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In vitro anti-inflammatory activity of 3-*O*-methyl-flavones isolated from *Siegesbeckia glabrescens*

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Abstract—Four flavones, 3,4′-*O*-dimethylquercetin (**1**), 3,7-*O*-dimethylquercetin (**2**), 3-*O*-methylquercetin (**3**) and 3,7,4′-*O*-trimethylquercetin (**4**) were isolated as the inhibitors of nitric oxide production in activated microglia (IC_{50} values: 11.1, 4.2, 3.8, and 25.1 μM, respectively). They suppressed the expression of protein and mRNA of inducible nitric oxide synthase. Furthermore, compounds **2** and **3** showed scavenging activity of peroxynitrite with SC_{50} values of 1.75 and 0.77 μM, respectively.

Microglia are resident macrophage-like cells that play a role in host defense and tissue repair in CNS.[1] They can be activated in response to a variety of neurodegenerative and neuroinflammatory conditions resulting in the amplication of inflammation and the mediation of cellular degeneration.[2] Activated microglia produce proinflammatory and neurotoxic materials such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), nitric oxide (NO), prostaglandins (PGs) and reactive nitrogen species (RNS).[3,4] These mediators are involved in the pathogenesis of various neuronal diseases including Alzheimer's disease and Parkinson's disease.[5]

NO is produced from L-arginine by nitric oxide synthase (NOS). There are three isoforms of NOS. The constitutive NOS (cNOS) found in neuronal tissues (nNOS, type I) and vascular endothelium (eNOS, type III) release the small amounts of NO required for physiological functions.[6] As a neuromodulator in CNS, NO participates in brain development, pain perception, memory, neuronal plasticity and behavior.[7] The inducible NOS (iNOS, type II) is induced by several stimuli and produces large amounts of NO for extended periods of time.[8] NO activates the inducible cyclooxygenase (COX-2) resulting in the markedly increased release of proinflammatory PGs.[9] Large amounts of NO can be converted into peroxynitrite ($ONOO^-$) in the presence of superoxide anion (O_2^-). These neurotoxic RNS mediates neurodegenerative diseases.[10]

Therefore, the inhibition of NO production by activated microglia and the scavenging of peroxynitrite might be beneficial for the treatment of neuronal inflammatory diseases. In the course of our efforts to discover plant-derived anti-inflammatory agents as inhibitors of NO production in activated microglia, *Siegesbeckia glabrescens* was selected as a research target from the activity-screening of medicinal plant extracts.

Siegesbeckia glabrescens (Compositae), well-known as 'Hi-Chum' in Korea is an annual herb growing in Korea. The aerial parts of *Siegesbeckia glabrescens* have been used as a traditional medicine to treat rheumatoid arthritis, asthma, paralysis and allergic disorders. It has been reported that the extracts of *Siegesbeckia glabrescens* exhibit antioxidative, antiallergic, antihypertension, antitumor, and anti-inflammatory activities.[11,12] The present study described the isolation of four flavones from *Siegesbeckia glabrescens* as inhibitors of NO production in activated microglia, and their peroxynitrite scavenging activity.

BV-2 cells were stimulated with 0.1 μg/mL of LPS and the production of NO was induced by the enzymatic reaction of iNOS.[13] The amounts of NO released into culture media were determined by the Griess method.[14] The EtOAc soluble fraction of *Siegesbeckia glabrescens* exhibited 98% inhibition of NO production at 50 μg/mL

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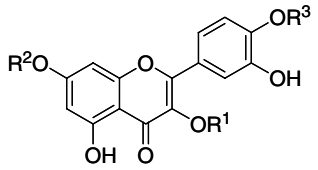

	R^1	R^2	R^3	IC_{50} (μM)
1	CH_3	H	CH_3	11.1 ± 1.5
2	CH_3	CH_3	H	4.2 ± 1.1
3	CH_3	H	H	3.8 ± 1.8
4	CH_3	CH_3	CH_3	25.1 ± 3.1
5	H	H	H	29.6 ± 2.9
L-NMMA				13.2 ± 1.8

Figure 1. Inhibition of NO production by compounds from *Siegesbeckia glabrescens* in LPS-activated BV-2 cells. Samples were treated during LPS-activation (0.1 μg/mL) and the nitrite assay was performed after 20 h incubation.

