPS in BV2. The protein levels of iNOS and COX-2 in cell lysates were determined by western blot analysis. Similar results were shown in two independent experiments.

the comparison of spectroscopic values to those reported in the literature (Lavoie *et al*., 2005; Lee *et al*., 2007; Lee *et al*., 2005; Paulo *et al*, 2000; Yang *et al*., 2007; Yoo *et al*., 2006), and all values were nearly identical to the experimental data obtained. According to the above data, **5** was identified as daucosterol (β-sitosterol-3-O-β-D-glucopyranoside). To the best of our knowledge, this is the first time that the two compounds have been isolated from *Pyrus pyrifolia*.

Methanol extracts of three parts of *Pyrus pyrifolia* fruit (peel, pulp, and core) were initially compared on the production of nitric oxide. The water insoluble part of the MeOH extract of *P. pyrifolia* fruit peel (PEWI) markedly suppressed LPS-induced nitrite production in macrophages and microglia dose-dependently (Fig. 2.). The inhibitory effects of PEWI in both cell lines were comparable to L-NMMA, NO synthase inhibitor. This study also showed that PEWI inhibited nitric oxide more effectively in RAW 264.7 than in BV2. MTT assay revealed that PEWI showed cytotoxicity at concentrations of higher than 10 μg/mL in microglia (data not shown).

Western blot analysis was performed to determine whether the inhibitory effect of PEWI on NO is related to changes in the protein levels of iNOS and COX-2. As shown in Fig. 3, the protein levels of iNOS and COX-2 were obviously increased by stimulation with LPS, and PEWI markedly inhibited these protein expressions in a dose-dependent manner over a concentration range from 2.5 to 10 μg/mL. The expression of iNOS was almost decreased to that of control level by concomitant treatment with PEWI at the concentrations of 5 μg/mL in macrophages (Fig. 3.A.). The suppression of nitric oxide by PEWI could be explained by the reduction of iNOS protein expression in macrophages, as PEWI treatment was more effective in suppressing iNOS than COX-2 protein expression. In microglia, however, PEWI showed superior suppressing effects on COX-2 than iNOS. These findings suggest that PEWI could exert its NO inhibitory

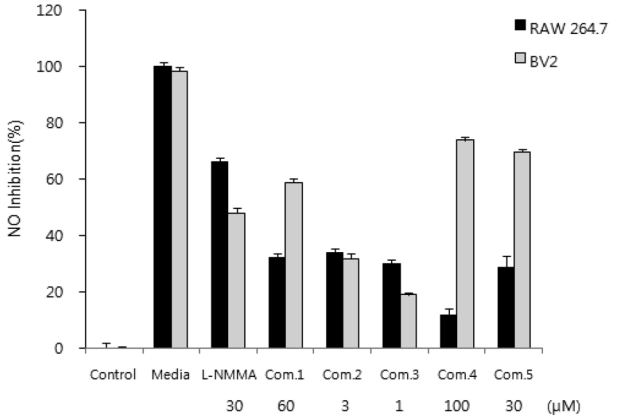

Fig. 4. Effect of compounds **1 - 5** on the nitric oxide production in LPS-activated RAW 264.7 macrophages and BV2 microglia. Cells were treated with LPS (1 μg/mL in RAW 264.7 and 0.1 μg/mL in BV2) in the presence of compounds **1 - 5** followed by the nitrite assay using Griese reagent. Data are means ± S.E.M. from three independent experiments. L-NMMA, NO synthase inhibitor, was used as a positive control. In each experiment, triplicate determinations were made for each treatment. All values were significantly different from control (LPS alone).

