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Inhibition of lipopolysaccharide-induced inducible nitric oxide synthase and cyclooxygenase-2 expression by xanthanolides isolated from *Xanthium strumarium*

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Abstract—Three sesquiterpenoids, xanthatin (**1**), xanthinosin (**2**), and 4-oxo-bedfordia acid (**3**) were isolated from *Xanthium strumarium* as inhibitors of nitric oxide synthesis in activated microglia (IC_{50} values: 0.47, 11.2, 136.5 μM, respectively). Compounds **1** and **2** suppressed the expression of iNOS and COX-2 and the activity of NF-κB through the inhibition of LPS-induced I-κB-α degradation in microglia.

Microglia, the immune regulatory and inflammatory cells of central nervous system (CNS), can be activated in response to a variety of neurodegenerative and neuro-inflammatory conditions. Activated microglia produce inflammatory mediators such as nitric oxide (NO), prostaglandins (PGs), IL-1β, IL-6, and TNF-α.[1,2] Overproduction of NO and PGs, the products of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) are responsible for brain injuries and neurodegenerative diseases including Alzheimer's disease and Parkinson's disease.[3]

Nitric oxide (NO), a gaseous free radical, is produced through the oxidation of L-arginine by nitric oxide synthase (NOS).[4] The calcium-regulated constitutive isoforms (eNOS, endothelial NOS; nNOS, neuronal NOS) have important roles in the regulation of blood pressure, neurotransmission,[5] platelet aggregation, and other homeostatic mechanisms.[6] The inducible isoform (iNOS) is calcium-independent and can be induced by lipopolysaccharide (LPS) and various cytokines such as IFN-γ, IL-1β, and TNF-α.[7] As a neuromodulator in CNS, NO participates in brain development, pain perception, memory, neuronal plasticity, and behavior.[8] Generally the iNOS along with the release of NO by microglia contributes to progress neurodegeneration and aggravate diseases. Large amounts of NO can be converted into a neurotoxic peroxynitrite ($ONOO^-$) in the presence of superoxide anion O_2^-.[9] Cyclooxygenase (COX) has been observed in mammalian brain and the inducible COX-2 plays a significant role in the production of inflammatory PGs.[10] The inhibition of COX-2 attenuates the levels of inflammatory mediators including TNF-α, IL-1β, and iNOS in injured brain tissue.[11] The modulation of iNOS and COX-2 expression can be a good strategy for the management of CNS inflammation.

As a part of our on-going screening program to evaluate the anti-inflammatory potentials of natural compounds, we have investigated the inhibitors of iNOS from the extract of *X. strumarium* L (Compositae). *X. strumarium* has been used in traditional Chinese medicine for the treatment of inflammatory diseases like rhinitis, empyema, and rheumatoid arthritis.[12] The genus *Xanthium* is a well-known source of xanthanolide sesquiterpenes, which has been known to possess antiviral, antibacterial,[13] antitrypanosomal,[14] antimalarial,[15] fungicidal[16] and cytotoxic activities against cancer cell lines.[17] Recently the extracts of *X. strumarium* semen were reported to possess anti-inflammatory activity by

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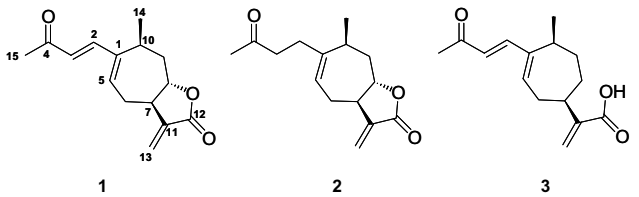
blocking NF-κB activation.[18] There has been, however, no report on the bioactive principles responsible for the anti-inflammatory activity of this plant. This article describes the identification of three sesquiterpenes from *X. strumarium* and their inhibitory activities of iNOS and COX-2 expression in LPS-activated microglial cells.

Among the extracts from different parts of *X. strumarium*, leaves showed the most potent activity (data not shown). The treatment of EtOAc soluble fraction of leaves (30 μg/mL) into the cells reduced the production of NO to 0.9 ± 0.3 μM, while that of LPS control was 28.1 ± 0.9 μM.