(data not shown). Four active principles were isolated from EtOAc fraction by activity-guided purification.[15] The structures of active compounds were elucidated as 3-*O*-methoxyflavones, 3,4′-*O*-dimethylquercetin (**1**), 3,7-*O*-dimethylquercetin (**2**), 3-*O*-methylquercetin (**3**), 3,7,4′-*O*-trimethylquercetin (**4**) (ajanin) by the analysis of Mass, NMR and IR spectral data, and confirmed by the comparison of reported data (Fig. 1). Compound **2** was reported from *Siegesbeckia orientalis*,[16] and **1**, **3**, and **4** were not yet reported from *Siegesbeckia* species. Copies of the original spectra are obtainable from the author of correspondence.

Compounds **1**–**4** inhibited NO production dose-dependently in LPS-activated microglia. As shown IC_{50} values in Figure 1, the methylation of 3-hydroxyl group enhanced the activity of quercetin (**5**). The flavones with hydroxyl group at 4′ (**2** and **3**) showed stronger activities than those methylated at 4′ position (**1** and **4**). The methylation at 7 position makes the activity further weaker (**1** vs. **4**). The methylation effect of quercetin on the activity of NO production was compatible with the results of flavonols reported previously.[17] Cell viabilities were assessed to be above 90% by MTT method[18] at the treated concentrations of compounds for nitrite assay. (N^G-monomethyl-L-arginine (L-NMMA), a well-known NOS inhibitor was used as positive control for nitrite assay.

Western blot and RT-PCR analyses were performed to clarify the underlying mechanism for the inhibition of NO production in LPS-activated microglia.[19] The cell lysates of vehicle treated cells did not show detectable iNOS protein. The treatment of **1**, **2**, and **3** (20 μM) decreased the expression of iNOS protein (Fig. 2A) in LPS-activated microglia. The results of RT-PCR analysis also showed that **1**, **2** and **3** (20 μM) inhibited the expression of iNOS mRNA (Fig. 2B) in LPS-activated microglia. Among the quercetin derivatives, **3** was the most potent. It has been reported that **3** isolated from *Rhamnus nakaharai* Hayata (Rhamnaceae) inhibited the expression of iNOS in LPS-activated murine macrophage cell.[20] The amounts of protein and mRNA expression were quantitated from the volume intensity of the bands corresponding to iNOS and actin.