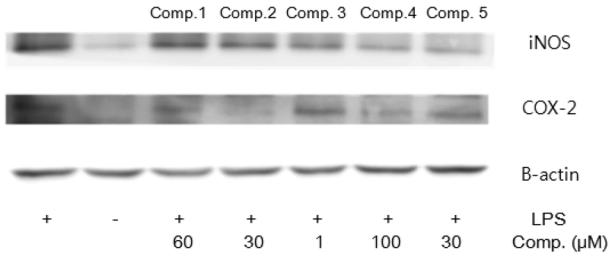

Fig. 5. Modulation of LPS-induced iNOS and COX-2 expression by compounds **1 - 5** in BV2 microglia. BV2 cells were treated with compounds **1 - 5** (60, 3, 1, 100 and 30 μM, respectively) in the presence of 0.1 μg/mL LPS or with LPS alone for 20 h. The protein levels of iNOS and COX-2 in cell lysates were determined by western blot analysis. Similar results were shown in two independent experiments.

effect by different mechanism in macrophages and microglia.

We investigated whether compounds **1 - 5** isolated from PEWI affect NO production in LPS- stimulated RAW 264.7 macrophages and BV2 microglial cells. As shown in Fig. 4., compounds **1**, **4** and **5** showed stronger effects in BV2 than in RAW 264.7. The difference among compounds **1**, **2** and **3** in their abilities to suppress nitric oxide production might be associated with their structural characteristics, considering the fact that these three compounds differ only with respect to the functional group(-CH_3, -CH_2OH and –COOH , respectively) of C-28 in lupanoid triterpene skeleton. As it is known that the high affinity of compounds for proteins and a possible subsequent conformational change in the enzyme might be related to the observed inhibitory effects (Kobuchi *et al*., 1999), the difference in the activities among compounds **1 - 3** could be explained by the different affinity of functional groups of C-28 for iNOS and COX-2 enzymes. In addition, a glycoside, daucosterol (compound **5**) was more potent than its aglycone (compound **4**) in RAW 264.7, but not in BV2. Under the experiment conditions, no cytotoxicity was observed at the test concentrations. Western blot analysis was performed to clarify mechanism of **1 - 5** for the NO inhibition in microglia. As shown in Fig. 5, the expression of iNOS and COX-2 in LPS-stimulated BV2 microglial cells were reduced by compounds **1 - 5** with 60, 3, 1, 100 and 30 μM, respectively. The suppression of nitric oxide in microglia by compounds **1 - 5**, therefore, could be explained by the reduction of iNOS and COX-2 protein expressions.

The individual compounds that we isolated from *P. pyrifolia* have been tested for the antioxidant and anti-inflammatory activities before; lupeol in RAW 264.7 (Jin *et al*., 2012), betulin in human cartilage culture (Huh *et al*., 2009), betulinic acid in RAW 264.7 and lung inflammation in rats (Yun *et al*., 2003; Nader et al., 2012), β-sitosterol and daucosterol in mouse bone marrow-derived mast cells (Kim *et al*., 2006). However, it is the first time that the effects of compounds **1 - 5** on nitric oxide, iNOS and COX-2 in microglia have been evaluated and that the activities of three lupane triterpenoids, compounds **1 - 3**, have been compared. Furthermore, the inhibitory effect of methanolic extract of *Pyrus pyrifolia* fruit peel on NO, iNOS and COX-2 has not been studied before.

In summary, our present study suggest that methanol extract of *Pyrus pyrifolia* fruit peel (PEWI) significantly exerted its anti-inflammatory actions by suppressing nitric oxide, iNOS and COX-2 in macrophages and microglia. Western blot analysis showed that the NO inhibitory effect of PEWI is more mediated through iNOS in macrophages and COX-2 in microglia. Upon treatment with compounds **1 - 5**, the NO production in the two cell lines was inhibited. Lupeol (**1**), β-sitosterol (**4**) and daucosterol (**5**) showed more inhibition against LPS-induced NO production in BV2 than in RAW 264.7. The iNOS and COX-2 protein levels were also decreased in microglia by concomitant treatment with compounds **1 - 5** with 60, 3, 1, 100 and 60 μM, respectively. These results suggest that the five compounds from *P. pyrifolia* attenuated inflammatory responses in microglia through the inhibition of overproduced nitric oxide and suppression of iNOS and COX-2 expression. Taken together, these

findings indicate that *P. pyrifolia* and its constituents may be a potential approach to neurodegenerative diseases, and further studies on their precise mechanism of anti-inflammatory actions can be encouraged to provide insights into such novel approaches.

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