The EtOAc soluble fraction of leaves was subjected to the activity-guided purification.[19] Compounds **1**–**3** were isolated as inhibitors of NO production and their purities were confirmed by the reverse phased HPLC analyses and NMR spectrum. From the ^{1}H and ^{13}C NMR spectra of **1**–**3**, we found overall spectral pattern of typical xanthanolide from *Xanthium* species. The molecular mass $[M]^{+}$ of **1** was observed as 246 from EIMS data. Two methyl groups at δ 1.13 (d, J = 8.0 Hz) and 2.27 (s) were observed. We found five olefinic protons ranging from δ 5.47 to 7.05 including two *trans*-coupled protons at δ 7.05 and δ 6.17 (J = 16.0 Hz), two coupled protons at δ 6.17 and δ 5.47 (J = 2.8 Hz), and another methine proton at δ 6.27 (dd, J = 9.2, 3.2 Hz). Additional five sp_3-type carbon peaks from the HSQC spectra including three methines (δ 81.4, δ 47.4, δ 29.1), and two methylenes (δ 36.6, δ 27.2) were found. The presence of seven-membered ring and α,β-unsaturated lactone ring, and the position of side chain was confirmed from the correlation of ^{1}H–^{1}H COSY and HMBC spectra. Terminal sp_2-methylene protons (13-H_2) coupled with 7-C/12-C, and the protons of 9-CH_2 coupled with 14-C/8-C together. Carbonyl 4-C coupled with 15-H_3/2-H, 3-H coupled with 1-C, respectively. From the above-mentioned evidences and comparison of reported data,[20] compound **1** was identified as xanthatin. Compound **2** showed very similar spectral pattern with those of **1**. Additional two methylene protons (δ 2.26, δ 2.5) and carbons (δ 35.4, δ 42.7) were observed instead of two olefinic methines (2-C, 3-C) of **1**. Compound **3** lacks oxymethine peak of 8-C due to the cleavage of lactone ring. From the above-mentioned data, the structures of **2** and **3** were identified as respective xanthinosin and 4-oxo-bedfordia acid. All the spectral data were confirmed by the comparison with those of published in literature.[21]



1 2 3

When the purified compounds were treated to the cell culture media at the time of stimulation of cells, they inhibited the production of NO in a dose-dependent manner.[22] The IC_{50} values of **1**–**3** were found to be 0.47, 11.2, and 136.5 μM, respectively. Curcumin was used as a positive control in nitrite assay. Cell viability was above 85% at the concentrations of nitrite assay when assessed by MTT method. Compounds **1** and **2** have a lactone ring conjugated with exomethylene group, while **3** has a free carboxylic acid group. As reported previously,[23] α,β-unsaturated lactone was suggested as an essential moiety of sesquiterpene for the iNOS-dependent inhibition of NO. The most potent **1** has an extra α,β-unsaturated carbonyl group at the side chain that was reported as an additional enhancer of biological activity of sesquiterpene lactone. These compounds had no significant effects on the NO and PGE_2 production when treated after completion of cell activation by LPS. This result suggested that **1**–**3** might not direct enzymatic inhibitors of iNOS and COX-2.

In order to determine whether **1** and **2** would inhibit the expression of iNOS and COX-2, Western blot and reverse transcription-polymerase chain reaction (RT-PCR) analyses were performed.[24] The induction of iNOS protein was maximized by 18 h stimulation of BV-2 cells with 0.1 μg/mL of LPS. Compounds **1** and **2** suppressed significantly the expression of iNOS at 20 μM (Fig. 1) in activated microglia. They also significantly suppressed the induction of COX-2 that is another inducible enzyme that produces pro-inflammatory PGs in activated microglia (Fig. 1). The results of RT-PCR analysis revealed that the levels of iNOS and COX-2 mRNA were markedly increased by LPS-activation for 4 h. The induction of iNOS and COX-2 mRNA