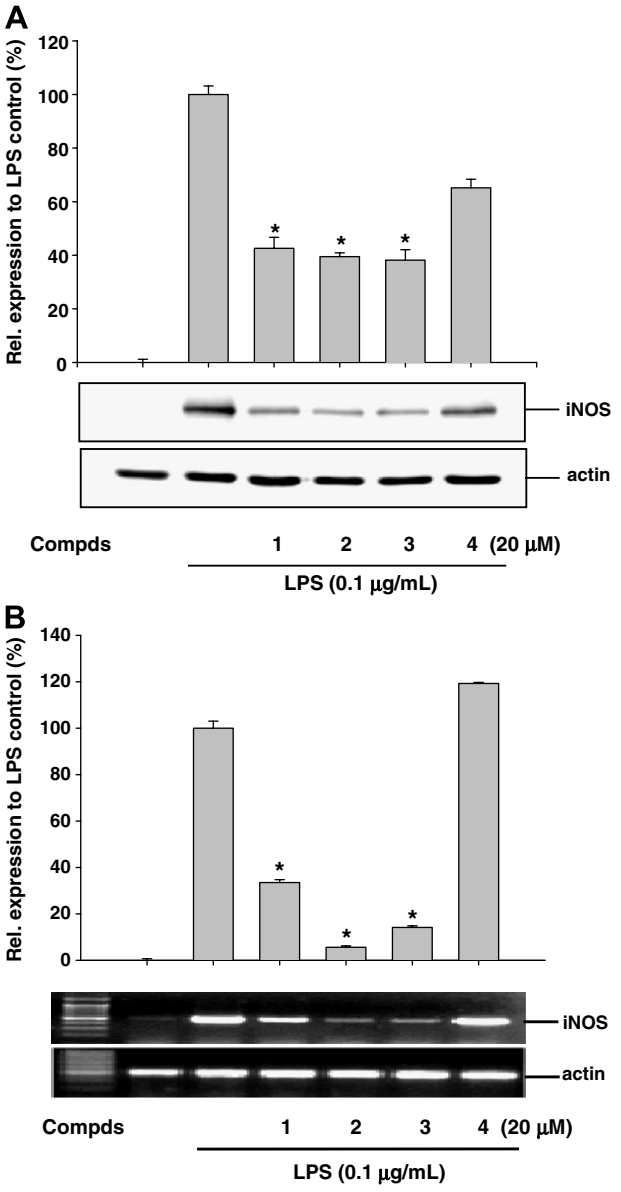

Figure 2. The effects of compounds on the expression of iNOS in LPS-activated BV-2 cells. (A) Cells were treated with compounds (20 μM) during LPS activation for 20 h. The protein expressional levels of iNOS were determined by Western blotting. (B) Cells were pretreated with compounds (20 μM) for 30 min prior to LPS (0.1 μg/mL) treatment. After 4 h of LPS treatment, total RNA was prepared for the RT-PCR analyses of iNOS gene expression in microglia cells. Quantitations of iNOS expression were normalized to actin using densitometry. Values are means ± SD of three experiments. Values with asterisk represent significant ($p < 0.05$) differences of protein and mRNA expression compared with LPS-media control.

Large amounts of NO produced by iNOS can rapidly react with superoxide anion radical ($\bullet O_2^-$) to produce peroxynitrite anion ($ONOO^-$).[21] Peroxynitrite is a highly reactive molecule capable of oxidizing proteins, lipids, and DNA and homolytical decomposing to yield even more potent neurotoxins like the hydroxyl radical.[7] These RNS might be involved in the pathogenesis of various neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and ischemia.[5] The scavenging activity of $ONOO^-$ was measured by mon-

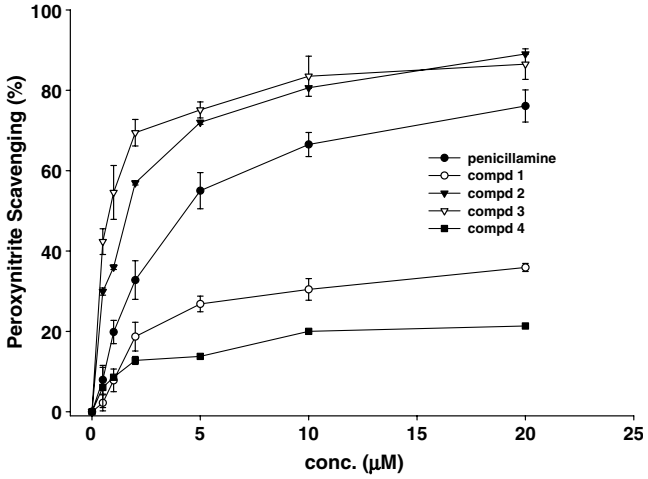

Figure 3. Peroxynitrite scavenging activity of compounds **1**–**4** and penicillamine (20, 10, 5, 2, 1, and 0.5 μM) in vitro cell free system. The level of peroxynitrite was measured by monitoring the oxidation of DHR 123. Values are expressed as means ± SD of tree measurements.

itoring the fluorescence of the oxidation product of dihydrorhodamine 123 (DHR 123) by authentic peroxynitrite.[22] $ONOO^-$ scavenging activity (%) of samples was expressed as the ratio of the decreased fluorescence to the fluorescence of control. Compounds **2** and **3** showed stronger activity of $ONOO^-$ scavenging than positive control, penicillamine (Fig. 3) in cell free assay system. The SC_{50} values of **2**, **3** and penicillamine, the concentrations required for 50% scavenging of $ONOO^-$, were 1.75, 0.77, and 5.13 μM, respectively. Compound **3** was reported to fail to scavenge the NO released from sodium nitroprusside.[20] The $ONOO^-$ scavenging activity of compounds **2** and **3** might explain further the anti-inflammatory activity of *Siegesbeckia glabrescens*.

