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 al*., 2003). We have previously identified three lupane triterpenoids from the fruits of *P. pyrifolia* (Yoo et
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, 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°C 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, 30.7 and 15 kg), respectively. The brown extract (4.5 g). The ethyl acetate extract (3.5 g) was chromatographed on silica gel (5 × 5 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, 1 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$^+$]; IR (NaCl neat) cm$^{-1}$: 3350(–OH), 1734(C = C); $^1$H-NMR (500 MHz, CD$_2$D$_2$N, 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, CD$_2$D$_2$N, 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°C 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 24 h. 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% naphthylethylenediamine 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.