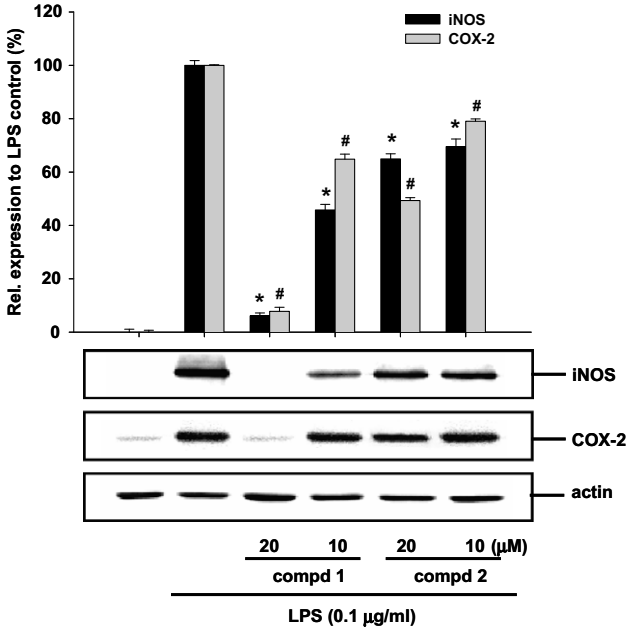


Figure 1. Effects of compounds **1** and **2** on the expression of LPS-induced iNOS and COX-2 protein in activated microglia. BV-2 cells were treated for 20 h with compounds (20 μM, 10 μM) during LPS (0.1 μg/mL) activation. Cell lysates were prepared and the iNOS, COX-2, and actin protein levels were determined by Western blotting. The blot is the representative results of three separate experiments. The relative intensity of iNOS/COX-2 to actin bands was measured by densitometry. Values represent means ± SD (n = 3). $^{*,\#}p < 0.05$ indicate significant difference (*iNOS, $^{\#}$COX-2) between LPS alone control and sample treatment.

was significantly suppressed by **1** and **2** (20 μM) during the LPS activation of BV-2 cells (Fig. 2). The inhibition of NO production by sesquiterpenes from *X. strumarium* was resulted from the suppression of iNOS expression.

The gene expression of iNOS and COX-2 can be modulated by NF-κB.[25] NF-κB can be activated by the degradation of inhibitor-κB (I-κB) through its phosphorylation. I-κB-α was fully degraded by 30 min exposure of LPS (0.1 μg/mL) and followed by the recovery in microglia.[26] To elucidate the further mechanism of **1** and **2** for the inhibition of iNOS and COX-2 expression in microglia, we determined the effects of compounds on NF-κB activity and I-κB-α degradation in LPS-activated microglia. As shown in Figure 3, **1** and **2** (20 μM) suppressed the LPS-induced degradation of I-κB-α. Compound **1** (10 μM) suppressed significantly NF-κB activity as determined by NF-κB (2×)-luciferase reporter gene assay[27] as shown in Figure 4.

Microglia are resident macrophage-like cells that play a role in host defense and tissue repair in CNS.[28] However, activated microglia play a pivotal role in the amplification of inflammation and the mediation of cellular degeneration.[29] NO and PGs released from activated microglia participates in the process of neuronal cell death through DNA damage, mitochondrial disruption,

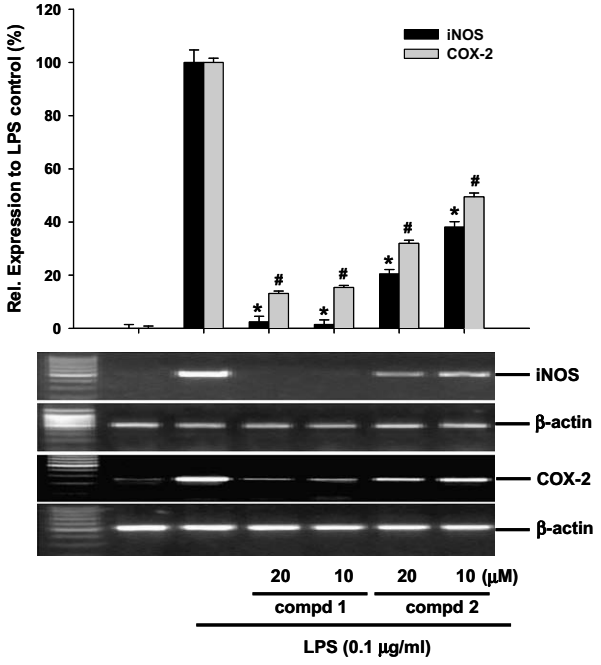


Figure 2. Effects of compounds **1** and **2** on the expression of LPS-induced iNOS and COX-2 mRNA in activated microglia. BV-2 cells were treated for 4 h with compounds (20 μM, 10 μM) during LPS (0.1 μg/mL) activation. The mRNA levels for iNOS, COX-2, and β-actin were determined by RT-PCR from total RNA extracts. The gel shown is the representative results of three separate experiments. The relative intensity of iNOS/COX-2 to β-actin bands was measured by densitometry. Values represent means ± SD ($n = 3$). $^{*,\#}p < 0.05$ indicate significant difference (*iNOS, $^{\#}$COX-2) between LPS alone control and sample treatment.