In summary, four flavones, 3,4′-*O*-dimethylquercetin (**1**), 3,7-*O*-dimethylquercetin (**2**), 3-*O*-methylquercetin (**3**) and 3,7,4′-*O*-trimethylquercetin (**4**) were isolated from *Siegesbeckia glabrescens* as inhibitors of NO production in LPS-activated microglia. They exerted their activities through the inhibition of protein and mRNA expression of iNOS. These results imply that *Siegesbeckia glabrescens* might be beneficial for the treatment of neuro-inflammatory diseases through the inhibition of overproduction of NO and peroxynitrite scavenging.

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13. *Cell culture and nitrite assay*. The murine microglial cell line (BV-2) was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM containing 10% fetal bovine serum, 2 mM glutamine, 1 mM pyruvate, penicillin (100 U/mL) and streptomycin (10 μg/mL) at 37 °C in a humidified incubator with 5% CO_2. Cells were stimulated with 0.1 μg/mL of LPS in the presence of test samples for 20 h. NO released from BV-2 cells was assessed by the determination of NO_2^- concentration in culture supernatant. Samples (100 μL) of culture media were incubated with an 150 μL of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine in 2.5% phosphoric acid solution) at room temperature for 10 min in 96-well microplate.[14] Absorbance at 540 nm was read using an ELISA plate reader. Standard calibration curves were prepared using sodium nitrite as standard.
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15. *Procedures of extraction and isolation*. The whole plant of *Siegesbeckia glabrescens* was collected from Wan-Do, Jeolla-Namdo province, Korea in November 2005 and authenticated by Prof. K.S. Yang at the College of Pharmacy, Sookmyung Women's University (SMU). A voucher specimen (No. SPH 05007) was deposited in the herbarium of SMU. The air-dried material (2.7 kg) was extracted with methanol (3 × 2 L) to yield crude methanol extract (190 g) which was successively partitioned with *n*-hexane, EtOAc, $CHCl_3$ and BuOH. The EtOAc soluble fraction was followed by activity-guided chromatography for the purification of active principles. The EtOAc fraction (15 g) was subjected to CC (SiO_2, 70-230 mesh, 200 g, 4.5 × 23 cm) eluting with a gradient mixture of *n*-hexane: EtOAc (contained 10% *i*-PrOH) (15:1, 10:1, 5:1, 1:1, 1:2, 2 L each) to give 17 fractions. Fr. 7 (820 mg) was further separated by CC (SiO_2, 230–400 mesh, 70 g, 2.8 × 23 cm) eluting with *n*-hexane:acetone (3:1, 2.5 L) as eluent to give Fr. 7–10 (32 mg) that was further purified by semi-preparative HPLC (μ-Bondapak C18 column, 10 × 300 cm, 70% MeOH, 2 mL/min, 254 nm) to yield compounds **1** (5.0 mg) and **2** (9.9 mg). Fr. 9 (632 mg) was subjected to CC (SiO_2, 230-400 mesh, 60 g, 2.8 × 22 cm) eluting with *n*-hexane: acetone (4:1, 2:1, 1:1, 1 L each) as eluent to give Fr. 9–3 (56 mg). Fr. 9–3 was further chromatographed on RP-C18 column (40–63 μm, 2 × 7 cm) with a gradient elution (30, 50, 60% MeOH, 100 mL each)

to afford compound **3** (42 mg). Fr. 6 (137 mg) was chromatographed on RP-C18 column (40–63 μm, 2×7 cm) with a gradient elution (50, 70, 90% MeOH, 100 mL each) to afford compound **4** (37 mg).

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