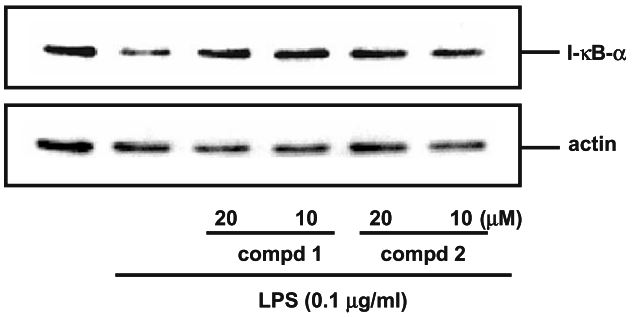


Figure 3. Effects of compounds **1** and **2** on the degradation of I-κB-α in LPS-activated microglia. Cells were treated with compounds (20 μM, 10 μM) and LPS (0.1 μg/mL) for 30 min. Cell lysates were prepared and the I-κB-α and actin protein levels were determined by Western blotting. The blot is the representative results of three separate experiments.

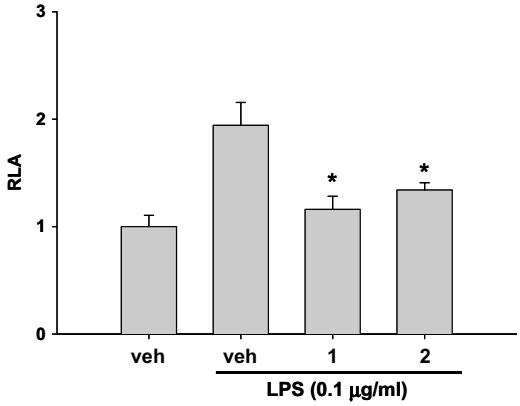


Figure 4. Effects of compounds **1** and **2** on the activation of NF-κB in LPS-activated microglia. BV-2 cells were transfected with luciferase reporter plasmid containing NF-κB binding site. After cells were pretreated with compounds (10 μM) for 1 h, cells were further stimulated with LPS (0.1 μg/mL) for 6 h. Relative luciferase activity (RLA) was measured by normalization with β-galactosidase activity. Values represent means ± SD ($n = 3$). $^{*}p < 0.05$ indicate significant difference between LPS alone control and sample treatment.

and induction of apoptosis. Therefore, inhibitors of these mediators might be beneficial for the treatment of neuro-inflammatory diseases. The genus *Xanthium* was characterized to contain xanthanolides as its common secondary metabolites. Compounds **1**–**3** might be at least in part responsible for the anti-inflammatory activity of *X. strumarium* through the inhibition of iNOS and COX-2 expression.

In conclusion, xanthatin, xanthinosin, and 4-oxo-bedfordia acid were isolated from *X. strumarium* as inhibitors of NO production in LPS-activated microglial BV-2 cells. The α-methylene–γ-lactone ring was identified as the essential moiety for their biological activity. They exerted their activity through the inhibition of I-κB-α degradation, NF-κB activation, and subsequent suppression of iNOS and COX-2 expression. These results imply that *X. strumarium* may be beneficial for the treatment of neuro-inflammatory diseases through

down regulation of such inflammatory enzymes as iNOS and COX-2.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.12.076.

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19. *Procedures of extraction and isolation*. The dried whole plant of *Xanthium strumarium* collected from Wando island, Jeolla-Namdo province was extracted at room temperature for 24 h with methanol. The extracts of leaves (45 g) were dispersed in water and partitioned with EtOAc. The EtOAc soluble fraction (12 g) was subjected to column chromatography (SiO_2, 70–230 mesh, 300 g) eluting with a gradient mixture of *n*-hexane/EtOAc (20:1–1:1) to give 12 fractions. The active Fr. 9 (722 mg) was further separated by column chromatography (SiO_2, 230–400 mesh, 35 g) eluting with *n*-hexane/acetone (5:1) to give Fr. 9–3 (385 mg) that was further chromatographed on RP-C18 column (40-63 μm, 3 × 25 cm) to yield compound **2** (101 mg). Another active Fr. 11 (2.4 g) was further separated by column chromatography (SiO_2, 230–400 mesh, 75 g) eluting with *n*-hexane/acetone (3:1) to give Fr. 11–2 (1.38 g) and Fr. 11–3 (674 mg). Fr. 11–2 was further chromatographed on RP-C18 column (40–63 μm, 3 × 25 cm) with a gradient elution using 40–90% MeOH to yield compound **1** (468 mg). A part of Fr. 11–3 (50 mg) was further purified by semi-preparative HPLC (μ-Bondapak C18 column, 10 × 300 mm, 70% MeOH) to yield compound **3** (28 mg).
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